Assisted Reproductive Technologies and Their Influence on Epigenetic Regulation and Embryonic Development

by

Sydney Opfer

B.S. Genetics and Cell Biology, Washington State University, 2020

Submitted to the Graduate Faculty of the

Department of Human Genetics

School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2024

UNIVERSITY OF PITTSBURGH

SCHOOL OF PUBLIC HEALTH

This essay is submitted

by

Sydney Opfer

on

April 23, 2024

and approved by

Essay Advisor: F. Yesim Demirci, MD, Department of Human Genetics, School of Public Health, University of Pittsburgh

Essay Reader: Laura Runnels, MPH, Public Health Consultant

Essay Reader: Akira Sekikawa, MD, PhD, MPH, Department of Epidemiology, School of Public Health, University of Pittsburgh

Assisted Reproductive Technologies and Their Influence on Epigenetic Regulation and Embryonic Development

Sydney Opfer, MPH

University of Pittsburgh, 2024

Abstract

While assisted reproductive technologies (ARTs) are now widely used and considered to be generally safe, concerns have been raised about their potential effects on epigenetic regulation in gametes/embryos and their potential impact on offspring health. The use of ARTs has been associated with various epigenetic effects and potential impact on embryonic development. While this is mostly supported by animal studies, the results are relatively less consistent across human studies. The external environment of ART can cause disruptions in DNA methylation or other epigenetic regulation mechanisms, which can result in altered gene expression. ART processes such as ovarian stimulation, cryopreservation, and culture media/conditions are a few of the many that can interfere with epigenetic regulation mechanisms. Epigenetics is an emerging field and various aspects of epigenetic regulation, beyond DNA methylation, remain understudied as related to ART. However, since the number of women using ART is only increasing, it is extremely important for researchers to comprehensively study various aspects of epigenetic regulation that can be influenced by ARTs in future and larger studies given the vulnerability of early embryos to such influences due to epigenetic reprogramming. There is a significant public health relevance to these effects because they can lead to future disease risk later in the child's life. Because ART is a relatively new development in the field of reproductive health, researchers have not yet been able to follow ART-conceived children into later adulthood to monitor long-term health outcomes

compared to the naturally conceived population. Therefore, it is of significant public health importance to determine the long-term consequences of ARTs and identify ways to render the related techniques safer and as close to the natural conception and in-utero environment as possible for the most advantageous health results.

Table of Contents

List of Figures

Preface

I gratefully acknowledge the contributions of all individuals who have supported and assisted me in the completion of this literature review. A special thank you to my essay advisor Dr. Yesim Demirci at the University of Pittsburgh, as well as my essay readers, Laura Runnels and Dr. Akira Sekikawa. Thank you to my friends and family who have supported me along the way.

1.0 Introduction and Aims

While assisted reproductive technologies (ARTs) are now widely used and considered to be generally safe, concerns have been raised about their potential effects on epigenetic regulation in gametes/embryos and their potential impact on offspring health. The purpose of this essay is to provide a literature review on the epigenetic effects that are at play as related to ARTs and potential health consequences. The public health relevance of understanding the potential epigenetic effects and consequences of commonly used ARTs are also discussed. Below are the specific aims of this review:

1. To define epigenetic regulation mechanisms and commonly used ARTs and review the current knowledge on the epigenetic effects of ARTs on gametes/embryos and their potential impact on offspring health.
2. To identify the current knowledge gaps and discuss future directions as well as how the advanced knowledge can benefit the ART field and perinatal outcomes thus overall public health.

2.0 Background on Epigenetic Mechanisms

Epigenetics is defined as alterations of gene expression that are not caused by mutation or other sequence variation of the actual DNA [1]. The DNA is simply adjusted, into a tight formation where no transcription can occur or into a more open formation which allows the cell machinery to transcribe and create more gene products. This alteration in gene expression is a normal process controlled in the body and it regulates which genes are expressed in a tissue-specific and context-dependent manner [1]. Although epigenetic mechanisms occur naturally in a controlled manner, they can also occur in a disorderly manner when influenced by certain environmental or other factors. Epigenetic regulation can occur at both the DNA and the RNA levels through a number of processes. At the DNA level, there are three main mechanisms, which include DNA methylation, histone modifications/variants, and chromatin remodeling. Epigenetic regulation at the RNA level includes altered effects of non-coding RNAs (ncRNAs), like long ncRNAs (lncRNAs) and small/short ncRNAs, as well as RNA modifications [1-3]. As stated before, these mechanisms occur naturally to regulate gene expression, but sometimes, however, can lead to an over- or under-expression of certain gene(s) that contribute to disease pathogenesis [1-3]. Because epigenetic modifications do not change the actual sequence of DNA and can be reversible, they have become a good target for new therapies that focus on changing the levels of gene expression, such as new cancer treatments.

As we get older our risk of acquiring pathogenetic epigenetic effects increases due to a number of factors, like environmental exposures, poor diet, and other lifestyle choices [4]. These may play a role in the development of serious diseases like cancer and other issues such as infertility [4]. Although they do not change the DNA sequence, some epigenetic effects may be

passed down to offspring, through the so-called ‘epigenetic inheritance’. Epigenetic inheritance is believed to go hand in hand with germline cell reprogramming [5]. While the data are currently limited, epidemiological observations suggest that transgenerational epigenetic inheritance may also occur in humans [6]. During embryonic development, there are two phases of epigenetic reprogramming, one occurring after fertilization (pre-implantation) and one later down the line when the cells differentiate [7]. The reprogramming allows the cells to return to a totipotent state where they are then capable of differentiating into one of the many different cell types [5]. Because of this development-associated epigenetic reprogramming, the early embryo period is considered to be very critical in terms of potential impacts on long-lasting epigenetic marks that may increase disease risk later in life [5, 8-10].

2.1 Epigenetic Mechanisms at the DNA level

Epigenetic regulation that occurs at the DNA level includes DNA methylation, histone modifications/variants, and chromatin conformation changes/remodeling (Figure 1).

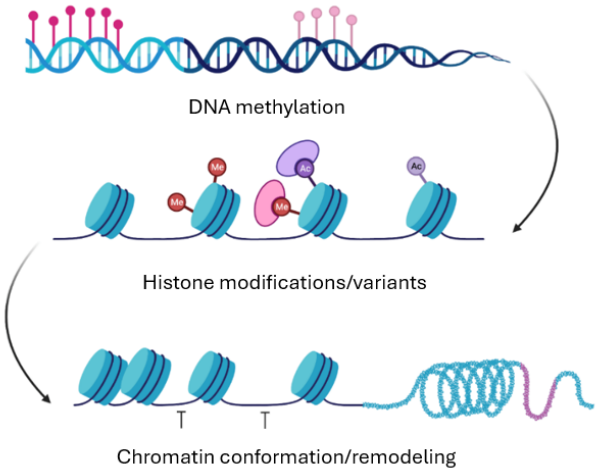

Figure 1 Epigenetic regulation at the DNA level

(Created with BioRender.com)

In mammals, DNA methylation commonly occurs in cytosines, especially at CpG sites where cytosine is followed by guanine on the same DNA strand connected to one another with a phosphate bond [1]. These CpG sites can be grouped together on what are called CpG islands, which are usually located in the promoter region of the expressed genes [11]. When this promoter region is methylated, transcription from the DNA will be repressed, and when the methylation is lost the gene can become activated and transcribed [12]. Like all epigenetic processes, DNA methylation is a natural process that plays a role in the development and several cellular processes like imprinting, X-chromosome inactivation, DNA replication and transcription [1]. In methylated DNA, where the methyl groups are added at the C-5 position of the Cytosine (5), the regulatory molecules that participate in DNA replication or transcription cannot access their binding sites to activate these processes. Dysregulation in DNA methylation can contribute to the development of diseases such as cancer [1, 13]. Many studies have noted the effects of specific environmental factors and lifestyle choices such as diet on certain genes being methylated or unmethylated [11].

