Heart transplant, current state and future

Shechter, Avi

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Inbar Shafran

The role of DNA methylation in human spermatogenesis

GRADUATE THESIS

Zagreb, 2022.

This graduate thesis was done at the Department of Medical Biology School of Medicine University of Zagreb, under the mentorship of Assoc. Professor Ana Katušić Bojanac, was submitted for evaluation in the academic year 2021/2022.

ABBREVIATIONS

DMRs- Differentially Methylated Region

DNMT- DNA methyltransferase

TET- ten-eleven translocation

AID/APOBEC- activation-induced cytidine deaminase/apolipoprotein B mRNA editing enzyme complex)

TDG- thymine DNA glycosylate

UHRF- ubiquitin-like PHD and RING finger domain

5-mC- 5-methylcytosine

5-hmC- 5-hydroxymethylcytosine

5-fC- 5-formylcytosine

5-caC- 5-carboxylcytosine

PGC- Primordial germ cells

SRY- Sex determining region of chromosome y

TDF- testis determining factor

SSC- Spermatogonial Stem Cell

ICR- Imprinting Control Regions

DPC- day post coitum

MEST- mesodermal-specific transcript

PEG1- paternally expressed gene 1.

ICSI- Intracytoplasmic sperm injection

SNRPN- small nuclear ribonucleoprotein polypeptide N

NOA- non-obstructive azoospermia

OA – obstructive azoospermia

Zdbf2 - the zinc finger DBF type containing 2

TABLE OF CONTENTS

ABSTRACT

What is the story behind the term “germ cell epigenetics”, what can we learn from the changes in methylation during gametogenesis, and how could it be put to good use? This thesis aims to explain how changes in the DNA methylation affects the development of primordial cells into mature sperm.

Methylation is the epigenetic process controlling what genes will be expressed and what genes will be silenced. Epigenetics can be viewed as the environmental inheritance we get from our ancestors. It reflects changes that occur due to normal aging (development) but also environmental influences. Alarmingly, our epigenome reflects not only our lifestyle choices but also the experiences of our parents and perhaps also grandparents.

Some methylation processes are inherited from the father and some from the mother, part of which reflects the imprinting process. Today, it is known that some genes in specific sites will be maternally imprinted and others paternally imprinted, making each parent contribute to various specific cells. This is all done by the process of methylation.

The development of the oocyte and sperm starts in the embryonic period and varies drastically. Male developing germ cells show changes in their methylation process already during the fetal period and continue during spermatogenesis. Sperm of infertile males have been shown to carry methylation defects and there is a danger of passing the methylation errors to the next generation. Thus, understanding the male germ cell methylome can help us understand its vulnerabilities, as well as identify factors that may influence its methylation.

I hope that by learning more about these processes, we will be able to understand ourselves better and create better diagnostic and treatment options for men with infertility problems, as well as help us create personalized preventive approaches for those problems.

SAŽETAK

Metilacija DNA u humanoj spermatogenezi

Što stoji iza riječi “epigenetika spolnih stanica”, što možemo naučiti iz promjena u metilaciji tijekom gametogeneze i kako se to može dobro iskoristiti? Ovaj rad će objasniti razvoj primordijalnih stanica u zrele spermije u kontekstu promjena u procesu DNA metilacije.

Metilacija je epigenetski proces koji kontrolira koji će geni biti izraženi, a koji geni će biti utišani. Epigenetika se može promatrati kao ekološko naslijeđe koje dobivamo od naših predaka. Odražava također i promjene do kojih dolazi zbog normalnog starenja (razvoja), ali i utjecaja okoline. Zaprepaštujuće, naš epigenom odražava ne samo naš način života, već i iskustva naših roditelja, a možda i baka i djedova.

Neki procesi metilacije su naslijeđeni od oca, a neki od majke, od kojih dio odražava proces utiskivanja. Danas je poznato da će neki geni na određenim mjestima biti utisnuti s majčine, a drugi s očeve strane, čineći da svaki roditelj doprinosi ra~~u~~zličit~~i~~osti specifičnih stanica Sve se to postiže procesom metilacije.

Razvoj oocita i spermija počinje u embrionalnom razdoblju i drastično varira. Muške zametne stanice u razvoju pokazuju promjene metilacije već tijekom fetalnog razdoblja i kasnije u spermatogenezi. Pokazalo se da spermiji neplodnih muškaraca imaju defekte DNA metilacije. Međutim, postoji opasnost od prenošenja pogrešnih metilacijskih obrazaca na sljedeću generaciju. Dakle, razumijevanje metiloma muških zametnih stanica može nam pomoći razumjeti njihovu ranjivost kao i čimbenike koji na nju mogu utjecati.

Nadam se da ćemo, učeći više o tim procesima, moći bolje razumjeti sebe i stvoriti bolje dijagnostičke i terapijske mogućnosti, pomažući muškarcima s problemima neplodnosti kao i u razvoju personalizirane preventivne medicine.

INTRODUCTION

Genetics is the study of genes, heritage, and how a trait is transmitted from parents to offspring by the inheritance of the parental DNA. It was first described in peas by the famous German monk Gregor Mendel, who discovered dominant and recessive traits and defined classical genetics.

Epigenetics is the study of gene expression caused by structural modifications, without alternating the underlying DNA nucleotide sequences (Cui et al., 2016). Instead, it is influenced by substances that bind DNA or other modifiers within the cell, regulating the expression of genetic material. Histone modification, control of non-coding RNA, chromatin remodelling and, the best-known, DNA methylation all play a role in epigenetic gene expression. Epigenetic changes can occur due to environmental or lifestyle changes in a tissue-specific fashion but could also affect gametes and be passed from one generation to another. This somewhat contradicts the classical genetic model of heredity as we inherit not only our DNA but the consequences of what we do with it.

The regulations of these processes are linked to fertility outcomes and various disorders and dysfunctions, affecting embryogenesis, perinatal mortality, and fetal outcomes. In this review, we will focus on the methylation within the sperm cells. Understanding this subject matter can offer the basis for prevention and perhaps future treatment of male infertility and reduction of epigenetic diseases, thus allowing the selection of the fittest sperm for assisted reproductive technologies**.**

1. DNA METHYLATION

DNA methylation most commonly refers to the addition of a methyl group to the fifth carbon of cytosine in a CpG sequence, hence creating a 5-methylcytosine (Moore et al., 2013). Methylation is part of the epigenetic process that allows genes to be switched on or off, thus controlling the process of transcription (Bird, 2002). The addition of the methyl group causes a structural alteration in the chromatin, changing the availability of the binding site for transcriptional factors and other proteins. In most places, adding a methyl group silences the gene being expressed; however, this rule has certain exceptions (Schübeler, 2015).

CG dyads are methylated symmetrically on both DNA strands; hence their methylation can be passed along during cell division, allowing for "epigenetic memory" (Sendžikaitė & Kelsey, 2019). Furthermore, in sperm, methylome has been found to respond to environmental and physiological changes, such as dietary changes and exercise (Denham et al., 2015; Donkin et al., 2016; Franzago et al., 2021; Ingerslev et al., 2018), toxins, and even child abuse and stress (Denham et al., 2015; Roberts et al., 2018), thus allowing any of these events to lead to epigenetic changes in the offspring (Guerrero-Bosagna et al., 2012; Huypens et al., 2016; Wei et al., 2014; Youngson et al., 2016).

Enzymes involved in DNA methylation can be divided into three groups: writers, erasers, and readers (Moore et al., 2013).

Writers, the enzymes involved in writing are DNA methyltransferases (DNMTs), and they do so by transferring a methyl group from S-adenyl methionine (SAM). During embryogenesis, DNA methylation is erased and then re-established in unique patterns according to the cell type. This re-establishment of DNA methylation patterns is referred to as de novo methylation. DNMT3a and DNMT3b lead to these de novo methylation patterns (F. Guo et al., 2015). The maintenance of DNA methylation patterns after they have been formed is the role of DNMT1. DNMT1 also plays a role in the inheritance of methylation, copying the paternal methylation pattern to the synthesized daughter strand, as well as in the DNA repair (Goll & Bestor, 2005; A. Hermann et al., 2004; Mortusewicz et al., 2005). During male germ cell development, DNMT3C participates in the methylation of retrotransposons (Barau et al., 2016). DNMT3C and P-element induce wimpy testis/interacting RNA (PIWI), which handles retrotransposon promoter

methylation within the male germline. piRNAs are little non-coding RNAs that interact with PIWI. These RNA–protein complexes are fundamental for the LINE1 retrotransposon methylation forms in male germ cells (F. Yang & Wang, 2016). LINE-1 account for around 10% of human genome retrotransposons (A. S. Yang et al., 2004).

All DNMTs share a similar structure, possessing an N-terminal regulatory domain and a C catalytic terminal site. The C terminal site binds DNA and acts upon it (Goll & Bestor, 2005). The N-terminal regulatory domains of DNMT1 and DNMT3 proteins differ significantly and play a key role in determining their activity across the genome. Domains that facilitate anchoring to the replication fork are found in the N-terminal region of DNMT1 (Leonhardt et al., 1992), while in DNMT3, N-terminus has an ADD domain interacting with histone's H3 tail (Ooi et al., 2007). DNMTs3 A and B have a second domain PWWP, which is believed to play a role in their repression (Kim et al., 2007; Shikauchi et al., 2009). During early development, a DNMT stimulator, known as DNMT3L, stimulates DNMT3a and DNMT3b; on its own, it lacks the catalytic site and is believed to only act as a stimulator (Bourc'his & Bestor, 2004; Jia et al., 2007; Ooi et al., 2007). During embryonic development and implantation, DNMT3B targets promoter regions, setting up the genome-wide DNA methylation (Okano et al., 1999). Without DNMT3, spermatogonia are lost (la Salle et al., 2007).

Erasers, demethylation, or erasing is done by deamination and oxidation of the 5-methyl cytosine (5-mc), followed by base excision repair to cytosine. (Bhutani et al., 2011). When replication is carried out in the absence, down-regulation, or suppression of DNMT1, DNA demethylation can be a passive process. Cells need DNMT1 to maintain methylation; in its absence, methylation patterns will start to disappear (Ficz & Hoppeler, 2015). TET protein, a hydroxylase, actively demethylates DNA. It adds a hydroxyl group in three successive oxidation reactions, converting 5mC to 5hmC, 5fC, or 5caC (Ito et al., 2010, 2011; Tahiliani et al., 2009). These can then be further catalysed actively or passively by different enzymes to achieve a naked cystine. For example, ID/APOBEC deamination forms a 5hm-uracil, which can replace cysteine with the enzyme thymine TDG (Cortellino et al., 2011). There are three known TET enzymes: TET1, TET2, and TET3. TET1 and TET3t have the CXXC DNA binding domain at the N-terminus, whereas TET2 has the IDAX CXXC4 domain (Iyer et al., 2009; Ko et al., 2013). These domains prefer to bind to unmethylated CpG islands (An et al., 2017).

The readers are represented by 3 different families: Methyl Binding Domain (MBD) containing protein (n=11), Methyl CpG binding zinc fingers (n=8), and the Set and Ring associated (SRA) domain containing proteins (n=2) (Mahmood & Rabbani, 2019). The first to be discovered is MeCP2, an MBD, that can silence transcription. MeCP2 is known for its role in Rett syndrome. Other MBD subtypes include HMT and HAT MBDs. The Methyl CpG binding zinc fingers can bind both methylated and unmethylated DNA, they include Kaiso, ZBTB4 and ZBTB38 among others (Filion et al., 2006). The third family, SRA containing proteins, include URHF 1 and URHF 2. Immediately after replication, UHRF1 recruits DNMT1 to the replication fork by binding SRA domains on the hemi methylated DNA allowing for methylation (Arita et al., 2008; Avvakumov et al., 2008; Kimura & Shiota, 2003). UHRF2 on the other hand can enhance TET expression and bind 5mC and H3K9me as well as 5hmC.

2. HUMAN GERM CELL DEVELOPMENT

2.1. The appearance of human primordial germ cells (hPGCs)

The primordial germ cells are the common origin of both spermatozoa and oocytes. Their differentiation and proliferation begin during early embryogenesis. These cells will go through a series of evolutionary changes to become what we know as sperm. In general, male primordial germ cells will migrate to become the actively proliferating gonocytes, which will later become the prespermatogonia (Gaskell et al., 2004). However, not all cells are transient at the same time, allowing for multiple populations to coexist.

The primordial germ cells (PGC) are proliferatively active, reaching more than 100.000 numbers in the first eight weeks. They start to develop from the posterior end of the developing primitive streak, by separating from the proximal epiblast (D. Chen et al., 2019; Y. Zheng et al., 2019). They then move to the extraembryonic tissue and are found at the base of the yolk sac, at the end of the primitive streak and the connecting stalk (Raz, 2004). This location allows them to be minimally influenced by the induction from other somatic cells in early development. At the beginning, their morphology is remarkably similar to the cells around them. However, as they enlarge and become rounder, with their nuclei more prominent, sometimes having two of them, they grow differently from one another.

Around the 5th week, at the beginning of gastrulation, they migrate by amoeboid movement and growth displacement dorsocranially through the dorsal mesentery towards the genital ridge, forming the gonads (Hara et al., 2009). Between them, the primordial germ cells have cytoplasmatic bridges with long processes, and they share junctional complexes with nearby somatic cells, allowing for their modification by the local environment. Extracellular matrix molecules may also interact with them via different binding sites, controlling both proliferation and migration (de Felici, 2013). Not all PGC migrate, and those that fail to do so either degenerate or have the potential to form tumors (Hutson et al., 2013; Runyan et al., 2006; Tien et al., 2020).

PGC are derived from embryonic cells that have already been partially committed to a somatic destiny, implying that they have undergone DNA methylation. Global DNA methylation ablation occurs during hPGC proliferation and re-localization. This process is called epigenetic reprogramming, as almost all methylation marks are erased in hPGC to be ready for new, de novo DNA methylation gain after the specification of their sex.

In males, gene in the Sex-Determining Region of chromosome Y, i.e., *SRY* gene, codes for the protein "testis determining factor" (TDF), which around the 6th week initiates the development of the testis. At the same time, methylation takes place in most imprinted control regions, exhibiting a sex specific pattern (L. Li et al., 2020). The genital ridge is influenced by the SYR sex-determining region to from the somatic cells and the testicular niche (Hanley et al., 2000). The testicular niche will form the seminiferous tubules with elongation and lumen acquired in adolescence (J. Guo et al., 2020). Within the primordial gonads, it is the testis-determining factor, SRY, which causes medullary cords derived from the coelomic epithelium to elongate. The PGC and gonocytes are embedded within those cords. The growth of cords creates the straight tubules, and later, when meeting with the mesonephric mesenchyme, the rete testis. The tunica albuginea is formed from mesenchymal cell proliferation beneath the coelomic epithelium.

