DNA METHYLATION AT IMPRINTED AND NON-IMPRINTED GENES IN THE SPERM OF MEN AFFECTED BY SEVERE MALE FACTOR INFERTILITY

by

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ABSTRACT

Abnormal DNA methylation at imprinted and non-imprinted genes has been associated with spermatogenesis failure. However, little information is available regarding DNA methylation at those genes in men affected by severe male factor infertility. We hypothesized a higher incidence of aberrant DNA methylation would be present in the ejaculate and testicular sperm of men affected by severe male factor infertility compared to that in fertile control men. Furthermore, we hypothesized abnormal DNA methylation would also affect non-imprinted genes in the sperm of men affected by severe oligozoospermia.

DNA methylation at the differentially methylated regions (DMRs) of imprinted genes, *H19*, *IG-GTL2* and *MEST,* was studied in the ejaculate sperm of men affected by severe and very severe oligozoospermia, in the testicular sperm of men affected by obstructive azoospermia (OA) and non-obstructive azoospermia (NOA), and having undergone vasectomy reversal. The results were compared to that in the sperm of control men of proven fertility. Methylation at the DMRs was evaluated by bisulphite sequencing of multiple unique clones, representative of single sperm. DNA methylation was also studied at non-imprinted genes in sperm of men affected by severe and very severe oligozoospermia. DNA methylation was analyzed at 1,505 CpG sites using the Illumina GoldenGate methylation Cancer Panel I with the results at selected CpG sites being confirmed using pyrosequencing.

We found the *H19* DMR to be most susceptible to methylation abnormalities and the *IG-GTL2* DMR to be the most robust. We found a higher incidence of aberrant DNA methylation in the sperm of men affected by severe oligozoospermia, OA and in men undergoing vasectomy reversal compared to control men. The presence of aberrant imprinting in men with obstruction suggests that abnormal methylation at imprinted genes may not only be related to spermatogenesis failure, as seen in patients affected by severe oligozoospermia, but also to changes in testicular environment that may occur in response to obstruction. Lastly, our analysis of a limited number of samples suggests that abnormal DNA methylation in the sperm of men affected by severe oligozoospermia may also affect non-imprinted genes. Our results warrant further analysis of a larger sample size.

PREFACE

The experiments in Chapter 2 were conceived of by Dr. Sai Ma and Agata Minor. The experiments, data analysis, figures and tables were performed and prepared by Agata Minor. Dr. Victor Chow provided some of the samples analyzed and clinical data for the patients. A version of Chapter 2 has been submitted for publication. Minor A, Chow A and Ma S (2010). Evaluation of DNA methylation at imprinted genes in the sperm of men with severe male factor infertility. The manuscript was written by Agata Minor with guidance from Dr. Sai Ma. The experiments presented in Chapter 3 were conceived of by Dr. Sai Ma and Agata Minor. The experiments, data analysis, figures and tables were performed and prepared by Agata Minor. The experiments presented in Chapter 4 were conceived of by Dr. Sai Ma and Agata Minor. The experiments, data analysis, figures and tables were performed and prepared by Agata Minor. Dr. Victor Chow performed testicular biopsies and provided clinical data for the patients. The manuscript was written by Agata Minor with guidance from Dr. Sai Ma. A version of Chapter 4 has been submitted for publication. Minor A, Chow A and Ma S (2010). Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with azoospermia.

Ethical approval for the experiments presented was obtained from the University of British Columbia Clinical Research Ethics Board and from the UBC and C&W Research Ethics Board (certificate number H06-03547).

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LIST OF ABBREVIATIONS

In this thesis genes are named following rules established by the Human Genome Organization (HUGO). Names of genes in humans are reported using all capital letters, while names of genes in mice have the first letter capitalized. Genes or RNA, either human or mouse, are indicated by italicized letters, while proteins are indicated by non-italicized letters.

ABP; androgen binding protein
ART; assisted reproductive technologies
AS; Angelman syndrome
AZF; azoospermia factor
ASO; allele specific oligonucleotide
BORIS; brother of the regulator of imprinted sites
BWS; Beckwith Wiedemann syndrome
CBAVD; congenital bilateral absence of vas deferens
CFTR; Cystic fibrosis transmembrane regulator
COBRA; combined bisulphite restriction analysis
CTCF; CCCCTC binding factor
CTCFL; CTCF-like
DES; estrogen diethylstilbestrol
DLK1; delta, Drosophila, homolog-like 1
DMR; differentially methylated region
DNA; deoxyribonucleic acid
DNMT; DNA methyltransferase
FSH; follicle stimulating hormone
GnRH; gonadotropin releasing hormone
GTL2; gene trap locus 2
GV; germinal vesicle
HDAC; histone deacetylase
hCG; human chorionic gonadotropin
hMG; human menopausal gonadotropin

HPA; hypothalamic-pituitary-adrenal

IAP; intracisternal A particle

ICF; immunodeficiency, centromere instability and facial anomalies

ICR; imprinting control region

ICSI; intracytoplasmic sperm injection

IG; intragenic

IGF2; insulin-like growth factor 2

IVF; *in vitro* fertilization

IPTG; Isopropyl β-D-1 thiogalactopyranoside

IUGR; intrauterine growth restriction

KSMO; potassium simplex optimized medium

LH; leutinizing hormone

LINE; long interspersed transposable element

LOS; large offspring syndrome

LSO; locus-specific oligonucleotide

MI; Meiosis I

MBD; methyl-CpG domain-binding domain

MeCP2; methyl-CpG binding protein 2

MEST; mesoderm-specific transcript

MSP; methyl sensitive PCR

MTHFR; 5,10-methylenetetrahydrofolate reductase

NOA; non-obstructive azoospermia

OA; obstructive azoospermia

OAT; Oligoastenoteratozoospermia

PCR; polymerase chain reaction

PGC; primordial germ cell

ROS; reactive oxygen species

SAM; S-adenosyl-L-methionine

SCOS; Sertoli cell only syndrome

SINE; short interspersed transposable element

SNP; single nucleotide polymorphism

SRS; Silver Russel syndrome

SRY; sex-determining region Y

STS; single tagged site

TRD; transcriptional repression domain

UPD; uniparental disomy

WHO; World Health Organization

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CHAPTER 1: INTRODUCTION

Infertility, defined as the inability to achieve pregnancy within one year of unprotected intercourse, affects an estimated 15% of couples today. Male factor infertility is identified in roughly half of the couples undergoing evaluation for infertility. While there are well known factors that contribute to male factor infertility, the etiology remains unknown in 50% of cases. Recently an association between spermatogenesis failure seen in male infertility and aberrant DNA methylation at imprinted genes has been suggested (Marques et al., 2008; Kobayashi et al., 2007; Poplinski et al., 2009). DNA methylation is a chemical modification of DNA that marks the parental alleles, establishing parent-specific gene expression of imprinted genes. However, limited information is available regarding DNA methylation at imprinted genes in severe male factor infertility. Men affected by severe male factor infertility can still contribute to pregnancy through the use of intracytoplasmic sperm injection (ICSI), where a single sperm can be used to achieve pregnancy. This thesis will try to assess the role DNA methylation, primarily at imprinted genes, plays in severe male factor infertility, including severe and very severe oligozoospermia, and obstructive and non-obstructive azoospermia. Possible causes as well as consequences of abnormal DNA methylation at imprinted genes in the sperm retrieved from infertile men will also be discussed.

1.1 SPERMATOGENESIS

Gametogenesis is a process by which haploid cells capable of fertilization are created from diploid cells. Gametogenesis initiates with the appearance of primoridal gem cells (PGCs) in the yolk sac about 24 days post fertilization. PGCs migrate through the dorsal mesentery to the gonadal ridge (Sadler, 2006). At this stage in development, cells that line the gonadal ridge can give rise to either the female or male gonads. It is the expression of the sex-determining region Y (SRY) gene in the pre-Sertoli cells that begins the differentiation of the male gonads (Sadler, 2006). Sertoli cells and Leydig cells are two types of diploid cells that have a supporting function in spermatogenesis. Sertoli cells are located within the seminiferous tubules, while Leydig cells lie between the tubules. Early in development Sertoli cells secrete anti-Müllerian hormone which inhibits the development of the Müllerian duct. As a result the female gonads do not develop and the adjoining Wolffian ducts give rise to the male

reproductive tract including the vas efferens, the epididymis, the vas deferens, the ejaculatory ducts and the seminal vesicles (Bullock et al., 2001). Leydig cells secrete testosterone, initiating sexual differentiation of internal and external male genitalia (Sadler, 2006). Testosterone has an androgenic effect on external genitalia in its reduced dihydrotestosterone form (Bullock et al., 2001). After puberty, Sertoli and Leydig cells are involved in the hormonal control of spermatogenesis.

Spermatogenesis is the process through which diploid spermatogonia divide and differentiate into haploid spermatids, a process that takes approximately 74 days (Sadler, 2006). Spermatogenesis occurs in seminiferous tubules where PGCs differentiate into spermatogonial stem cells: spermatogonia type A dark. Spermatogonia type A dark enter mitotic arrest and cell division is resumed at puberty. Spermatogonia type A dark can undergo cell division to give rise to a larger population of spermatogonia type A dark or they can differentiate into spermatogonia type A pale and spermatogonia type B (Figure 1.1). Spermatogonia type B are committed to giving rise to primary spermatocytes that enter meiosis. Meiosis consists of two rounds of specialized cell divisions, meiosis I and meiosis II, that reduce the number of chromosomes to a haploid complement. In the first meiotic division primary spermatocytes duplicate their DNA content and give rise to secondary spermatocytes (Clermont, 1972). During prophase of the first meiotic division, chromosomes start to condense during leptotene and double strand breaks start to form; these will be the sites of recombination. At zygotene, sister chromatids start pairing and forming the synaptonemal complex. Synapsis is complete at pachytene when recombination occurs. This is also the stage when the XY body is formed and undergoes silencing (Handel et al., 2004). At diplotene, chromosomes start to separate. Prophase is followed by metaphase, anaphase and telophase to give rise to two secondary spermatocytes per every primary spermatocyte. There is a cell division at metaphase that gives rise to secondary spermatocytes. In the second meiotic division the secondary spermatocytes divide and become round spermatids (Figure 1.1). In the end, four haploid spermatids are generated for every spermatocyte (Cobb and Handel, 1998; Clermont 1972).

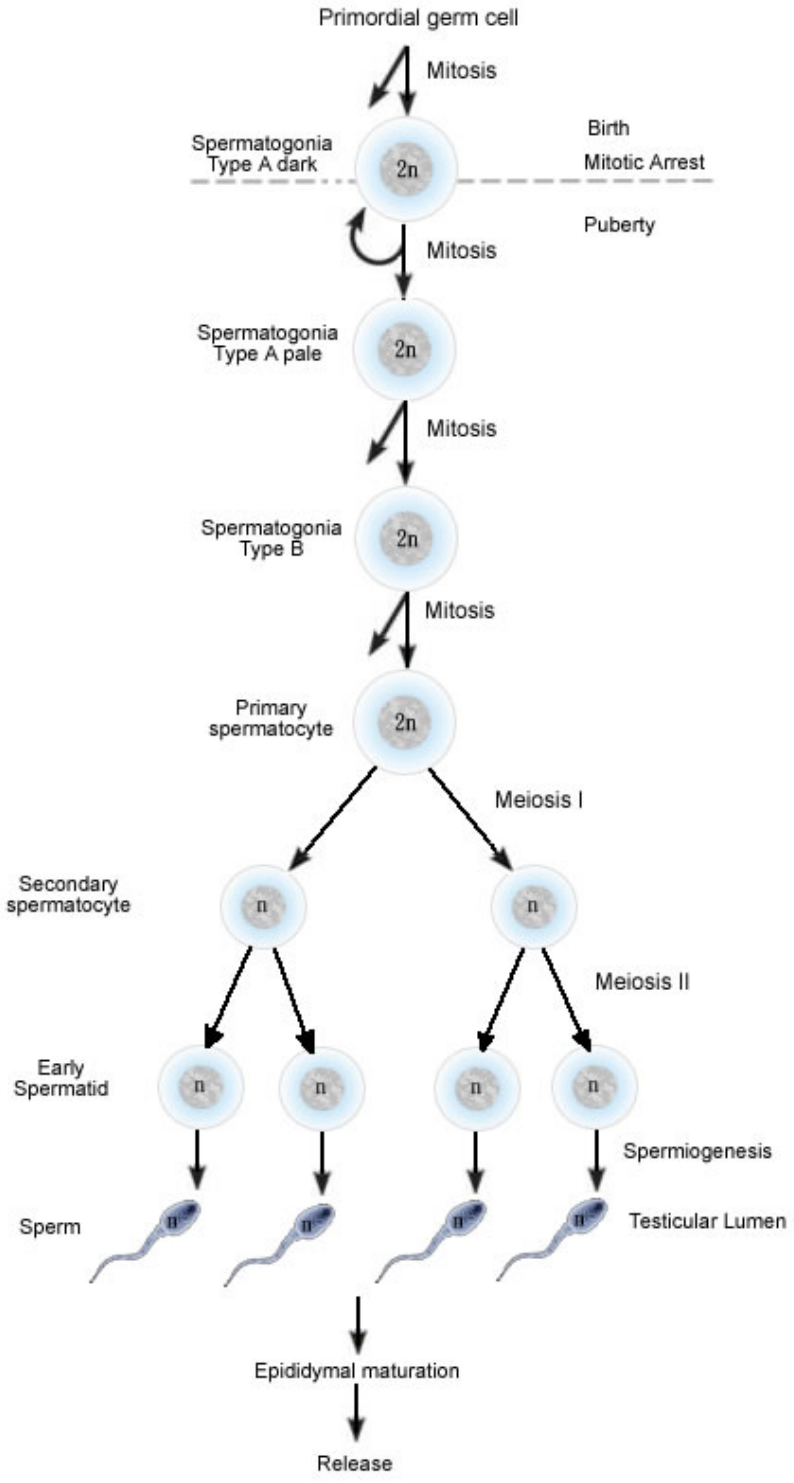


Figure 1.1 Spermatogenesis. Diploid germ cells first undergo mitosis to establish a germ cell population and then meiosis to give rise to haploid male germ cells. Sperm are then released into the lumen and are transported to the epididymis to undergo final maturation. They are then ready to be released into the ejaculate.

After the completion of meiosis, the haploid cells undergo spermiogenesis. Spermiogenesis is characterized by the development of the acrosome cap, the flagellum, shedding of excess cytoplasm and chromatin compaction so that elongated spermatozoa can form (Holstein et al. 2003). Histones undergo a progressive replacement, first to transition proteins then to protamines, although some histones may be retained (Gusse et al., 1986; Vu et al., 2004; Delaval et al., 2007). The ratio of protamine 1 to protamine 2 is around one in fertile men (Aoki et al., 2005; Oliva, 2006). An altered protamine 1 to protamine 2 ratio has been associated with infertility and susceptibility of sperm DNA to damage (Oliva, 2006; Aoki et al., 2005). The transition process is facilitated through histone hyperacetylation in elongating spermatids, which opens up the chromatin structure allowing for the replacement to occur (Hazzouri et al., 2000). A decrease of histone acetylation has also been associated with infertility (Sonnack et al., 2002).

From type A spermatogonia to the spermatid cell stage, the differentiating cells are connected through cytoplasmic bridges due to incomplete cytokenesis allowing for the synchronization of spermatogenesis. Spermatogenesis progresses within the seminiferous tubules from the basal lamina toward the lumen. Following spermiogenesis, immature spermatozoa are released into the lumen and are transported to the epididymis where they acquire motility and the ability to fertilize an oocyte (Sadler, 2006). As part of the maturation process, some spermatogenesis-specific non-imprinted genes undergo changes in DNA methylation in the epididymis (Ariel et al., 1994). Around 12 to 21 days are required for spermatozoa to travel through the epididymis and the vas deferens to reach the ejaculatory duct (Bullock et al., 2005).

1.1.1 Hormonal control of spermatogenesis

Spermatogenesis is under hormonal control. The acruate neurons in the hypothalamus release gonadotropin-releasing hormone (GnRH) (Figure 1.2). The pulsatile release of GnRH stimulates the pituitary gland to synthesize luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH stimulates the Leydig cells to produce testosterone, and creates a negative feedback on the hypothalamus and the pituitary to control hormone release. The released testosterone binds Sertoli cells, stimulating spermatogenesis. FSH stimulates Sertoli cells to

produce testicular fluid androgen-binding protein (ABP). ABP binds testosterone and allows it to pass through the Sertoli junctions. FSH also stimulates Sertoli cells to synthesize activin and inhibin which stimulate and inhibit the production of GnRH, respectively, and the release of LH and FSH by the pituitary gland (Brehm and Klaus, 2005). Neighboring Sertoli cells are interconnected through junctions forming the blood-testis barrier and it is on the surface of Sertoli cells that spermatogenesis occurs. The close contact enables the Sertoli cells to nourish germ cells during spermatogenesis and expose them to hormonal control. Sertoli cells are also responsible for absorbing waste and abnormal germ cells, and secreting fluid that enables transport of the immature spermatozoa from the seminiferous tubules to the epididymis for the final stages of maturation (Sikka et al., 2005).