DNA methylation is tissue-specific as it assists in cell differentiation, a process that is extremely important during the in-utero and postnatal stages [11].

Similarly, histone modification (or variant histone usage) is another regulatory process for gene stabilization and transcription/replication [1]. Various interactions can occur between different epigenetic processes including those happening between histone modifications and DNA methylation [1]. For example, DNA methylation can trigger the recruitment of histone deacetylases, which change the conformation of the chromatin and repress transcription. Histones are proteins freely found in the cell and attached to DNA [12]. There are five types of canonical histones, four of which are called "core histones" being HB2A, HB2B, H3, and H4, and one additional referred to as a "linker histone" being H1 [14]. These histones make up the nucleosome, which is the basic unit of the chromatin [14]. Each nucleosome is formed with ~147 base pairs of DNA that double-wraps around two sets of the core histones, called a histone octamer. The linker histone H1 binds to the DNA outside the nucleosome to function as a clamp that keeps the DNA wrapped around the core histones in place. When the histones are tightly bound, no DNA transcription will take place. Acetylation of histones will alter chromatin conformation, opening it up and allowing the DNA-binding proteins to interact with DNA and start the transcription process. Acetylation and other post-translational modifications (e.g., methylation, phosphorylation, ubiquitylation, and ADP-ribosylation) usually occur at the N-terminal tail of the histones (at specific amino acid residues such as Lysine and Arginine) and may lead to transcriptional activation or repression depending on the cohort of modifications [15].

In addition to the enzymes involved in DNA methylation or histone modifications, the proteins that can recognize methylated DNA or modified histones, as well as the ATP-dependent chromatin remodeling complexes that can reposition nucleosomes or mediate variant histone

swapping within the nucleosomes, can also regulate chromatin conformation and accessibility [16, 17]. Changes in chromatin conformation, by condensing or opening up, would in turn regulate gene expression by changing the accessibility of DNA for transcription factors and other regulators.

2.2 Epigenetic Mechanisms at the RNA level

Epigenetic modifications at the RNA level occur through the regulatory effects of non-coding RNAs including small/short or long ncRNAs (such as microRNAs (miRNAs), PIWI-interacting RNA (piRNAs), small-interfering RNAs (siRNAs), promoter-associated RNAs (paRNAs), enhancer RNAs (eRNAs), and other lncRNAs) as well as RNA modifications [2, 3, 18].

LncRNAs make up the majority of the "non-protein coding transcripts" [18]. RNA transcripts are considered "long" if they are over 200 nucleotides in length [18]. LncRNAs can play diverse roles in gene regulation, both transcriptionally and post-transcriptionally, through their interactions with various molecules. They can regulate nuclear organization, chromatin structure, enhancer function, *cis* or *trans* gene expression, RNA processing/stability/translation, as well as act as miRNA sponges and interact with the proteins [19-22].

Short/small ncRNAs are about 20-24 nucleotides in length and are evolutionarily conserved [18]. MicroRNAs (miRNAs) are the most common form of short ncRNAs that assist in post-transcriptional gene expression regulation by suppressing the translation of or enabling the degradation of mature RNAs [18]. MiRNAs work with an RNA-Induced Silencing Complex (RISC) to execute their functions [19]. A miRNA recognizes its mature RNA target by base pairing

of the 3' untranslated region (UTR) and may target multiple mature RNAs for suppression/downregulation. Likewise, a mature RNA can be targeted by multiple microRNAs for regulation [19]. As one can suspect, faulty translational regulation or degradation by miRNA under- or over-expression can lead to an over- or under-production of target mRNA transcript(s) and contribute to disease pathogenesis.

Another layer of post-transcriptional epigenetic regulation involves RNA modifications, the most common being N6-methyladenosine (m6A) in eukaryotic cells [2]. Post-transcriptional modifications of mRNAs or ncRNAs can modulate their biogenesis, functions, and interactions with other molecules, and if dysregulated, can contribute to the development of various diseases [2, 3, 23].

2.3 Imprinting as an Epigenetic Process

Imprinting is an epigenetic process in which gene expression is regulated based on the parental origin of the gene. This means that either the maternal or paternal gene will be expressed in the offspring and the other will be epigenetically repressed [24]. This expression of just one of the parental alleles is called monoallelic expression, however, imprinting may also be partial with one of the parental alleles being expressed at a lower level [24]. During gametogenesis, the imprints are reset by erasing and replacing the epigenetic marks based on the sex of the parent. These imprints end up escaping the epigenetic reprogramming in the early zygote, where the parental allele to be expressed is unmethylated and the parental allele to be silenced remains methylated limiting accessibility for transcription factors and other regulators [24]. In addition to DNA methylation, genomic imprinting involves ncRNA and histone modifications to regulate

gene expression at the imprinted loci [25]. Imprinted loci are often complex and harbor an imprinting control region and often multiple genes, some maternally or paternally imprinted and some non-imprinted. The specific alleles that are either expressed or silenced based on parental origin are chosen by the natural selection processes, meaning whichever is more favorable is chosen. Imprinting can regulate embryonic and fetal development as well as lead to disease if regulated improperly [25]. Many imprinted genes are directly related to important developmental pathways, making their dysregulation detrimental in some situations [25]. While the imprints are erased and reestablished in germline cells, they are maintained through mitoses in somatic cells [24]. Problems with imprinting occur when both parental copies are activated, or both are deactivated. This can lead to problems in development and metabolism, resulting in one of several imprinting disorders such as Beckwith-Wiedemann syndrome or Silver-Russell syndrome [26].

2.4 Epigenetic Dysregulation and Human Diseases

Epigenetic abnormalities have the potential to cause various health problems, like cancer and other diseases and infertility issues. However, much is still unclear about epigenetics and its potential effects as well as the potential inheritance of epigenetic marks. Studies suggest that epigenetic effects can follow into adulthood [27]. Multiple studies over the last decade have implicated epigenetic effects in various disorders such as cancer, autoimmune diseases, addiction, psychiatric diseases, and neurodegenerative diseases [28]. Furthermore, epigenetics has also been shown to have effects on fertility and potentially contribute to infertility in some [29]. The use of ART has also been linked to some epigenetic effects and it is known to be associated with complications such as hypertension, gestational diabetes, placental abruption, preterm birth, low

birth weight, and perinatal mortality [29]. However, the extent to which epigenetic changes contribute to those outcomes remains unclear and is the subject of active research.