During the 8th week of development, some cells in the walls of medullary cords develop into the Sertoli cells, while some central PGCs develop into gonocytes (J. Guo et al., 2021). The Sertoli cells secrete anti-Mullerian hormone (AMH), which causes the degeneration of the Mullerian duct, previously called the paramesonephric duct, into the appendix testis. The Sertoli cells act to support the gonocytes, allowing

for extensive gonocytes proliferation. They will continue to do so until week 19, when their division declines significantly (Wang et al., 2022a). Each Sertoli cell will support a limited number of spermatogonia, so their number established at this stage is crucial for sperm count (Sharpe, 2003). Some of the cells in the genital primordium develop into the mesenchymal cells, along the coelomic epithelium, and later form the fetal Leydig cell population, responsible for the secretion of testosterone and insulin-like factor 3 to support the gonocyte-to-prespermatogonia differentiation (DeFalco et al., 2011; Wen et al., 2016).

2.2. Prespermatogonia and testis development

Around the 13th weeks of development, the gonocytes start to gradually migrate into the basal lamina and become the prespermatogonia. At the same time, secretion of hormones leads to the first hormonal peak in males, with maximal testosterone levels between the 11th and 14th gestational weeks. Following this testosterone peak, the subpopulation of the hPGC later exits mitosis and transitions into a state that is presumably the first appearance of state 0 spermatogonia.

Under the testosterone influence, the mesonephric duct elongates to form the reproductive ducts outside the testis. The remaining glomerular capsule connects the rete testis to some cranial mesonephric tubules. These, under the influence of testosterone, become convolute, creating the epididymis where sperm will mature. The previously primitive ureter, now the mesonephric duct, also elongates under the same effect to become the vas deferens. Later, a seminal gland, responsible for sperm nourishing, is formed where the end of each vas deferens forms the ejaculatory duct. The enzyme 5α-reductase converts testosterone to dihydrotestosterone, which is responsible for developing the external male genitalia from the urogenital sinus.

The prespermatogonia can be visualized in the testis as small round cells within a homogenous population. They are more pronounced at the periphery, with few cells having cytoplasmic bridges connecting them (L. Li et al., 2017). Although the morphological changes have started, the markers of early gonocytes are kept until 12th week. It is only at late second trimester when most gonocytes lose

their mitotic ability and are then arrested at the transitional prespermatogonia phase. (Culty, 2009; J. Guo et al., 2021; McCarrey, 2017; Overeem et al., 2021; Wang et al., 2022b).

In humans, following the testosterone peak, initially mitotically active gonocytes show a decrease in methylation. Between the 7th week until the 17th week of development, there is a 76% decrease in methylation. Prespermatogonia will acquire its specific methylation in utero via re-methylation (Ohinata et al., 2009; Sasaki & Matsui, 2008; Smallwood & Kelsey, 2012b). After the 17th week, de novo DNA methylation takes places, allowing methylation to increase and becomes stable and heterogenous (L. Li et al., 2020). It appears that DNA methylation correlates more with fetal age than cell developmental stage, suggesting that it involves an interplay of more than one mechanism to function (L. Li et al., 2020).

2.3. Postnatal spermatogonia development – mini puberty

Mini puberty is the transitory sex-specific activation of the hypothalamic-pituitary-gonadal axis during the neonatal age. It results in elevated gonadotropin and sex hormone levels.

At birth, a great LH increase in boys of about ten times leads to a 12-hour peak in testosterone level. Due to the loss of the negative feedback loop (by the fall in placental steroids), there is a reactivation of the hypothalamyc-pituitary-gonadal axis. The gonadotropins themselves rise between 6 and 10 days after birth. In boys, LH rise begins in the first week and reaches pubertal levels within ten days. The hormonal peak will occur between 4-8 weeks postnatally and, within six months, will decrease to typical prepubertal values (Kuiri-Hänninen et al., 2014; Maisnam Indira, 2021).

These peak changes cause growth and development of the male genitalia and are thought to lead to masculine brain development (Lamminmäki et al., 2012). The LH level increases the number of Leydig cells and testosterone secretion, leading to testicular descent and penile growth of about 1 mm per month in the first three months (Boas et al., 2006). The FSH surge causes Sertoli cells proliferation and elongation of seminiferous tubules, leading to an increased testicular volume.

Prespermatogonia transforms into a spermatogonial stem cells (SSC) at 3-6 months of age(Hutson et al., 2013). This transformation is believed to be achieved by priming followed by activation of SSC fate. Later

in adulthood, SSC cells can either maintain their fate, thereby renewing themselves, or go through priming of differentiation. They transform into spermatogonia by activating genes responsible for cell differentiation (B. P. Hermann et al., 2015, 2018; McCarrey, 2013; Schaefer et al., 2007), and in the process lose their pluripotency, as shown by changes in their morphology and gene expression patterns (Stukenborg et al., 2014).

Spermatogenesis develops from different cell linage. The first wave of spermatogenesis starts from the SOHOLO1 expressing cells, producing prespermatogonia and type A spermatogonia (Ballow et al., 2006). The SSC are located at the basal membrane of the tubule until committing to spermatogenic development. Migration may be due to Sertoli cell contact, platelet-derived growth factors, and Notch signalling (Shima, 2022). As they migrate up and mature, they lose *SSEA4* expression and gain *c-KIT* expression. Single-cell genomic profiling suggests that as cells grow from SSC to sperm, their function changes by process of methylation (J. Guo et al., 2017). At the beginning, SSC are quiescent and show high expression of *TXNIP*, which inhibits glucose uptake (J. Guo et al., 2017). They also express stem cell signalling factors such as FGFR. Later, the cell cycle is up-regulated as *FGFR3 e*xpression is lost, *TXNIP* is downregulated, and zinc finger motifs are up-regulated. At this point, we see a change in the DMRs of *SNRPN1 & 2*, RNA splicers needed for cell development, which start to become hypomethylated. *TXNIP* downregulation, along with zinc finger factors up-regulation, the attenuation of stem cell signalling, and the upregulation of mitochondria, allow SSC to grow and complete the transition. *SNRPN, PEG*, and *KCNQ1Q1* are examples of maternally expressed genes that gain function and become completely unmethylated, while *MEG3* and *H19* are paternally imprinted genes that become entirely methylated at the level of the sperm (See further for imprinting explanation and those specific genes in details). *SNRPN* is different because it is the first to be 100% unmethylated and does so already at the FGFR+ to FGFR- transition(Fend-Guella et al., 2019). Knowing how to distinguish between SSC and progenitor cells is essential as it can help identify and treat the fertility problems in males.

The regulation of hormonal axes is believed to play a critical role in the proper development of the SSC. In cryptorchidism, there is an impaired migration and transformation of gonocytes to SSC (Huff et al., 1991). This is a low testosterone state in which GnRH treatment can help save fertility, and it is only sensible that these (testosterone levels and cell migration/transformation) are causally related

(Hadziselimovic & Hoecht, 2008; Rodprasert et al., 2020). If androgenic theory is in fact true, it should be influenced by testosterone receptors, yet recent studies in mice show no correlation between testosterone receptor knockout and cell migration or differentiation. Biopsies of androgen insensitive patients showed early development of SCC, supporting the controversy regarding testosterone's accountability (Hadziselimovic & Huff, 2002; R. Li et al., 2015; Su et al., 2014). The role of FSH is also questionable. Namely, FSH receptor mouse knockouts show reduced testicular volume and reduced spermatogenesis (Kumar et al., 1997), but in mice, knockout of FSH means normal spermatogenesis and only a reduction in testicular size (Abel et al., 2000; R. Li et al., 2015), raising the possibility of more players involved. Further research in rats shows that the regulation of SSC growth is achieved by a combination of FSH and follistatin action on spermatogenesis (Meehan et al., 2000).

2.4. Pubertal testis – gametogenesis

Spermatogenesis, which begins at puberty, is the transformation of spermatogonia into spermatozoa which takes approximately 74 days. It starts just before puberty, when seminiferous tubules acquire a lumen, and the stem cells, at regular intervals, begin to divide into type A spermatogonia. It is worth mentioning that, unlike other gametocytes, spermatogonial stem cells can self-renew (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). During puberty, LH and FSH surge takes place. LH stimulates the Sertoli cells to generate testosterone, which acts on the Leydig cells to induce spermatogenesis. FSH stimulates Sertoli cells to synthesize intracellular androgen receptors and sperm cell precursor supporting fluids. Until the formation of spermatozoa, cell division is incomplete, allowing cytoplasmic bridges to connect the cell colony. This entire process occurs while the cells are embedded in the Sertoli cells that supply protection and nutrition for cell maturation. Each type A spermatogonia will go through a limited number of mitotic divisions, eventually resulting in type B spermatogonia. These will further divide to become the primary spermatocyte. The primary spermatocytes rest in prolonged prophase for around 22 days. Next, quick completion of meiosis I and secondary spermatocyte generation occur. Secondary spermatocytes go through a fast second meiotic division forming haploid spermatids, which then undergo spermiogenesis to become spermatozoa. Spermiogenesis is the process of producing sperm structure, including a tail, neck, and acrosome, condensing the nucleus, and shedding the cytoplasm. The acrosome protects the anterior nuclear surface and includes enzymes that aid

penetration (Sadler T.W & Langman J., 2004). Chromatin also undergoes reorganization during the spermatogenesis, e.g. DNA methylation marks are preferentially placed at intergenic sequences and transposons (Smallwood & Kelsey, 2012b).

3. DNA METHYLATION IN DEVELOPMENT

3.1 Imprinting

The concept of genomic imprinting is fundamental for understanding non-genetic inheritance. Imprinting is the process by which specific genes are expressed only from one parent. The imprinted patterns, maternal or paternal, are the same for specific genes in all humans. We have two sets of most genes, one from each parent. Imprinting describes the process by which one gene is silenced by methylation of imprinted control regions, leaving the other copy to be expressed as the parent of origin. In other words, the gene being imprinted is silenced, allowing the parent of origin to be expressed. It was previously thought of as a random selection, but now it is known that specific genes are imprinted according to paternal or maternal inheritance patterns, allowing for specific gene expression of a paternal or maternal origin.

During the 80-ies, a few crucial discoveries were published, allowing us to better understand what mother and father are contibuting (McGrath & Solter, 1983a, 1983b, 1984; Surani et al., 1984; Surani & Barton, 1983). Before that time, it was unrecognized that each parent's genome is expressed differently. One experiment used the pronuclear transfer of two maternal or two paternal genes. When two gynogenetic, female, genomes were transplanted and allowed to develop, no extraembryonic tissue developed, so there was no placenta to maintain a pregnancy, leading to an abortion. On the other hand, when two androgenetic of male origin, genomes were transplanted, poor embryonic development took place. Both gynogenetic and androgenetic genomes lead to a non-viable offspring. It was concluded that perhaps a male and a female genome together are required for healthy embryonic development, needing paternal expression for proper placental growth and maternal expression for proper foetal development.

In theory, pregnancy is an invasive process sometimes described as having similar mechanisms to tumour growth, so having an 'intruder' effort to allow growth and invasion makes sense. Nevertheless, could paternal imprinting potentially cause diseases affecting placental development, such as preeclampsia or abruptio placentae? These are just some of the questions raised by understanding the different responsibilities in gynogenetic and androgenetic imprinting. Parental imprinting has been shown to affect not only placental and foetal growth but also homeostasis and specific brain functions, and this is just the beginning of the extensive research in this field.

Interestingly, mice chimeras experiments shows that different areas in brain development are differently imprinted, suggesting that maternal imprinting plays a more significant role in the cortex, hippocampus, and striatum. In contrast, paternal imprinting is highly expressed in the hypothalamus (Keverne et al., 1996). So, could this imply that intelligence comes from the mother? Furthermore, if that is so, is it an environmental effect? Or that of inheritance, or perhaps things are not merely as black and white as we used to think when it comes to the IQ?

Today we know that some tissues exhibit differences in their imprinting patterns - some take a maternal expression, and others take paternal. A well-known example is Prader Willi and Angelman syndromes (Cassidy & Schwartz, 1998). Both syndromes are due to imprinting disorder on chromosome 15. 15q11-13 chromosome codes for the small nuclear ribonucleoprotein polypeptide N, *SNRPN*-ICR, an important spliceosome for mRNA processing. It is highly expressed in both the brain and heart (Reed & Leff, 1994). In Prader Willi, while maternal genes are silenced due to imprinting, deletion or mutation of the paternal allele occurs, resulting in intellectual disability, obesity, hyperphagia, and hypogonadism. In Angelman syndrome, paternal imprinting of *UBE3A* and maternal deletion or mutation ensue, leading to excessive laughter, ataxia, and seizures.

Interestingly, *SNRPN* also plays a role in the development of medulloblastoma (Jing et al., 2015) and its hypermethylation was reported as a risk for infertility (Botezatu et al., 2014). One study evaluated different CpGs at the *SNRPN* sequence, of which two (on positions 2 and 7) were most valuable in identifying oligospermia. The hypermethylation of *SNRPN* correlates with decreased sperm motility and normal morphology (Dong et al., 2017).

Imprinted genes are regulated by common regulatory elements such as non-coding RNA and differentially methylated regions (DMR), which are regions that show different DNA methylation patterns in various samples. These regulatory elements, controlling one or more imprinted gene, are known as the imprinted control regions (ICR).

We can demonstrate this by the known example of Beckwith Wiedemann (BWS) and Silver Russell syndromes. Beckwith Wiedemann imprinting is done on two imprinted control regions (ICR), paternal imprinting on ICR1 and maternal imprinting on ICR2. The first ICR1 is the biallelic *H19* paternally imprinted and the *IGF2* maternally imprinted, both located on 11p15 (Gicquel et al., 2005), and the second ICR2 includes *CDKN1C* and *KCNQ1*. While, among other things, BWS is characterized by intrauterine overgrowth, Silver Russel syndrome is characterized by intrauterine growth retardation (Õunap, 2016). Whereas Silver Russel is most commonly due to paternal hypomethylation in the ICR1 region, BWS is most commonly due to loss of maternal imprinting or by uniparental disomy, whereas most familial cases are due to some mutation in *CDKN1C*.