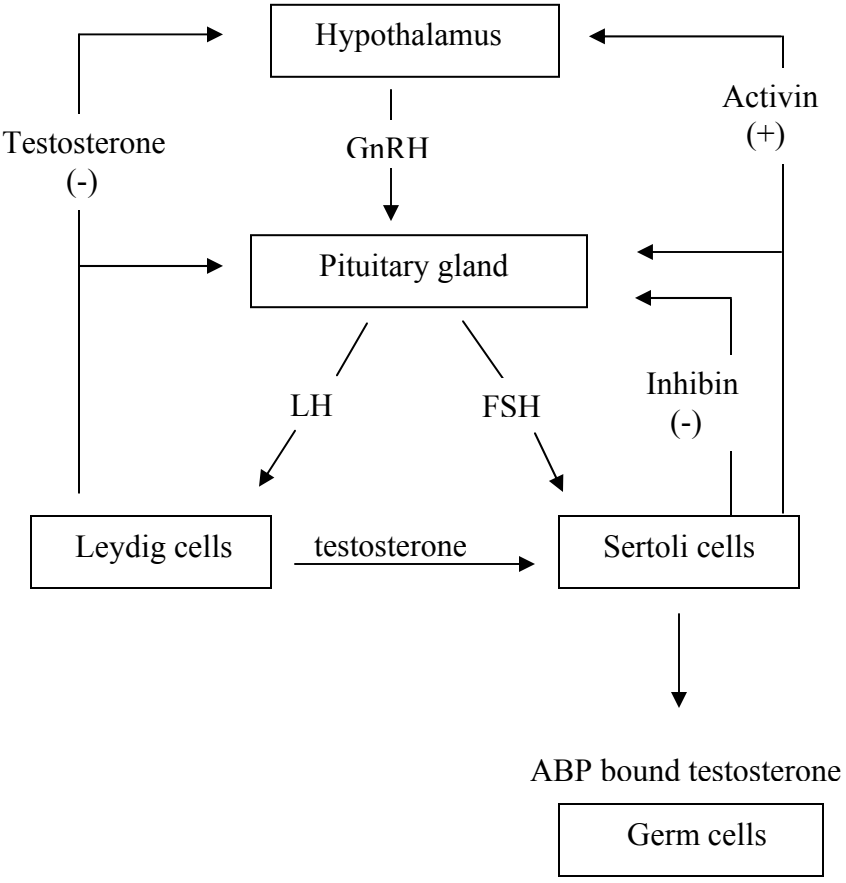


Figure 1.2 Hormonal control of spermatogenesis. The pituitary gland secretes LH and FSH hormones in response to GnRH release by the hypothalamus. LH and FSH control spermatogenesis by acting on Leyding cells and Sertoli cells and regulating androgen production. Spermatogenesis is in turn controlled through negative and positive feedback exerted by Leyding and Sertoli cells on the pituitary gland and the hypothalamus (Bullock et al., 2001; Halvorson and Chin, 1999).

1.2. MALE FACTOR INFERTILITY

Infertility is defined as the inability to conceive after one year of unprotected intercourse. About 15% of couples experience infertility, which may result from either male or female factors. In 35% of the cases infertility is due to female factor, 30% of cases are typically due to male factor alone, while 20% of cases are due to male and female factors. Male factor infertility is multifactorial and etiologies can be grouped into genetic and non-genetic. However, in about 50% of the cases the cause of infertility remains unknown (de la Calle et al., 2001). Male infertility is diagnosed based on sperm parameters.

1.2.1 Sperm parameters

The World Health Organization (WHO, 1999) has established criteria for evaluating semen parameters of infertile men based on sperm concentration, motility and morphology (Table 1.1). Oligozoospermia is diagnosed based on reduced sperm concentration, and based on severity can be further subdivided into moderate, severe and very severe (Table 1.1). Asthenozoospermia is diagnosed based on reduced sperm motility and teratozoospermia is diagnosed based on abnormal morphology. Oligoastenoteratozoospermia (OAT) is diagnosed when sperm concentration, motility and morphology are below normal semen parameters (Table 1.1). Patients without sperm in the ejaculate are diagnosed with azoospermia, but sperm in testicular tissue may be present. Azoospermia is divided into obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). Histological analysis of testicular tubules can help to differentiate between OA and NOA. Normal spermatogenesis in the tubules would be expected in OA patients. These patients have an obstruction in the urogenital tract that prevents transport of sperm to the ejaculate. NOA patients can have spermatogenesis but at reduced levels (hypospermatogenesis), spermatogenesis arrest at a specific cell type (maturation arrest) or they may have a complete absence of germ cells with only Sertoli cells being present (Sertoli-cell only syndrome; SCOS) (McLachlan et al., 2007). Severe male factor infertility refers to infertility due to a low sperm concentration, such as severe and very severe oligozoospermia, or due to an absence of sperm in the ejaculate, such as azoospermia.

Table 1.1 WHO criteria for diagnosis of semen parameters

Type of infertility	Semen Parameter		
	Concentration (10^6/ml)	Motility (%)	Normal Morphology (%)
Oligozoospermia	<20	Normal	Normal
Moderate	≥5 but <20	Normal	Normal
Severe	<5 but ≥1	Normal	Normal
Very severe	<1	Normal	Normal
Asthenozoospermia	Normal	<50	Normal
Teratozoospermia	Normal	Normal	<30
Oligoastenoteratozoospermia (OAT)	<20	<50	<30
Azoospermia		no sperm in ejaculate	
Aspermia		no ejaculate	
Normal sperm parameters	≥20	≥50	≥30

1.2.2 Etiologies of male factor infertility

Male factor infertility has been associated with non-genetic and genetic factors. Non-genetic factors of male infertility may include hormonal imbalances, undescended testis, acquired obstruction, varicocele, chronic illness, immunological factors and impotency, recreational drug use, chemotherapy and radiation. More recently exposure to environmental factors and chemicals are also thought to play a role in male infertility (Namiki, 2000) and will be discussed in a later section (section 1.5.1). Genetic factors of male factor infertility include somatic and sperm chromosome abnormalities, Y chromosome microdeletions and single gene mutations.

The incidence of somatic chromosome abnormalities in infertile men ranges between 2.2 to 8.6% (Antonelli et al., 2000). As many as 13.7% of azoospermic men and 4.6% of oligozoospermic men have a chromosome abnormality (Van Assche et al, 1996), compared to a rate of 0.38% in the general population. Anomalies involving the autosomes are more common in oligozoospermic men, and include chromosome translocations, chromosome inversions and chromosome markers (Van Assche et al., 1996; Antonelli et al., 2000; Peschka et al., 1999; Scholtes et al., 1998, Tuerlings et al., 1998). Sex-chromosome anomalies are more prevalent in azoospermic men (Van Assche et al., 1996) with the most common being 47,XXY (Van Assche et al., 1996; Peschka et al., 1999; Tuerlings et al., 1998). Men with somatic chromosome abnormalities are at a higher risk for generating chromosomally abnormal spermatozoa. However, infertile men with normal somatic chromosomes can also have chromosomally abnormal sperm. This association has been observed in relation to severe oligozoospermia

(Bernardini et al., 1997; Pang et al., 1999; Tang et al., 2004; Kirkpatrick et al., 2008), poor morphology and motility (Hristova et al., 2002; Templado et al., 2002; Tang et al., 2010), as well as azoospermia (Bernardini et al., 2000; Rodrigo et al., 2004). Asynapsis and reduced recombination during meiosis have been associated with increased rates of chromosome aneuploidy in infertile men (Ma et al., 2006; Ferguson et al., 2007). Use of aneuploid sperm to achieve pregnancy may result in a chromosome abnormality in the progeny (Moosani et al., 1999; Tang et al., 2004).

Microdeletions within the azoospermia factor (AZF) region have also been associated with male infertility. The AZF region has been mapped to three intervals on Yq11, designated as AZFa, AZFb and AZFc (Vogt et al., 1996). The rate of microdeletions can range between 11.5% and 25% in men affected by azoospermia and between 1.5% and 7.9% in men affected by oligozoospermia (Oliva et al., 1998; Krausz et al., 2001; Fujisawa et al., 2001). Deletions of the AZFa region are often associated with SCOS, deletions of the AZFb region are associated with spermatogenic arrest, while deletions of the AZFc region range in phenotypes from azoospermia to oligozoospermia (Vogt et al., 1996; Kamp et al. 2000b; Krausz et al., 2001). Diagnostic testing for Y chromosome microdeletions can be performed by analyzing sequence tagged sites (STSs) specific to each interval (Simoni et al., 2004; Minor et al., 2008), and is offered as part of routine infertility screening to infertile men at some fertility centers.

Finally, single gene mutations such as mutations in the Cystic fibrosis transmembrane regulator (CFTR) gene have been associated with male factor infertility. CFTR gene mutations are associated with congenital bilateral absence of the vas deferens (CBAVD). CBAVD occurs in 1-2% of infertile men that are not affected by cystic fibrosis (Blau et al., 2002), and may occur in up to 25% of men with OA (Vogt, 2004). Spermatogenesis in these men is usually normal but due to blockage sperm cannot reach the ejaculate.

1.2.3 Assisted reproductive technologies

The use of assisted reproductive technologies (ART) as treatment for infertility is responsible for up to 1% of births worldwide. One such technique is *in vitro* fertilization (IVF). IVF produced the first live birth in 1978 (Steptoe and Edwards, 1978). In this procedure, an oocyte is incubated with purified sperm in a petri dish in order to achieve fertilization. However,

IVF has not been successful in treating severe male factor infertility, as often not enough sperm are present for this procedure. Intracytoplasmic sperm injection (ISCI) has been specifically developed to treat male factor infertility and has been used since 1992. ICSI consists of the direct injection of a single immobilized sperm into an oocyte (Palermo et al., 1992; Ma and Ho Yuen, 2001). In cases where sperm in not available in the ejaculate, sperm for ICSI can be retrieved from the epididymis through microepididymal sperm aspiration, percutaneous epididymal sperm aspiration or from the testes through testicular sperm extraction or testicular sperm aspiration (ASRM, 2008). However, due to the bypass of natural fertilization barriers, the use of ICSI has been associated with negative pregnancy outcomes (Bonduelle et al., 2002; Hansen et al., 2005; Sutcliffe et al., 2001; Maher 2003; Debaun et al., 2003).

A number of steps are involved in IVF and ICSI, which include ovarian stimulation, egg maturation and embryo transfer. Ovarian stimulation induces the growth of multiple follicles so that multiple eggs can be retrieved. Ovarian stimulation can be achieved through the administration of hormones such as FSH or human menopausal gonadotropin (hMG). Once the follicles are ready for oocyte retrieval, oocyte maturation is stimulated by human chorionic gonadotropin (hCG), and oocytes are aspirated transvaginally under ultrasound guidance (ASRM, 2008). Fertilization is achieved either through the incubation of many sperm with the retrieved oocytes in IVF or by the direct injection of a sperm into an oocyte in ICSI. Fertilization is confirmed by the presence of two pronuclei. Embryos are then transferred into the uterus usually on day three or day five at the blastocyst stage. The transferred embryo will then hatch and implant into the uterine lining (ASRM, 2008).

Although the use of ART accounts for around one million births each year, procedures involved in ART present risks to the ART pregnancy. While there are known factors associated with abnormalities found in babies born through ART, aberrant DNA methylation may also contribute to the negative outcome of ART pregnancies. Higher rates of chromosome abnormalities have been reported in ART babies, detected during prenatal diagnosis (Gjerris et al., 2008; Kolibianakis et al., 2003; Bonduelle et al., 2002; Lam et al., 2001) and after birth (Bonduelle et al., 2002; Gjerris et al., 2008). The rate of chromosome abnormalities detected prenatally in IVF pregnancies is about half of what it is for ICSI (Gjerris et al., 2008). ICSI is mainly associated with a higher rate of *de novo* sex chromosome abnormalities and inherited

abnormalities, most of which have come from the father and may be related to errors in paternal meiosis (Bonduelle et al., 2002). A systemic review of ART pregnancy outcomes found that singleton ART pregnancies have a thirty to forty percent increased risk for birth defects compared to spontaneous births (Hansen et al., 2005). Abnormalities found most often in ART babies are of a neural, cardiac, renal, and genital nature (Merlob et al., 2005; Katalinic et al., 2004; Kallen et al., 2005).

Low birth weight and preterm birth have been consistently observed in IVF and ICSI singleton pregnancies (Sutcliffe et al., 2001; Merlob et al., 2005; Bonduelle et al., 2004; Katalinic et al., 2004; Stromberg et al., 2002). These may be associated with higher rates of surgical interventions and therapy in ICSI babies (Bonduelle et al., 2004). Low birth weight may be associated with adult onset disease (Barker et al., 1989; Barker, 1998; Gluckman et al., 2007). An increased risk for neuro-developmental problems was observed in IVF and ICSI babies, but may be due to twinning in some cases (Stromberg et al., 2002; Bowen et al., 1998). However, other studies failed to report the same risk in children born through ART at two (Sutcliffe et al., 2001) and five years of age (Leslie et al., 2003; Bonduelle et al., 2004). In addition, ART pregnancies may also be at a higher risk for complications such as placenta previa, pre-eclampsia, gestational hypertension and diabetes (Romunstad et al., 2006; Bonduelle et al., 2004; Katalinic et al., 2004). The risk for miscarriage for ICSI compared to IVF and spontaneous pregnancies has also been reported to be higher (Katalinic et al., 2004). In addition, a higher rate of imprinting syndromes has been reported in children born through ART (Sutcliffe et al., 2006; Maher 2003; Debaun et al., 2003). Imprinting syndromes are very rare disorders that are associated with abnormalities affecting one of the parental alleles and may involve DNA methylation. These will be discussed in more detail in a later section (section 1.4.1).

Although negative pregnancy outcomes have been associated with IVF and ICSI, the causative factors are largely unknown but may be procedure dependent. However, correction for maternal and paternal characteristics, such as maternal age and genetic background of the parents, diminished some risks associated with ART (Katalinic et al., 2004; Kallen et al., 2005), suggesting that risks may be associated with parental background.

1.3 GENOMIC IMPRINTING

Epigenetic modifications refer to modifications at the chromatin or DNA level that affect chromatin function without altering the genetic code. Epigenetic modifications are heritable, reversible and allow the epigenome to respond to environmental factors. Two more widely studied epigenetic modifications are histone modifications and DNA methylation. These two modifications modulate genomic imprinting; however, DNA methylation will primarily be discussed here.

1.3.1 DNA methylation

Chromatin consists of DNA packaged with histones into nucleosomes. Each nucleosome contains 146 base pairs of DNA wrapped around an octamer of core histones (Kouzarides, 2007). Nucleosomes are connected by linker histones, H1. Histone tails extend from the nucleosome and can be modified to modulate chromatin compaction (Bannister and Kouzarides, 2005). A relaxed chromatin structure is conducive to gene expression, while tightly packaged chromatin is associated with gene silencing (Jenuwein and Allis, 2001). Chromatin structure is further modulated by DNA methylation. In mammals, DNA methylation consists of the covalent addition of a methyl group at the 5 prime position of a cytosine located within CpG dinucleotides. The binding of methyl-CpG binding protein 2 (MeCP2) to methylated DNA initiates transcriptional silencing and the recruitment of histone deacetylases (HDACs) (Jones et al., 1998; Nan et al., 1998). MeCP2 is made up of two domains: the methyl-CpG domain-binding domain (MBD) and the transcriptional repression domain (TRD). MBD recognizes methylated CpGs in the nucleosome through contact with the major groove in the double helix. TRD interacts with other regulatory factors including the Sin3a adaptor protein. The interaction of TRD with regulatory factors is associated with repression of gene expression (Bird and Wolffe, 1999). Binding of MeCP2, DNA methylation and histone deacetylation are associated with the compaction of chromatin; chromatin changes related to suppressed transcription (Jones et al., 1998; Nan et al., 1998). MeCP2 can also inhibit transcription without conferring changes to chromatin structure by physically blocking access of basal transcriptional machinery to DNA (Bird and Wolffe, 1999). Most CpG dinucleotides can be found in CpG islands which are defined as stretches of DNA enriched in CpG dinucleotides (Bird et al., 1984). CpG islands are

usually located within or near promoters or the first exon of genes, and are associated with an open chromatin structure that contains mostly acetylated histones (Bird and Wolffe, 1999). These characteristics are consistent with active transcription and gene expression, therefore methylation at CpG islands influences the expression of genes.

Although about 70% of all CpG sites in the genome are methylated, most CpG islands are unmethylated, with the exception of those associated with imprinted genes or within the inactive X chromosome in females (Antequera and Bird, 1993). Around 90% of methylation in the genome occurs at repetitive sequences such as satellite DNA and parasitic elements such as long interspersed transposable elements (LINEs), short interspersed transposable elements (SINEs), endogenous retroviruses, and intracisternal A particle (IAPs) (Yoder et al., 1997). DNA methylation may have evolved as a host defense mechanism to protect the genome against activation of these parasitic sequences and prevent them from spreading, which may interfere with homologous recombination and proper gene expression (Yoder et al., 1997). DNA methylation at promoters of these sequences keeps them inactive. DNA methylation also plays an important role in promoter silencing of imprinted genes, and of genes that undergo X chromosome inactivation.

1.3.2 Genomic Imprinting

Imprinted genes show mono-allelic parent-specific gene expression. The parent-specific expression is established through the presence of an epigenetic modification. The epigenetic mark of many imprinted genes is DNA methylation at differentially methylated regions (DMRs) (it is also referred to as an imprinting control region (ICR)). Enrichment of histone methylation has also been found at imprinted genes: methylation of histone H3 at lysine 4 has been associated with the expressed allele, while methylation of histone H3 at lysine 9 has been associated with the silenced allele (Delaval et al., 2007; Vu et al., 2004). Imprinted genes are often found in clusters and methylation at DMRs can affect the expression of surrounding genes. The DMRs of imprinted genes are found within CpG islands and near direct repeats. The presence of direct repeats may mark the location of imprinted genes (Khatib et al., 2007). Imprinted genes may also show asynchronous replication of the parental alleles during the cell

cycle when the paternal allele replicates before the maternal allele (Kitsberg et al., 1993; Knoll et al., 1994).