3.0 Background on Natural Conception, Reproduction, and Assisted Reproduction

3.1 Stages of Reproduction

The stages of reproduction that ultimately lead to pregnancy start with the process of gametogenesis. Gametogenesis is a natural process that happens in both females and males and involves individual maturation of primordial germ cells into mature germ cells: oocytes or spermatozoa [30]. Gametes are the reproductive cells that undergo mitosis and meiosis to become either mature eggs or sperm during gametogenesis [30]. Once the germ cells are mature and individuals have gone through puberty, their bodies allow them to reproduce. Reproduction happens when an ovum and a spermatozoon are united to make a zygote either through the natural process of coitus or with the assistance of various reproductive technologies. Natural conception is the process where a sperm and egg meet in the fallopian tubes, followed by their fusion to form a zygote (fertilization), and the implantation of the early embryo (blastocyst) into the uterine lining, overall inducing a pregnancy [29]. The zygote goes through multiple rounds of cell divisions to create a morula, then a blastocyst, then an embryo, and ultimately a fetus [29]. Embryonic development, also known as embryogenesis, begins with the fertilization process and continues to embryo formation. During this development, the blastocyst splits into two masses: the inner and outer mass. The inner mass develops into the embryo and the outer mass into the placenta and yolk sac [31]. The placenta serves as a connection between the mother and fetus through which oxygen and nutrients can be passed to the fetus, and it also functions as an immune and endocrine organ enabling the healthy growth of the fetus [31].

3.2 Assisted Reproductive Technologies

ARTs are techniques that help families conceive a child when they are infertile and/or incapable of reproducing one naturally. Infertility can be present in both females and males and be caused by a number of factors such as problems with ovulation and endometriosis in women and low testosterone levels and low sperm count in men [32, 33]. Infertility is defined as a lack of a confirmed pregnancy after one full year of trying to conceive [33]. Numerous ARTs have been used to help families conceive children including ovarian stimulation (OS), intrauterine insemination, intracytoplasmic sperm injection (ICSI), in-vitro fertilization (IVF), embryo culture, and cryopreservation of gametes or embryos. Ovarian stimulation (OS) usually requires women to receive injectable gonadotrophins, with or without prior oral hormone therapy, to enable the harvest of multiple oocytes to increase the chances of successful IVF treatment [34]. Gonadotrophins involved in ovarian stimulation are follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that aid in releasing eggs from the ovary [34]. Being able to collect multiple eggs in one take will increase the overall likelihood that at least one egg will be fertilized and make it through to the implantation stage. Intrauterine insemination (artificial insemination) is done by placing sperm directly into the uterus, significantly cutting the time it takes the sperm to travel to the egg, and increasing the chance of pregnancy occurring [35]. Artificial insemination is often combined with ovarian stimulation treatment to ensure that insemination occurs at the time of ovulation [35]. Intracytoplasmic sperm injection (ICSI) is regularly done when the sperm produced by the male partner has low motility or low abundance [36]. The process involves the use of a needle to inject the sperm into the egg, making fertilization much more likely to occur. The use of ICSI has significantly increased during recent years and has also involved indications for non-male factor infertility; however, this recent trend has been controversial and led to

concerns about the overuse of ICSI [37, 38]. ICSI is often used in combination with other ARTs such as IVF. ICSI comes with some ethical considerations as reproducing with low-motility sperm that would not reach the egg through natural conception could potentially lead to offspring with health issues by enabling the fertilization with abnormal sperm, which would otherwise be subjected to natural selection. In-vitro fertilization (IVF) involves fertilization outside of the body, as the mature eggs are harvested and directly fertilized with sperm in a culture medium in the lab. IVF also comes with some ethical considerations due to the same reason discussed above for ICSI, and multiple eggs harvested during this procedure can also be frozen for later usage [39]. Proper disposal of the remaining frozen eggs or embryos also causes ethical implications. During IVF, the created embryos are allowed to further mature before they are implanted in the uterus. This is possible with the use of embryo culture, which provides the embryo with the right environment and nutrients for growth. Cryopreservation is the process of freezing cells or other biological materials with liquid nitrogen at -196 degrees Celsius [40]. Cryopreservation can be used for reproductive purposes to freeze sperm, eggs, and embryos. Cryopreservation done with gametes and embryos can result in epigenetic changes due to the vulnerable stage at which they are frozen [40]. All of these ARTs have been used for a number of years, but they can be associated with some adverse outcomes in the offspring due to the ART procedures themselves or as related to infertility that led to the use of ART [40].

3.3 Infertility Treatment, Donor Eggs/Sperm Usage, and Surrogacy

Infertility treatments are broken into three groups: medicine, surgical procedures, and assisted conception [41]. Assisted conception covers all of the ARTs mentioned above. Infertility

medication consists of clomiphene, tamoxifen, metformin, gonadotrophins, gonadotrophin-releasing hormone, and dopamine agonists [42]. These medications can provide a variety of functions from stimulating ovulation to regulating ovulation [42]. In most cases, fertility problems are treated with a conventional approach such as medication or surgery. In cases where infertility remains a problem despite the available treatments and ART procedures, the usage of donor eggs/sperm and surrogacy is recommended. However, donor eggs/sperm can also be used in the absence of infertility conditions, by women and men who wish to start a family at their own pace. Donor sperm can be acquired through a donor bank or through social media sites like Facebook with little to no regulation. The issue with the latter is that the sperm being sold would be unregulated and not checked for sexually transmitted infections or overall quality. Sperm and egg banks are typically regulated by each state and on a federal level by the FDA. When a person donates sperm or eggs, they can opt to be de-identified or identified when the child turns 18. Regardless of whether a child wishes to meet their biological parent, it is important to consider the missing hereditary health information these children will have to deal with and how it could affect their future health. In addition to donor usage, surrogacy is another way through which those struggling to have children can accomplish their goal. Surrogacy is when a woman carries an embryo to term for another woman or couple who are unable to do so. The agreement with the surrogate involves legal documentation and legal consequences if it is breached in any way.

3.4 Current State of ARTs and Donor Usage and Their Public Perception

Currently, in the United States, ARTs are becoming more widely used and accepted, although, they are met with some ethical implications as mentioned earlier in this review.

According to the CDC, in 2021, there were 97,128 live infants born with the help of ARTs (https://www.cdc.gov/art/artdata/index.html). The data for donor usage is harder to estimate due to the lack of regulation of donated sperm and the reselling of that sperm when it is acquired. Some reports state that donor gametes account for 10,000 births per year in the United States and others say 40,000 births [43]. Additionally, in the United States, births by surrogacy have significantly increased from 727 live births in 1999 to 3,432 live births in 2013 [44]. ARTs, donor usage, and surrogacy assistance all received positive attitudes from the public in a study conducted across Europe [45]. There was also support for public funding for the necessary ARTs since the cost of these technologies is astronomical, leaving only wealthy individuals able to afford them [45]. This leaves those of lower socioeconomic status, including the minority populations, at a disadvantage in receiving such infertility care.

4.0 Epigenetic Changes and Imprinting Disorders Associated with ART

In recent years, assisted reproductive technologies (ARTs) have been widely used to create almost 10 million children worldwide [46]. While these techniques are advantageous for creating children when the families wouldn't naturally be able to do so, there are also well-documented concerns of potential epigenetic effects and an increased risk for imprinting disorders from ART usage as reviewed in the following sections.