Some genes show transit imprinting, allowing for paternal expression at one point of development, and for maternal expression at later point. One such example is the zinc finger DBF type containing 2. The long isoform of *Zdbf2* is shortly expressed during preimplantation and it is found on pluripotent embryonic cells. This long isoform is paternally expressed in a mono allelic fashion (Duffié et al., 2014). After implantation, it is methylated, and the maternal germline DMR is activated. Lacking the long isoforms has long-term consequences such as growth defects, failure of proper brain development, and defects in the hypothalamyc-pituitary axes (Greenberg et al., 2016).

We know today that imprinting is a mono-allelical expression of genes, which is controlled by epigenetic modifications such as DNA methylation (Tucci et al., 2019). DNA methylation could be considered a heritable epigenetic stamp related to transcriptional suppression when happening on regulatory sequences. The parent-of-origin-specific germline methylation is fundamental for the monoallelic expression of imprinted genes and is held as the memory of parental origin after fertilization. Gene regions that exhibit different methylation patterns according to parental imprinting are called differentially methylated regions.

3.2 DNA methylation reprogramming in germ cell development

Following fertilization, there is first the embryonic reprogramming or rest phase, which includes demethylation and imprinted re-methylation. At the start, most methylation patterns are demethylated, followed by global re-methylation at the blastocyst stage. The re-methylation is retained in a parent-specific fashion (i.e., imprinted). Starting with gastrulation, the next stage is somatic programming and reprogramming. The gastrula gives rise to both somatic cells and germ cells. The somatic cell methylome is tissue-specific and does not change drastically. The germ cell, however, undergoes a more extensive genome-wide erasure of methylation, followed by a re-methylation *(see figure 1).* The demethylation phase happens between 8- and 13.5-days post coitum (dpc) in mice (Hajkova et al., 2002; Santos & Dean, 2004) and in the first 6-17 weeks in humans. Demethylation at this point is achieved both passively and actively - passively due to a lack of DNMT1 action and actively by the actions of TET enzymes. During germline reprogramming, cells experience the epigenetic ground state, the lowest level of methylation, which is shorter in males (Lee et al., 2014; Smallwood & Kelsey, 2012a, 2012c). Re-methylation starts at approximately day 15.5 dpc, the point at which parental imprinting occurs along the differentially methylated regions (DMR) (Boissonnas et al., 2013; Davis et al., 2000). At the PGC level, the leading players of methylation are DNMT3a and DNMT3L; as mentioned earlier, DNMT3L, or ligand, induces and recruits DNMT3a de novo methylation action at ICR germline DMRs within those cells. DNMT3 knockout impairs spermatogonia paternal imprinting, leading to impaired spermatogenesis (Kaneda et al., 2004). In foetal human testis, recent single cell methylome analysis showed that foetal germ cells, e.g. gonocytes undergo reprogramming in the first trimester. There, the methylation level was shown to decrease from 14.5% in 6-week's testes to 4.9% in 17-week's testes. DNA methylation level later gradually increased and reached 7.2% in 24-week's testes, indicating that the global de novo DNA methylation process in male germ cells had been initiated. Also, DNMT3A and DNMT1 show highest expression around the 22nd week of development (Galetzka et al., 2007), pointing to the fact that de novo methylation happens from about 20 weeks onward (L. Li et al., 2020) .

Most genes lose their methylation throughout the process of global demethylation and regain it at a tissue-specific pattern. Imprinted control regions are unique in maintaining methylation patterns during post-fertilization reprogramming. This is theorized to be the control of epigenetic memory. Few studies

have highlighted the importance of ZFP57 binding sites which are abundant in the ICR regions. ZFP57 is not expressed in oocytes and thus must have a paternal origin. ZFP57 recruits KAP1 heterochromatic complex to maintain methylation (Quenneville et al., 2011). At the blastocyst stage, where global re-methylation follows, ZFP57 and KAP1 keep their parent-specific DNA methylation. After implantation, nearly all ICRs are lost except for canonical 20 or so ICRs. Interestingly, the placenta retains its oocyte-derived imprinted patterns, suggesting that the epigenetic commitment is fixed within the placenta at an earlier stage (Hanna et al., 2016; Sanchez-Delgado et al., 2016). Recessive *ZFP57* mutations lead to imprinting associated phenotypes such as growth defects and, most notably, transient neonatal diabetes (Mackay et al., 2008).

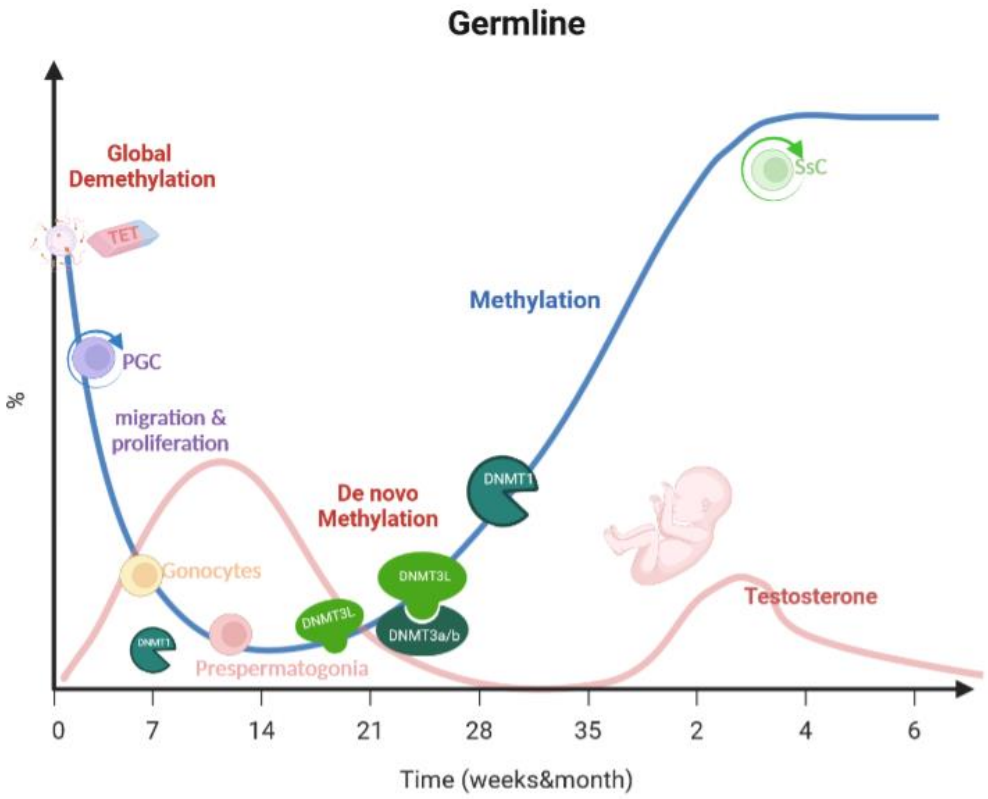

Figure 1. Germline methylation: at the start, as PGC turn into gonocytes and prespermatogonia, we have the process of global demethylation, done actively using TET and passively, by the lack of methylation maintenance. Some of the genome, mostly in imprinted regions, is kept by Dnmt1. Next, de novo methylation occurs by the action of Dnmt3L, Dnmt3a, and Dnmt3b. The methylation process continues after birth and into mini puberty, with the peak in methylation correlating with testosterone.

Created and adapted from BioRender.com (2022). Retrieved from https://app.biorender.com

4 HUMAN SPERM DNA METHYLOME PATHOLOGIES

4.1 Fertility status

4.1.1 Pathophysiology of infertility

Male fertility has been declining drastically within the past 50 years, with a decrease of 50% in sperm count (Carlsen et al., 1992; Levine et al., 2017), which is likely due to environmental exposures. The WHO defines infertility as the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. It affects somewhere between 48 and 186 million individuals worldwide (*Infertility*, n.d.). The European association of urology attempted to divide the causes according to etiological categories. It is important to note that it is difficult to conclude the percentages of all etiological categories as the data are poor, and there are multiple confounders. Likewise, a male can be infertile due to more than one cause simultaneously so that the numbers may be even higher.

Producing a healthy sperm can be looked at as a three-part process. In the first one, mitosis allows for the spermatogonia multiplication, whereas in the second one the number of chromosomes in cells change from diploid to a haploid. Lastly, spermiogenesis allows round spermatids to become spermatozoa (de Kretser et al., 1998). Infertility can be affected by any of these steps. Moreover, problems can occur at the PGC level or at the stage of developing of the testis and supporting cells. Therefore, any alteration during these steps can cause a reduction in quality or quantity, affecting the male fertility status.

We can divide infertility abnormalities into idiopathic infertility, in which there is a normal seminal fluid, and idiopathic dyspermatogenesis, in which semen quality or quantity is impaired. Semen abnormalities include oligospermia (defined as low semen volume of <1.5 ml) , azoospermia (no sperm in ejaculate), oligozoospermia (low sperm concentration of <15 million spermatozoa/ml), asthenozoospermia (the decrease in sperm motility, with <40%) and teratozoospermia (abnormal morphology, with <4% normal) (Cooper et al., 2010).

According to the European Association of Urology, 10-20% of infertile males suffer from idiopathic infertility, which is the infertility with no apparent cause (Jungwirth et al., 2012). One can look at this

category for methylation changes or epigenetic factors as the sole causes. However, other suggested reasons such as primary testicular defects, hypogonadotropic hypogonadism, or sperm transport disorders may also have an epigenetic causation. Many epigenetic changes are set at spermatogenesis stage, making spermatogenesis especially vulnerable and male idiopathic infertility has been, according to recent studies, associated with changes in epigenetic modifications and the methylation process. Dysregulations within the DNA methylation can result in the irregular expression of target genes, leading to infertility. In conclusion, although numerous epigenetic anomalies causing male infertility are still unknwon, most cases of idiopathic infertility, may likely be accounted for by methylation defects (ovo je vec 3 puta u ovom paragrafu da kaze de je idiopatska infertilnost uzrokovana metilacijkim defektima!) (Aston et al., 2012; Jenkins & Carrell, 2011). Recent research has also aimed to identify how methylation abnormalities result in abnormal chromatin condensation at the head of the sperm, making it more vulnerable to damage and leading to impairment of fertilization(Benchaib et al., 2005; McSwiggin & O'Doherty, 2018; Miller et al., 2010).

4.1.2 Epigenetics of azoospermia

Azoospermia, as an unfavourable pathological diagnosis of male infertility, can be due to obstructive or non-obstructive causes. Obstructive azoospermia (OA) accounts for 40% of all azoospermia cases and is, as its name suggests, due to an obstruction in the reproductive tracts. One gene that may be involved in many cases of OA is the *CFTR* gene. *CFTR* mutation can lead to the bilateral absence of the vas deference.

Non-obstructive azoospermia (NOA) is due to unknown failure of spermatogenesis. Unlike OA, in NOA, sperm retrieval is reduced, with only a 50% success rate(Carpi et al., 2009; Nicopoullos et al., 2004). Using histology, we can further classify NOA according to sperm maturation: NOA with Sertoli cells only; NOA without spermatocytes present; NOA without spermatids present; and NOA without spermatozoa. This classification is essential for diagnostics. Today we understand that different genes act at each stage, so screening and treatment should be aimed accordingly.

Trying to identify a correlation with infertility, some studies have looked at global methylation status, rather than at specific genes. One such study using methylation arrays found 2752 dmCpGs difference

between fertile and infertile men (Urdinguio et al., 2015a). Infertile men had hypermethylation of 1447 CpGs and hypomethylation of 1305 CpGs. Hypomethylated CpGs were predominant in CGIs, but hypermethylated CpGs favoured CGI shores found along the edge up to 2kbp away from the CGIs. Promoter regions of infertile individuals showed a lower percentage of both hyper- and hypomethylated CpGs. Hypermethylated CpG regions of infertile men correlated with the abundance of H3K4me3.

On the global genomic level, there is a lower level of methylation and higher gene expression in OA, while in NOA, it is the opposite (X. Wu et al., 2020). Overall, however, there is a consistency of methylation patterns seen in OA which does not exist in NOA. Moreover, NOA group showed higher epigenetic modifications in genes responsible for metabolic and hormonal pathways. For example, when comparing NOA and OA, one study found 5,832 DMR differences between them (Han et al., 2020), majority of which were in intergenic regions or introns.

One study showed hypermethylation of promoter regions on chromosomes 3, 18 and 5 (Han et al., 2020). Of worth noting is the *SOX30* promoter region, which is hypermethylated in most NOA patients (Han et al., 2020). *SOX30* expression correlates not only with sperm count but with testicular volume, suggesting its role in spermatogenesis and testicular development. When mutated SOX30 is restored to wild-type gene in adult mice, they regained their ability to produce healthy sperm. Another worth mentioning genetic mark is Alu Yb8, an Alu short, interspersed elements (SINEs), which shows lower methylation levels in infertile sperm.

DDR1, disciodin domain receptor 1, a non-integrin-type receptors for collagen, oversees cell adhesion, proliferation, and extracellular matrix remodelling. It is a potential marker for methylation defects, both hyper- and hypomethylated, in idiopathic infertility. In one small study, DDR1 was overexpressed (2-fold increase) in 19% and underexpressed (1.8 fold) in 25% of idiopathically infertile men with non-obstructive azoospermia (Ramasamy et al., 2014).

C-MYC is a well-known regulator of cellular proliferation. Its induction is controlled by the expression of zinc-finger CCHC-type containing 13 (ZCCHC13). ZCCHC13 is found in the nucleus of the testicular germ cells. It is hypermethylated and under-expressed in infertile NOA men and is present in both OA and fertile men (Z. Li et al., 2018). Male infertility is also associated with changes in methylation of MLH1 and MSH2, which are crucial for DNA mismatch repair and crossing over during meiosis (Gunes et al., 2018)

. In sperm of oligozoospermic men, *MLH1* was significantly more methylated compared to normozoospermic men and showed a positive correlation with the level of reactive oxygene species.

H19 is the frequent target of studies, with the aim of using it as a diagnostic tool. It is a paternally imprinted gene and its epimutations or hypomethylation can lead to reduced sperm count, oligozoospermia and azoospermia (di Persio et al., 2021; Kobayashi et al., 2007a; Marques et al., 2004, 2008; Minor et al., 2011). Teratozoospermia and asthenozoospermia are not affected by H19 abnormal methylation, suggesting its expression affects only quantity (Montjean et al., 2013).