The observation that the parental genomes do not contribute equally to embryonic development was made based on the finding that gynogenotes, embryos that contain a diploid maternal contribution, die at mid-gestation, but show some fetal development with poor placental development, while androgenotes, embryos that contain a diploid paternal contribution, lack fetal development but show proper placental development (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984). These experiments suggest that a maternal component is needed to support embryo development and a paternal component is needed to support the development of the placenta (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984). Hydatidiform moles and ovarian teratomas are the equivalents of androgenotes and gynegenotes in humans, respectively, and show a similar pattern of either placental or fetal development, respectively (Jacobs et al, 1980; Surti et al., 1990). Parent-specific gene expression, such as seen for imprinted genes, may be the mechanism that regulates the different maternal and paternal functions in development discussed above. Many imprinted genes that are paternally expressed (often maternally methylated) promote fetal growth, while maternally expressed genes (often paternally methylated) restrict it (Moore and Haig, 1991).

Around 80 imprinted genes have been identified to date (www.otago.ac.nz/IGC). The majority of imprinted genes that have been identified are methylated in the oocyte. Very few imprinted genes have been identified to carry the methylation mark in the sperm, these include *H19*, *Gtl2*, *Rasgfr1,* and *Gpr1-Zdbf2* in mice (Davis et al., 2000; Shibata et al., 1998; Takada et al., 2002; Hiura et al., 2010) and *H19* and *GTL2* in humans (Kerjean et al., 2000; Geuns et al., 2007a).

1.3.3 Genome reprogramming

The genome undergoes two rounds of DNA demethylation and *de novo* methylation at gametogenesis and during preimplantation development (Okano et al., 1999; Kafri et al., 1992). However, imprinted genes escape demethylation at preimplantation development (Okano et al., 1999; Kafri et al., 1992). After birth, changes in DNA methylation are associated with ageing

and cancer (Maegawa et al., 2010). The process is highly regulated by DNA methyltransferases (DNMTs); DNMT1 is the main DNA maintenance enzyme, while DNMT3A and DNMT3B are primarily responsible for establishing methylation. The enzymes and their roles will be discussed in further detail in an upcoming section (section 1.3.4). The steps involved in genome reprogramming are mainly supported by data from mouse studies.

1.3.3.1 Genome reprogramming during gametogenesis

1.3.3.1.1 Genome-wide demethylation

Before entering the gonads, PGCs are highly methylated (Hajkova et al., 2002). PGCs are derived from the posterior primitive streak and migrate from the base of the allantois to the gonadal ridge where they undergo the first round of genome-wide DNA demethylation or erasure (Szabo et al., 2002; Davis et al., 2000; Kafri et al., 1992; Lee et al., 2002). In the mouse, erasure of methylation in single copy genes and imprinted genes begins at embryonic day (E) E11.5 to E12.5 and is fully complete by E13 to E14 (Karfi et al., 1992; Davis et al., 2000). Erasure of methylation at repetitive sequences, such as IAPs, LINEs and minor satellite DNA, is protracted and partial. The remaining methylation may therefore be passed on to the next generation (Hajkova et al., 2000; Lees-Murdock et al., 2003; Lane et al., 2003). The gonadal ridge is also where the inactive X-chromosome is re-activated in female PGCs but this modification may occur gradually (Tam et al., 1994; Chuva et al., 2008).

Erasure is likely to be an active process as it occurs in the presence of DNMT1 in the nuclei of PGCs (Hajkova et al., 2002). However, it is not clear how active demethylation occurs as the mechanism or enzymes involved have not yet been identified. The exact timing of erasure at imprinted genes may be gene dependent. For example, Li et al. (2004) showed erasure of methylation at *Rasgrf1* and *H19* to be complete in E12.5 cells; however, methylation at *Gtl2* was still present in about 50% of cells analyzed. A later study demonstrated erasure at *Gtl2* to have occurred by E14.5 (Hiura et al., 2007). Incomplete erasure has also been demonstrated at some imprinted genes. Demethylation of *H19* was incomplete in some male PGCs and the imprint was actually preserved in a proportion of cells (Ueda et al., 2000). However, all maternally imprinted genes were fully demethylated in male and female PGCs (Ueda et al., 1992). The incomplete erasure may serve as a mark to differentiate between the maternal and

paternal alleles for when the parental alleles undergo asynchronous *de novo* methylation (Ueda et al., 2000). The paternal allele becomes remethylated first during fetal stages while the maternal allele becomes fully methylated before the onset of meiosis (Ueda et al., 2000). In humans, complete erasure at *MEST* and *H19* was seen in fetal spermatogonia (Kerjean et al., 2000) and erasure at *SNRPN* was complete in mature spermatogonia (Manning et al., 2001a). After completion of demethylation, the male cells enter mitotic arrest while female cells enter meiotic arrest (Reik et al, 2001; Ueda et al., 2000).

1.3.3.1.2 Genome-wide de novo methylation in the male germ line

In the male germline, remethylation of repetitive sequences, single copy genes and imprinted genes occurs in prospermatogonia before birth (at E15-E17.5 in mice) (Li et al., 2004; Hiura et al., 2007; Lees-Murdock et al., 2003). Although demethylation of *Gtl2* may be slower than demethylation at *Rasgrf1* or *H19*, all three genes show a high degree of methylation at E17.5, with *Gtl2* having more complete methylation compared to *Rasgrf1* and *H19* (Li et al., 2004). The authors suggested that the higher methylation at *Gtl2* may be related to the presence of repetitive stretches of DNA in the gene, which the cell may recognize and suppress as it similarly does to repetitive sequences (Li et al., 2004). At the *H19* DMR in the sperm, the paternal and maternal alleles undergo *de novo* methylation asynchronously, with the paternal allele undergoing *de novo* methylation before the maternal allele (Ueda et al., 2000). In humans, methylation at *H19* is almost complete in spermatogonia with all cells showing methylation at the spermatocyte stage, and is maintained in round spermatids, elongated spermatids and spermatozoa (Kerjean et al., 2000). Less information is available about the methylation dynamics at the human *GTL2*, only that it is fully methylated in sperm (Geuns et al., 2007; Kobayashi et al., 2007). *MEST* and *SNRPN* remain unmethylated in sperm (Kerjean et al., 2000, Manning et al., 2001).

1.3.3.1.3 Genome-wide de novo methylation in the female germ line

De novo methylation of the female germline occurs after birth during oocyte growth corresponding to diplotene or dictyotene stage of meiotic prophase I. The establishment of imprints coincides with the physical growth of the oocyte (Bao et al., 2000). Studies have reported a sequential imprint establishment in oocytes so that different imprinted genes become

methylated at different stages during oocyte development (Obata and Kono, 2002; Sato et al., 2006). For example *Snrpn, Znf127* and *Ndn* acquire their imprint at the primordial to primary follicle stages, *Peg3, Igf2r* and $p57^{KIP2}$ acquire their imprint at the secondary follicle stage and the *Peg1/Mest* acquires its imprint in the tertiary to early antral follicle stage (Obata and Kono, 2002). *H19* was unmethylated at all stages of oocyte development (Sato et al., 2006). Asynchronous methylation of imprinted genes in the oocyte has also been reported, as in sperm for *H19* (Ueda et al., 2000): the maternal allele is methylated prior to the paternal allele at the *Snrpn* DMR in mice (Davis et al., 2000; Lucifero et al., 2004). A similar pattern of methylation progression was observed in human oocytes for *LIT1, ZAC* and *PEG1* genes, with methylation being fully set at the germinal vesicle (GV) stage human oocyte. Similarly, *H19* stayed unmethylated throughout oogenesis (Sato et al., 2006). Methylation at the *SNRPN* DMR is also already established in human oocytes by the GV stage (Geuns et al., 2007; Geuns et al., 2003).

1.3.1.2 Genome reprogramming during preimplantation

1.3.1.2.1 Genome wide demethylation

Upon fertilization, before the second round of reprogramming can occur, sperm protamines are replaced by acetylated histones. After sperm chromatin remodeling takes place, genome-wide demethylation occurs during preimplantation development (Mayer et al., 2000). Demethylation is complete by the 16-cell morula stage at many DNA sequences (Kafri et al., 1992), while imprinted genes are protected from this wave of demethylation and maintain their methylation marks (Olek and Walter, 1997; Tremblay et al., 1997). It is not known how methylation at imprinted genes is protected from the genome-wide wave of demethylation. Demethylation on the paternal genome occurs before the demethylation of the maternal genome, within a few hours of fertilization in an active manner before replication begins (Mayer et al., 2000; Oswald et al., 2000). However, the mechanism or enzymes that would catalyze the reaction have not yet been identified. Passive demethylation occurs subsequently on the maternal genome, characterized by the progressive loss of methylation over a number of cell divisions (Mayer et al., 2000). During cell divisions DNMT1 is excluded from the nucleus (Grohmann et al., 2005).

1.3.3.2.2 Genome-wide de novo methylation

De novo methylation occurs after the morula stage (Monk et al., 1987), in the inner cell mass of murine blastocyst and at the 8-16 cell stage bovine embryo (Reik et al., 2001). The extraembryonic lineage becomes methylated to a lesser extent than the embryonic lineage (Popp et al., 2010).

1.3.4 DNA methyltransferases

DNMTs are a class of enzymes essential for the establishment and maintenance of methylation of DNA. DNMTs catalyze the addition of methyl groups to the 5 prime position of cytosine using S-adenosyl-L-methionine (SAM) as methyl donor. Five DNMTs have been classified according to the homology of their catalytic domains at the C-terminus. These include DNMT1, DNMT3A, DNMT3B, that are catalytically active, and DNMT2 and DNMT3L, that are catalytically inactive. In addition, there are also DNMT splice variants in mice and humans (La Salle and Trasler, 2006; Sakai et al., 2004; Watanabe et al., 2002; Robertson et al., 1999; Huntriss et al., 2004).

DNMT1 is the primary enzyme responsible for the maintenance of DNA methylation (Li et al., 1992). Mutations in the *Dnmt1* gene are associated with genome-wide demethylation and are embryonic lethal (Li et al., 1992). DNMT1 also has high affinity for hemi-methylated DNA (Bestor, 1992; Yoder et al., 1997) and localizes to DNA replication foci presumably re-establishing methylation on newly synthesized DNA strands (Leonhardt et al., 1992). Two sex-specific *Dnmt1* variants have been identified: *Dnmt1o* and *Dnmt1p*. The somatic *Dnmt1* variant is called *Dnmt1s*. DNMT1o is an enzymatically active truncated protein present only in oocytes (Mertineit et al., 1998), while DNMT1p is a protein that is nuclear in leptotene and zygotene stages and disappears in pachytene-stage spermatocytes (Jue et al., 1995).

DNMT3A and DNMT3B are the primary enzymes responsible for *de novo* methylation (Okano et al., 1999). DNMT3A is necessary for establishing methylation at some paternally imprinted genes and for proper embryo development (Kaneda et al., 2004). DNMT3B specifically methylates centromeric repeats in chromosomes (Okano et al. 1999). Deletions in

DNMT3B are associated with immunodeficiency, centromere instability and facial anomalies (ICF) syndrome in humans that is characterized by hypomethylation of centromeric repeats (Okano et al., 1999; Xu et al., 1999). ICF patients have point mutations in the C-terminal catalytic domain of DNMT3B resulting in partial loss of function (Xu et al., 1999; Okano et al., 1999). ICF patients show demethylation of pericentric regions correlated with chromosome instability, and centromeric breakage (Tagarro et al., 1994). Up-regulation of *Dnmt3b* expression has been observed in tumors; further suggesting it may be involved in chromosome stability (Robertson et al., 1999). DNMT3L may act as co-factor for *de novo* methylation, primarily by interacting with DNMT3A (Chedin et al., 2002). The primary function of DNMT3L may be to silence repetitive sequences in the genome, such as unique non-pericentric heterochromatin, IAPs, interspersed repeats and retrotransposons (Bourc'his and Bestor, 2004; Webster et al., 2005, Hata et al., 2006). DNMT3L may also be involved in chromatin packaging (Webster et al., 2005) and in establishing maternal imprints in females (Bourc'his et al., 2001; Hata et al., 2002) and males (Webster et al., 2005). However, methylation at imprinted genes by DNMT3L may be mediated through its interaction with DNMT3A. Co-expression of *Dnmt3l* with *Dnmt3a* stimulated *de novo* methylation at DMRs of maternally imprinted genes (Chedin et al., 2002). DNMT2 does not appear to participate in either *de novo* methylation or maintenance methylation (Okano et al., 1998), and its function is currently unknown.

1.4 IMPRINTING ABNORMALITIES ASSOCIATED WITH ART PREGNANCIES

1.4.1 Imprinting disorders found in ART births

It is estimated that children born through ART may be 3 to 6 times more likely to be affected by an imprinting disorder compared to the general population (Sutcliffe et al., 2006; Maher 2003; Debaun et al., 2003). While Beckwith Wiedemann syndrome (BWS) and Angelman syndrome (AS) are the most frequently reported (Maher 2003; Debaun et al., 2003; Orstavik et al., 2003; Cox et al., 2002, Sutcliffe et al., 2006), Prader-Willi and Silver Russell syndromes have also been found (Doornbos et al., 2007; Sutcliffe et al., 2006; Kagami et al., 2007; Kanber et al., 2009). BWS is an overgrowth syndrome and AS is characterized by mental retardation, speech impairment and behavioral problems (Maher, 2005; Buiting et al., 1999). Both syndromes are associated with a loss of function of the maternal allele: *LIT1* at 11p15 in BWS and *UBE3A* from the *SNRPN* imprinting center at 15q (11-13) in AS. While the

syndromes can occur either through a deletion, a mutation, uniparental disomy or the loss of methylation on the maternal allele, what is apparent from reports in the literature on imprinting syndromes in ART children is that most cases are associated with the loss of methylation at the maternal allele (Maher et al., 2003; Debaun et al., 2003; Gicquel et al., 2003; Orstravik et al., 2003; Sutcliffe et al., 2006; Doornbos et al., 2007). This is highly significant as in the general population the loss of methylation is expected in about 40 to 50% of BWS cases and in less than 5% of AS cases (Maher et al., 2005). No association with BWS and factors such as *in vitro* culture conditions, including the type of media used and length of culture, ART method used and type of infertility has been identified (Chang et al., 2005). It has been suggested that parental infertility itself may be associated with the increased risk of imprinting abnormalities in children born through ART. Ludwig et al. (2005) found a strong association between parental subfertility and the risk of AS. In addition, the initial risk of imprinting disorders found in ART pregnancies was non-existent after correction for parental infertility (Doornbos et al., 2007). Therefore, it is possible that parental infertility is associated with a risk of imprinting disorder and that infertility treatment may further increase this risk (Ludwig et al., 2005; Doornbos et al., 2007; Chang et al., 2005).

Since it is the maternal allele that is improperly methylated in BWS and AS, these two syndromes are unlikely to be associated with male infertility; however, Silver Russell syndrome (SRS) may be. SRS is characterized by growth retardation, poor feeding, and digit and limb abnormalities. SRS has been associated with abnormalities at multiple genes (Kotzot, 2008). Hypomethylation at *H19* was reported in one girl born after ICSI (Bliek et al., 2006). Normally the maternal *H19* allele is unmethylated while the paternal allele is methylated; therefore for a loss of methylation to occur the paternal allele would have to have been affected. In addition, hypermethylation at *MEST* has been described in one child born through IVF (Kagami et al., 2007) and in one child born through ICSI who also showed hypermethylation at *KCNQ1OT1* (Kanber et al., 2009). Normally the paternal *MEST* and *KCNQ1OT*1 alleles are unmethylated, while the maternal alleles are methylated, therefore for a gain of methylation to occur, the paternal allele would have to have been hypermethylated. A similar pattern of abnormal methylation was found in the blood of the IVF child's father, but the sperm was not analyzed (Kagami et al., 2007). The abnormalities found in the children could have been present in the

fathers' sperm and been passed on through ART to their children. A recent paper demonstrated parental origin of improper methylation at two imprinted genes, *H19* and *GTL2*, in abortuses from IVF and ICSI treatment (Kobayashi et al., 2009), supporting the idea that abnormal methylation in the gametes may be passed on to progeny through the use of ART and have a detrimental effect on the pregnancy outcome.

1.4.2 Etiology of imprinting abnormalities in ART pregnancies

ART involves hormonal stimulation of ovulation followed by *in vitro* culture and embryo manipulation, procedures that have been associated with changes in DNA methylation at imprinted genes (Sato et al., 2006; Geuns et al., 2007b; Doherty et al., 2000; Mann et al., 2004). The procedures involved in ART may give rise to abnormal DNA methylation that has been reported at imprinted genes in children born through the use of ART (Maher 2003; Debaun et al., 2003; Orstavik et al., 2003; Cox et al., 2003, Sutcliffe et al., 2006). However, a recent report also shows that some abnormalities may originate in the sperm (Kobayashi et al., 2009), supporting the hypothesis of abnormal DNA methylation at imprinted genes in infertile men affected by severe male factor infertility.

1.4.2.1 Ovulation induction

Ovulation induction has been associated with changes in DNA methylation at imprinted genes in women undergoing infertility treatment as well as in mice (Sato et al., 2006; Geuns et al., 2007b). Imprinting abnormalities in harvested oocytes may be present either due to the release of oocytes that have not yet completed imprint establishment or oocytes with improperly set imprints that would have otherwise not been ovulated. It is also possible that the hormones used for ovulation induction may interfere with the proper maintenance of imprints. For example, a gain of methylation at *H19* and a loss of methylation at *PEG1* and the *KvDMR1* were observed in superovulated immature GV stage and metaphase I (MI) oocytes obtained from infertile women (Sato et al., 2006; Geuns et al., 2007b). The gain of methylation at *H19* was also seen in superovulated mouse oocytes (Sato et al., 2006). It is unclear at this time whether the high hormone doses required to stimulate ovulation are responsible for the imprinting abnormalities found in superovulated oocytes (Market-Velker et al., 2010; Anckaert et al., 2009). Furthermore, *in vitro* maturation of superovulated immature oocytes has been

associated with the presence of imprinting abnormalities at *H19* in oocytes in women (Borghol et al., 2006). One study reported a decrease in implantation rates and an increase in the number of embryos showing delayed development following superovulation in mice (Fortier et al., 2008). Superovulation resulted in changes in *Igf2* expression that particularly affected placental tissues in the developing embryo (Fortier et al., 2008), demonstrating that effects of superovulation may be maintained throughout development but may also be tissue specific.