4.1 Epigenetic Alterations Linked to ART

Usage of ARTs has been shown to be associated with an increased risk for epigenetic alterations when compared to natural conception, as shown in animal studies and less conclusively in human studies [47-50]. During the two stages of epigenetic reprogramming in the embryo, the epigenome is highly susceptible to alterations, which can lead to long-lasting epigenetic effects with negative impacts later on in a child's life and health [51]. It is well known that environmental factors/exposures can interact with epigenetic regulation, and the ART processes such as IVF leave embryos exposed to external environmental conditions that they otherwise would not be exposed to. Among those processes, ovarian stimulation (OS), intracytoplasmic sperm injection (ICSI), IVF, embryo culture medium/conditions, cryopreservation of gametes/embryo, storage length, as well as the stage of development at which the embryo was frozen can all increase the likelihood of epigenetic aberrations to occur [46, 52]. ART procedures can therefore be associated with disruption of normal DNA methylation patterns and histone modifications, ultimately leading to

altered gene expression [51]. While ARTs have been shown to be variably associated with short- and long-term perinatal, developmental, and medical (e.g., cognitive, behavioral, cardiovascular, and metabolic) adverse outcomes, the potential contribution of ART-induced epigenetic modifications to those outcomes is currently an active area of research [51, 53, 54].

The culture medium is where an embryo grows until it can be implanted into the uterus and serves as the protein and nutrient source for development [55]. Several studies have investigated the effect of culture medium on perinatal outcomes to date. Kleijkers et al. (2015) looked at the effects of two different culture media (G5 Vitrolife and human tubal fluid HTF) on gene expression in preimplantation embryos (after being frozen and thawed) and they found that the G5 medium served as ideal for development [55]. Not only did the embryos in the G5 medium have an increased cell count, but the oxidative phosphorylation pathway was also more active, and the implantation success rate was overall higher [55]. Along with its effects on gene expression, culture medium used in IVF processes can also have epigenetic effects, especially on DNA methylation and genomic imprinting; however, according to the human studies published to date based on the analyses of placental tissues, cord blood, or buccal/saliva samples obtained later in childhood, there are no significant differences between the global epigenetic effects caused by different culture media [56-59]. It is therefore believed that the commercial culture media in general do not support the appropriate DNA methylation necessary for the development [56, 60].

In addition to culture media, the oxygen concentration during *in vitro* culturing may have epigenetic effects on embryos. Standard *in vivo* oxygen concentration for embryos is about 5% whereas oxygen concentration during *in vitro* culture can be as high as 20% [61]. This increase in concentration can affect embryo development, and cause changes in the embryo's proteome, transcriptome and epigenome as shown in animal studies [61-63]. These changes can influence the

cell's fate, meaning that a cell designated to become a certain cell type will eventually become a different cell type [64]. Cell programming is a very specific process and deviation from this process can result in abnormalities in critical pathways within the body [65].

Once an embryo is implanted, the trophoblast invasion process can be another point where epigenetic error can occur [66]. Trophoblast invasion is the communication that occurs between mother and child before the placenta starts to form. During this "invasion" the child's cells migrate to the mother's endometrium and attach to the mother's spiral arteries, forming the bond between the child and the placenta [67]. Reproductive technologies can produce epigenetic effects on both the embryo due to the outside environment and on the mother from the necessary hormone treatments required for ART [66]. Epigenetic regulation is required for a properly functioning placenta and overall fetal development, therefore suboptimal ART-related environment may disturb the epigenetic regulation in both the extra-embryonic tissues and the embryo [66, 68]. One *in vitro* study, looking at the disruption of DNA methylation on placental development, observed an inability of trophoblast invasion to occur due to drug-induced changes in DNA methylation [69]. The human epigenetic studies that used extra-embryonic tissues (e.g., placenta) or specimens from ART-conceived embryos/children (e.g., cord or peripheral blood, buccal or saliva samples) have primarily focused on DNA methylation analyses to date and have overall yielded inconsistent results including those from more recent studies [47-49, 58, 66, 70-76]. These inconclusive results have been mainly attributed to the differences in analytical approaches, the types of samples/tissues investigated, and different ART populations examined with varying sample sizes [48, 77]. The limited overlap observed in methylation changes across these different studies (even across those using similar tissue types/populations) might suggest that the ART-induced epigenomic modifications may be minimal; however, additional studies are warranted given the

ongoing improvements in analytical technologies/approaches and increasing sample sizes with the use of larger collections [48, 49, 77].

Apart from the studies focusing on ART-related changes in epigenetic regulation at the DNA level (especially methylation analyses), a limited number of human studies have also evaluated potential epigenetic effects at the RNA level (especially miRNA studies) [78, 79]. These studies have implicated a role for cryopreservation on alterations in miRNA expression profiles of human semen, especially after long-term storage, with subsequent potential effects on fertilization and embryonic development [78, 79].

4.2 Imprinting Disorders Associated with ART

Along with an increased risk of epigenetic errors with the usage of ARTs, an increased risk of imprinted disorders has also been suggested by various studies [49, 50, 54, 80-85]. ART usage has been shown to induce misregulation of multiple imprinted genes in animal tissues such as the kidney, brain, muscle, and liver [86]. Misregulation of imprinted genes is known to be associated with the development of imprinting disorders such as Beckwith-Wiedemann syndrome, Silver-Russell syndrome, Prader-Willi syndrome, and Angelman syndrome [87]. Phenotypes of Large Offspring syndrome (LOS), a fetal overgrowth syndrome, can occur in higher rates with births using ART than natural births [86]. LOS is an imprinting disorder that shows many of the same gene expression patterns as Beckwith-Wiedemann syndrome. Although not all cases of LOS are related to ART, ART-induced LOS is believed to be caused by misregulation of imprinted genes that occurs during ART processes [86]. Using allele-specific expression and methylation analyses, Chen et al. (2015) have demonstrated misregulation of multiple imprinted genes in *Bos taurus*

LOS fetuses vs. controls, and they also detected a correlation between the severity of LOS and the number of misregulated imprinted genes [86]. Although research is still being conducted, accumulating data from animal/cell models and epidemiological human studies suggest a link between ART and an increased risk for imprinting disorders through ART procedures and epigenetic modifications [49, 81, 83, 84]. Imprinting disorders were shown to be higher within the population conceived by ART, which is believed to interfere with the maintenance of imprints in the early embryo [84]. Superovulation, which is the process of taking drugs to facilitate ovulation was seen to cause methylation errors at imprinted sites and dysregulate gene expression in mouse models [84, 88]. In a recent human study, the ovulation drug Clomifene and ART were shown to interfere with genomic imprinting by causing sex-specific aberrant methylations of imprint control regions (ICRs) in cord blood leukocytes from the ART-conceived population [83]. The affected regions/genes have been linked to cardiovascular, metabolic, and behavioral outcomes [83]. Imprinted genes affected by altered DNA methylation included *PEG1/MEST*, *PEG3*, *KCNQ1OT1*, *H19/IGF2* and *GNAS* in related studies [29, 49, 77, 83, 89]. Apart from hormone therapy, the cryopreservation procedure and culture media may also cause disruptions in genomic imprinting, although further studies are needed in humans [13, 50]**.** Therefore, ART procedures including cryopreservation, culture media, and hormone therapies need to be reevaluated for fetus safety as they appear to be directly associated with an increased risk of epigenetic and imprinting alterations with potential impacts on offspring health later on in life [54, 85].