One study tried to measure the severity of methylation loss at *H19-DMR*. A correlation was found between the degree of methylation loss and decrease in sperm concentration, with the majority of oligozoospermia correlating to 0-50% loss in methylation (B. Li et al., 2013). This was supported by a meta-analysis (Santi et al., 2017a) concluding that infertile men have a significantly lower level of methylation on the *H19* DMR (2.94%, 95% CI: 2.44–3.44%, $p < 0.001$). Furthermore, it showed an association between oligozoospermia and H19/IGF2-CTCF6, IGF-DMR2, MEG2/DLK, and SNPRN locuses.

Infertile men with hypermethylated MTHFR gene promoters have significant methylation abnormalities at the *H19* imprinted gene (Rotondo et al., 2013). The *MTHFR* promoter region is involved in folate metabolism and, most importantly, remethylating reactions. *MTHFR* has been found to be hypermethylated in both sperm and testicular samples of NOA (Ferfouri et al., 2013; W. Wu et al., 2010). The CTCF-binding site 6 area, hypomethylated or entirely unmethylated, is where most of these methylation errors occur. Hypomethylation influences the CTCF-binding site 6, which acts as a regulator for *IGF2*, a maternal alleles' expression (Marques et al., 2010). *IGF2* expression is necessary for preimplantation and embryo development (Fedoriw et al., 2004; Olek & Walter, 1997).

Thus, one study compared normal sperm methylation to asthenoteratozoospermia and oligoasthenozoospermia on 16 CpG sites. 8 sites from *H19-ICR* and 8 from *SNRPN-ICR* were selected to be tested for decreased methylation. Using all 16 CpG sites, 88% could be successfully classified, the success rate of each being 74% and 72%, respectively(Peng et al., 2018). This hints at the importance of combining multiple methylation regions to achieve better accuracy and precision.

A critical study was published recently comparing the whole genome methylome of testicular germ cells of cryptozoospermia and OA men with normal spermatogenesis. They stated the importance of

differentiating between somatic cells within the tested sample as they can interfere with the methylation analysis (di Persio et al., 2020). Trying to find methylation differences within the whole genome, 271 DMRs were found, most of which were hypermethylated. 61 of those genes were in cryptozoospermia men, and majority were hypermethylated (di Persio et al., 2021b).

4.1.3 Imprinting pathologies

As mentioned above, imprinting pathologies can lead to pathologies of spermatogenesis. Here we will concentrate on the abnormal DNA methylation in imprinted genes associated with abnormal spermatogenesis and infertility.

The embryonic mesoderm development is controlled by a gene called Mesodermal-specific transcript (*MEST*). located on 7q32, that codes for an alpha/beta hydrolase family protein. It is a monoallelic paternally expressed gene, sometimes referred to as the paternally expressed gene 1 (PEG1). The *PEG*1 gene methylation correlates with early abortion rate (H. Y. Zheng et al., 2011).

Another gene worth mentioning is found on 6q24 and codes for ZAC protein that induces G1 cell cycle arrest and apoptosis. It is also a paternally expressed gene in most cells and plays a significant role in transient neonatal diabetes mellitus 1(Kamiya et al., 2000). *PEG3* inactivation in neuronal cell culture represses P53-mediated apoptosis, which is imperative for the brain's early development and sexual differentiation. It is found chiefly in mesodermal tissues during embryogenesis (Broad et al., 2009; Hammoud et al., 2010).

MEST DNA aberrant methylation, a maternally imprinted region, has been shown to correlate with low testicular volume, sperm concentration, and total sperm count. DNA methylation level is drastically higher among infertile men (Poplinski et al., 2010; Santi et al., 2017b) and *MEST, DIRAS3*, and *PLAG1* have all shown low concentrations in the semen of infertile men. Therefore, measures of all have the potential to be used as diagnostic tools (Santi et al., 2017a). *SNRPN*, another maternally imprinted gene, also has promising diagnostic potential, with methylation significantly higher in infertile men (Santi et al., 2017b).

Protamines are important in condensing the chromatin within the sperm's head. During sperm condensation, they replace the histones. We have two types of protamine that should be in a ratio of 1:1. This is known as the P1:P2 ratio. This ratio is somewhat investigated, with higher or lower levels correlating with infertility (Aoki et al., 2005, 2006; CARRELL & LIU, 2001; Oliva, 2006). Methylation at the *CREM* imprinted gene seems to correlate with an abnormal P1:P2 ratio and with oligozoospermia (Nanassy & Carrell, 2011). Moreover, density gradient centrifugation seems to select sperms with lower methylation of the *CREM* gene accordingly.

4.1.4 Risks of Assisted Reproductive Technology (ART)

One way to overcome male infertility is using assisted reproduction. When a male factor is identified, an in vitro fertilization or intracytoplasmic sperm injection (ICSI) can be used. Some evidence suggests that ART technology may alter epigenetic reprogramming and, ultimately, embryogenesis (Esteves et al., 2018; Laprise, 2009). Intracytoplasmic sperm injection can overcome morphological problems, but since epigenetic changes can occur due to external factors, it may not eliminate the risk of transferring an abnormal epigenome to the embryo.

Interestingly, some studies reported inheritance of methylation defects in embryos conceived by ART. It is hypothesized that the environment created by ART may play a role contributed by the different hormonal, nutrition, and innate immaturity. Most research conducted up to this date has been on infertile men using IVF/ICSI to conceive, and so it is problematic to avoid confounders and figure out which of these changes are the result due to the technique and which due to the infertility problem itself. Using epidemiological data analyses, we can see that children born via in vitro methods show a higher incidence of rare imprinting disorders. This could, somewhat, be explained by imprinted genes' vulnerability (Gosden et al., 2003). H. Not all investigators, however, agree on the mechanism of the methylation defects in ART. Some specific markers have been shown to differ; for example, placentas from ICSI children, but not form other classical IVF methods, have been shown to have wide H3K4me3 compared to those of regularly conceived children. DNA methylation at the *PLAG1* DMR was shown to be higher in cord blood of IVF and ICSI children as well (Vincent et al., 2016). Studies suggest that IVF

and ICSI components can induce epigenetic changes impacting embryonic development (Bloise et al., 2014; M. Chen & Heilbronn, 2017). Other studies showed no differences in LINE1, Alu Yb8, NBK2, or D4Z4 between ICSI and IVF-conceived individuals (Urdinguio et al., 2015b). Concerns include the risk of obesity, poor cardiometabolic health later in life, and changes in body composition during adolescence, especially among females conceived via ICSI (Ombelet et al., 2005). All those metabolic changes can thus be seen as the long-term consequences of epigenetic reprogramming (Heijmans et al., 2008). *IGF2-H19* methylation defects have been observed in ART placental tissue (Hattori et al., 2019; Pinborg et al., 2016) and *IGF2-H19* and *SNRPN* epigenetic dysregulations were found in human fetuses conceived using ART (Lou et al., 2019). Another concern is reduced sperm quality among ICSI-conceived males (Belva et al., 2016), with the overall median sperm count, median total sperm count, and total motile sperm count being half that of their spontaneously born contemporaries. The existence of sperm abnormalities in adult men and the metabolic changes among females conceived by ICSI calls for additional research. This highlights the importance of evaluating the safety of ART and suggests that sperm cell methylation should be taken into account as well.

5 FUTURE PERSPECTIVES

Using the knowledge of cell's epigenetic modifications to develop new diagnostic methods slowly emerges as a promising research field. I hope future research will focus on developing a collective understanding of normal methylation in terms of qualitative and quantitative measures. When it comes to sperm epigenetic characteristics, we should aim to learn how to identify men that might benefit from lifestyle changes, as well as which, when, and how these changes might be helpful for their health or for establishing their healthy progeny. Also, we need not only to understand the extent of the epigenetic control, our habits, or environmental modifiers but to develop novel diagnostic and treatment tools.

Below are summarized some of the insights in the epigenetic patterns observed in sperm during the life cycle of a human male.

5.1 Lifestyle

With our knowledge today, I believe we should aim to learn how lifestyle changes can benefit our health, not only as a general saying, but how our lifestyle adjustments can benefit specific problems.

We all know smoking is dangerous, but just how bad is it? Cigarette smoking decreases sperm concentration, morphology, and motility (Meri et al., 2013; Xu et al., 2013). A few studies have tried to assess the relationship between smoking and methylation abnormalities. They demonstrate that smoking increases male infertility risk and show how it influences hypermethylation at NRPN-ICR and hypomethylation at *H19*-ICR (Dong et al., 2017). More specifically, *H19* hypermethylation correlates significantly with oligozoospermia or azoospermia, while *SNRPN* hypermethylation correlates with azoospermia or teratozoospermia. Should men avoid smoking three months prior to conception? Is this a chronic effect or an acute one?

Epigenetics is the capability to adapt and change due to an environmental influence. As such, we can affect it, for better or worse. It is only reasonable to see how some of our bad and good habits affect our fertility and, more importantly, our children. There are numerous studies on how the sperm is affected by lifestyle changes, how obesity causes change in metabolism epigenetics in sperm, and how a vegan diet contributes to methylation changes. However, we are yet to know what it actually means. Most genome methylation is re-methylated, and we have just started to build our understanding of which imprinted genes are related to what outcome. Nevertheless, the transgenerational impact of sperm epigenome is a poorly studied topic of great importance

In mice, offspring of males fed a low-protein diet show increased hepatic expression of multiple genes involved in lipid and cholesterol production and lower levels of cholesterol esters (Carone et al., 2010). In humans, spermatozoa from obese men have a distinct epigenetic signature, particularly in genes regulating brain function and development (Donkin et al., 2016). These epigenetic changes could be somewhat corrected after gastric bypass-induced weight loss. The vegan diet also contributes to a change in sperm epigenetics. *FTO* and *MC4R* genes are found to be hypermethylated in sperm of vegans (Franzago et al., 2021). FTO is abundantly expressed in the hypothalamus, visceral fat, and liver, and *MC4R* is responsible for controlling appetite and regulating energy balance. Another exciting study teaches us that endurance training can affect schizophrenia and Parkinson's disease associated genes in

the sperm(Denham et al., 2015; Ingerslev et al., 2018). Many studies that show transgenerational effects in mouse models, likely have similar counterpart in humans. However, more research is needed to investigate methylation as a transgenerational effect in humans. Based on these findings, could we see a future with personalized approach to reproductive health that involves regulating the methylation in germ cells?

This is perhaps the most critical field of research regarding methylation. In epigenetics, we have the potential to be our own medicine. I hope that by further investigating and understanding the effectors of methylation and those of the transgenerational effect, we will be able to live a healthier lifestyle.

5.2 Diagnosis and Beyond

Methylation can be used in the attempt to develop a DMR-based molecular analysis tool to aid in identifying those with idiopathic infertility. Individuals with idiopathic infertility were shown to have different DNA methylation regions (DMRs) hallmarks. This can be implemented in diverse ways. The use of endocrine therapies, equivalent to treating female infertility, is a potential strategy for the clinical management of male infertility (Luján et al., 2019). Nevertheless, the FSH treatment success rate falls short, leading to the recruitment of DMRs. The DMRs of FSH treatment responders was compared to the DMRs of non-responders, allowing for the identification of specific DMRs that could anticipate the success rate of the treatment (Luján et al., 2019). If this approach can be implemented, it may aid in better treatment of infertility. This is the start and proof of concept, stressing the importance of seeking diagnostic and therapeutic epigenetic biomarkers.

We can also use the knowledge about genes affected at different stages of spermatogenesis futher treatmet options. We know that not all azoospermia etiologists are the same. When looking for a diagnostic tool, we can use measurement of genes that correlate with the developmental stage of the problem. Lastly, it is essential to see how we can develop diagnostic tools that are minimally invasive and so perhaps focus on sperm and seminal fluid rather than testicular biopsy.

We can use methylation changes not only to identify how to improve our next generation or our fertility but to help us in identifying psychological problems. Sperm from men who have gone through child

abuse shows variation in DNA methylation when compared to men not being abused. Of those different DMRs, neural function, fat cell regulation, and immune modulators genes were affected. Using machine learning, three probes were identified that could predict the likelihood of abuse with a 71% success rate (Roberts et al., 2018). Could we use these markers to test for therapy success? Or perhaps methylation can speak where we fail? To help diagnose trauma or trauma severity, or even identify if someone went through sexual harassment. Could this be used forensically in the future as credible evidence? These are just some of the questions and mysteries waiting to be discovered.

5.3 Aging

One interesting study showed that there are certain methylation patterns correlated to aging. The age of fatherhood is increasing worldwide, and it carries the risk of lower fertility and the risk of offspring developing neurodevelopmental consequences (Oluwayiose et al., 2021) . Male age is associated with alteration in sperm methylation. Genome-based research found 2844 methylation alterations, of which 1698 on CpG sites. *PLCH2, TPI1P3, DLGAP2*, and *FRFB126* showed age-related differential methylation related to sperm aging and could be used to identify 64% of low fertility aged men. Of all CpG identified, 62% were hypermethylated and hypomethylated. The hypomethylated CpGs were located in the vicinity of the gene, while the hypermethylated CpG were positioned distally. Clusters of age associated CpG were found also on PGC1*α* CpG on chromosome 4 and RBFOX1 and they code for proteins involved in metabolism and neurodevelopmental disease, respectively. What else could those genes tell us? Could we perhaps learn how to avoid the aging process? This is still a distant goal, but for now we can, map the epigenome and try to understand if it holds any significance for the aging process.

Altogether, many genes on the global level are known to us, and new ones are being identified all the time. One challenge still present are the ethical difficulties regarding research on human sperm and embryo and that is the reason that, for example, the data on germ cell DNA methylation in fetal period, or the data on DNA methylation of early human embryos, are scarce. However, having more extensive databases would certainly benefit the research findings by making them more likely to be of clinical significance. That is why I believe it is most important to create reference values and definitions we can all work with to allow for better collaboration of whatever data is available.