1.4.2.2 Embryo culture

Following oocyte retrieval and fertilization, the embryo is cultured for three to five days until being transferred to the womb for implantation. The possible deleterious effects of ART procedures on the development of the fetus were first shown in animal studies, particularly sheep and cattle. The growth abnormalities observed were termed large offspring syndrome (LOS) characterized by an overall increase in the size and weight of the calf and larger internal organs (Young et al., 2001; Bertolini et al., 2002). Embryo culture in sheep was associated with 20 to 80% heavier fetuses than normal that correlated with a loss of methylation and reduced expression of *IGF2R* in the embryo-cultured fetuses (Young et al., 2001). Further research demonstrated that specific media additives were associated with LOS, while supplementation with bovine serum albumin or amino acids did not affect growth (Thompson et al., 1995). Media supplementation with serum was associated with LOS and prolonged gestation in sheep (Thompson et al., 1995), abnormal physiology, and malformations such as abnormal organ and skeletal development in sheep and cattle (Sinclair et al., 1999; Farin et al., 2001), as well as placental abnormalities and a higher rate of perinatal mortality (Sinclair et al., 1999).

In mice, *in vitro* embryo culture has been associated with a decrease in fetal size. The observed reduction in size was correlated with the culture of 1-cell embryos until transfer in the presence of serum in the media. In addition, the presence of serum correlated with the lack of development of 1-cell embryos into blastocysts, and fewer live-born animals (Khosla et al., 2001). These abnormalities were associated with a reduction in expression of two imprinted genes, *H19* and *Igf2*, and the increase in expression of *Grb10*, while the expression of *Mest* was not affected (Khosla et al., 2001). Embryo culture in specific media has been associated with loss of methylation and biallelic expression of *H19* (Doherty et al., 2000; Mann et al., 2004) and

loss of methylation at *Snrpn* (Mann et al., 2004) in embryos cultured to the blastocyst stage in Whitten's medium, while being normal when cultured in potassium simplex optimized medium (KSMO) medium (Doherty et al., 2000; Mann et al., 2004). Biallelic expression of *H19* was limited to the trophectoderm cells but was normal in the inner cell mass (Doherty et al., 2000). Furthermore, later in development hypomethylation and abnormal expression of *H19* and *Snrpn* persisted in placental tissue, but the DMRs were properly methylated in embryonic tissues (Mann et al., 2004). These results suggest that the trophectoderm, which gives rise to the placenta, may be more prone to acquiring methylation abnormalities due to its direct contact with the medium (Doherty et al., 2000) and that it may be less able to maintain genomic imprints compared to embryonic tissue (Mann et al., 2004). Other studies in relation to embryo culture and superovulation have reported similar observations of placental tissues being specifically prone to aberrant imprinting (Rivera et al., 2008). *In vitro* culture has also been associated with changes in development and behavior (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004) but an epigenetic association has not yet been established.

1.4.2.3 Sperm

Studies have also tried to correlate the presence of aberrant methylation at imprinted genes in the sperm of infertile men with the outcome of pregnancies achieved through ART. A decrease in the fertilization rate was observed in men showing a decrease in methylation at the *H19* DMR and *IGF2* DMR2 (Boissonnas et al., 2010). However, no difference was observed in the rate of early cleavage, implantation rate, delivery rate, pregnancy rate, term delivery and birth weight (Boissonnas et al., 2010). Aberrant methylation at imprinted genes in the sperm has also been associated with the presence of aberrant methylation in abortuses. Abnormal methylation at the *H19* DMR and *IG-GTL2* DMR in abortuses following ART was traced back to the father's sperm based on the presence of a polymorphism (Kobayashi et al., 2009). Some of these men had gene variations in the *DNMT3L* gene, but no clear mutations could be determined (Kobayashi et al., 2009). The abortuses and sperm samples showed an almost complete loss of methylation at either one of the paternally imprinted genes. In one abortus and paternal sperm sample there was a complete lack of methylation at both paternal DMRs. DNA methylation at paternally imprinted genes was abnormal in four sperm samples but was normal in the abortus. However, the methylation in these sperm samples was not as severely decreased

as it was in the sperm samples where the abnormality was passed on to the conceptus (Kobayashi et al., 2009), suggesting that in order for the abnormality to be passed on, a large proportion of sperm have to carry the abnormal imprint. However, the birth of a normal girl conceived through ICSI using sperm with decreased methylation at *H19* DMR was reported (Kobayashi et al., 2007). In the sperm used, only one out of ten clones of sequenced strands analyzed carried the proper methylation, the remaining clones were completely unmethylated. In addition, improper methylation at *MEST* and *ZAC* were also present in the sperm, but were normal in the child (Kobayashi et al., 2007). These studies suggest that abnormal methylation in the sperm at the single cell level may be more relevant to pregnancy outcome than is methylation at a small number of selected CpG sites. The data also emphasize the importance of reporting more than the mean methylation levels for each gene either for a group or an individual.

1.5 GENOMIC IMPRINTING IN THE MALE GAMETE

H19 and *GTL2* are the two known genes in humans to be methylated at the DMR in the sperm (Kerjean et al., 2000; Geuns et al., 2007a). *H19* maps to p15.5 on human chromosome 11 (chromosome 7 in mice), a region that contains two imprinted domains that control the expression of several imprinted genes (Figure 1.3: ICR1 (also known as *H19* DMR) is more telomeric and controls the expression of *H19* and *IGF2*, while ICR2 (also known as *KvDMR1*) is more centromeric and controls the expression of *CDKN1C* (also known as $p57^{KIP2}$), *KCNQ1* and *KCNQ1OT1* (also known as *LIT1*). *H19* and *IGF2* are reciprocally imprinted. *H19* is an untranslated RNA and is maternally expressed, while *IGF2*, insulin-like growth factor 2, is paternally expressed (Rainier et al., 1993). The parent-specific expression of *H19* and *IGF2* is regulated by methylation at ICR1 (*H19* DMR) (Thorvaldsen et al., 1998). When unmethylated on the maternal allele, a chromatin insulator protein CCCCTC binding factor (CTCF) can bind ICR1 preventing access of the *IGF2* promoter to shared enhancer elements located downstream of *H19* (Figure 1.4. This prevents *IGF2* expression, but enables the shared enhancer elements to interact with the *H19* promoter that correlates with *H19* expression. When ICR1 is methylated on the paternal allele, CTCF cannot bind, thus allowing the *IGF2* promoter to interact with the enhancer elements, which correlates with *IGF2* expression and *H19* silencing (Bell and Felsenfeld, 2000; Hark et al., 2000; Hark etal., 1998). There is also a testis specific CTCF-like

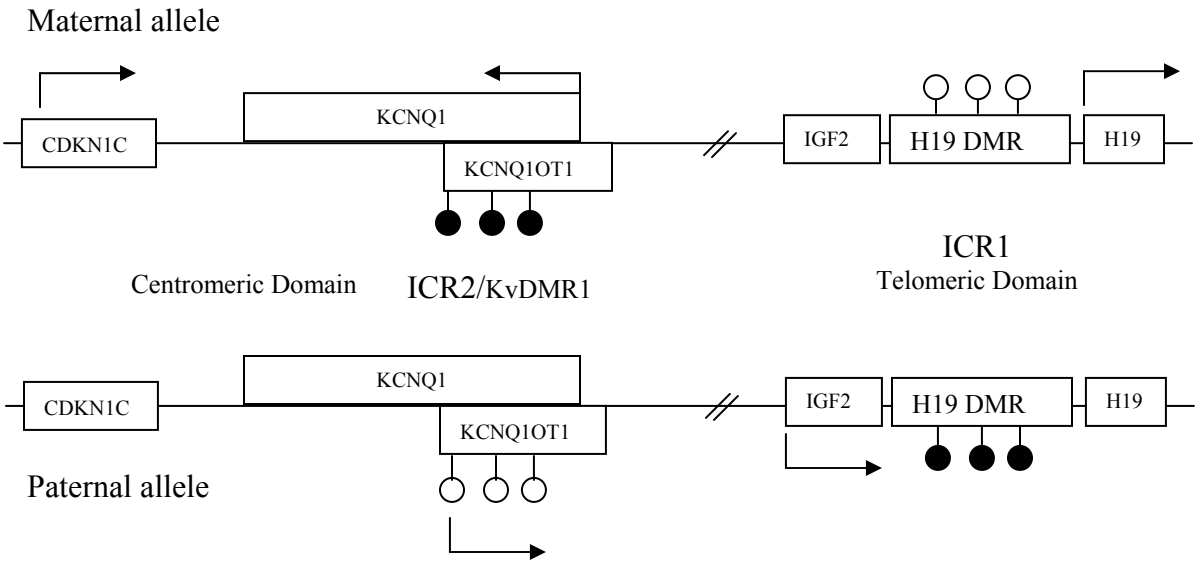


Figure 1.3 Imprinting cluster on the human chromosome 11p15.5. The two control regions, *H19* DMR and *KvDMR1*, are shown. The *H19* DMR is methylated on the paternal allele and unmethylated on the maternal allele, while the *KvDMR*1 is methylated on the maternal allele and unmethylated on the paternal allele. Absence of methylation is indicated by empty lollipops while presence of methylation is indicated by black lollipops. Allele-specific gene expression resulting from the differential methylation is indicated by arrows. Not drawn to scale.

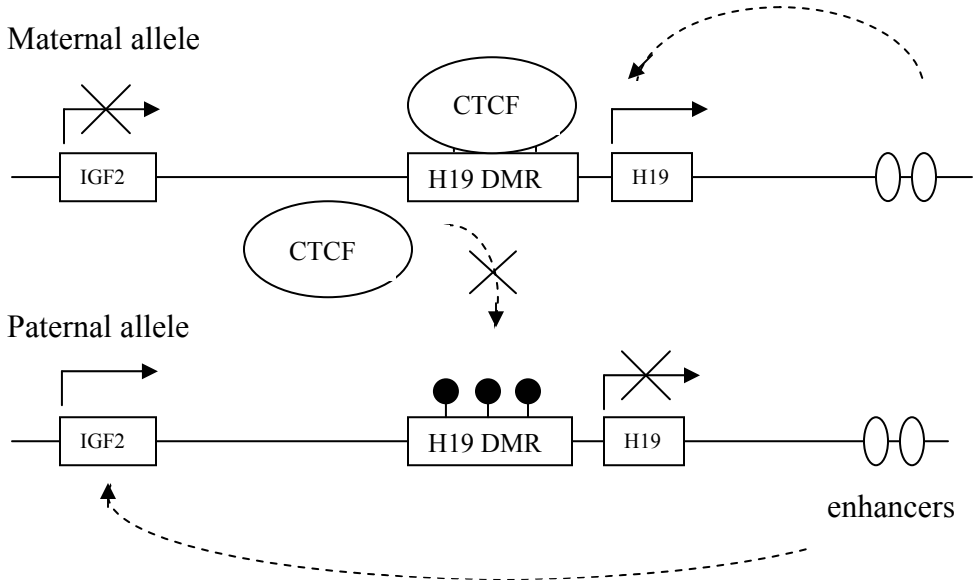


Figure 1.4 Control at the *H19/IGF2* locus. On the maternal allele the CTCF protein binds the unmethylated *H19* DMR, enabling the enhancer elements to interact with the *H19* promoter resulting in expression of *H19* from the maternal allele. On the paternal allele the CTCF protein cannot bind the methylated *H19* DMR, enabling the enhancer elements to interact with the *IGF2* promoter resulting in the expression of *IGF2* from the paternal allele. Not drawn to scale.

(CTCFL) protein, called BORIS (brother of the regulator of imprinted sites). This protein is only expressed during embryonic development as well as in some cancers and binds either the methylated or unmethylated *H19* DMR (Klenova et al., 2002; Nguyen et al., 2008).

GTL2, gene trap locus 2, maps to q32 on human chromosome 14 (chromosome 12 in mice). *GTL2* and *DLK1* (delta, Drosophila, homolog-like 1) are reciprocally imprinted and show similarity to the *H19/IGF2* imprinted region. *GTL2*, like *H19*, is an untranslated RNA that is maternally expressed, while *DLK1* is paternally expressed (Schmidt et al., 2000; Wylie et al., 2000; Takada et al., 2000). *DLK1* encodes a trans-membrane protein that resembles the delta notch family of signaling molecules and is important for cellular differentiation (Laborda, 2000). The parent-specific expression of *GTL2* and *DLK1* is regulated by methylation at the *GTL2* DMR. The CTCF protein-binding sequence has been identified in the *GTL2* DMR (Takada et al., 2002), therefore, expression of *GTL2* and *DLK1* may be regulated in the same manner as *H19* and *IGF2* are. However, the DMR that shows germ-cell-specific methylation and undergoes epigenetic reprogramming is the intragenic (IG) DMR (referred to in this thesis as *IG-GTL2* DMR) (Geuns et al., 2007a; Takada et al., 2002) (Figure 1.5. The *IG-GTL2* DMR does not interact with the CTCF protein as it lacks the binding sequence (Paulsen et al., 2001).

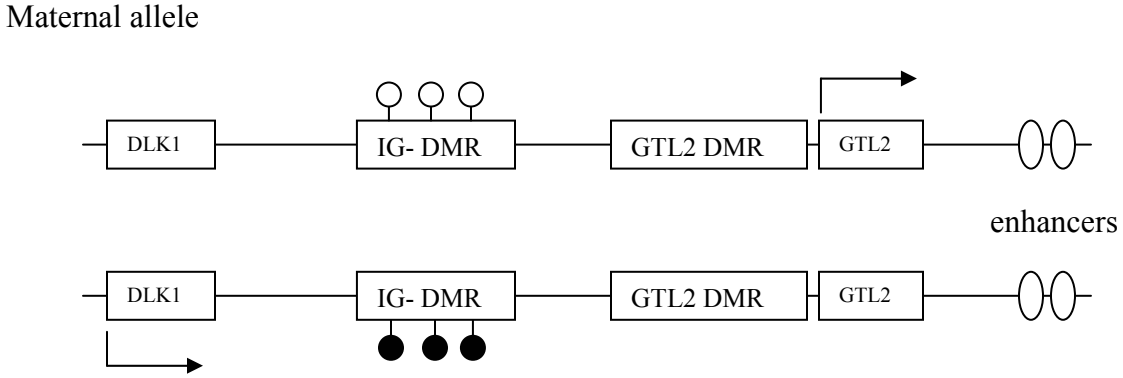


Figure 1.5 Imprinted *IG-GTL2* DMR on the human chromosome 14q32. The *IG-GTL2* DMR is unmethylated on the maternal allele and methylated on the paternal allele, as indicated by the white and black lollipops, respectively. The *GTL2* gene shows maternal expression while the *DLK1* gene is paternally expressed, as indicated by arrows. Although the *IG-GTL2* DMR shows gamete specific methylation, it may not control the expression of *GTL2* and *DLK1* on the paternal allele.

The *IG-GTL2* DMR is methylated in the sperm and unmethylated in the oocyte (Takada et al., 2002; Geuns et al., 2007). However, it is uncertain whether this DMR controls expression of the nearby imprinted genes (Lin et al., 2003). Both the *H19/IGF2* and *GTL2/DLK1* regions have been involved in the regulation of prenatal growth (DeChiara et al., 1990; Georgiades et al., 2000; Georgiades et al., 2001). The genes are also expressed in the same tissues during development (Takada et al., 2000). *Igf2* is important for proper fetal and placental growth. Lack of *Igf2* expression is associated with a reduction in fetal weight of up to 40% (De Chiara et al., 1990), as well as placental growth retardation (Constancia et al., 2002). One possible mechanism of growth restriction may be decreased nutrient transfer across the placenta (Constancia et al., 2002). Loss of methylation at the *H19* DMR has been associated with small for gestational age placentae in humans (Guo et al., 2008). On the other hand, over-expression of *Igf2* has been associated with fetal overgrowth (Leightonet al., 1995), and has also been involved in human imprinting syndromes such as BWS (Brown et al., 1996). Abnormal *Igf2* expression has also been linked to cancer (Sievers et al., 2005; Randhawa et al., 1998). With respect to *GTL2/DLK1*, mouse embryos that have maternal or paternal uniparental disomy (UPD) for chromosome 12 exhibit growth defects and are not viable (Georgiades et al., 2000; Georgiades et al., 2001). UPD occurs when both chromosomes in the offspring were inherited from one parent. Growth retardation in relation to UPD 14 has also been observed in humans (Georgiades et al., 1998).

MEST (mesoderm-specific transcript) is one of the maternally methylated and paternally expressed imprinted genes that are often studied. It is also known as the paternal expressed gene 1 (*PEG1*). In humans it has been mapped to 7q32 and is mainly expressed in mesodermal cells (Kobayashi et al., 1997). It encodes an enzyme belonging to the alpha hydrolase fold family, and its function may affect the growth and maintenance of mesodermal cells (Kobayashi et al., 1997). The *MEST* DMR extends over the promoter and shows a straightforward control of expression. It is silenced on the methylated maternal allele and expressed from the unmethylated paternal allele (Reule et al., 1998; Kobayashi et al., 1997). Its expression can be detected in oocytes as well as in four-cell stage embryos. It is also a candidate gene for the growth restriction seen with SRS in humans (Kaneko-Ishino et al., 1995). In mice, *Mest* maps to chromosome 6. It has been shown to play an important role in early development as a maternal

duplication of this gene is embryonic lethal (Lefebvre et al., 1997). It also plays a role in maternal behavior such that mothers lacking *Mest* fail to feed their young and are neglectful towards them (Lefebvre et al., 1998).