4.3 Environmental and Demographic Factors That May Influence ART-associated Epigenetic Changes

In addition to ART exposure, demographic and environmental factors such as parental age and diet have been shown to influence the presence of epigenetic effects [90]. Increased age in both parents can affect epigenetic mechanisms from DNA methylation to histone modifications [90]. A positive relationship between age and alterations in CpG methylation patterns has been shown, including either hypermethylation or hypomethylation of a subset of genes [90, 91]. Along with contributing to the accumulation of epigenetic markers, age can also contribute to the overall quality of the sperm and oocyte retrieved for the ART processes [92]. It is well known that a maternal age after 35 can have several adverse effects on reproductive outcomes, from chromosome abnormalities of the child to birth complications for the mother and increased risk of miscarriages. It is also reasonable to expect that older fathers would have more time to develop genetic and epigenetic abnormalities in their reproductive tissues/cells [93]. One study concluded that advanced paternal age may also increase the risk of spontaneous miscarriage [93].

Additionally, the diet of the mother and father can affect the child's development. Maternal diet holds significant weight on the development of the fetus because of the direct line of nutrient flow through the placenta from mother to child. There is evidence that maternal diet can affect epigenetic mechanisms, specifically methyl donors like folate and choline, as well as bioactive compounds like polyphenols [94, 95]. Nutritional factors have been well documented to affect genomic DNA through DNA methylation [96]. The consistency of nutrients as well as the amount of methyl donors consumed can alter epigenetic patterns and change gene expression [94, 95]. Nutrients can also influence chromatin remodeling and alter the expression of miRNAs [94, 97]. The breakdown of nutrients and their epigenetic effects were studied during pregnancy. It was

found that those who consumed low amounts of protein while pregnant could influence their children's tolerance to glucose, their expression of cholesterol, and their DNA methylation patterns [98-100]. Additionally, it was found in mice that a high-fat diet during pregnancy can lead to non-alcoholic fatty liver, changes in gene expression and DNA methylation, leading to abnormal feeding behaviors and an increased risk of obesity [98, 100, 101]. Overall, different nutritional stressors such as parental malnutrition, various diets (seasonal, low-protein, or high-fat), and folic acid supplementation may all have epigenetic effects and can interact with those induced by ARTs as shown in human or animal studies [100, 102]. These epigenetic modifications related to nutritional status and diet have the potential to negatively impact the fetal development by influencing the ART-associated epigenetic changes in gametes and/or embryo.

5.0 Public Health Relevance

5.1 Public Health Relevance of Studying Epigenetic Effects

Epigenetic effects are very relevant to public health as they can contribute to many health effects but are potentially modifiable/preventable due to their dynamic and reversible nature. Moreover, these effects can be brought about by interactions with environmental toxins, nutrition, lifestyle choices, and other factors, providing opportunities for prevention and better management upon a better understanding of these interactions and underlying mechanisms. They are important factors for the public to consider for their own health and also for the health of their future children given the important role of epigenetic regulation during early development. Epigenetic effects can influence offspring's health outcomes, especially if introduced during the critical periods associated with epigenetic reprogramming in early embryonic life.

5.2 Public Health Relevance of ART-related Epigenetic Changes

Reproductive health is extremely important to study as infertility is steadily increasing in the United States and worldwide [103]. Infertility can have other health impacts on those affected, causing depression, emotional distress, and social stigma. People dealing with infertility often seek ARTs to help them conceive a child and extend their families.

Additional research on the ART processes is important to make sure that patients and their future children are going through the safest procedures possible. We discussed that multiple factors

such as oxygen concentration and the type of culture media used are very important in creating viable, healthy embryos. Further research can render these procedures even safer, with fewer complications in children conceived using ART as they age. Additionally, since there has been a shift for people to have children later in life, more women are freezing their eggs, and researchers must ensure that ART processes like cryopreservation are safe for both the gametes and embryos. The increased use of ARTs has also increased the concerns about the negative health outcomes in the offspring that are believed to result from the epigenetic changes induced during those processes. Because the first ART-created child was born in 1978, making them about 46 years old today, we have yet to see if there are any related health impacts later in life. It is therefore important to continue to follow this population and determine if their conception process adds to their overall risk of health conditions like cancer later in life. A recent study reported an increased risk of childhood cancers among ART-conceived children, and it remains to be determined whether adult cancers will also be more common in this population [104]. We discussed that ART with fresh embryo transfers can contribute to low birth weight and preterm delivery, which may be related to ART-induced epigenetic changes [80]. Children with a low birth weight can have a harder time fighting off infections and gaining weight which may negatively impact their mental and physical development. Low birth weight and preterm birth are also associated with neonatal mortality [105]. On the other hand, based on the study of frozen and thawed bovine embryos, ART-induced epigenetic changes in imprinted genes can have the reverse effect in some and contribute to Large Offspring Syndrome (LOS) and related health issues [80, 86]. Additional research should be done with fresh and frozen embryos to determine the safety of ART procedures and understand the related epigenetic changes, which can then guide the measures to be put in place to make these procedures safer with fewer adverse effects and outcomes.

Furthermore, ART has also the potential to affect the mothers by increasing the risk of preeclampsia, which should also be considered in future research [106]. Although this increase in preeclampsia may or may not be related to epigenetic effects, it can be seen as a confounding factor, affecting both the mother's and child's health. The more difficulties the mother deals with during pregnancy, the more likely the baby is to experience adverse health outcomes. Therefore, mothers looking to use ART should be informed about the increased risks of preeclampsia and the increased risks of potential epigenetic effects on their offspring.

ART-induced epigenetic errors and imprinting disorders are relevant to public health because they can create adverse health effects, especially in offspring. The public should be aware of the extent to which ARTs can induce such changes, how likely they are to become permanent and lead to pathological conditions, the factors contributing to those epigenetic changes and resulting conditions, and the potential ways to limit the negative health outcomes. In addition to potentially contributing to low birth weight and imprinted disorders, ART-induced epigenetic changes have also been suspected to increase the risk for other non-communicable diseases such as cardiovascular and metabolic diseases and neurodevelopmental disorders [107]. ART-related epigenetic changes can also cause placental abnormalities and lead to abnormal nutrient and molecular exchange, which could be another mechanism contributing to these disorders becoming more prevalent in the ART-conceived population. ART-conceived individuals should be aware of these potential effects on their health that need to be monitored closely.

Upon further and larger studies in humans that will help to generate consistent results and establish ART-induced and health-relevant epigenetic changes/mechanisms, the field can move forward with the development of necessary preventive measures or potential treatments such as the Epi-drugs. Epi-drugs are chemical compounds that can alter the gene expression processes

through their effects on DNA methylation, histone modifications, and chromatin structure as well as on ncRNA-mediated mechanisms [108]. Epi-drugs are a newer development and are currently actively studied in various fields, however, they have already been increasingly used for therapy in the cancer field [108].