6 REFERENCES

Abel, M. H., Wootton, A. N., Wilkins, V., Huhtaniemi, I., Knight, P. G., & Charlton, H. M. (2000). The Effect of a Null Mutation in the Follicle-Stimulating Hormone Receptor Gene on Mouse Reproduction. *Endocrinology*, *141*(5), 1795–1803. https://doi.org/10.1210/ENDO.141.5.7456

An, J., Rao, A., & Ko, M. (2017). TET family dioxygenases and DNA demethylation in stem cells and cancers. *Experimental & Molecular Medicine 2017 49:4*, *49*(4), e323–e323. https://doi.org/10.1038/emm.2017.5

Aoki, V. W., Liu, L., Jones, K. P., Hatasaka, H. H., Gibson, M., Peterson, C. M., & Carrell, D. T. (2006). Sperm protamine 1/protamine 2 ratios are related to in vitro fertilization pregnancy rates and predictive of fertilization ability. *Fertility and Sterility*, *86*(5), 1408–1415. https://doi.org/10.1016/J.FERTNSTERT.2006.04.024

Aoki, V. W., Moskovtsev, S. I., Willis, J., Liu, L., Mullen, J. B. M., & Carrell, D. T. (2005). DNA integrity is compromised in protamine-deficient human sperm. *Journal of Andrology*, *26*(6), 741–748. https://doi.org/10.2164/JANDROL.05063

Arita, K., Ariyoshi, M., Tochio, H., Nakamura, Y., & Shirakawa, M. (2008). Recognition of hemi-methylated DNA by the SRA protein UHRF1 by a base-flipping mechanism. *Nature 2008 455:7214*, *455*(7214), 818–821. https://doi.org/10.1038/nature07249

Aston, K. I., Punj, V., Liu, L., & Carrell, D. T. (2012). Genome-wide sperm deoxyribonucleic acid methylation is altered in some men with abnormal chromatin packaging or poor in vitro fertilization embryogenesis. *Fertility and Sterility*, *97*(2), 285-292.e4. https://doi.org/10.1016/J.FERTNSTERT.2011.11.008

Avvakumov, G. v., Walker, J. R., Xue, S., Li, Y., Duan, S., Bronner, C., Arrowsmith, C. H., & Dhe-Paganon, S. (2008). Structural basis for recognition of hemi-methylated DNA by the SRA domain of human UHRF1. *Nature 2008 455:7214*, *455*(7214), 822–825. https://doi.org/10.1038/nature07273

Ballow, D., Meistrich, M. L., Matzuk, M., & Rajkovic, A. (2006). Sohlh1 is essential for spermatogonial differentiation. *Developmental Biology*, *294*(1), 161–167. https://doi.org/10.1016/J.YDBIO.2006.02.027

Barau, J., Teissandier, A., Zamudio, N., Roy, S., Nalesso, V., Hérault, Y., Guillou, F., & Bourc'his, D. (2016). The DNA methyltransferase DNMT3C protects male germ cells from transposon activity. *Science*, *354*(6314), 909–912. https://doi.org/10.1126/SCIENCE.AAH5143/SUPPL_FILE/BARAU.SM.PDF

Belva, F., Bonduelle, M., Roelants, M., Michielsen, D., van Steirteghem, A., Verheyen, G., & Tournaye, H. (2016). Semen quality of young adult ICSI offspring: the first results. *Human Reproduction*, *31*(12), 2811–2820. https://doi.org/10.1093/HUMREP/DEW245

Benchaib, M., Braun, V., Ressnikof, D., Lornage, J., Durand, P., Niveleau, A., & Guérin, J. F. (2005). Influence of global sperm DNA methylation on IVF results. *Human Reproduction*, *20*(3), 768–773. https://doi.org/10.1093/HUMREP/DEH684

Bhutani, N., Burns, D. M., & Blau, H. M. (2011). DNA Demethylation Dynamics. *Cell*, *146*(6), 866–872. https://doi.org/10.1016/J.CELL.2011.08.042

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes & Development*, *16*(1), 6–21. https://doi.org/10.1101/GAD.947102

Bloise, E., Feuer, S. K., & Rinaudo, P. F. (2014). Comparative intrauterine development and placental function of ART concepti: implications for human reproductive medicine and animal breeding. *Human Reproduction Update*, *20*(6), 822–839. https://doi.org/10.1093/HUMUPD/DMU032

Boas, M., Boisen, K. A., Virtanen, H. E., Kaleva, M., Suomi, A. M., Schmidt, I. M., Damgaard, I. N., Kai, C. M., Chellakooty, M., Skakkebæk, N. E., Toppari, J., & Main, K. M. (2006). Postnatal penile length and growth rate correlate to serum testosterone levels: a longitudinal study of 1962 normal boys. *European Journal of Endocrinology*, *154*(1), 125–129. https://doi.org/10.1530/EJE.1.02066

Boissonnas, C. C., Jouannet, P., & Jammes, H. (2013). Epigenetic disorders and male subfertility. *Fertility and Sterility*, *99*(3), 624–631. https://doi.org/10.1016/J.FERTNSTERT.2013.01.124

Botezatu, A., Socolov, R., Socolov, D., Iancu, I. V., & Anton, G. (2014). Methylation pattern of methylene tetrahydrofolate reductase and small nuclear ribonucleoprotein polypeptide N promoters in oligoasthenospermia: a case-control study. *Reproductive BioMedicine Online*, *28*(2), 225–231. https://doi.org/10.1016/J.RBMO.2013.10.010

Bourc'his, D., & Bestor, T. H. (2004). Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature 2004 431:7004*, *431*(7004), 96–99. https://doi.org/10.1038/nature02886

Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(24), 11303–11307. https://doi.org/10.1073/PNAS.91.24.11303

Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(24), 11298–11302. https://doi.org/10.1073/PNAS.91.24.11298

Broad, K. D., Curley, J. P., & Keverne, E. B. (2009). Increased apoptosis during neonatal brain development underlies the adult behavioral deficits seen in mice lacking a functional paternally expressed gene 3 (Peg3). *Developmental Neurobiology*, *69*(5), 314–325. https://doi.org/10.1002/DNEU.20702

Carlsen, E., Giwercman, A., Keiding, N., & Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *British Medical Journal*, *305*(6854), 609–613. https://doi.org/10.1136/BMJ.305.6854.609

Carone, B. R., Fauquier, L., Habib, N., Shea, J. M., Hart, C. E., Li, R., Bock, C., Li, C., Gu, H., Zamore, P. D., Meissner, A., Weng, Z., Hofmann, H. A., Friedman, N., & Rando, O. J. (2010). Paternally Induced Transgenerational Environmental Reprogramming of Metabolic Gene Expression in Mammals. *Cell*, *143*(7), 1084–1096. https://doi.org/10.1016/J.CELL.2010.12.008

Carpi, A., Sabanegh, E., & Mechanick, J. (2009). Controversies in the management of nonobstructive azoospermia. *Fertility and Sterility*, *91*(4), 963–970. https://doi.org/10.1016/J.FERTNSTERT.2009.01.083

CARRELL, D. T., & LIU, L. (2001). Altered Protamine 2 Expression Is Uncommon In Donors of Known Fertility, but Common Among Men With Poor Fertilizing Capacity, and May Reflect Other Abnormalities of Spermiogenesis. *Journal of Andrology*, *22*(4), 604–610. https://doi.org/10.1002/J.1939-4640.2001.TB02220.X

Cassidy, S. B., & Schwartz, S. (1998). Prader-Willi and Angelman syndromes. Disorders of genomic imprinting. *Medicine*, *77*(2), 140–151. https://doi.org/10.1097/00005792-199803000-00005

Chen, D., Sun, N., Hou, L., Kim, R., Faith, J., Aslanyan, M., Tao, Y., Zheng, Y., Fu, J., Liu, W., Kellis, M., & Clark, A. (2019). Human Primordial Germ Cells Are Specified from Lineage-Primed Progenitors. *Cell Reports*, *29*(13), 4568-4582.e5. https://doi.org/10.1016/J.CELREP.2019.11.083

Chen, M., & Heilbronn, L. K. (2017). The health outcomes of human offspring conceived by assisted reproductive technologies (ART). *Journal of Developmental Origins of Health and Disease*, *8*(4), 388–402. https://doi.org/10.1017/S2040174417000228

Cooper, T. G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H. W. G., Behre, H. M., Haugen, T. B., Kruger, T., Wang, C., Mbizvo, M. T., & Vogelsong, K. M. (2010). World Health Organization reference values for human semen characteristics. *Human Reproduction Update*, *16*(3), 231–245. https://doi.org/10.1093/HUMUPD/DMP048

Cortellino, S., Xu, J., Sannai, M., Moore, R., Caretti, E., Cigliano, A., le Coz, M., Devarajan, K., Wessels, A., Soprano, D., Abramowitz, L. K., Bartolomei, M. S., Rambow, F., Bassi, M. R., Bruno, T., Fanciulli, M., Renner, C., Klein-Szanto, A. J., Matsumoto, Y., … Bellacosa, A. (2011). Thymine DNA Glycosylase Is Essential for Active DNA Demethylation by Linked Deamination-Base Excision Repair. *Cell*, *146*(1), 67–79. https://doi.org/10.1016/J.CELL.2011.06.020

Culty, M. (2009). Gonocytes, the forgotten cells of the germ cell lineage. *Birth Defects Research Part C: Embryo Today: Reviews*, *87*(1), 1–26. https://doi.org/10.1002/BDRC.20142

Davis, T. L., Yang, G. J., McCarrey, J. R., & Bartolomei, M. S. (2000). The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. *Human Molecular Genetics*, *9*(19), 2885–2894. https://doi.org/10.1093/HMG/9.19.2885

de Felici, M. (2013). Origin, Migration, and Proliferation of Human Primordial Germ Cells. *Oogenesis*, 19–37. https://doi.org/10.1007/978-0-85729-826-3_2

de Kretser, D. M., Loveland, K. L., Meinhardt, A., Simorangkir, D., & Wreford, N. (1998). Spermatogenesis. *Human Reproduction (Oxford, England)*, *13 Suppl 1*(SUPPL. 1), 1–8. https://doi.org/10.1093/HUMREP/13.SUPPL_1.1

DeFalco, T., Takahashi, S., & Capel, B. (2011). Two distinct origins for Leydig cell progenitors in the fetal testis. *Developmental Biology*, *352*(1), 14–26. https://doi.org/10.1016/J.YDBIO.2011.01.011

Denham, J., O'Brien, B. J., Harvey, J. T., & Charchar, F. J. (2015). Genome-wide sperm DNA methylation changes after 3 months of exercise training in humans. *Http://Dx.Doi.Org/10.2217/Epi.15.29*, *7*(5), 717–731. https://doi.org/10.2217/EPI.15.29

di Persio, S., Leitão, E., Wöste, M., Tekath, T., Cremers, J. F., Dugas, M., Li, X., zu Hörste, G. M., Kliesch, S., Laurentino, S., Neuhaus, N., & Horsthemke, B. (2021a). Whole-genome methylation analysis of

testicular germ cells from cryptozoospermic men points to recurrent and functionally relevant DNA methylation changes. *Clinical Epigenetics*, *13*(1), 1–19. https://doi.org/10.1186/S13148-021-01144-Z/TABLES/2

di Persio, S., Leitão, E., Wöste, M., Tekath, T., Cremers, J. F., Dugas, M., Li, X., zu Hörste, G. M., Kliesch, S., Laurentino, S., Neuhaus, N., & Horsthemke, B. (2021b). Whole-genome methylation analysis of testicular germ cells from cryptozoospermic men points to recurrent and functionally relevant DNA methylation changes. *Clinical Epigenetics*, *13*(1). https://doi.org/10.1186/S13148-021-01144-Z

di Persio, S., Leitão, E., Wöste, M., Tekath, T., Cremers, J.-F., Dugas, M., Li, X., Meyer zu Hörste, G., Kliesch, S., Laurentino, S., Neuhaus, N., & Horsthemke, B. (2020). Hypermethylation and decreased expression of TMEM240 are potential early-onset biomarkers for colorectal cancer detection, poor prognosis, and early recurrence prediction. *Clinical Epigenetics*, *2021*(1), 160. https://doi.org/10.1186/s13148-021-01144-z

Dong, H., Wang, Y., Zou, Z., Chen, L., Shen, C., Xu, S., Zhang, J., Zhao, F., Ge, S., Gao, Q., Hu, H., Song, M., & Wang, W. (2017). Abnormal Methylation of Imprinted Genes and Cigarette Smoking: Assessment of Their Association with the Risk of Male Infertility. *Reproductive Sciences*, *24*(1), 114–123. https://doi.org/10.1177/1933719116650755

Donkin, I., Versteyhe, S., Ingerslev, L. R., Qian, K., Mechta, M., Nordkap, L., Mortensen, B., Appel, E. V. R., Jørgensen, N., Kristiansen, V. B., Hansen, T., Workman, C. T., Zierath, J. R., & Barrès, R. (2016). Obesity and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans. *Cell Metabolism*, *23*(2), 369–378. https://doi.org/10.1016/J.CMET.2015.11.004

Duffié, R., Ajjan, S., Greenberg, M. v., Zamudio, N., del Arenal Escamilla, M., Iranzo, J., Okamoto, I., Barbaux, S., Fauque, P., & Bourc'his, D. (2014). The Gpr1/Zdbf2 locus provides new paradigms for transient and dynamic genomic imprinting in mammals. *Genes & Development*, *28*(5), 463–478. https://doi.org/10.1101/GAD.232058.113

Esteves, S. C., Roque, M., Bedoschi, G., Haahr, T., & Humaidan, P. (2018). Intracytoplasmic sperm injection for male infertility and consequences for offspring. *Nature Reviews Urology 2018 15:9*, *15*(9), 535–562. https://doi.org/10.1038/s41585-018-0051-8

Fedoriw, A. M., Stein, P., Svoboda, P., Schultz, R. M., & Bartolomei, M. S. (2004). Transgenic RNAi Reveals Essential Function for CTCF in H19 Gene Imprinting. *Science*, *303*(5655), 238–240. https://doi.org/10.1126/SCIENCE.1090934/SUPPL_FILE/FEDORIW.SOM.REV1.PDF

Fend-Guella, D. L., von Kopylow, K., Spiess, A. N., Schulze, W., Salzbrunn, A., Diederich, S., Hajj, N. el, Haaf, T., Zechner, U., & Linke, M. (2019). The DNA methylation profile of human spermatogonia at single-cell- and single-allele-resolution refutes its role in spermatogonial stem cell function and germ cell differentiation. *Molecular Human Reproduction*, *25*(6), 283–294. https://doi.org/10.1093/MOLEHR/GAZ017

Ferfouri, F., Boitrelle, F., Ghout, I., Albert, M., Molina Gomes, D., Wainer, R., Bailly, M., Selva, J., & Vialard, F. (2013). A genome-wide DNA methylation study in azoospermia. *Andrology*, *1*(6), 815–821. https://doi.org/10.1111/J.2047-2927.2013.00117.X

Ficz, G., & Hoppeler, H. H. (2015). New insights into mechanisms that regulate DNA methylation patterning. *Journal of Experimental Biology*, *218*(1), 14–20. https://doi.org/10.1242/JEB.107961