1.5.1 Environmental disruption of genomic imprinting

It is thought that the waves of demethylation and remethylation that occur during embryonic development are the stages when the embryo is most susceptible to acquiring methylation abnormalities. Aberrant methylation may occur due to environmental changes during embryonic development, which may be induced through processes such as ovarian hyperstimulation, *in vitro* embryo culture and embryo manipulation, as well as diet and chemicals present in the environment. Exposure during embryo development may not only affect the developing fetus but may also correlate with abnormalities in the germ cells of the developing fetus, indirectly affecting future generations. Adult exposure may induce epigenetic abnormalities in the germ cells, which when contributing to conception are passed on and may affect fetal development.

1.5.1.1 In utero development

The Barker hypothesis postulates that the risk for hypertension, cardiovascular disease and diabetes may be acquired *in utero* due to a stressor, such as poor nutrition, maternal illness or severe *in utero* stress, creating an unfavorable early growth environment. It is believed that the fetus will adapt to the unfavorable environment through physiological and metabolic changes and that these adaptations will later hinder its ability to function properly in a different environment after birth, putting it at a greater risk of developing chronic disease later in life (Barker et al., 1989; Barker, 1998; Gluckman et al., 2007). The one factor that is constantly associated with increased risk for chronic disease is low birth weight. For example, a decrease in birth weight, or in some cases intrauterine growth restriction (IUGR), has been associated with an increased risk for cardiovascular disease (Lawlor et al., 2005; Rich-Edwards et al., 2005), stroke (Lawlor et al., 2005; Rich-Edwards et al., 2005) and hypertension (Gortner, 2007). One proposed mechanism for the development of hypertension and cardiovascular disease in response to *in utero* stress is renal insufficiency (Mackenzie et al., 1995; Brenner and Chertow,

1994). Renal insufficiency has been associated with hypomethylation at *p53* and at *DNMT1* in IUGR rats (Pham et al., 2003), providing a possible epigenetic mechanism for kidney insufficiency in IUGR pregnancies. In addition, poor development of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress during development has been associated with low birth weight, and increased risk for type 2 diabetes and cardiovascular disease (Jaddoe and Witteman, 2006). A mouse study provided evidence of the possible involvement of epigenetic modifications in the HPA response (Weaver et al., 2004; Weaver et al., 2005). Maternal nurturing was associated with DNA hypomethylation of the glucocorticoid receptor promoter and consequently higher expression of the glucocorticoid receptor in the offspring (Weaver et al., 2004). Offspring of these mothers showed a more modest response to stress in their adult life (Weaver et al., 2004). The stress response in the adult rats could be reversed by dietary supplementation with methionine, a methyl donor, showing that acquired epigenetic modifications could be reversed by environmental factors (Weaver et al., 2005). Is has been shown that maternal psychological stress in humans can be associated with preterm birth and decreased birth weight, although from the study it was not clear whether the decrease in birth weight was due to preterm birth or maternal stress (Nordentoft et al., 1996; Precht et al., 2007). These studies demonstrate that early *in utero* development can affect DNA methylation in the developing fetus suggesting a mechanism that can induce aberrant imprinting before birth. *In utero* stressors may potentially also affect DNA methylation in the gametes; however, this has not yet been shown.

1.5.1.2 Diet

Nutrients such vitamin B12, methionine, betaine, folate and choline serve as methyl donors and co-factors needed to make SAM, the primary donor for methylation, and have been shown to affect methylation status of DNA (Kraunz et al, 2006). The viable yellow Agouti mouse model was the first model to show the effects of maternal diet supplementation on the fetal epigenome. A specific semi-dominant mutation at the agouti locus, the metastable epiallele A^{vy}, is caused by the insertion of an IAP element upstream of the Agouti gene start site (Duhl et al., 1994). Methylation at the IAP determines expression of the A^{vy} allele. Expression of the A^{vy} allele results in yellow fur, obesity and tumorigenesis, while silencing through methylation protects the mice from obesity and tumorigenesis and is associated with brown fur

(Miltenberger et al., 1997; Morgan et al., 1999). Dietary exposure of pregnant mice to folate, a methyl donor, or genistein, a phytoestrogen in soy, resulted in hypermethylation of the IAP of the A^{vy} allele in the fetus (Waterland and Jirtle, 2003; Dolinoy et al., 2006), showing that maternal diet could influence gene methylation in the fetus. In these mice, increased DNA methylation was associated with healthy, longer-living mice (Cooney et al., 2002). DNA hypomethylation and increased expression of the glucocorticoid receptor and peroxisomal proliferator-activated receptor was observed in rat fetuses after maternal dietary protein restriction. The rat fetuses were protected from these epigenetic modifications when the maternal protein restriction diet was supplemented with folate (Lillycrop et al., 2005).

There is little direct evidence to link dietary intake with male infertility; however, studies have shown that spermatogenesis is sensitive to alterations in DNA methylation (Raman and Narayan, 1995; Doerksen and Trasler 1996, Doerksen et al., 2000; Kelly et al., 2003; Oakes et al., 2007). Furthermore, mutations in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, an enzyme responsible for homocystein metabolism, were associated with abnormal spermatogenesis and infertility in male mice (Kelly et al., 2005). The phenotype was significantly improved by maternal administration of betaine during pregnancy and nursing, followed by direct administration of betaine to the offspring. Prolonged betaine supplementation resulted in improved testicular histology, increased sperm numbers and fertility (Kelly et al., 2005). In humans, increased seminal plasma levels of vitamin B12 and folate correlated with increased sperm concentration and decreased sperm DNA damage, respectively (Boxmeer et al., 2007; Boxmeer et al., 2009). In addition, administration of zinc sulfate and folic acid to subfertile and fertile men correlated with increased sperm counts in both groups of men (Wong et al., 2002). These studies suggest a possible effect of DNA methyl donors on spermatogenesis and male fertility in both animals and humans.

1.5.1.3 Endocrine disruptors of spermatogenesis

Endocrine disruptors are natural or synthetic molecules that mimic steroid hormones. They may act on the endocrine system by binding to hormone receptors evoking or inhibiting the proper hormonal response, or they may bind to other receptors, alter production or breakdown of hormone receptors and elicit an inappropriate hormonal action (Sikka et al.,

2005). Vinclozolin binds the androgen receptor preventing androgen from binding, estrogen diethylstilbestrol (DES) has estrogenic activity, and dioxins impair testosterone biosynthesis and normal sexual differentiation (Sikka et al., 2005). There is evidence in the literature to suggest a link between some of the compounds and a risk for developing disease (Wu et al., 2004; Bullock et a., 1988; Walker and Haven 1997; Newbold et al., 2000; Turusov et al., 1992), and that in some cases this risk may be modulated through an epigenetic mechanism (Wu et al., 2004). However, much more research is necessary in order to establish risks and mechanisms of action.

In vitro exposure of mouse embryos from the one-cell to blastocyst stage to a dioxin, a persistent by-product of paper production, polyvinyl chloride plastics and chlorinated pesticides, led to hypermethylation at the *H19* DMR, increased methyltransferase activity and decreased fetal growth (Wu et al., 2004). In humans, exposure to a dioxin is mainly dietary (Edwards and Myers, 2007). Post-natal exposure of mice to DES resulted in DNA demethylation upstream of the estrogen-response element of the lactoferrin promoter (Li et al., 1997), also affecting methylation in estrogen-responsive genes involved in proper reproductive organ development (Li et al, 2003). *In utero* exposure to DES in mice and humans (given to prevent miscarriage) has been associated with higher prevalence of cancers affecting male and female reproductive tracts of children and grandchildren of the exposed generation (Bullock et a., 1988; Walker and Haven 1997; Newbold et al., 2000; Turusov et al., 1992). These studies suggest that epigenetic changes may have a trans-generational effect as they may be transmitted through the germline and affect more than one generation. A trans-generational effect was confirmed by observations of male infertility in mice, characterized by a reduction in sperm numbers, motility and increased cell apoptosis, in F1 through F4 generations following exposure to the endocrine disruptor vinclozolin and methoxyclor of the gestating mother of the F1 generation (Anway et al., 2005). With ageing, the males also had a higher risk of cancer, prostate and kidney disease and immune abnormalities (Anway et al., 2006). The effects were transmitted through the paternal germ line and alterations in DNA methylation were found in the sperm (Anway et al., 2005). These studies also imply that there must be an epigenetic modification that can escape the epigenetic reprogramming process and be passed on to the next generation. They also

suggest that exposure to certain environmental chemicals may affect DNA methylation and in some cases affect male fertility.

1.5.2 DNA Methylation in infertile men

Proper progression of spermatogenesis, in mice and humans, is sensitive to DNA methylation. Improper DNA methylation, genome-wide and at the level of imprinted genes, has been associated with male infertility. Changes in DNA methylation may not only affect fertility, but also be passed on to the next generation through the use of ART and affect pregnancy outcome.

1.5.2.1 DNA methylation in spermatogenesis

The importance of proper DNA methylation for spermatogenesis and male fertility was first demonstrated by the administration of DNA hypomethylating agents to male mice and rats (Raman and Narayan, 1995; Doerksen and Trasler 1996, Doerksen et al., 2000; Kelly et al., 2003; Oakes et al., 2007). 5-Azacytidine and 5-Aza-deoxycytidine are cytidine analogues that incorporate into replicating DNA and irreversibly bind DNMTs. Administration of cytidine analogues has been correlated with DNA hypomethylation (Gabbara and Bhagwat, 1995). Administration of 5-Aza-deoxycytidine to neonatal male mice affected the differentiation of spermatogonia into spermatocytes (Raman and Narayan, 1995). Administration of these agents for the length of spermatogenesis in adults was consistently associated with decreased testes and epididymal weights and decreased sperm counts in rats and mice (Doerksen and Trasler, 1996, Doerksen et al., 2000; Kelly et al., 2003; Oakes et al., 2007). Germ cell loss was also observed through increased germ cell apoptosis and sloughing of immature germ cells (Doerksen et al., 2000; Kelly et al., 2003). Pregnancy outcome was also affected, including decreased pregnancy rates, increased preimplantation loss in females, increased rates of abnormal embryo development and embryo death (Doerksen and Trasler, 1996; Kelly et al., 2003). Decreased global DNA methylation levels in sperm were also detected (Doerdsen et al., 2000; Kelly et al., 2003). There is little information available demonstrating the importance of DNA methylation for spermatogenesis in humans. Reduced sperm DNA methylation levels in infertile men were associated with decreased pregnancy rates (Benchaib 2003; Benchaib 2005).

Furthermore, the importance of DNA methylation for spermatogenesis and male fertility has also been demonstrated through animal experiments involving DNMT mutations. *Dnmt3a* mutant male mice are infertile. They have smaller testes with only few round spermatids (Yaman and Grandjean, 2006) or spermatogonia (Kaneda et al., 2004). Analysis of spermatogonia showed a loss of methylation at the *H19* DMR and at the *IG-Gtl2* DMR, while methylation at *Rasgrf1* DMR and at retrotransposon sequences was not affected (Kaneda et al., 2004; Yaman and Granjean, 2006). *Dnmt3a* mutant germ cells showed delayed entry into meiosis, but completion was normal once entry occurred (Yaman and Grandjean, 2006). *Dnmt3a/Dnmt3b* double mutant male mice showed hypomethylation of repetitive sequences (SineB1) and their germ cells failed to differentiate to produce mature sperm (Takashima et al., 2009). *Dnmt3b* deficient prospermatogonia showed normal DNA methylation at the *H19* and *Dlk/Gtl2* DMRs, but displayed a lower DNA methylation level of 80% at the *Rasgrf1* DMR (Kato et al., 2007). *Dnmt3l* mutant male mice are affected by meiotic abnormalities such as non-homologous synapsis and presence of unpaired regions associated with meiotic germ cell arrest (Webster et al., 2005; Bourc'his and Bestor, 2004; Hata et al., 2006). An increase in germ cell apoptosis, and a progressive loss of germ cells (Bourc'his and Bestor, 2004) and an absence of meiotic germ cells (Hata et al., 2006) were observed in older adults. In addition, *Dnmt3l* mutant males showed a loss of methylation at the *Rasgrf1* DMR and a mosaic pattern of methylation at the *H19* DMR (Webster et al., 2005).

Although it has been demonstrated that paternal genomic imprints are almost fully set in human germ cells by the spermatogonia stage and are complete in spermatocytes in fertile males (Kerjean et al., 2000; Hartman et al., 2006), the status of genomic imprints in infertile males may be altered.

1.5.2.2 Methods for studying DNA methylation

A DNA sample undergoes sodium bisulphite conversion prior to studying DNA methylation. Bisulphite conversion is a chemical modification of DNA where unmethylated cytosines are converted to uracils, while methylated cytosines remain unchanged (Frommer et al., 1992). The conversion thus allows differentiation between methylated and unmethylated cytosines. The methodologies that have been used to study DNA methylation in sperm include

methylation sensitive PCR (MSP) (Manning et al., 2001a; Manning 2001b), bisulphite sequencing (direct and with cloning) (Marques et al., 2004; Marques 2008; Kobayashi et al., 2007, Hammoud et al., 2009), dye terminator methylation analysis (Poplinski et al., 2009) and pyrosequencing (Boissonnas et al., 2010). Direct bisulphite sequencing, dye terminator methylation analysis and pyrosequencing are similar in that these methods provide a measure of average methylation at each of the multiple CpG sites analyzed in a DNA sample, with pyrosequencing providing a more quantitative measure. MSP provides an average measure of DNA methylation in a sample without providing a quantitative measure of DNA methylation at each CpG site (Herman et al., 1996), while COBRA provides a measure of DNA methylation at a single CpG site (Xiong and Laird, 1997). All of these methods measure average DNA methylation in a sample, at multiple CpG sites or at a single CpG site. Bisulphite sequencing with cloning allows visualization of DNA methylation at each CpG site being analyzed and allows the simultaneous analysis of multiple CpG sites. This technique allows the study of DNA methylation at the single cell level.

Array based methodology has been developed for the high throughput study of DNA methylation in the genome. Array based technology provides the advantage of simultaneous analysis of methylation at multiple CpG sites when insufficient information is available regarding potentially informative targets. In one assay developed by Illumina Inc. DNA methylation at 1,505 CpG sites can be evaluated (Biblikova et al., 2006; Biblikova and Fan et al., 2009). Following a high throughput approach, DNA methylation at selected targets is confirmed using specific single gene analysis.

1.5.2.3 Determining the origin of abnormal methylation

There are three mechanisms that are associated with abnormal methylation at imprinted genes: improper erasure, establishment or maintenance. During early development, maternal and paternal imprints are erased in the primordial germ cells (Kafri et al., 1992; Davis et al., 2000) and are re-established in a sex-specific manner. The imprints are then maintained throughout development. During spermatogenesis, the re-establishment of methylation is almost complete in spermatogonia (Li et al., 2004; Kerjean et al., 2000) and is complete before germ cells enter meiosis (Ueda et al., 2000). The imprints are fully set in post-meiotic male germ cells. Improper

erasure of methylation at imprinted genes methylated in the sperm, such as the *H19* DMR and the *IG-GTL2* DMR, followed by correct re-establishment of methylation cannot be differentiated from the proper occurrence of both steps in sperm by examining DNA methylation at cloned sequences. In such situations sperm carrying the maternal allele would have acquired methylation while sperm carrying the paternal allele would have maintained the methylation they already had. However, when proper erasure is followed by faulty establishment, sperm carrying either the maternal or paternal allele will not have the correct methylation and the imprint will resemble that of the oocyte. Presumably, correct methylation may still be established in some cells resulting in proper methylation being present in some sperm cells but not all. Also, the alleles affected may either be of paternal or maternal origin. Imprinted genes that are unmethylated in sperm, such as the *MEST* DMR, should have the methylation mark erased from the maternal allele with no re-establishment. Improper erasure would result in methylation being present in sperm carrying the maternal allele only, while improper establishment could affect both maternal and paternal alleles and result in sperm carrying methylation. The improper establishment may affect only some but not all sperm.

The presence of an informative single nucleotide polymorphism (SNP) within the sequence and knowing the parental origin of the two alleles could be used to identify the mechanism responsible for the abnormality. Methylation of only the maternal allele within the *MEST* DMR would imply improper erasure while methylation of both parental alleles would imply improper establishment. In the case of the *H19* and *IG-GTL2* DMRs, the presence of SNPs would help to determine the parental alleles on which methylation is not being properly reset. Errors in maintenance of methylation could also result in the presence of improper methylation in the sperm.