6.0 Gaps in Current Knowledge and Future Directions

Many of the ART processes have been studied and shown to produce adverse epigenetic effects, especially in animal studies. The results from human studies are relatively less consistent and warrant further and larger studies. Moreover, most studies have focused on changes in DNA methylation while other aspects of epigenetic regulation remain significantly understudied. Therefore, there is still much research to be done regarding the extent to which ARTs can induce epigenetic changes and whether/how these epigenetic effects can affect children's health over time. Epigenetics, being an emerging field, has been left open for false interpretation by the public. Much is still unknown about what factors can cause the epigenetic effects, when exactly these modifications take place, and to what extent they may be inherited.

An additional gap in the research is related to minimal work being done with eggs. The reproductive research present is mainly done with sperm, due to its ease of being collected and studied. Eggs are limited in numbers, and harder to extract and study. However, this is a significant lack of knowledge in the reproductive research realm, since women are just as important in the reproductive landscape.

Another significant limitation is the newness of ART processes and procedures. Currently, they cannot be evaluated for long-term consequences because there is not yet a large older population of ART-conceived individuals.

Bibliography

1. Jin, B., Y. Li, and K.D. Robertson, *DNA methylation: superior or subordinate in the epigenetic hierarchy?* Genes Cancer, 2011. **2**(6): p. 607-17.

2. Gatsiou, A. and K. Stellos, *RNA modifications in cardiovascular health and disease.* Nat Rev Cardiol, 2023. **20**(5): p. 325-346.

3. Wang, K., et al., *Epigenetic regulation of aging: implications for interventions of aging and diseases.* Signal Transduct Target Ther, 2022. **7**(1): p. 374.

4. Erdogan, K., N.T. Sanlier, and N. Sanlier, *Are epigenetic mechanisms and nutrition effective in male and female infertility?* J Nutr Sci, 2023. **12**: p. e103.

5. Heard, E. and R.A. Martienssen, *Transgenerational epigenetic inheritance: myths and mechanisms.* Cell, 2014. **157**(1): p. 95-109.

6. Mc Auley, M.T., *An evolutionary perspective of lifespan and epigenetic inheritance.* Exp Gerontol, 2023. **179**: p. 112256.

7. Messerschmidt, D.M., B.B. Knowles, and D. Solter, *DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos.* Genes Dev, 2014. **28**(8): p. 812-28.

8. Safi-Stibler, S. and A. Gabory, *Epigenetics and the Developmental Origins of Health and Disease: Parental environment signalling to the epigenome, critical time windows and sculpting the adult phenotype.* Semin Cell Dev Biol, 2020. **97**: p. 172-180.

9. Lynch, F., et al., *Epigenetics and DOHaD: how translation to predictive testing will require a better public understanding.* J Dev Orig Health Dis, 2022. **13**(4): p. 424-430.

10. Azoulay, L., C. Bouvattier, and S. Christin-Maitre, *Impact of intra-uterine life on future health.* Ann Endocrinol (Paris), 2022. **83**(1): p. 54-58.

11. Sant, K.E., M.S. Nahar, and D.C. Dolinoy, *DNA methylation screening and analysis.* Methods Mol Biol, 2012. **889**: p. 385-406.

12. Irvine, R.A., I.G. Lin, and C.L. Hsieh, *DNA methylation has a local effect on transcription and histone acetylation.* Mol Cell Biol, 2002. **22**(19): p. 6689-96.

13. Farsetti, A., B. Illi, and C. Gaetano, *How epigenetics impacts on human diseases.* Eur J Intern Med, 2023. **114**: p. 15-22.

14. Li, X., et al., *Histones: The critical players in innate immunity.* Front Immunol, 2022. **13**: p. 1030610.

15. Alaskhar Alhamwe, B., et al., *Histone modifications and their role in epigenetics of atopy and allergic diseases.* Allergy Asthma Clin Immunol, 2018. **14**: p. 39.

16. Jiang, C. and B.F. Pugh, *Nucleosome positioning and gene regulation: advances through genomics.* Nat Rev Genet, 2009. **10**(3): p. 161-72.

17. Jansen, A. and K.J. Verstrepen, *Nucleosome positioning in Saccharomyces cerevisiae.* Microbiol Mol Biol Rev, 2011. **75**(2): p. 301-20.

18. Kaikkonen, M.U., M.T. Lam, and C.K. Glass, *Non-coding RNAs as regulators of gene expression and epigenetics.* Cardiovasc Res, 2011. **90**(3): p. 430-40.

19. Catalanotto, C., C. Cogoni, and G. Zardo, *MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions.* Int J Mol Sci, 2016. **17**(10).

20. Nemeth, K., et al., *Non-coding RNAs in disease: from mechanisms to therapeutics.* Nat Rev Genet, 2024. **25**(3): p. 211-232.

21. Statello, L., et al., *Gene regulation by long non-coding RNAs and its biological functions.* Nat Rev Mol Cell Biol, 2021. **22**(2): p. 96-118.

22. Tang, Y., et al., *The role of long non-coding RNAs in rheumatic diseases.* Nat Rev Rheumatol, 2017. **13**(11): p. 657-669.

23. Boulias, K. and E.L. Greer, *Biological roles of adenine methylation in RNA.* Nat Rev Genet, 2023. **24**(3): p. 143-160.

24. Bajrami, E. and M. Spiroski, *Genomic Imprinting.* Open Access Maced J Med Sci, 2016. **4**(1): p. 181-4.

25. Eggermann, T., et al., *Imprinting disorders.* Nat Rev Dis Primers, 2023. **9**(1): p. 33.

26. Eggermann, T., et al., *Imprinting disorders: a group of congenital disorders with overlapping patterns of molecular changes affecting imprinted loci.* Clin Epigenetics, 2015. **7**: p. 123.

27. Butler, A.A., W.M. Webb, and F.D. Lubin, *Regulatory RNAs and control of epigenetic mechanisms: expectations for cognition and cognitive dysfunction.* Epigenomics, 2016. **8**(1): p. 135-51.

28. Moosavi, A. and A. Motevalizadeh Ardekani, *Role of Epigenetics in Biology and Human Diseases.* Iran Biomed J, 2016. **20**(5): p. 246-58.

29. Rhon-Calderon, E.A., et al., *The effects of Assisted Reproductive Technologies on genomic imprinting in the placenta.* Placenta, 2019. **84**: p. 37-43.

30. Larose, H., et al., *Gametogenesis: A journey from inception to conception.* Curr Top Dev Biol, 2019. **132**: p. 257-310.

31. Herrick, E.J. and B. Bordoni, *Embryology, Placenta*, in *StatPearls*. 2024: Treasure Island (FL).

32. Xu, B., et al., *Oocyte quality is decreased in women with minimal or mild endometriosis.* Sci Rep, 2015. **5**: p. 10779.

33. Carson, S.A. and A.N. Kallen, *Diagnosis and Management of Infertility: A Review.* JAMA, 2021. **326**(1): p. 65-76.

34. Farquhar, C., et al., *Management of ovarian stimulation for IVF: narrative review of evidence provided for World Health Organization guidance.* Reprod Biomed Online, 2017. **35**(1): p. 3-16.