Filion, G. J. P., Zhenilo, S., Salozhin, S., Yamada, D., Prokhortchouk, E., & Defossez, P.-A. (2006). A Family of Human Zinc Finger Proteins That Bind Methylated DNA and Repress Transcription. *Molecular and Cellular Biology*, *26*(1), 169–181. https://doi.org/10.1128/MCB.26.1.169-181.2006/ASSET/08A8FEA1-15CA-4D2A-88A4-379293B462D4/ASSETS/GRAPHIC/ZMB0010656190008.JPEG

Franzago, M., Sabovic, I., Franchi, S., de Santo, M., di Nisio, A., Luddi, A., Piomboni, P., Vitacolonna, E., Stuppia, L., & Foresta, C. (2021). Sperm DNA Methylation at Metabolism-Related Genes in Vegan Subjects. *Frontiers in Endocrinology*, *12*. https://doi.org/10.3389/FENDO.2021.633943

Galetzka, D., Weis, E., Tralau, T., Seidmann, L., & Haaf, T. (2007). Sex-specific windows for high mRNA expression of DNA methyltransferases 1 and 3A and methyl-CpG-binding domain proteins 2 and 4 in human fetal gonads. *Molecular Reproduction and Development*, *74*(2), 233–241. https://doi.org/10.1002/MRD.20615

Gaskell, T. L., Esnal, A., Robinson, L. L. L., Anderson, R. A., & Saunders, P. T. K. (2004). Immunohistochemical Profiling of Germ Cells Within the Human Fetal Testis: Identification of Three Subpopulations. *Biology of Reproduction*, *71*(6), 2012–2021. https://doi.org/10.1095/BIOLREPROD.104.028381

Gicquel, C., Rossignol, S., Cabrol, S., Houang, M., Steunou, V., Barbu, V., Danton, F., Thibaud, N., le Merrer, M., Burglen, L., Bertrand, A. M., Netchine, I., & le Bouc, Y. (2005). Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nature Genetics*, *37*(9), 1003–1007. https://doi.org/10.1038/NG1629

Goll, M. G., & Bestor, T. H. (2005). EUKARYOTIC CYTOSINE METHYLTRANSFERASES. *Http://Dx.Doi.Org/10.1146/Annurev.Biochem.74.010904.153721*, *74*, 481–514. https://doi.org/10.1146/ANNUREV.BIOCHEM.74.010904.153721

Gosden, R., Trasler, J., Lucifero, D., & Faddy, M. (2003). Rare congenital disorders, imprinted genes, and assisted reproductive technology. *The Lancet*, *361*(9373), 1975–1977. https://doi.org/10.1016/S0140-6736(03)13592-1

Greenberg, M. V. C., Glaser, J., Borsos, M., Marjou, F. el, Walter, M., Teissandier, A., & Bourc'his, D. (2016). Transient transcription in the early embryo sets an epigenetic state that programs postnatal growth. *Nature Genetics 2016 49:1*, *49*(1), 110–118. https://doi.org/10.1038/ng.3718

Guerrero-Bosagna, C., Covert, T. R., Haque, M. M., Settles, M., Nilsson, E. E., Anway, M. D., & Skinner, M. K. (2012). Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reproductive Toxicology*, *34*(4), 694–707. https://doi.org/10.1016/J.REPROTOX.2012.09.005

Gunes, S., Agarwal, A., Henkel, R., Mahmutoglu, A. M., Sharma, R., Esteves, S. C., Aljowair, A., Emirzeoglu, D., Alkhani, A., Pelegrini, L., Joumah, A., & Sabanegh, E. (2018). Association between promoter methylation of MLH1 and MSH2 and reactive oxygen species in oligozoospermic men—A pilot study. *Andrologia*, *50*(3), e12903. https://doi.org/10.1111/AND.12903

Guo, F., Yan, L., Guo, H., Li, L., Hu, B., Zhao, Y., Yong, J., Hu, Y., Wang, X., Wei, Y., Wang, W., Li, R., Yan, J., Zhi, X., Zhang, Y., Jin, H., Zhang, W., Hou, Y., Zhu, P., … Qiao, J. (2015). The Transcriptome and DNA Methylome Landscapes of Human Primordial Germ Cells. *Cell*, *161*(6), 1437–1452. https://doi.org/10.1016/J.CELL.2015.05.015

Guo, J., Grow, E. J., Yi, C., Mlcochova, H., Maher, G. J., Lindskog, C., Murphy, P. J., Wike, C. L., Carrell, D. T., Goriely, A., Hotaling, J. M., & Cairns, B. R. (2017). Chromatin and Single-Cell RNA-Seq Profiling Reveal Dynamic Signaling and Metabolic Transitions during Human Spermatogonial Stem Cell Development. *Cell Stem Cell*, *21*(4), 533-546.e6. https://doi.org/10.1016/J.STEM.2017.09.003

Guo, J., Nie, X., Giebler, M., Mlcochova, H., Wang, Y., Grow, E. J., Kim, R., Tharmalingam, M., Matilionyte, G., Lindskog, C., Carrell, D. T., Mitchell, R. T., Goriely, A., Hotaling, J. M., & Cairns, B. R. (2020). The Dynamic Transcriptional Cell Atlas of Testis Development during Human Puberty. *Cell Stem Cell*, *26*(2), 262-276.e4. https://doi.org/10.1016/J.STEM.2019.12.005

Guo, J., Sosa, E., Chitiashvili, T., Nie, X., Rojas, E. J., Oliver, E., Plath, K., Hotaling, J. M., Stukenborg, J. B., Clark, A. T., & Cairns, B. R. (2021). Single-cell analysis of the developing human testis reveals somatic niche cell specification and fetal germline stem cell establishment. *Cell Stem Cell*, *28*(4), 764-778.e4. https://doi.org/10.1016/J.STEM.2020.12.004

Hadziselimovic, F., & Hoecht, B. (2008). Testicular histology related to fertility outcome and postpubertal hormone status in cryptorchidism. *Klinische Padiatrie*, *220*(5), 302–307. https://doi.org/10.1055/S-2007-993194/ID/21

Hadziselimovic, F., & Huff, D. (2002). Gonadal differentiation--normal and abnormal testicular development. *Advances in Experimental Medicine and Biology*, *511*, 15–23. https://doi.org/10.1007/978-1-4615-0621-8_2

Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J., & Surani, M. A. (2002). Epigenetic reprogramming in mouse primordial germ cells. *Mechanisms of Development*, *117*(1–2), 15–23. https://doi.org/10.1016/S0925-4773(02)00181-8

Hammoud, S. S., Purwar, J., Pflueger, C., Cairns, B. R., & Carrell, D. T. (2010). Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertility and Sterility*, *94*(5), 1728–1733. https://doi.org/10.1016/J.FERTNSTERT.2009.09.010

Han, F., Jiang, X., Li, Z. ming, Zhuang, X., Zhang, X., Ouyang, W. ming, Liu, W. bin, Mao, C. yi, Chen, Q., Huang, C. shu, Gao, F., Cui, Z. hong, Ao, L., Li, Y. feng, Cao, J., & Liu, J. yi. (2020). Epigenetic Inactivation of SOX30 Is Associated with Male Infertility and Offers a Therapy Target for Non-obstructive Azoospermia. *Molecular Therapy - Nucleic Acids*, *19*, 72–83. https://doi.org/10.1016/J.OMTN.2019.10.038

Hanley, N. A., Hagan, D. M., Clement-Jones, M., Ball, S. G., Strachan, T., Salas-Cortés, L., McElreavey, K., Lindsay, S., Robson, S., Bullen, P., Ostrer, H., & Wilson, D. I. (2000). SRY, SOX9, and DAX1 expression patterns during human sex determination and gonadal development. *Mechanisms of Development*, *91*(1–2), 403–407. https://doi.org/10.1016/S0925-4773(99)00307-X

Hanna, C. W., Peñaherrera, M. S., Saadeh, H., Andrews, S., McFadden, D. E., Kelsey, G., & Robinson, W. P. (2016). Pervasive polymorphic imprinted methylation in the human placenta. *Genome Research*, *26*(6), 756–767. https://doi.org/10.1101/GR.196139.115

Hara, K., Kanai-Azuma, M., Uemura, M., Shitara, H., Taya, C., Yonekawa, H., Kawakami, H., Tsunekawa, N., Kurohmaru, M., & Kanai, Y. (2009). Evidence for crucial role of hindgut expansion in directing proper migration of primordial germ cells in mouse early embryogenesis. *Developmental Biology*, *330*(2), 427–439. https://doi.org/10.1016/J.YDBIO.2009.04.012

Hattori, H., Hiura, H., Kitamura, A., Miyauchi, N., Kobayashi, N., Takahashi, S., Okae, H., Kyono, K., Kagami, M., Ogata, T., & Arima, T. (2019). Association of four imprinting disorders and ART. *Clinical Epigenetics*, *11*(1), 1–12. https://doi.org/10.1186/S13148-019-0623-3/FIGURES/2

Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., Slagboom, P. E., & Lumey, L. H. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(44), 17046–17049. https://doi.org/10.1073/PNAS.0806560105

Hermann, A., Goyal, R., & Jeltsch, A. (2004). The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites. *Journal of Biological Chemistry*, *279*(46), 48350–48359. https://doi.org/10.1074/JBC.M403427200/ATTACHMENT/E221B605-B491-4389-B1A2-F653AF39AC5D/MMC1.PDF

Hermann, B. P., Cheng, K., Singh, A., Roa-De La Cruz, L., Mutoji, K. N., Chen, I. C., Gildersleeve, H., Lehle, J. D., Mayo, M., Westernströer, B., Law, N. C., Oatley, M. J., Velte, E. K., Niedenberger, B. A., Fritze, D., Silber, S., Geyer, C. B., Oatley, J. M., & McCarrey, J. R. (2018). The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. *Cell Reports*, *25*(6), 1650-1667.e8. https://doi.org/10.1016/J.CELREP.2018.10.026

Hermann, B. P., Mutoji, K. N., Velte, E. K., Ko, D., Oatley, J. M., Geyer, C. B., & McCarrey, J. R. (2015). Transcriptional and translational heterogeneity among neonatal mouse spermatogonia. *Biology of Reproduction*, *92*(2). https://doi.org/10.1095/BIOLREPROD.114.125757

Houshdaran, S., Cortessis, V. K., Siegmund, K., Yang, A., Laird, P. W., & Sokol, R. Z. (2007). Widespread Epigenetic Abnormalities Suggest a Broad DNA Methylation Erasure Defect in Abnormal Human Sperm. *PLOS ONE*, *2*(12), e1289. https://doi.org/10.1371/JOURNAL.PONE.0001289

Huff, D. S., Hadziselimović, F., Snyder, H. M., Blyth, B., & Duckett, J. W. (1991). Early Postnatal Testicular Maldevelopment in Cryptorchidism. *The Journal of Urology*, *146*(2), 624–626. https://doi.org/10.1016/S0022-5347(17)37874-6

Hutson, J. M., Li, R., Southwell, B. R., Petersen, B. L., Thorup, J., & Cortes, D. (2013). Germ cell development in the postnatal testis: The key to prevent malignancy in cryptorchidism? *Frontiers in Endocrinology*, *3*(JAN), 176. https://doi.org/10.3389/FENDO.2012.00176/BIBTEX

Huypens, P., Sass, S., Wu, M., Dyckhoff, D., Tschöp, M., Theis, F., Marschall, S., de Angelis, M. H., & Beckers, J. (2016). Epigenetic germline inheritance of diet-induced obesity and insulin resistance. *Nature Genetics 2016 48:5*, *48*(5), 497–499. https://doi.org/10.1038/ng.3527

Infertility. (n.d.). Retrieved June 10, 2022, from https://www.who.int/news-room/fact-sheets/detail/infertility

Ingerslev, L. R., Donkin, I., Fabre, O., Versteyhe, S., Mechta, M., Pattamaprapanont, P., Mortensen, B., Krarup, N. T., & Barrès, R. (2018). Endurance training remodels sperm-borne small RNA expression and methylation at neurological gene hotspots. *Clinical Epigenetics*, *10*(1), 1–11. https://doi.org/10.1186/S13148-018-0446-7/FIGURES/4

Ito, S., Dalessio, A. C., Taranova, O. v., Hong, K., Sowers, L. C., & Zhang, Y. (2010). Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature 2010 466:7310*, *466*(7310), 1129–1133. https://doi.org/10.1038/nature09303

Ito, S., Shen, L., Dai, Q., Wu, S. C., Collins, L. B., Swenberg, J. A., He, C., & Zhang, Y. (2011). Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*, *333*(6047), 1300–1303. https://doi.org/10.1126/SCIENCE.1210597/SUPPL_FILE/ITO.SOM.PDF

Iyer, L. M., Tahiliani, M., Rao, A., & Aravind, L. (2009). Prediction of novel families of enzymes involved in oxidative and other complex modifications of bases in nucleic acids. *Https://Doi.Org/10.4161/Cc.8.11.8580*, *8*(11), 1698–1710. https://doi.org/10.4161/CC.8.11.8580

Jenkins, T. G., & Carrell, D. T. (2011). The paternal epigenome and embryogenesis: poising mechanisms for development. *Asian Journal of Andrology*, *13*(1), 76. https://doi.org/10.1038/AJA.2010.61

Jia, D., Jurkowska, R. Z., Zhang, X., Jeltsch, A., & Cheng, X. (2007). Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. *Nature 2007 449:7159*, *449*(7159), 248–251. https://doi.org/10.1038/nature06146

Jing, J., Zhao, Y., Wang, C., Zhao, Q., Liang, Q., Wang, S., & Ma, J. (2015). Effect of small nuclear ribonucleoprotein-associated polypeptide N on the proliferation of medulloblastoma cells. *Molecular Medicine Reports*, *11*(5), 3337–3343. https://doi.org/10.3892/MMR.2015.3148/HTML

Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., & Krausz, C. (2012). European Association of Urology guidelines on Male Infertility: the 2012 update. *European Urology*, *62*(2), 324–332. https://doi.org/10.1016/J.EURURO.2012.04.048

Kamiya, M., Judson, H., Okazaki, Y., Kusakabe, M., Muramatsu, M., Takada, S., Takagi, N., Arima, T., Wake, N., Kamimura, K., Satomura, K., Hermann, R., Bonthron, D. T., & Hayashizaki, Y. (2000). The cell cycle control gene ZAC/PLAGL1 is imprinted—a strong candidate gene for transient neonatal diabetes. *Human Molecular Genetics*, *9*(3), 453–460. https://doi.org/10.1093/HMG/9.3.453

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, H., Li, E., & Sasaki, H. (2004). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature 2004 429:6994*, *429*(6994), 900–903. https://doi.org/10.1038/nature02633