1.5.2.4 DNA methylation in oligozoospermic men

1.5.2.4.1 Analysis of DNA methylation at imprinted genes in sperm of oligozoospermic men

A number of studies have analyzed DNA methylation at imprinted genes in the sperm obtained from infertile men affected by oligozoospermia (Table 1.2) and have shown a higher

Table 1.2 Summary of studies examining the incidence of DNA methylation at imprinted genes in sperm of infertile men affected by oligozoospermia

Study	Methodology	Designation of improper methylation	Microscopic examination (10^6 sperm/ml)	Gene Analyzed								
				H19	GTL2	MEST	SNRPN	ZAC	LIT1	PEG3	IGF2	IGF2R
Reported proportion of men with abnormalities												
Manning et al., 2001a	M-PCR Analyzed 50 sperm	Presence of maternal allele	Normal				0/4					
			OAT				0/8					
Manning et al., 2001b	M-PCR; heminested PCR	Presence of maternal allele	Normal				3/30 (10); 19/30 (63.3)					
			5-20				3/30 (10); 17/30 (56.7)					
			<5				4/30 (13); 18/30 (60)					
	Single sperm analysis		All infertile				7/60 (11.7); 35/60 (58.3)					
			Normal				0/17[1]					
			5-20				0/17[1]					
			<5				0/16[1]					
			All infertile				0/33 (0)					
Marques et al., 2004	Direct bisulphite sequencing	Number of improperly methylated CpGs per sample	Normal	0/27 (0)		0/27 (0)						
			5-20	8/46 (17)[2]		0/46 (0)						
			<5	15/50 (30)[3]		0/50 (0)						
			All infertile	23/96 (23.9)		0/96 (0)						
Kobayashi et al., 2007	COBRA/bisulphite sequencing		Normal	0/79 (0)	5/79 (6)	7/79 (9)	1/79 (1.3)	0/79 (0)	3/79 (3.8)	4/79 (5.1)		
			5-20	1/7 (14.3)	2/7 (28.6)	2/7 (28.6)	2/7(28.6)	0/7	0/7	1/7(14.3)		
			<5	3/9 (33.3)	4/9 (44.4)	3/9 (33.3)	1/9 (11.1)	3/9 (33.3)	1/9	0/9		
			All infertile	4/16 (25)	6/16 (37.5)	5/16 (31.3)	3/16(18.8)	3/16 (18.8)	1/16 (6.3)	1/16 (6.3)		
Marques et al., 2008	Bisulphite sequencing with cloning	Hypomethylated[4] H19/ Hypermethylated[5] MEST	Normal	0/5 (0)		0/5 (0)						
			10-20	0/5 (0)		0/5 (0)						
			5-10	2/5 (40)		1/5 (20)						
			1-5	2/5 (40)		1/5 (20)						
			<1	1/5 (20)		2/5 (40)						
			All infertile	5/20 (25)		4/20 (20)						
Reported mean methylation level												
Hammond et al., 2009	Bisulphite sequencing with cloning	Mean methylation in patients vs. controls	Control (n=5)[7]	(97)		(1)	(3)	(2)	(0)	(2)	(9)	
			<10 (n=10)	(90)		(8)	(7)	(4)	(12)	(4)	(10)	
			protamine[6] (n=10)	(92)		(3)	(14)	(5)	(21)	(6)	(10)	
Poplinski et al., 2009	Dye terminator methylation analysis and direct sequencing	Mean methylation in patients vs. controls	normal + control (n=33)[7]	(90)		(7)						
			>20 (n=45)	(84)		(8)						
			10-20 (n=34)	(84)		(11)						
			4.8-10 (n=69)	(82)		(20)						
Boissonnas et al., 2010	pyrosequencing	Patient methylation below control methylation - 2SD for H19	Normal (n=17)[7]	(83.7±7.7); 0/17 (0)						(4.6±0.9)	(88.7±1.9)	(7±2.5)
			>20 (n=7)	(76.7±3.6); 0/7 (0)								
			5-10 (n=3)	(27.3±12.7); 3/3 (100)								
			1-5 (n=6)[8]	(41.4±29.2); 6/6 (100)								
			<1 (n=6)[8]	(31.6±19.9);6/6 (100)								
			All infertile	(53±26.3); 15/22 (68)						(5.8±3)	(78±15.2)	(9.1±3.9)

Normal: ≥20 million sperm /ml; male partner of couple undergoing ART
Control: ≥20 million sperm /ml; fertile sperm donor
Percentages shown in brackets
[1] total number of sperm analyzed from a total of 30 men
[2] 1-5 CpGs were unmethylated per patient
[3] 1-4 CpGs were unmethylated per patient
[4] presence of clones with >50% of unmethylated CpGs
[5] presence of clones with >50% of methylated CpGs
[6] patients with abnormal protamine replacement (altered P1:P2 ratio), average sperm count 73 million sperm/ml
[7] mean methylation levels were estimated from graphs,
[8] data on graphs was shown only for 5 of the 6 patients

incidence of imprinting abnormalities in the sperm of these men. Mostly infertile men affected by moderate oligozoospermia have been studied. Studies have also used different methodologies to gather and analyze the data, making comparisons in some cases difficult. The studies summarized in Table 1.2 are subdivided into studies that reported the proportion of men with imprinting abnormalities and those that reported mean methylation levels in the control and study groups.

DNA methylation has been evaluated at genes that are methylated in the sperm, such as *H19* and *GTL2*, and at genes that are unmethylated in the sperm, including *MEST, SNRPN, ZAC, LIT1, PEG3, IGF2* and *IGFR* (Table 1.2). However, DNA methylation has been most often analyzed at *H19, MEST* and *SNRPN*. Overall, studies have shown that the rate of imprinting errors in the sperm of men affected by oligozoospermia is higher than it is for normozoospermic men; furthermore the rate is higher in men affected by severe oligozoospermia compared to men affected by moderate oligozoospermia (Table 1.2). Both methylated and unmethylated imprinted genes in the sperm are affected and show the same trend of higher rates of abnormalities in severe oligozoospermia compared to moderate oligozoospermia. Analysis of genes that are methylated in the sperm included mostly evaluation of the *H19* DMR, while only one study examined the other known DMR to be methylated in sperm, the *IG-GTL2* DMR (Table 1.2). Abnormal DNA methylation in sperm at the *H19* DMR has been detected in as many as 20% of men affected by moderate oligozoospermia and in as many as 33.3% of men affected by severe oligozoospermia (Marques et al., 2004; Kobayashi et al., 2007;Marques et al., 2008), and in 40% of men affected by very severe oligozoospermia (Marques et al., 2008). Abnormal methylation at the *H19* DMR was detected in 100% of samples analyzed obtained from men affected by moderate, severe and very severe oligozoospermia (Boissonnas et al., 2010). Significantly reduced mean methylation at the *H19* DMR was also reported (Hammond et al., 2009; Poplinski et al., 2009; Boissonnas et al., 2010). Compared to other studies Boissonnas et al. (2010) reported very low mean methylation at the *H19* DMR of 27.3%, 31.6% and 41.4% in three groups of infertile men with varying severity of sperm parameters, while mean methylation level of 90% was detected by another study (Hammond et al., 2009). The results shown are not consistent. Methylation at *H19* DMR and *IGF2* DMR2 correlated with poor sperm parameters, confirming that the studies to date have

been analyzing a DMR that is specifically affected by poor sperm parameters (Boissonnas et al., 2010). Only one study to date has examined the imprint of the *IG-GTL2* DMR. Abnormal DNA methylation at the IG-GTL2 DMR was found in the sperm of 28.6% of men affected by moderate oligozoospermia and in 44.4% of men affected by severe oligozoospermia (Kobayashi et al., 2007). However, this analysis involved only one CpG site and may not have been representative of methylation at the surrounding CpG sites in the DMR. This is also true of analysis done for *PEG3* and *SNRPN*. The reported rates of abnormal DNA methylation at the *MEST* DMR have also varied considerably among studies, and the reported rates of abnormal methylation have ranged between 0 to 31.3% in men affected by oligozoospermia (Marques et al., 2004; Kobayashi et al., 2007; Marques et al., 2008). Abnormal methylation at the *MEST* DMR has been observed in between 0 and 28.6% of men affected by moderate oligozoospermia and in between 0 to 33.3% of men affected by severe oligozoospermia (Marques et al., 2004; Kobayashi et al., 2007; Marques et al., 2008) and in 40% of men affected by very severe oligozoospermia.

Some studies have also reported the presence of imprinting abnormalities or low methylation at imprinted genes in the sperm of control patients (Manning et al., 2001b; Kobayashi et al., 2007; Poplinski et al., 2009; Boissonnas et al., 2010). All of these studies included men in their control group that had sperm counts of more than 20 million sperm per milliliter, but that were partners of couples undergoing infertility treatment. These individuals may also be subfertile. It has been suggested that subfertile individuals may be at a higher risk for having children with imprinting disorders (Ludwig et al., 2005); therefore, these controls may not be the best controls to use. This is further supported by the fact that pregnancies conceived through ART using sperm from men with apparently normal sperm parameters, such as those used as controls for some of these studies, ended in spontaneous abortions. The abortuses had abnormal methylation at imprinted genes such as *LIT1, MEST, PEG3, SNRPN* and *GTL2* (Kobayashi et al., 2009). Some of these men also had variations in the *DNMT3L* and *DNMT3A* genes, but the significance of these variants is currently unknown (Kobayashi et al., 2009). Only one study included fertile donor men in their control group, and the methylation levels in this group were close to the expected values for paternally and maternally methylated genes (Hammond et al., 2009).

Although Marques et al. (2004) reported a higher incidence of imprinting errors at the *H19* DMR, an abnormality in the patient was defined as the presence of at least one unmethylated CpG site, and typically patients with abnormal methylation had between 1 and 5 CpGs unmethylated within the seventeen CpGs analyzed. The consequences of having a few selected unmethylated CpGs are currently unknown, while consequences associated with improper methylation at the single sperm level have been reported. Abnormal methylation at the single sperm level was passed on to the conceptus and associated with a negative pregnancy outcome (Kobayashi et al., 2009). In addition, Marques et al. (2008) reported to have only performed one amplification reaction on a small quantity of sperm with subsequent analysis of non-unique clones. This approach may be problematic for the analysis of a limited number of sperm cells obtained from men affected by severe and very severe oligozoospermia. Sperm in these patients was present in small quantities and was isolated by micromanipulation. Amplification of small amounts of starting material may be subject to preferential amplification, where DNA originating from just one cell may become over-represented in the final analysis. In such cases, it may become important to analyze individual clones, recognized by single nucleotide differences in the sequence, to confirm that DNA methylation at more than one sperm is analyzed.

In addition to analyzing methylation at imprinted genes in men affected by oligozoospermia, Hammond et al. (2009) also analyzed methylation at imprinted genes in men with abnormal protamine replacement. Protamine exchange affects sperm chromatin packaging and it is possible that patients with an altered protamine 1 to protamine 2 ratio may be at a higher risk for aberrant methylation due to improper chromatin packaging (Hammond et al., 2009). The study found that men with abnormal protamine replacement were more prone to having aberrant imprinting in their sperm compared to men affected by oligozoospermia. For example, higher mean methylation was found at *LIT1, SNRPN,* and *PEG3* in men with abnormal protamine replacement compared to oligozoospermic men. However, methylation at *H19* and *MEST* was more severely affected in oligozoospermic men compared to men with abnormal protamine exchange, suggesting there may be gene specific effects. No differences were found for *ZAC* or *IGF2*. The study also suggested that abnormalities at *H19* and *MEST* may be specific to oligozoospermia. Poplinski et al. (2010) tried to correlate imprinting abnormalities in the

sperm with mutations in the *CTCFL* gene that encodes the CTCF-like binding factor BORIS. The authors analyzed the *CTCFL* gene in twenty patients with abnormal methylation either at the *H19* DMR or the *MEST* DMR, but failed to detect any mutations (Poplinski et al., 2009).

1.5.2.4.2 Analysis of methylation at non-imprinted genes in sperm of oligozoospermic men

In addition to analyzing DNA methylation at imprinted genes, DNA methylation has also been analyzed at non-imprinted genes and at *LINE1* and *Alu* sequences in the sperm of men affected by oligozoospermia. Analysis of DNA methylation at repetitive sequences, such as *LINE1* and *Alu*, has been used as an estimate of global DNA methylation levels. No differences in methylation were identified in *LINE1* and *Alu* sequences between control and infertile men affected by oligozoospermia (Marques et al., 2008; Kobayashi et al., 2007; Boissonnas et al., 2010). Houshdaran et al. (2007) did report a correlation between poor sperm concentration and poor motility, and an increase in methylation at the repetitive element Satellite 2 (*SAT2CHRM1*). This study also identified a trend for increased methylation at non-imprinted genes with worsening sperm parameters. Increased methylation at *NTF2, MT1A* and *PAX8* correlated with poor sperm concentration, motility and morphology, while increased methylation at *HRAS* and *SNR* correlated with poor sperm concentration and motility (Houshdaran et al., 2007). The authors also subjected seven sperm samples obtained from partners of women undergoing evaluation for infertility to high throughput DNA methylation analysis using the Illumina GoldenGate Methylation Cancer Panel I. The GoldenGate assay involves the simultaneous analysis of methylation at 1,505 CpGs. The sperm concentration of the seven samples ranged between 20 and 95 million sperm per milliliter. The sperm sample with the most CpG sites showing abnormal DNA methylation had a concentration of 20 million sperm per milliliter. However, the results were not confirmed by specific single gene analysis (Houshdaran et al., 2007). This study suggests that in addition to imprinting errors, men with lower sperm counts may also be at risk of being affected by abnormal methylation at non-imprinted genes.

1.5.2.5 Analysis of DNA methylation in azoospermic men

1.5.2.5.1 Analysis of DNA methylation at imprinted genes in sperm of azoospermic men

There is little data available on the methylation of imprinted genes in men affected by azoospermia, either OA or NOA. Relatively few samples and genes have been analyzed (Table1.3). Germ cells other than sperm have also been analyzed. Analysis of spermatogonia and spermatocytes retrieved from three patients with spermatogenic arrest at the spermatogonia stage and from six patients with spermatogenic arrest at the spermatocyte stage, respectively, demonstrated proper methylation at the *H19* DMR (Hartman et al., 2006). Single cell analysis of the *SNRPN* DMR in thirty testicular sperm and five round spermatids obtained from four men affected by OA and two men affected by incomplete testicular failure (NOA) showed the expected lack of methylation (Manning et al., 2001a). Abnormal methylation at the *H19* DMR was found in one out of nine men affected by hypospermatogenesis (NOA) and at the *MEST* DMR in one out of five men affected by secondary OA due to inflammation (Marques et al., 2009). No abnormalities were found in anejaculatory men or men affected by OA due to CBAVD (Marques et al., 2009). The authors reported a lower mean methylation level at the *H19* DMR for the CBAVD group (96.3%) and for the NOA group (89.9%), while a higher mean methylation level was reported at the *MEST* DMR for the ANJ group (2.2%) and primary OA

Table 1.3 Summary of studies examining the incidence of DNA methylation at imprinted genes in sperm of infertile men affected by azoospermia.

Study	Method	Designation of improper methylation	Testicular Pathology	DMR Analyzed *H19*	*MEST*	*SNRPN*
Hartman et al., 2006	M-PCR	Presence of maternal allele	3 NOA: arrest at spermatogonia 6 NOA: arrest at spermatocyte	0/9		
Manning et al., 2001a	M-PCR Analyzed 35 single sperm	Presence of maternal allele	4 OA 2 NOA			0/35[1]
Marques et al., 2009	Bisulphite sequencing with cloning	Number of improperly methylated CpGs per sample; Hypomethylated[2] H19/ Hypermethylated[3] MEST	ANJ[4] OA - 2° (OAZI) - 1° (CBAVD) NOA (HS)	(97.6);0/5 0/10 (98.1); 0/5 (96.3)*; 0/5 (89.8)*; 1/9 (11)	(2.2)*; 0/5 1/10 (10) (2.4)*; 1/5 (20) (1.2); 0/5 (0.9); 0/9	

Percentages shown in brackets
* significantly different from other groups
[1] number of sperm analyzed
[2] presence of clones with >50% of unmethylated CpGs
[3] presence of clones with >50% of methylated CpGs
[4] ANJ (anejaculation mainly due to spinal cord injury); OAZI (obstructive azoospermia due to inflammatory epididymal disease); CBAVD (obstructive azoospermia due to CBAVD); HS (hypospermatogenesis)

(OAZI) group (2.4%), compared to the other groups analyzed (Table 1.3). It is not known at this time whether small changes in methylation at the examined DMRs would affect pregnancy outcome. For this analysis only one amplification reaction was set up per gene for each patient using a small amount of starting material (Marques et al., 2008). As already discussed before, such results may be subject to preferential amplification and over-representation of a few cells. The *IG-GTL2* DMR has not yet been analyzed in azoospermic men.

1.5.2.6 Selection of proper control samples for analysis of DNA methylation at imprinted genes in men affected by severe oligozoospermia and azoospermia.

Of the eight published papers evaluating DNA methylation at imprinted genes in the sperm of men affected by oligozoospermia (Table 1.2), six studies used sperm obtained from normozoospermic men undergoing evaluation for infertility (Manning et al., 2001a; Manning et al., 2001b; Marques et al., 2004; Kobayashi et al., 2007; Marques et al., 2008; Boissonnas et al., 2010) and two studies used sperm obtained from men of proven fertility as controls in their data sets (Poplinski et al., 2009; Hammond et al., 2009). Abnormal methylation or a relatively low rate of methylation at imprinted genes was reported in the sperm of normozoospermic men used as controls by two studies (Kobayashi et al., 2007; Boissonnas et al., 2010; Table 1.2). The presence of abnormal methylation in the sperm of normozoospermic men suggests that better controls may be sperm retrieved from men of proven fertility. Normozoospermic men may be sub-fertile since they are undergoing evaluation for infertility. Sperm retrieved from men of proven fertility were selected as controls for the severe oligozoospermia group analyzed in this study.

The three published papers that evaluated DNA methylation at imprinted genes in the sperm of men affected by azoospermia did not include control samples in their data set (Hartman et al., 2006; Marques et al., 2009; Manning et al., 2001a). The lack of controls may be in part associated with the difficulty in obtaining testicular sperm from fertile men. Appropriate controls may include sperm retrieved from testicular tissue of fertile men undergoing vasectomy or vasectomy reversal. A vasectomy is a surgical procedure that is used as male contraception to prevent further pregnancies; therefore most men undergoing a vasectomy have already fathered children and are fertile. During the procedure the vas deferens are severed bilaterally to prevent sperm from reaching the ejaculate. A vasectomy reversal may be performed to reconnect the

severed vas deferens. Return of sperm in the ejaculate is dependent on the duration of the vasectomy (Silber, 1977; Belker et al., 1991). It has been shown that after vasectomy sperm may undergo resorbtion (Jones, 2004) or apoptosis (Shiraishi et al., 2001; O'Neil et al., 2007) and that the testicular tissue may undergo destruction (Aydos et al., 1998) to decrease sperm production. In addition, obstruction resulting from the vasectomy may increase production of reactive oxygen species (ROS) (Aydos et al., 1998). Excessive presence of ROS has been associated with oxidative damage to DNA (Oschsendorf, 1999). ROS induced DNA damage has been associated with loss of DNA methylation (Weitzman et al., 1994; Turk et al., 1995; Hepburn et al., 1991; Tan et al., 1990) and could also affect chromatin condensation (Valinluck et al., 2004; Henkel et al., 2010). These results suggest that changes that occur in response to obstruction post vasectomy may affect the epigenome. Because of the possibility that DNA methylation may be affected in men post vasectomy other controls may be needed, such as ejaculate sperm of proven fertile men, when studying DNA methylation at imprinted genes in testicular sperm of men with azoospermia.