35. Schorsch, M., et al., *Success Rate of Inseminations Dependent on Maternal Age? An Analysis of 4246 Insemination Cycles.* Geburtshilfe Frauenheilkd, 2013. **73**(8): p. 808-811.

36. Practice Committees of the American Society for Reproductive, M. and a.a.o. the Society for Assisted Reproductive Technology. Electronic address, *Intracytoplasmic sperm injection (ICSI) for non-male factor indications: a committee opinion.* Fertil Steril, 2020. **114**(2): p. 239-245.

37. Balli, M., et al., *Opportunities and Limits of Conventional IVF versus ICSI: It Is Time to Come off the Fence.* J Clin Med, 2022. **11**(19).

38. Haddad, M., et al., *Thoughts on the popularity of ICSI.* J Assist Reprod Genet, 2021. **38**(1): p. 101-123.

39. Asplund, K., *Use of in vitro fertilization-ethical issues.* Ups J Med Sci, 2020. **125**(2): p. 192-199.

40. Estudillo, E., et al., *Cryopreservation of Gametes and Embryos and Their Molecular Changes.* Int J Mol Sci, 2021. **22**(19).

41. Doody, K.J., *Infertility Treatment Now and in the Future.* Obstet Gynecol Clin North Am, 2021. **48**(4): p. 801-812.

42. Brown, J. and C. Farquhar, *Clomiphene and other antioestrogens for ovulation induction in polycystic ovarian syndrome.* Cochrane Database Syst Rev, 2016. **12**(12): p. CD002249.

43. Peyser, A., et al., *The evolving landscape of donor egg treatment: success, women's choice, and anonymity.* J Assist Reprod Genet, 2021. **38**(9): p. 2327-2332.

44. Tsai, S., et al., *Surrogacy Laws in the United States: What Obstetrician-Gynecologists Need to Know.* Obstet Gynecol, 2020. **135**(3): p. 717-722.

45. Fauser, B., et al., *Beliefs, attitudes and funding of assisted reproductive technology: Public perception of over 6,000 respondents from 6 European countries.* PLoS One, 2019. **14**(1): p. e0211150.

46. Sciorio, R., et al., *Risk of genetic and epigenetic alteration in children conceived following ART: Is it time to return to nature whenever possible?* Clin Genet, 2023. **103**(2): p. 133-145.

47. Carlsen, E.O., et al., *An examination of mediation by DNA methylation on birthweight differences induced by assisted reproductive technologies.* Clin Epigenetics, 2022. **14**(1): p. 151.

48. Schaub, A.M., et al., *A systematic review of genome-wide analyses of methylation changes associated with assisted reproductive technologies in various tissues.* Fertil Steril, 2024. **121**(1): p. 80-94.

49. Rahimi, S., et al., *Capturing sex-specific and hypofertility-linked effects of assisted reproductive technologies on the cord blood DNA methylome.* Clin Epigenetics, 2023. **15**(1): p. 82.

50. Chen, H., et al., *Advantages of vitrification preservation in assisted reproduction and potential influences on imprinted genes.* Clin Epigenetics, 2022. **14**(1): p. 141.

51. Zeng, Z., et al., *The Association between Assisted Reproductive Technologies and Neurodevelopmental Disorders in Offspring: An Overview of Current Evidence.* J Integr Neurosci, 2024. **23**(1): p. 15.

52. Sciorio, R. and N. El Hajj, *Epigenetic Risks of Medically Assisted Reproduction.* J Clin Med, 2022. **11**(8).

53. Graham, M.E., et al., *Assisted reproductive technology: Short- and long-term outcomes.* Dev Med Child Neurol, 2023. **65**(1): p. 38-49.

54. Menezo, Y., et al., *Biochemical Hazards during Three Phases of Assisted Reproductive Technology: Repercussions Associated with Epigenesis and Imprinting.* Int J Mol Sci, 2022. **23**(16).

55. Kleijkers, S.H., et al., *Differences in gene expression profiles between human preimplantation embryos cultured in two different IVF culture media.* Hum Reprod, 2015. **30**(10): p. 2303-11.

56. Menezo, Y. and K. Elder, *Epigenetic remodeling of chromatin in human ART: addressing deficiencies in culture media.* J Assist Reprod Genet, 2020. **37**(8): p. 1781-1788.

57. Koeck, R.M., et al., *At age 9, the methylome of assisted reproductive technology children that underwent embryo culture in different media is not significantly different on a genome-wide scale.* Hum Reprod, 2022. **37**(11): p. 2709-2721.

58. Ducreux, B., et al., *Genome-Wide Analysis of DNA Methylation in Buccal Cells of Children Conceived through IVF and ICSI.* Genes (Basel), 2021. **12**(12).

59. Barberet, J., et al., *Do assisted reproductive technologies and in vitro embryo culture influence the epigenetic control of imprinted genes and transposable elements in children?* Hum Reprod, 2021. **36**(2): p. 479-492.

60. Market-Velker, B.A., A.D. Fernandes, and M.R. Mann, *Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance.* Biol Reprod, 2010. **83**(6): p. 938-50.

61. Van Montfoort, A.P.A., et al., *Reduced oxygen concentration during human IVF culture improves embryo utilization and cumulative pregnancy rates per cycle.* Hum Reprod Open, 2020. **2020**(1): p. hoz036.

62. Mani, S., et al., *Epigenetic changes and assisted reproductive technologies.* Epigenetics, 2020. **15**(1-2): p. 12-25.

63. Gaspar, R.C., et al., *Oxygen tension affects histone remodeling of in vitro-produced embryos in a bovine model.* Theriogenology, 2015. **83**(9): p. 1408-15.

64. Marcho, C., W. Cui, and J. Mager, *Epigenetic dynamics during preimplantation development.* Reproduction, 2015. **150**(3): p. R109-20.

65. Selvaraj, V., et al., *Switching cell fate: the remarkable rise of induced pluripotent stem cells and lineage reprogramming technologies.* Trends Biotechnol, 2010. **28**(4): p. 214-23.

66. Choux, C., et al., *The placenta: phenotypic and epigenetic modifications induced by Assisted Reproductive Technologies throughout pregnancy.* Clin Epigenetics, 2015. **7**(1): p. 87.

67. Moser, G., et al., *Human trophoblast invasion: new and unexpected routes and functions.* Histochem Cell Biol, 2018. **150**(4): p. 361-370.

68. Xiang, M., et al., *Placental diseases associated with assisted reproductive technology.* Reprod Biol, 2021. **21**(2): p. 100505.

69. Rahnama, F., et al., *Epigenetic regulation of human trophoblastic cell migration and invasion.* Endocrinology, 2006. **147**(11): p. 5275-83.

70. Romanowska, J., et al., *The X-factor in ART: does the use of assisted reproductive technologies influence DNA methylation on the X chromosome?* Hum Genomics, 2023. **17**(1): p. 35.

71. Dolinko, A.V., et al., *Disrupted methylation patterns at birth persist in early childhood: a prospective cohort analysis.* Clin Epigenetics, 2022. **14**(1): p. 129.