Keverne, E. B., Fundele, R., Narasimha, M., Barton, S. C., & Surani, M. A. (1996). Genomic imprinting and the differential roles of parental genomes in brain development. *Developmental Brain Research*, *92*(1), 91–100. https://doi.org/10.1016/0165-3806(95)00209-X

Kim, S. H., Park, J., Choi, M. C., Kim, H. P., Park, J. H., Jung, Y., Lee, J. H., Oh, D. Y., Im, S. A., Bang, Y. J., & Kim, T. Y. (2007). Zinc-fingers and homeoboxes 1 (ZHX1) binds DNA methyltransferase (DNMT) 3B to

enhance DNMT3B-mediated transcriptional repression. *Biochemical and Biophysical Research Communications*, *355*(2), 318–323. https://doi.org/10.1016/J.BBRC.2007.01.187

Kimura, H., & Shiota, K. (2003). Methyl-CpG-binding Protein, MeCP2, Is a Target Molecule for Maintenance DNA Methyltransferase, Dnmt1 *. *Journal of Biological Chemistry*, *278*(7), 4806–4812. https://doi.org/10.1074/JBC.M209923200

Ko, M., An, J., Bandukwala, H. S., Chavez, L., Äijö, T., Pastor, W. A., Segal, M. F., Li, H., Koh, K. P., Lähdesmäki, H., Hogan, P. G., Aravind, L., & Rao, A. (2013). Modulation of TET2 expression and 5-methylcytosine oxidation by the CXXC domain protein IDAX. *Nature 2013 497:7447*, *497*(7447), 122–126. https://doi.org/10.1038/nature12052

Kuiri-Hänninen, T., Sankilampi, U., & Dunkel, L. (2014). Activation of the Hypothalamic-Pituitary-Gonadal Axis in Infancy: Minipuberty. *Hormone Research in Paediatrics*, *82*(2), 73–80. https://doi.org/10.1159/000362414

Kumar, T. R., Wang, Y., Lu, N., & Matzuk, M. M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nature Genetics 1997 15:2*, *15*(2), 201–204. https://doi.org/10.1038/ng0297-201

la Salle, S., Oakes, C. C., Neaga, O. R., Bourc'his, D., Bestor, T. H., & Trasler, J. M. (2007). Loss of spermatogonia and wide-spread DNA methylation defects in newborn male mice deficient in DNMT3L. *BMC Developmental Biology*, *7*(1), 1–18. https://doi.org/10.1186/1471-213X-7-104/FIGURES/7

Lamminmäki, A., Hines, M., Kuiri-Hänninen, T., Kilpeläinen, L., Dunkel, L., & Sankilampi, U. (2012). Testosterone measured in infancy predicts subsequent sex-typed behavior in boys and in girls. *Hormones and Behavior*, *61*(4), 611–616. https://doi.org/10.1016/J.YHBEH.2012.02.013

Laprise, S. L. (2009). Implications of epigenetics and genomic imprinting in assisted reproductive technologies. *Molecular Reproduction and Development*, *76*(11), 1006–1018. https://doi.org/10.1002/MRD.21058

Lee, H. J., Hore, T. A., & Reik, W. (2014). Reprogramming the Methylome: Erasing Memory and Creating Diversity. *Cell Stem Cell*, *14*(6), 710–719. https://doi.org/10.1016/J.STEM.2014.05.008

Leonhardt, H., Page, A. W., Weier, H. U., & Bestor, T. H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell*, *71*(5), 865–873. https://doi.org/10.1016/0092-8674(92)90561-P

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., & Swan, S. H. (2017). Temporal trends in sperm count: a systematic review and meta-regression analysis. *Human Reproduction Update*, *23*(6), 646–659. https://doi.org/10.1093/HUMUPD/DMX022

Li, B., Li, J. bo, Xiao, X. feng, Ma, Y. fei, Wang, J., Liang, X. xin, Zhao, H. xi, Jiang, F., Yao, Y. qing, & Wang, X. hong. (2013). Altered DNA Methylation Patterns of the H19 Differentially Methylated Region and the DAZL Gene Promoter Are Associated with Defective Human Sperm. *PLoS ONE*, *8*(8). https://doi.org/10.1371/JOURNAL.PONE.0071215

Li, L., Dong, J., Yan, L., Yong, J., Liu, X., Hu, Y., Fan, X., Wu, X., Guo, H., Wang, X., Zhu, X., Li, R., Yan, J., Wei, Y., Zhao, Y., Wang, W., Ren, Y., Yuan, P., Yan, Z., … Qiao, J. (2017). Single-Cell RNA-Seq Analysis Maps Development of Human Germline Cells and Gonadal Niche Interactions. *Cell Stem Cell*, *20*(6), 858-873.e4. https://doi.org/10.1016/J.STEM.2017.03.007

Li, L., Li, L., Li, Q., Liu, X., Ma, X., Yong, J., Gao, S., Wu, X., Wei, Y., Wang, X., Wang, W., Li, R., Yan, J., Zhu, X., Wen, L., Qiao, J., Yan, L., & Tang, F. (2020). Dissecting the epigenomic dynamics of human fetal germ cell development at single-cell resolution. *Cell Research 2020 31:4*, *31*(4), 463–477. https://doi.org/10.1038/s41422-020-00401-9

Li, R., Vannitamby, A., Meijer, J., Southwell, B., & Hutson, J. (2015). Postnatal Germ Cell Development during Mini-Puberty in the Mouse Does Not Require Androgen Receptor: Implications for Managing Cryptorchidism. *The Journal of Urology*, *193*(4), 1361–1367. https://doi.org/10.1016/J.JURO.2014.10.024

Li, Z., Chen, S., Yang, Y., Zhuang, X., & Tzeng, C. M. (2018). Novel biomarker ZCCHC13 revealed by integrating DNA methylation and mRNA expression data in non-obstructive azoospermia. *Cell Death Discovery 2018 4:1*, *4*(1), 1–15. https://doi.org/10.1038/s41420-018-0033-x

Lou, H., Le, F., Hu, M., Yang, X., Li, L., Wang, L., Wang, N., Gao, H., & Jin, F. (2019). Aberrant DNA Methylation of IGF2-H19 Locus in Human Fetus and in Spermatozoa From Assisted Reproductive Technologies. *Reproductive Sciences*, *26*(7), 997–1004. https://doi.org/10.1177/1933719118802052

Luján, S., Caroppo, E., Niederberger, C., Arce, J. C., Sadler-Riggleman, I., Beck, D., Nilsson, E., & Skinner, M. K. (2019). Sperm DNA Methylation Epimutation Biomarkers for Male Infertility and FSH Therapeutic Responsiveness. *Scientific Reports 2019 9:1*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-52903-1

Mackay, D. J. G., Callaway, J. L. A., Marks, S. M., White, H. E., Acerini, C. L., Boonen, S. E., Dayanikli, P., Firth, H. v., Goodship, J. A., Haemers, A. P., Hahnemann, J. M. D., Kordonouri, O., Masoud, A. F., Oestergaard, E., Storr, J., Ellard, S., Hattersley, A. T., Robinson, D. O., & Temple, I. K. (2008). Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in ZFP57. *Nature Genetics 2008 40:8*, *40*(8), 949–951. https://doi.org/10.1038/ng.187

Mahmood, N., & Rabbani, S. A. (2019). DNA methylation readers and cancer: Mechanistic and therapeutic applications. *Frontiers in Oncology*, *9*(JUN). https://doi.org/10.3389/FONC.2019.00489

Maisnam Indira, & Asish Kurmar Basu. (2021, April 20). *Minipuberty and its Implications*. Endocrine Society of Bengal. https://www.youtube.com/watch?v=gitlG3JdYbo&t=2421s

Marques, C. J., Francisco, T., Sousa, S., Carvalho, F., Barros, A., & Sousa, M. (2010). Methylation defects of imprinted genes in human testicular spermatozoa. *Fertility and Sterility*, *94*(2), 585–594. https://doi.org/10.1016/J.FERTNSTERT.2009.02.051

McCarrey, J. R. (2013). Toward a more precise and informative nomenclature describing fetal and neonatal male germ cells in rodents. *Biology of Reproduction*, *89*(2), 47–48. https://doi.org/10.1095/BIOLREPROD.113.110502/2514289

McCarrey, J. R. (2017). Transition of prenatal prospermatogonia to postnatal spermatogonia. *The Biology of Mammalian Spermatogonia*, 23–38. https://doi.org/10.1007/978-1-4939-7505-1_2/COVER/

McGrath, J., & Solter, D. (1983a). Nuclear Transplantation in the Mouse Embryo by Microsurgery and Cell Fusion. *Science*, *220*(4603), 1300–1302. https://doi.org/10.1126/SCIENCE.6857250

McGrath, J., & Solter, D. (1983b). Nuclear transplantation in mouse embryos. *Journal of Experimental Zoology*, *228*(2), 355–362. https://doi.org/10.1002/JEZ.1402280218

McGrath, J., & Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, *37*(1), 179–183. https://doi.org/10.1016/0092-8674(84)90313-1

McSwiggin, H. M., & O'Doherty, A. M. (2018). Epigenetic reprogramming during spermatogenesis and male factor infertility. *Reproduction*, *156*(2), R9–R21. https://doi.org/10.1530/REP-18-0009

Meehan, T., Schlatt, S., O'Bryan, M. K., de Kretser, D. M., & Loveland, K. L. (2000). Regulation of Germ Cell and Sertoli Cell Development by Activin, Follistatin, and FSH. *Developmental Biology*, *220*(2), 225–237. https://doi.org/10.1006/DBIO.2000.9625

Meri, Z. B., Irshid, I. B., Migdadi, M., Irshid, A. B., & Mhanna, S. A. (2013). Does Cigarette Smoking Affect Seminal Fluid Parameters? A Comparative Study. *Oman Medical Journal*, *28*(1), 12. https://doi.org/10.5001/OMJ.2013.03

Miller, D., Brinkworth, M., & Iles, D. (2010). Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction*, *139*(2), 287–301. https://doi.org/10.1530/REP-09-0281

Minor, A., Chow, V., & Ma, S. (2011). Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with obstructive azoospermia and undergoing vasectomy reversal. *Reproduction*, *141*(6), 749–757. https://doi.org/10.1530/REP-11-0008

Montjean, D., Ravel, C., Benkhalifa, M., Cohen-Bacrie, P., Berthaut, I., Bashamboo, A., & McElreavey, K. (2013). Methylation changes in mature sperm deoxyribonucleic acid from oligozoospermic men: assessment of genetic variants and assisted reproductive technology outcome. *Fertility and Sterility*, *100*(5). https://doi.org/10.1016/J.FERTNSTERT.2013.06.047

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *38*(1), 23–38. https://doi.org/10.1038/NPP.2012.112

Mortusewicz, O., Schermelleh, L., Walter, J., Cardoso, M. C., & Leonhardt, H. (2005). Recruitment of DNA methyltransferase I to DNA repair sites. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(25), 8905–8909. https://doi.org/10.1073/PNAS.0501034102

Nanassy, L., & Carrell, D. T. (2011). Abnormal methylation of the promoter of CREM is broadly associated with male factor infertility and poor sperm quality but is improved in sperm selected by density gradient centrifugation. *Fertility and Sterility*, *95*(7), 2310–2314. https://doi.org/10.1016/J.FERTNSTERT.2011.03.096

Nicopoullos, J. D. M., Ramsay, J. W. A., Almeida, P. A., & Gilling-Smith, C. (2004). REVIEW: Assisted reproduction in the azoospermic couple. *BJOG: An International Journal of Obstetrics & Gynaecology*, *111*(11), 1190–1203. https://doi.org/10.1111/J.1471-0528.2004.00202.X

Ohinata, Y., Ohta, H., Shigeta, M., Yamanaka, K., Wakayama, T., & Saitou, M. (2009). A Signaling Principle for the Specification of the Germ Cell Lineage in Mice. *Cell*, *137*(3), 571–584. https://doi.org/10.1016/J.CELL.2009.03.014

Okano, M., Bell, D. W., Haber, D. A., & Li, E. (1999). DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for De Novo Methylation and Mammalian Development. *Cell*, *99*(3), 247–257. https://doi.org/10.1016/S0092-8674(00)81656-6

Olek, A., & Walter, J. (1997). The pre-implantation ontogeny of the H19 methylation imprint. *Nature Genetics 1997 17:3*, *17*(3), 275–276. https://doi.org/10.1038/ng1197-275

Oliva, R. (2006). Protamines and male infertility. *Human Reproduction Update*, *12*(4), 417–435. https://doi.org/10.1093/HUMUPD/DML009

Oluwayiose, O. A., Wu, H., Saddiki, H., Whitcomb, B. W., Balzer, L. B., Brandon, N., Suvorov, A., Tayyab, R., Sites, C. K., Hill, L., Marcho, C., & Pilsner, J. R. (2021). Sperm DNA methylation mediates the association of male age on reproductive outcomes among couples undergoing infertility treatment. *Scientific Reports 2021 11:1*, *11*(1), 1–14. https://doi.org/10.1038/s41598-020-80857-2

Ombelet, W., Peeraer, K., de Sutter, P., Gerris, J., Bosmans, E., Martens, G., Ruyssinck, G., Defoort, P., Molenberghs, G., & Gyselaers, W. (2005). Perinatal outcome of ICSI pregnancies compared with a matched group of natural conception pregnancies in Flanders (Belgium): a cohort study. *Reproductive BioMedicine Online*, *11*(2), 244–253. https://doi.org/10.1016/S1472-6483(10)60965-0

Ooi, S. K. T., Qiu, C., Bernstein, E., Li, K., Jia, D., Yang, Z., Erdjument-Bromage, H., Tempst, P., Lin, S. P., Allis, C. D., Cheng, X., & Bestor, T. H. (2007). DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature 2007 448:7154*, *448*(7154), 714–717. https://doi.org/10.1038/nature05987

Õunap, K. (2016). Silver-Russell Syndrome and Beckwith-Wiedemann Syndrome: Opposite Phenotypes with Heterogeneous Molecular Etiology. *Molecular Syndromology*, *7*(3), 110–121. https://doi.org/10.1159/000447413

Overeem, A. W., Chang, Y. W., Spruit, J., Roelse, C. M., & Chuva De Sousa Lopes, S. M. (2021). Ligand–Receptor Interactions Elucidate Sex-Specific Pathways in the Trajectory From Primordial Germ Cells to Gonia During Human Development. *Frontiers in Cell and Developmental Biology*, *9*, 1462. https://doi.org/10.3389/FCELL.2021.661243/BIBTEX