1.5.2.7 Statistical analysis of data

A probability value of equal to or less than 5% is widely considered statistically significant when comparing a single variable between two groups. It represents the type I alpha error and at the 5% level assumes that the null hypothesis will be inappropriately rejected 5% of the time. Significance at the 5% level may no longer be sufficient when multiple genes or treatments are tested in a data set. Corrections for multiple testing have been introduced when multiple hypotheses are tested in order to decrease the rate of false positives (Benjamini and Hochberg, 1995). At least eighteen methods exist that can be used to correct for multiple testing (Dudolt et al., 2003); however, there is little consensus in the literature as to how statistical analyses should be corrected (Benjamini and Hochberg, 1995; Slonim, 2002; Roeder and Wasserman, 2009). Correction for multiple testing has been accepted for statistical analysis of microarray data where hundreds or thousands of genes are simultaneously tested (Slonim, 2002; Roeder and Wasserman, 2009) and is mandatory for studies submitted to the Food and Drug Administration of the USA (Benjamini and Hochberg, 1995); however, its use is lacking in many published studies (Benjamini and Hochberg, 1995). The Bonferroni correction is the simplest correction that can be performed and the corrected P value is calculated by multiplying

the uncorrected P value by the number of tests performed (Slonim, 2002). Corrected P values <0.05 are then considered significant. Alternatively, Bonferroni corrected P values can be calculated by dividing 0.05 by the number of tests performed. The uncorrected P values must then be below the corrected P value to be considered significant (Benjamini and Hochberg, 1995). Correction for multiple testing can be incorporated into ANOVA analysis by performing the Dunn's multiple comparison post hoc test. For comparison of data obtained through microarray technology, such as the GoldenGate Illumina assay, the use of a false discovery rate (FDR) has been introduced by Benjamini and Hochberg (1995). The FDR is defined as the expected proportion of false rejections among the rejected hypotheses (Benjamini and Hochberg, 1995). An FDR <0.05 is an available option for statistical analyses performed using the BeadStudio software available from the manufacturer Illumina Inc. Tests that correct for multiple testing aim to control the rate of false positives, but due to their stringency, may increase the rate of false negatives.

1.6 RATIONALE

Infertility affects an estimated 15% of couples today. Male factor infertility contributes to the inability to conceive in 50% of couples. Although a number of physiological, hormonal and genetic factors are well known to contribute to male factor infertility, infertility remains idiopathic in 50% of cases. Recent reports have suggested that aberrant DNA methylation at imprinted genes may contribute to spermatogenesis failure seen in male factor infertility.

Imprinted genes undergo a process of genomic reprogramming. DNA methylation is erased from the genome in primordial germ cells (Szabo et al., 2002; Davis et al., 2000). It is then re-established at imprinted genes in a sex-specific manner. In the male, *de novo* DNA methylation at imprinted genes is initiated at the prospermatogonia stage (Li et al., 2004) and is fully set before germ cells enter meiosis (Kerjean et al., 2000). Potentially, errors in imprint erasure or establishment could correlate with abnormal DNA methylation at imprinted genes in the sperm. Alternatively, maintenance of DNA methylation could also be affected. Animal studies have shown that a loss of DNA methylation in the sperm was associated with male infertility (Doerksen and Trasler, 1996, Doerksen et al., 2000; Oakes et al., 2007). Mutations in DNMTs, *Dnmt3a* and *Dnmt3l,* were associated with male infertility and abnormal DNA

methylation at imprinted genes in the germ cells (Kaneda et al., 2004; Yaman and Granjean, 2006; Webster et al., 2005). Furthermore, DNA methylation may also be affected by environmental factors. Factors such as maternal diet have been shown to affect DNA methylation in the fetus (Waterland and Jirtle 2003; Dolinoy et al., 2006), and *in utero* exposure to endocrine disruptors has been associated with male infertility (Anway et al., 2005). Male gametes may be particularly vulnerable to perturbations of methylation during *in utero* development as it is during this time that genomic imprinting is established. Exposure to environmental factors after birth may also affect spermatogenesis (Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). Published studies show that DNA methylation is important for proper spermatogenesis and male fertility (Marques et al., 2008; Kobayashi et al., 2007; Raman and Narayan, 1995; Doerksen and Trasler, 1996). Studies also suggest abnormal DNA methylation may be acquired during *in utero* development or environmental exposure to certain factors (Anway et al., 2005; Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). Furthermore, abnormal DNA methylation in the sperm may be passed on through the use of ART and affect pregnancy outcome or the well being of the child (Anway et al., 2005; Kobayashi et al., 2009; Kanber et al., 2009; Orstavik et al., 2003). The use of ART has been associated with negative pregnancy outcomes (Sutcliffe et al., 2003; Katalinic et al., 2004) and there may be an epigenetic component to these complications.

The majority of DMRs of imprinted genes are methylated in the oocyte, including *MEST,* while only a few imprinted genes identified to date are methylated in the sperm, and include *H19* and the IG-*GTL2* in humans (Kerjean et al., 2000; Geuns et al., 2007). Most studies to date have primarily evaluated genomic imprinting in the sperm of men affected by mild to moderate oligozoospermia (Kobayashi et al., 2007; Marques et al., 2008; Marques et al., 2004, Poplinski et al., 2009; Boissonnas et al., 2010; Hammoud et al., 2009). Limited information is currently available on the status of methylation at imprinted genes in the sperm of men affected by severe and very severe oligozoospermia and the results vary considerably among studies. Aberrant imprinting at the *H19* and *MEST* DMRs was reported in 30 to 100% and 0 to 33.3% of men affected by severe oligozoospermia, respectively (Marques et al., 2008; Marques et al., 2004; Boissonnas et al., 2010; Kobayashi et al., 2007). Furthermore, only one study to date has evaluated methylation at the *IG-GTL2* DMR and this study reported aberrant imprinting in

44.4% of men affected by severe oligozoospermia (Kobayashi et al., 2007). However, methylation at only one CpG site was evaluated and may not have been representative of methylation at neighboring CpG sites. In addition, the effect of severe versus very severe oligozoospermia on DNA methylation at the DMRs of imprinted genes is not clear, and although results suggest an increased rate of aberrant imprinting with worsening sperm parameters, the association has not always been observed (Marques et al., 2008; Boissonnas et al., 2010).

Abnormal methylation at imprinted genes in the sperm of men has been primarily reported in men affected by moderate oligozoospermia (Marques et al., 2008; Kobayashi et al., 2007; Boissonnais et al., 2010; Houshdaran et al., 2007). While methylation at repetitive DNA sequences, such as *LINEs* and *Alus*, appears to be normal in infertile men (Marques et al., 2008; Kobayashi et al., 2007), data regarding methylation at non-imprinted genes in the sperm of infertile men remains limited. To date one study has evaluated DNA methylation at non-imprinted genes in the sperm of infertile men, but has only reported a trend for significant change in DNA methylation at five non-imprinted genes (Houshdaran et al., 2007). Therefore it is currently not known whether abnormal methylation in the sperm of infertile men is specific to imprinted genes or whether non-imprinted genes are also affected.

To date only two studies have evaluated DNA methylation at imprinted genes in the sperm retrieved from men affected by azoospermia. The few number of samples analyzed suggest a much lower rate of abnormal DNA methylation in the sperm of men affected by azoospermia, ranging between 0% to 5.3% (Marques et al., 2009; Hartmann et al., 2006) compared to the sperm of men affected by oligozoospermia, ranging between 20% to 68% (Marques et al., 2008; Kobayashi et al., 2007; Boissonnas et al., 2010). Factors that may account for the discrepancy in the rates of abnormal methylation at imprinted genes between the two groups of infertile men are unknown. Furthermore, analysis of DNA methylation at imprinted genes in the sperm retrieved from men with different etiologies, NOA and OA, would help in the understanding of factors that may disrupt DNA methylation such as spermatogenesis failure in NOA patients or obstruction in OA patients. Analysis of DNA methylation at imprinted genes in the testicular sperm retrieved from men undergoing vasectomy reversal may also show whether disruption of imprinting is associated with obstruction.

1.6.1 Hypotheses and specific objectives

1. Evaluation of DNA methylation at imprinted genes in men affected by severe and very severe oligozoospermia

***Hypotheses*:**

1(a) Aberrant DNA methylation at imprinted genes will be more prevalent in the sperm of men affected by oligozoospermia compared to control men.

1(b) Aberrant DNA methylation will be more prevalent in the sperm of men affected by very severe oligozoospermia compared to men affected by severe oligozoospermia.

Objectives:

1(a) Methylation at the DMRs of three imprinted genes, *H19, GTL2* and *MEST,* will be studied in the sperm of infertile men affected by severe and very severe oligozoospermia and compared to methylation in the sperm of control men of proven fertility. The study of these genes in human sperm will allow us to determine whether the three imprinted genes are equally affected by epigenetic abnormalities or whether there may be a gene specific effect where certain genes are more sensitive to methylation errors.

1(b) Where possible, based on the presence of a polymorphism in the DMR, the origin of a DNA methylation error will be evaluated to determine whether the error occurred due the lack of erasure or improper establishment

2. Evaluation of DNA methylation at non-imprinted genes in men affected by severe oligozoospermia

***Hypothesis*:**

2(a) Abnormal DNA methylation in the sperm of men affected by severe oligozoospermia will be associated with aberrant DNA methylation at non-imprinted genes.

Objective:

2(a) Analyze DNA methylation at non-imprinted genes in the sperm of men affected by severe oligozoospermia using a genome-wide approach. DNA methylation at 1,505 CpG sites will be

analyzed by the Illumina GoldenGate Methylation Cancer Panel I. Methylation at selected CpG sites will be confirmed using a gene-specific approach by pyrosequencing.

3. Evaluation of DNA methylation at imprinted genes in testicular sperm retrieved from men affected by azoospermia

Hypotheses:

3(a) We hypothesize a higher prevalence of imprinting abnormalities will be present in the sperm of men affected by azoospermia and in men undergoing vasectomy reversal compared to fertile control men.

3(b) We also hypothesize that sperm obtained from men affected by obstructive azoospermia will be more prone to aberrant DNA methylation at imprinted genes compared to sperm retrieved from men affected by non-obstructive azoospermia.

Objectives:

3(a) Methylation at the DMRs of three imprinted genes, *H19, GTL2* and *MEST,* will be studied in testicular sperm retrieved from men affected by azoospermia, obstructive and non-obstructive, and men undergoing a vasectomy reversal. Analysis of DNA methylation at imprinted genes in the sperm retrieved from men with different etiologies, NOA and OA, will help in the understanding of factors that may disrupt DNA methylation such as spermatogenesis failure in NOA patients or obstruction in OA patients. The study of three imprinted genes will allow us to determine whether all three genes are equally susceptible to changes in methylation.

3(b) Where possible, the origin of an error in DNA methylation will be assessed to determine whether the error occurred due the lack of erasure or improper establishment.

CHAPTER 2: EVALUATION OF DNA METHYLATION AT IMPRINTED GENES IN MEN AFFECTED BY SEVERE AND VERY SEVERE OLIGOZOOSPERMIA

2.1 INTRODUCTION

Inability to achieve pregnancy affects one out of seven couples. Female and male factors contribute equally to infertility. Factors contributing to male infertility remain unknown in about 50% of cases (de la Calle et al., 2001). Abnormal DNA methylation at imprinted genes has been associated with spermatogenesis failure (Kobayashi et al., 2007; Marques et al., 2008) and may be a contributing factor to some cases of male infertility.

DNA methylation is involved in the control of gene expression and genomic imprinting. Imprinted genes show mono-allelic parent-specific gene expression that is often regulated through oocyte and sperm specific DNA methylation at DMRs (Szabo et al., 2002; Davis et al., 2000). DNA methylation at imprinted genes is fully established before germ cells enter meiosis (Kerjean et al., 2000) and is maintained throughout development (Olek and Walter 1997; Tremblay et al., 1997). Animal and human data have suggested DNA methylation to be important for proper spermatogenesis. In rodents, mutations in *Dnmt3a* and *Dnmt3l* were associated with male infertility in otherwise healthy animals and disrupted DNA methylation at imprinted genes (Kaneda et al., 2004; Webster et al., 2005). Environmental factors, such as maternal diet and *in utero* exposure to endocrine disruptors, have been associated with modifications of DNA methylation (Waterland and Jirtle 2003; Dolinoy et al., 2006) and male infertility (Anway et al., 2005), respectively. Male gametes may be particularly vulnerable to perturbations of methylation during *in utero* development as it is during this time that genomic imprinting is established. Exposure to environmental factors after birth may also affect spermatogenesis. For example, higher seminal plasma levels of methyl donors in males correlated with increased sperm concentration and decreased sperm DNA damage in humans (Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). The data suggest that methylation acquired through environmental exposure may affect spermatogenesis and fertility.

Two genes have been identified that have a methylated DMR in human sperm, *H19* and *GTL2* (Kerjean et al., 2000; Geuns et al., 2007). The majority of DMRs of imprinted genes are

methylated in the oocyte, including *MEST* (Kerjean et al., 2000). Most studies that have correlated abnormal DNA methylation at imprinted genes with male infertility evaluated sperm of men affected by mild to moderate oligozoospermia (Kobayashi et al., 2007; Marques et al., 2008; Marques et al., 2004; Poplinski et al., 2009; Boissonnas et al., 2010; Hammoud et al., 2009). There is little information in the literature regarding the status of DNA methylation at imprinted genes in the sperm of men affected by severe oligozoospermia and the results vary considerably among studies. Studies have reported abnormal methylation at the *H19* DMR in the sperm of up to 100% of men affected by severe oligozoospermia (Marques et al., 2008; Marques et al., 2004; Boissonnas et al., 2010; Kobayashi et al., 2007). The incidence of abnormal DNA methylation at the *MEST* DMR was lower and was found in the sperm of up to 33.3% of men affected by severe oligozoospermia (Marques et al., 2008; Marques et al., 2004; Boissonnas et al., 2010; Kobayashi et al., 2007). Methylation at the *IG-GTL2* DMR in the sperm of men affected by severe oligozoospermia has been evaluated by one study, which found an incidence of 44.4% (Kobayashi et al., 2007). However, because DNA methylation was analyzed at only one CpG site, it may not have been representative of methylation at neighboring CpG sites. In addition, results suggest an increased rate of aberrant imprinting with worsening sperm parameters; however, this association has not always been observed (Marques et al., 2008; Boissonnas et al., 2010).

In this study DNA methylation of two paternally methylated DMRs, *H19* and *IG-GTL2*, and of one paternally unmethylated DMR, *MEST*, was studied by bisulphite sequencing in the sperm of infertile men affected by severe and very severe oligozoospermia. Sperm retrieved from men of proven fertility were used as controls. Bisulphite sequencing is considered the gold standard for the study of methylation as it allows the simultaneous analysis of DNA methylation at multiple CpG sites and the direct visualization of methylation at the single sperm level. While the consequences of abnormal methylation at single CpG sites are currently unknown, they have been documented for abnormalities at the single sperm level. The working hypothesis is that methylation abnormalities at imprinted genes will be more prevalent in the sperm of men affected by oligozoospermia compared to control men. We further hypothesized that methylation abnormalities at imprinted genes will be more prevalent in the sperm of men affected by very severe oligozoospermia compared to men affected by severe oligozoospermia.

In addition, where possible based on the presence of SNPs within the DMR, the origin of an error was evaluated, to determine whether the error occurred due the lack of erasure or improper establishment of the DNA methylation imprint. The study of three imprinted genes in human sperm allowed us to determine whether different imprinted genes are equally affected by epigenetic abnormalities or whether there may be a gene specific effect where certain genes are more sensitive to methylation errors. The data gathered will provide a better understanding of the frequency of abnormal methylation at imprinted genes present in the sperm of infertile men, which may be important in clinical counseling of couples attempting ART.

2.2 MATERIALS AND METHODS

2.2.1 Sample preparation

2.2.1.1 Sample collection

Semen samples were collected from control men who had normal semen parameters according to WHO criteria (WHO, 1999). The selected control men were also of proven fertility having had a child within one to two years prior to the collection of a semen sample. Semen samples were obtained from infertile men undergoing fertility evaluation at the University of British Columbia Centre for Reproductive Health. Leftover semen samples were obtained from men affected by severe oligozoospermia having sperm counts below 5 million sperm per milliliter. Samples also showed reduced motility and poor morphology, complications that are often seen in patients with reduced sperm counts. Ethical approval was obtained from the University of British Columbia Ethics Committee before initiating this study.

In total 35 samples were obtained: 9 samples were obtained from control men (C01-C09) and 26 samples were obtained from men with sperm counts below 5 million sperm per milliliter (Oligo; P01-P26). The infertile study group was further subdivided into two sub-groups: patients with a sperm count between 5 and 1 million sperm per milliliter (n=15) (Oligo-I; P01-P15) and patients with a sperm count below 1 million sperm per milliliter (n=11) (Oligo-II; P016-P26).