72. Haberg, S.E., et al., *DNA methylation in newborns conceived by assisted reproductive technology.* Nat Commun, 2022. **13**(1): p. 1896.

73. Sundrani, D.P. and S.R. Joshi, *Assisted reproductive technology (ART) and epigenetic modifications in the placenta.* Hum Fertil (Camb), 2023. **26**(3): p. 665-677.

74. Liu, Z., et al., *Whole-Genome Methylation Analysis Revealed ART-Specific DNA Methylation Pattern of Neuro- and Immune-System Pathways in Chinese Human Neonates.* Front Genet, 2021. **12**: p. 696840.

75. Caramaschi, D., et al., *Association of medically assisted reproduction with offspring cord blood DNA methylation across cohorts.* Hum Reprod, 2021. **36**(8): p. 2403-2413.

76. Penova-Veselinovic, B., et al., *DNA methylation patterns within whole blood of adolescents born from assisted reproductive technology are not different from adolescents born from natural conception.* Hum Reprod, 2021. **36**(7): p. 2035-2049.

77. Barberet, J., et al., *DNA methylation profiles after ART during human lifespan: a systematic review and meta-analysis.* Hum Reprod Update, 2022. **28**(5): p. 629-655.

78. Huang, C., et al., *Long-term storage modifies the microRNA expression profile of cryopreserved human semen.* Biomol Biomed, 2024. **24**(1): p. 51-60.

79. Xu, X., et al., *Effect of Sperm Cryopreservation on miRNA Expression and Early Embryonic Development.* Front Cell Dev Biol, 2021. **9**: p. 749486.

80. Sciorio, R. and S.C. Esteves, *Contemporary Use of ICSI and Epigenetic Risks to Future Generations.* J Clin Med, 2022. **11**(8).

81. Zhang, G., et al., *Assisted reproductive technology and imprinting errors: analyzing underlying mechanisms from epigenetic regulation.* Hum Fertil (Camb), 2023. **26**(4): p. 864-878.

82. Sciorio, R., et al., *Exploring the effect of cryopreservation in assisted reproductive technology and potential epigenetic risk.* Zygote, 2023. **31**(5): p. 420-432.

83. Lloyd, D.T., et al., *Clomifene and Assisted Reproductive Technology in Humans Are Associated with Sex-Specific Offspring Epigenetic Alterations in Imprinted Control Regions.* Int J Mol Sci, 2022. **23**(18).

84. Horanszky, A., et al., *Epigenetic Mechanisms of ART-Related Imprinting Disorders: Lessons From iPSC and Mouse Models.* Genes (Basel), 2021. **12**(11).

85. Ochoa, E., *Alteration of Genomic Imprinting after Assisted Reproductive Technologies and Long-Term Health.* Life (Basel), 2021. **11**(8).

86. Chen, Z., et al., *Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction.* Proc Natl Acad Sci U S A, 2015. **112**(15): p. 4618-23.

87. Litzky, J.F. and C.J. Marsit, *Epigenetically regulated imprinted gene expression associated with IVF and infertility: possible influence of prenatal stress and depression.* J Assist Reprod Genet, 2019. **36**(7): p. 1299-1313.

88. Chen, X., et al., *Effects of superovulation, in vitro fertilization, and oocyte in vitro maturation on imprinted gene Grb10 in mouse blastocysts.* Arch Gynecol Obstet, 2018. **298**(6): p. 1219-1227.

89. Xie, T., et al., *PEG10 as an oncogene: expression regulatory mechanisms and role in tumor progression.* Cancer Cell Int, 2018. **18**: p. 112.

90. Ashapkin, V., et al., *Age-associated epigenetic changes in mammalian sperm: implications for offspring health and development.* Hum Reprod Update, 2023. **29**(1): p. 24-44.

91. Jenkins, T.G., et al., *Age-associated sperm DNA methylation alterations: possible implications in offspring disease susceptibility.* PLoS Genet, 2014. **10**(7): p. e1004458.

92. Colaco, S. and D. Sakkas, *Paternal factors contributing to embryo quality.* J Assist Reprod Genet, 2018. **35**(11): p. 1953-1968.

93. du Fosse, N.A., et al., *Advanced paternal age is associated with an increased risk of spontaneous miscarriage: a systematic review and meta-analysis.* Hum Reprod Update, 2020. **26**(5): p. 650-669.

94. Chango, A. and I.P. Pogribny, *Considering maternal dietary modulators for epigenetic regulation and programming of the fetal epigenome.* Nutrients, 2015. **7**(4): p. 2748-70.

95. Niculescu, M.D. and S.H. Zeisel, *Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline.* J Nutr, 2002. **132**(8 Suppl): p. 2333S-2335S.

96. Socha, M.W., W. Flis, and M. Wartega, *Epigenetic Genome Modifications during Pregnancy: The Impact of Essential Nutritional Supplements on DNA Methylation.* Nutrients, 2024. **16**(5).

97. Hardy, T.M. and T.O. Tollefsbol, *Epigenetic diet: impact on the epigenome and cancer.* Epigenomics, 2011. **3**(4): p. 503-18.

98. Cai, S., et al., *Nutritional Status Impacts Epigenetic Regulation in Early Embryo Development: A Scoping Review.* Adv Nutr, 2021. **12**(5): p. 1877-1892.

99. Zambrano, E., et al., *Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation.* J Physiol, 2005. **566**(Pt 1): p. 225-36.

100. Peral-Sanchez, I., et al., *Epigenetics in the Uterine Environment: How Maternal Diet and ART May Influence the Epigenome in the Offspring with Long-Term Health Consequences.* Genes (Basel), 2021. **13**(1).

101. Dudley, K.J., et al., *Offspring of mothers fed a high fat diet display hepatic cell cycle inhibition and associated changes in gene expression and DNA methylation.* PLoS One, 2011. **6**(7): p. e21662.

102. Rahimi, S., et al., *Moderate maternal folic acid supplementation ameliorates adverse embryonic and epigenetic outcomes associated with assisted reproduction in a mouse model.* Hum Reprod, 2019. **34**(5): p. 851-862.

103. Sadecki, E., et al., *Fertility trends and comparisons in a historical cohort of US women with primary infertility.* Reprod Health, 2022. **19**(1): p. 13.

104. Weng, S.S., et al., *Assisted Reproductive Technology and Risk of Childhood Cancers.* JAMA Netw Open, 2022. **5**(8): p. e2230157.

105. Jana, A., et al., *Relationship between low birth weight and infant mortality: evidence from National Family Health Survey 2019-21, India.* Arch Public Health, 2023. **81**(1): p. 28.

106. Kornfield, M.S., S.B. Gurley, and L.A. Vrooman, *Increased Risk of Preeclampsia with Assisted Reproductive Technologies.* Curr Hypertens Rep, 2023. **25**(9): p. 251-261.

107. Li, J., et al., *Cardiovascular health of offspring conceived by assisted reproduction technology: a comprehensive review.* Front Cardiovasc Med, 2024. **11**: p. 1287060.

108. Yang, T., Y. Yang, and Y. Wang, *Predictive biomarkers and potential drug combinations of epi-drugs in cancer therapy.* Clin Epigenetics, 2021. **13**(1): p. 113.