Peng, H., Zhao, P., Liu, J., Zhang, J., Zhang, J., Wang, Y., Wu, L., Song, M., & Wang, W. (2018). Novel Epigenomic Biomarkers of Male Infertility Identified by Methylation Patterns of CpG Sites Within Imprinting Control Regions of H19 and SNRPN Genes. *Https://Home.Liebertpub.Com/Omi*, *22*(5), 354–364. https://doi.org/10.1089/OMI.2018.0019

Pinborg, A., Loft, A., Romundstad, L. B., Wennerholm, U. B., Söderström-Anttila, V., Bergh, C., & Aittomäki, K. (2016). Epigenetics and assisted reproductive technologies. *Acta Obstetricia et Gynecologica Scandinavica*, *95*(1), 10–15. https://doi.org/10.1111/AOGS.12799

Poplinski, A., Tüttelmann, F., Kanber, D., Horsthemke, B., & Gromoll, J. (2010). Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *International Journal of Andrology*, *33*(4), 642–649. https://doi.org/10.1111/J.1365-2605.2009.01000.X

Quenneville, S., Verde, G., Corsinotti, A., Kapopoulou, A., Jakobsson, J., Offner, S., Baglivo, I., Pedone, P. v., Grimaldi, G., Riccio, A., & Trono, D. (2011). In Embryonic Stem Cells, ZFP57/KAP1 Recognize a Methylated Hexanucleotide to Affect Chromatin and DNA Methylation of Imprinting Control Regions. *Molecular Cell*, *44*(3), 361–372. https://doi.org/10.1016/J.MOLCEL.2011.08.032

Ramasamy, R., Ridgeway, A., Lipshultz, L. I., & Lamb, D. J. (2014). Integrative DNA methylation and gene expression analysis identifies discoidin domain receptor 1 association with idiopathic nonobstructive azoospermia. *Fertility and Sterility*, *102*(4), 968-973.e3. https://doi.org/10.1016/J.FERTNSTERT.2014.06.028

Raz, E. (2004). Guidance of primordial germ cell migration. *Current Opinion in Cell Biology*, *16*(2), 169–173. https://doi.org/10.1016/J.CEB.2004.01.004

Reed, M. L., & Leff, S. E. (1994). Maternal imprinting of human SNRPN, a gene deleted in Prader–Willi syndrome. *Nature Genetics 1994 6:2*, *6*(2), 163–167. https://doi.org/10.1038/ng0294-163

Roberts, A. L., Gladish, N., Gatev, E., Jones, M. J., Chen, Y., MacIsaac, J. L., Tworoger, S. S., Austin, S. B., Tanrikut, C., Chavarro, J. E., Baccarelli, A. A., & Kobor, M. S. (2018). Exposure to childhood abuse is associated with human sperm DNA methylation. *Translational Psychiatry 2018 8:1*, *8*(1), 1–11. https://doi.org/10.1038/s41398-018-0252-1

Rodprasert, W., Virtanen, H. E., Mäkelä, J. A., & Toppari, J. (2020). Hypogonadism and Cryptorchidism. *Frontiers in Endocrinology*, *10*, 906. https://doi.org/10.3389/FENDO.2019.00906/BIBTEX

Rotondo, J. C., Selvatici, R., di Domenico, M., Marci, R., Vesce, F., Tognon, M., & Martini, F. (2013). Methylation loss at H19 imprinted gene correlates withmethylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples from infertile males. *Http://Dx.Doi.Org/10.4161/Epi.25798*, *8*(9), 990–997. https://doi.org/10.4161/EPI.25798

Runyan, C., Schaible, K., Molyneaux, K., Wang, Z., Levin, L., & Wylie, C. (2006). Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. *Development*, *133*(24), 4861–4869. https://doi.org/10.1242/DEV.02688

Sadler T.W, & Langman J. (2004). *Langman's medical embryology* (14th edition). Lippincott Williams & Wilkins.

Sanchez-Delgado, M., Court, F., Vidal, E., Medrano, J., Monteagudo-Sánchez, A., Martin-Trujillo, A., Tayama, C., Iglesias-Platas, I., Kondova, I., Bontrop, R., Poo-Llanillo, M. E., Marques-Bonet, T., Nakabayashi, K., Simón, C., & Monk, D. (2016). Human Oocyte-Derived Methylation Differences Persist in the Placenta Revealing Widespread Transient Imprinting. *PLOS Genetics*, *12*(11), e1006427. https://doi.org/10.1371/JOURNAL.PGEN.1006427

Santi, D., de Vincentis, S., Magnani, E., & Spaggiari, G. (2017a). Impairment of sperm DNA methylation in male infertility: a meta-analytic study. *Andrology*, *5*(4), 695–703. https://doi.org/10.1111/ANDR.12379

Santi, D., de Vincentis, S., Magnani, E., & Spaggiari, G. (2017b). Impairment of sperm DNA methylation in male infertility: a meta-analytic study. *Andrology*, *5*(4), 695–703. https://doi.org/10.1111/ANDR.12379

Santos, F., & Dean, W. (2004). Epigenetic reprogramming during early development in mammals. *Reproduction*, *127*(6), 643–651. https://doi.org/10.1530/REP.1.00221

Sasaki, H., & Matsui, Y. (2008). Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nature Reviews Genetics 2008 9:2*, *9*(2), 129–140. https://doi.org/10.1038/nrg2295

Schaefer, C. B., Ooi, S. K. T., Bestor, T. H., & Bourc'his, D. (2007). Epigenetic decisions in mammalian germ cells. *Science*, *316*(5823), 398–399. https://doi.org/10.1126/SCIENCE.1137544

Schübeler, D. (2015). Function and information content of DNA methylation. *Nature 2015 517:7534*, *517*(7534), 321–326. https://doi.org/10.1038/nature14192

Sendžikaitė, G., & Kelsey, G. (2019). The role and mechanisms of DNA methylation in the oocyte. *Essays in Biochemistry*, *63*(6), 691–705. https://doi.org/10.1042/EBC20190043

Sharpe, R. M. (2003). *Proliferation and functional maturation of Sertoli Cells, and their relevance to disorders of testis function in adulthood*. https://doi.org/10.1530/rep.0.1250769

Shikauchi, Y., Saiura, A., Kubo, T., Niwa, Y., Yamamoto, J., Murase, Y., & Yoshikawa, H. (2009). SALL3 Interacts with DNMT3A and Shows the Ability To Inhibit CpG Island Methylation in Hepatocellular Carcinoma. *Molecular and Cellular Biology*, *29*(7), 1944–1958. https://doi.org/10.1128/MCB.00840-08/SUPPL_FILE/SUPPLEMENTAL_FIG_S_1_1227.PDF

Shima, Y. (2022). Functional Importance of Mini-Puberty in Spermatogenic Stem Cell Formation. *Frontiers in Cell and Developmental Biology*, *10*. https://doi.org/10.3389/FCELL.2022.907989

Smallwood, S. A., & Kelsey, G. (2012a). Genome-Wide Analysis of DNA Methylation in Low Cell Numbers by Reduced Representation Bisulfite Sequencing. *Methods in Molecular Biology*, *925*, 187–197. https://doi.org/10.1007/978-1-62703-011-3_12

Smallwood, S. A., & Kelsey, G. (2012b). De novo DNA methylation: a germ cell perspective. *Trends in Genetics*, *28*(1), 33–42. https://doi.org/10.1016/J.TIG.2011.09.004

Smallwood, S. A., & Kelsey, G. (2012c). De novo DNA methylation: a germ cell perspective. *Trends in Genetics*, *28*(1), 33–42. https://doi.org/10.1016/J.TIG.2011.09.004

Stukenborg, J. B., Kjartansdóttir, K. R., Reda, A., Colon, E., Albersmeier, J. P., & Söder, O. (2014). Male Germ Cell Development in Humans. *Hormone Research in Paediatrics*, *81*(1), 2–12. https://doi.org/10.1159/000355599

Su, S., Szarek, M., Vooght, A., Hutson, J., & Li, R. (2014). Gonocyte transformation to spermatogonial stem cells occurs earlier in patients with undervirilisation syndromes. *Journal of Pediatric Surgery*, *49*(2), 323–327. https://doi.org/10.1016/J.JPEDSURG.2013.11.047

Surani, M. A. H., & Barton, S. C. (1983). Development of Gynogenetic Eggs in the Mouse: Implications for Parthenogenetic Embryos. *Science*, *222*(4627), 1034–1036. https://doi.org/10.1126/SCIENCE.6648518

Surani, M. A. H., Barton, S. C., & Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature 1984 308:5959*, *308*(5959), 548–550. https://doi.org/10.1038/308548a0

Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L. M., Liu, D. R., Aravind, L., & Rao, A. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*, *324*(5929), 930–935. https://doi.org/10.1126/SCIENCE.1170116/SUPPL_FILE/TAHILIANI_SOM.PDF

Tucci, V., Isles, A. R., Kelsey, G., Ferguson-Smith, A. C., Bartolomei, M. S., Benvenisty, N., Bourc'his, D., Charalambous, M., Dulac, C., Feil, R., Glaser, J., Huelsmann, L., John, R. M., McNamara, G. I., Moorwood, K., Muscatelli, F., Sasaki, H., Strassmann, B. I., Vincenz, C., & Wilkins, J. (2019). Genomic Imprinting and Physiological Processes in Mammals. *Cell*, *176*(5), 952–965. https://doi.org/10.1016/J.CELL.2019.01.043

Urdinguio, R. G., Bayón, G. F., Dmitrijeva, M., Toraño, E. G., Bravo, C., Fraga, M. F., Bassas, L., Larriba, S., & Fernández, A. F. (2015a). Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility. *Human Reproduction*, *30*(5), 1014–1028. https://doi.org/10.1093/HUMREP/DEV053

Urdinguio, R. G., Bayón, G. F., Dmitrijeva, M., Toraño, E. G., Bravo, C., Fraga, M. F., Bassas, L., Larriba, S., & Fernández, A. F. (2015b). Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility. *Human Reproduction*, *30*(5), 1014–1028. https://doi.org/10.1093/HUMREP/DEV053

Vincent, R. N., Gooding, L. D., Louie, K., Chan Wong, E., & Ma, S. (2016). Altered DNA methylation and expression of PLAGL1 in cord blood from assisted reproductive technology pregnancies compared with natural conceptions. *Fertility and Sterility*, *106*(3), 739-748.e3. https://doi.org/10.1016/J.FERTNSTERT.2016.04.036

Wang, R., Liu, X., Li, L., Yang, M., Yong, J., Zhai, F., Wen, L., Yan, L., Qiao, J., & Tang, F. (2022a). Dissecting human gonadal cell lineage specification and sex determination using a single-cell RNA-seq approach. *Genomics, Proteomics & Bioinformatics*. https://doi.org/10.1016/J.GPB.2022.04.002

Wang, R., Liu, X., Li, L., Yang, M., Yong, J., Zhai, F., Wen, L., Yan, L., Qiao, J., & Tang, F. (2022b). Dissecting human gonadal cell lineage specification and sex determination using a single-cell RNA-seq approach. *Genomics, Proteomics & Bioinformatics*. https://doi.org/10.1016/J.GPB.2022.04.002

Wei, Y., Yang, C. R., Wei, Y. P., Zhao, Z. A., Hou, Y., Schatten, H., & Sun, Q. Y. (2014). Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(5), 1873–1878. https://doi.org/10.1073/PNAS.1321195111

Wen, Q., Cheng, C. Y., & Liu, Y. X. (2016). Development, function and fate of fetal Leydig cells. *Seminars in Cell & Developmental Biology*, *59*, 89–98. https://doi.org/10.1016/J.SEMCDB.2016.03.003

Wu, X., Luo, C., Hu, L., Chen, X., Chen, Y., Fan, J., Cheng, C. Y., & Sun, F. (2020). Unraveling epigenomic abnormality in azoospermic human males by WGBS, RNA-Seq, and transcriptome profiling analyses. *Journal of Assisted Reproduction and Genetics 2020 37:4*, *37*(4), 789–802. https://doi.org/10.1007/S10815-020-01716-7

Xu, W., Fang, P., Zhu, Z., Dai, J., Nie, D., Chen, Z., Qin, Q., Wang, L., Wang, Z., & Qiao, Z. (2013). Cigarette smoking exposure alters pebp1 DNA methylation and protein profile involved in MAPK signaling pathway in mice testis. *Biology of Reproduction*, *89*(6), 142–143. https://doi.org/10.1095/BIOLREPROD.113.111245/2514167

Yang, A. S., Estécio, M. R. H., Doshi, K., Kondo, Y., Tajara, E. H., & Issa, J. P. J. (2004). A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Research*, *32*(3), e38–e38. https://doi.org/10.1093/NAR/GNH032

Yang, F., & Wang, P. J. (2016). Multiple LINEs of retrotransposon silencing mechanisms in the mammalian germline. *Seminars in Cell & Developmental Biology*, *59*, 118–125. https://doi.org/10.1016/J.SEMCDB.2016.03.001

Youngson, N., Lecomte, V., Maloney, C., Leung, P., Liu, J., Hesson, L., Luciani, F., Krause, L., & Morris, M. (2016). Obesity-induced sperm DNA methylation changes at satellite repeats are reprogrammed in rat offspring. *Asian Journal of Andrology*, *18*(6), 930–936. https://doi.org/10.4103/1008-682X.163190

Zheng, H. Y., Shi, X. Y., Wu, F. R., Wu, Y. Q., Wang, L. le, & Chen, S. L. (2011). Assisted reproductive technologies do not increase risk of abnormal methylation of PEG1/MEST in human early pregnancy loss. *Fertility and Sterility*, *96*(1), 84-89.e2. https://doi.org/10.1016/J.FERTNSTERT.2011.04.021

Zheng, Y., Xue, X., Shao, Y., Wang, S., Esfahani, S. N., Li, Z., Muncie, J. M., Lakins, J. N., Weaver, V. M., Gumucio, D. L., & Fu, J. (2019). Controlled modelling of human epiblast and amnion development using stem cells. *Nature 2019 573:7774*, *573*(7774), 421–425. https://doi.org/10.1038/s41586-019-1535-2

7 BIOGRAPHY

Inbar Shafran was born in Israel on 14th of August 1991. She served in the Israel Defence Forces from 2010-2012 during which she was a commander in the Navy school. Following her army service, she worked as an educator and training coordinator at Tzur Shalem a program. She enrolled to the School of Medicine University of Zagreb, Croatia 2016-2022. During her studies she was a demonstrator at Rebro Hospital, spent 2 months in the Galilee Medical Centre in Gynaecology and Obstetrics and Internal medical wards and was awarded the Dean's Award for excellence.