2.2.1.2 Karyotyping and screening for Y chromosome microdeletions

Chromosomal abnormalities and Y chromosome micordeletions are associated with male factor infertility. Clinical information regarding patient's chromosome analysis and presence of

Y chromosome microdeletions was obtained from patient's charts. When this information was not available patients were asked to donate a blood sample so that the analysis could be performed. A blood sample from control men was also obtained. Peripheral blood was drawn into a sodium heparinized vacutainer collection tube for blood culture. When possible blood was also drawn into an EDTA vacutainer collection tube for molecular analysis. Chromosome analysis was carried out on G-banded stimulated cultured whole blood samples using standard culture conditions. Metaphase spreads were analyzed under a light microscope (Zeiss) connected to a computer equipped with chromosome analysis software (Cytovision). Five metaphases were analyzed and karyotyped, and two additional metaphases were counted. Typically chromosomes at a band resolution of 500 were analyzed. To test for Y chromosome microdeletions a PCR based assay was designed that evaluated the presence of 15 STSs spanning the AZFa (sy84, sy86, sy625, sy117, sy127), AZFb (sy129, sy134, sy143) and AZFc (sy152, sy147, sy149, sy254, sy255, sy157) regions and the Y chromosome long arm heterochromatin (sy160). STSs were selected for analysis based on published reports of informative STSs associated with male factor infertility. Whole blood DNA was extracted using the standard salt extraction method. Deletion of an STS was confirmed if it did not amplify in three separate amplification reactions.

2.2.1.3 Purification of sperm

Sperm concentration for each sample analyzed was either obtained from a clinical chart or by counting sperm in unprepared semen using the Makler Counting Chamber (Sefi-Medical Instruments, Ltd, Haifa, Israel). After liquifaction at 37^{o}C in a humidified incubator for 30 min, sperm concentration was determined according to the manufacturer's instructions. Semen samples were washed two to three times in modified human tubule fluid (mHTF) (Irvine Scientific, Santa Ana, CA). Depending on sperm concentration and motility, sperm were either isolated by swim-up or micromanipulation. For swim-up, washed sperm were pelleted in a 1.5ml microfuge tube (Sarstedt Ltd, Montreal, QC) in a small amount of mHTF medium and incubated for one to two hours at 37^{o}C in a humidified incubator. After incubation, the top medium layer was carefully transferred to a 0.7ml microfuge tube (Sarstedt Ltd, Montreal, QC). Presence of isolated sperm and lack of other cells was assessed by phase contrast microscopy (Nikon, Tokyo, Japan). In cases of very low sperm count or low motility, 200-350 sperm were

isolated by micromanipulation using an inverted microscope (Nikon, Tokyo, Japan) equipped with Hoffman modulating optics, a thermal stage and micromanipulators (Narishige, Tokyo, Japan). Custom-made micropipettes were used to pick up sperm. 20µl droplets of sample and 10µl droplets of mHTF media were deposited in a 60x15mm petri dish (Corning, Lowell, MA) and overlaid with mineral oil (Sigma-Aldrich Canada Ltd, Oakville, ON). Sperm were picked and deposited into a clean droplet of medium. Attention was paid to only transfer clean sperm without any debris on the sperm or in the medium. Upon completion of isolation, the clean sperm sample was transferred to a thin-walled 0.7ml microfuge tube (Sarstedt Ltd, Montreal, QC) using a 10µl tip and a micropipette. Complete transfer was confirmed by looking for remaining sperm under the micromanipulator.

2.2.1.4 DNA isolation

DNA extraction from pure sperm isolated by swim-up was modified from Doerksen et al. (2000). Digestion was carried out in 3ml of sperm lysis buffer containing 20mM Tris (pH 8.0), 10mM dithio-threitol (DTT), 150mM NaCl and 10mM ethylenediaminetetraacetic acid (EDTA; pH8.0), 1ml of 10% sodium dodecyl sulfate (SDS) (all Sigma- Aldrich Canada Ltd, Oakville, ON) and 50µl of 5µg/ml proteinase K (Invitrogen Canada Inc., Burlington, ON). The sample was incubated at 60^{o}C in a water bath overnight or until complete digestion. DNA was extracted by standard salt extraction method, washed in 70% ethanol and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8). Sperm DNA concentration was determined by spectrophotometry and the samples were of adequate quality having a 260/280 ratio in the range of 1.7 to 2.0 (Eppendorf Canada, Mississauga, ON). Sperm picked up by micromanipulation were resuspended in 20µl of alkaline lysis buffer containing 200mM KOH (Sigma- Aldrich Canada Ltd, Oakville, ON) and 50mM of DTT (Invitrogen Canada Inc., Burlington, ON) to decondense the sperm DNA according to Manning et al. (2001). The cells were then frozen at –80^{o}C for at least three days. After thawing the cells were lysed at 80^{o}C for 15min on the thermoblock (Eppendorf Canada, Mississauga, ON) and 20µl of neutralization buffer was added containing 0.9M Tris-HCl, 0.3M KCl and 0.2M HCl (Sigma-Aldrich Canada Ltd, Oakville, ON).

2.2.1.5 Sodium bisulphite modification

Bisulphite modification of DNA consists of the deamination of unmethylated cytosine residues into uracil, while methylated cytosine residues are protected from this modification. Following PCR amplification and analysis of the modified sequences, the methylation status of the original DNA sample can be determined. Originally methylated cytosines will remain as cytosines, while unmethylated cytosines will read as thymidines. Bisulphite modification was either performed on 20µl containing 500ng of sperm DNA or on lysed sperm cells split into two aliquots of 20µl using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). The modification was carried out according to the manufacturer's instructions. However, the lysed sperm cells were incubated for a decreased amount of time of 2 hours to limit degradation of the small amount of DNA used. The samples were eluted in water. Bisulphite modified DNA was stored for short term at –20^{o}C or at –80^{o}C for long-term storage. Bisulphite modification consistently provided a conversion rate of over 95% of unmethylated cytosines to thymidine. Only sequences with a conversion rate of or above 95% were included in the results.

2.2.2 Analysis of DNA methylation

2.2.2.1 Sequences analyzed

Two DMRs methylated in the sperm, *H19* and *IG-GTL2*, and one DMR unmethylated in the sperm, *MEST,* were analyzed in the sperm. The three sequences are depicted in Figure 2.1, including the SNPs found within each sequence. Each genomic sequence (non-bisulphite modified) analyzed is presented in Table 2.1. CpG sites analyzed are indicated in brackets and the location of each SNP is indicated in bold font. Presence and location of SNPs in each genomic sequence analyzed was determined based on information displayed through the Basic Local Alignment Search Tool (BLAST) provided on the NCBI website. Four SNPs were identified in the *H19* sequence analyzed: C/T at nucleotide 67 (SNP #1073516), -/C at nucleotide 106 (SNP #34610866), C/A at nucleotide 109 (SNP #2071094) and C/A at nucleotide 112 (SNP #35678657) (Table 2.1). The C/T SNP is located within CG dinucleotide number 7, therefore methylation at CpG 7 is not informative with regard to the methylation status of the *H19* DMR and methylation at this CpG sites was excluded from the analysis of methylation. Within the *IG-GTL2* DMR sequence two SNPs were found: C/G at nucleotide 34

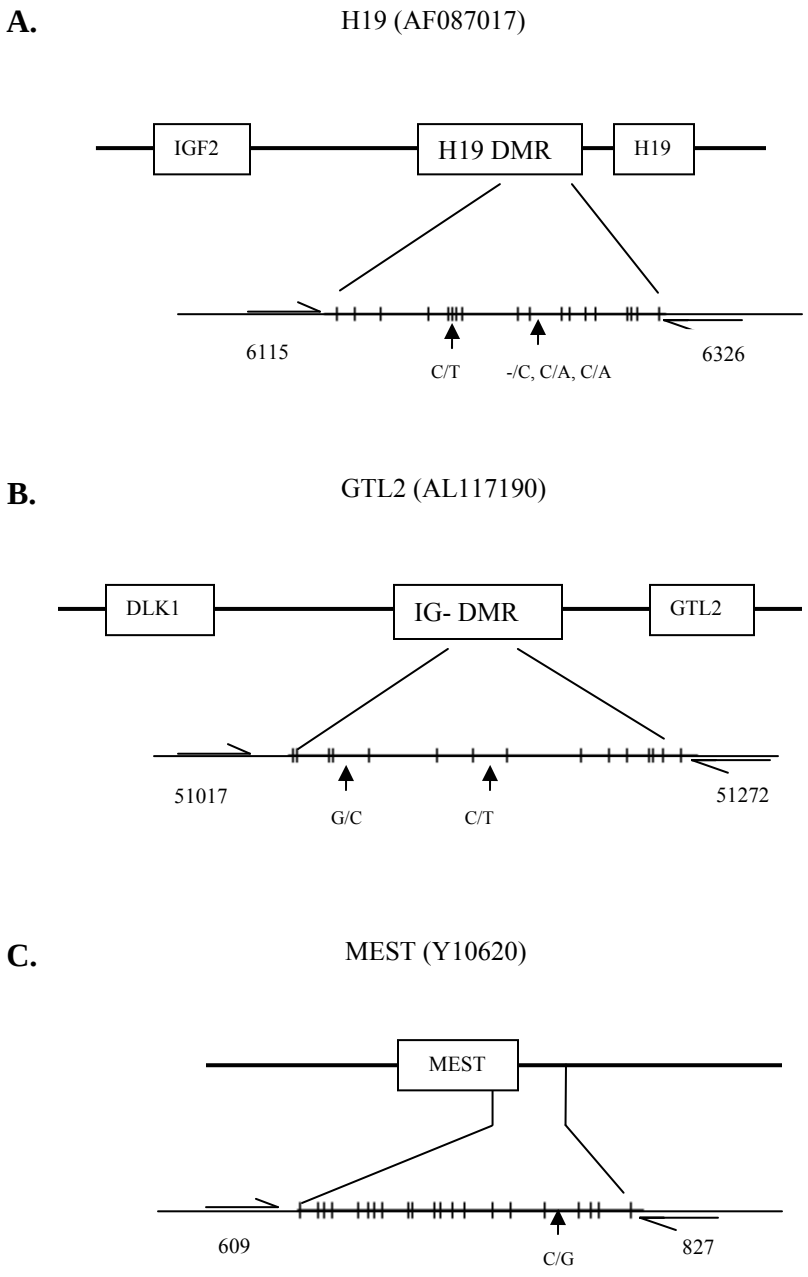


Figure 2.1. **Representation of the genomic sequences of analyzed DMRs.** Each region analyzed is depicted with the accession number indicated at the top for each sequence; (A) *H19* DMR, (B) *IG-GTL2* DMR and (C) *MEST* DMR. Location of primers is indicated within the accessioned sequence. Polymorphisms present within the sequences are indicated by arrows; (A) four polymorphisms are present including three between CpG number 10 and 11 and the C/T polymorphism at CpG number 7, (B) two polymorphisms are present but the C/T polymorphism is not informative for bisulphite modified DNA and (C) one C/G polymorphisms is present. Adapted from Kobayashi et al. (2007) and Geuns et al. (2007).

Table 2.1 Genomic sequences of analyzed imprinted genes.

DMR analyzed	CpG (N)	Genomic sequence analyzed
H19	17[1]	ctcctt[cg]gtctcac[cg]cctggatggca[cg]gaattggttgtagttgtggaat[cg]gaagtggc[cg][cg][**c**g][1]g[cg]gcagtgcaggctcacacatcacagcc[cg]agcc[cg]**c**cc**c**aa**c**tggggtt[cg]cc[cg]tggaaa[cg]tcc[cg]ggtcacccaagcca[cg][cg]t[cg]cagggttca[cg]gg
IG-GTL2	15[2]	cc[cg][cg]gctcaccagttgcc[cg][cg]actcaccag**g**tgcctg[cg]gctcaccagttgcctgtg gctcaccagctgcc[cg]tggctcaccagctgcc[cg]tggcttacagttg**c**c[cg]aggctcacagttgc ccatggcttgctaattgccag[cg]atttgccaattg[cg]agtggtt[cg]ccagttgcc[cg][cg]gtc[cg]ctaaacc[cg]taatcct
MEST	21[3]	g[cg]ggctctg[cg]g[cg]cc[cg]gtgctctgcaa[cg]ctg[cg]g[cg]gg[cg]gcatgggataa[cg][cg]gccatggtg[cg]c[cg]agat[cg]cctc[cg]caggtgagtgtg[cg]gtgggaa[cg]agggggtgtggctgg[cg]g**c**cctgggactaggg[cg]cagg[cg]ag[cg]gaggactgtgtgcc[cg]tgtcc

Location of SNPs is indicated in bold text

[1] the sequence contains 18 CpGs, however, CpG number 7 in the sequence is a known C/T polymorphisms and was therefore not taken into account when analyzing methylation within the sequence

[2] the sequence amplified contains 15 CpGs, however, methylation for the last 10 (CpG 6 to 15) was analyzed because of the presence of truncated sequences for the first five CpGs

[3] the sequence analyzed contains 21 CpGs that were analyzed in this project, however, the last C in the sequence is followed by a G and was analyzed as the 22nd CpG is some publications (for example Marques et al., 2008; Kerjean et al., 2000; Kobayashi et al., 2007).

(SNP #9671389) and C/T at nucleotide 108 (SNP #74455228). The C/T SNP is not informative for bisulphite modified DNA. In the *MEST* DMR sequence one SNP was found: C/G at nucleotide 127 (SNP #75706706).

2.2.2.2 DNA amplification

Semi-nested amplification was carried out to amplify the *H19* DMR, *IG-GTL2* DMR, and *MEST* DMR using tested published primer sequences (Table 2.2). Semi-nested polymerase chain reaction (PCR) involves two rounds of amplification, where one of the two primers used in the first round is re-used in the second round and is coupled with a primer that is specific to the pre-amplified sequence. This approach improves amplification success of small quantities of DNA and of degraded DNA, such as DNA after sodium bisulphite modification. The protocol for PCR amplification published by Kerjean et al. (2000) was followed for the amplification of all three sequences with minor modifications. Amplification was carried out in a 25µl volume containing 1X Buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs (Invitrogen Canada Inc., Burlington, ON), 0.5uM of each primer (Sigma-Genosys, Oakville, ON) and 0.5U of Taq polymerase (Invitrogen Canada Inc., Burlington, ON). 0.5-1µl of bisulphite modified DNA was added to each reaction.

Table 2.2. Primer sequences specific to imprinted genes analyzed.

Sequence analyzed		Primer Sequence	Size (bp)	CpG (N)	Reference
H19 DMR	F1	aggtgttttagttttatggatgatgg	172	18	Kerjean et al., 2000
	R1	tcctataaatatcctattcccaaataacc			
	Fnes	tgtatagtatatgggtatttttggaggttt			
IG-GTL2 DMR	F1	gtggatttgtgagaaatgattygt	205	15	Geuns et al., 2007
	R1	ccattataaccaattacaataccac			
	Fnes	gttagttgtttgtggtttattagttg			
MEST DMR	F1	tygttgttggttagttttgtayggtt	173	21	Kerjean et al., 2000
	R1	aaaaataacacccccctcctcaaat			
	Rnes	cccaaaaacaaccccaactc			

nes refers to nested primer

Amplification was performed using the following conditions: initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 45 sec, 59 ^{o}C for 45 sec, 72 ^{o}C for 60 sec, and a final extension step at 72 ^{o}C for 10 min. One to two microliters from the first amplification were added to the second amplification reaction. 30 to 35 amplification cycles were carried out for the second amplification round using the same cycling conditions that were used for the first round. Reagent controls were included in each round of amplification. On average five PCR reactions were set up for each gene per sample.

The primer sequences used were obtained from publications that had demonstrated a lack of amplification bias toward the paternal or maternal allele, indicated by a 50:50 ratio of methylated to unmethylated alleles seen after amplification of blood, fibroblast or amniocyte DNA (Kerjean et al., 2000; Geuns et al., 2007). At the beginning of the study primer sequences for the *IG-GTL2* DMR were obtained from Astuti et al., (2005); however, these primers were later changed to the current primers (Geuns et al., 2007) because they showed poor amplification efficiency. The sequence amplified by the new primers overlapped with the sequence amplified by the Astuti et al. (2005) primers, and sequences amplified using the old primers were trimmed to correspond to the same fragment size amplified by the new primers.

2.2.2.3 Cloning

The PCR products were run on a one percent agarose gel (Invitrogen Canada Inc., Burlington, ON) containing 2µl ethidium bromide (Sigma-Aldrich Canada Ltd, Oakville, ON) and the products were visualized on a gel trans-illuminator. 3µl of bench top 100bp DNA ladder (Promega, Madison, WI) were loaded on each gel and bands of appropriate size were cut out. DNA fragments were purified using the GenElute Gel Extraction Kit (Sigma-Aldrich Canada Ltd, Oakville, ON) following the manufacturer's instructions. The fragments were then cloned into the pGEM-T Easy Vector System (Promega, Madison, WI) according to the manufacturer's protocol with changes. Each reaction was set up in a third of the protocol volume using 1.68µl of 2X rapid ligation buffer, 0.4µl of T4 DNA ligase and 0.33µl of pGEM-T Easy Vector and 1µl of purified PCR product. The ligation reactions were incubated at 4^{o}C overnight and 20µl of JM109 high efficiency competent cells (Promega, Madison, WI) were added. Following the resting period, the cells were shocked at 42^{o}C for 2 min, and then cooled on ice for another 2 min. 380µl of Luria-Bertani (LB) broth (Invitrogen Canada Inc., Burlington, ON) were added and samples were incubated at 37^{o}C. Samples were occasionally shaken throughout the 1.5-hour incubation period. The samples were then plated on agar (Sigma-Aldrich Canada Ltd, Oakville, ON) plates containing ampicillin, IPTG and X-Gal (all Invitrogen Canada Inc., Burlington, ON) to allow for blue/white colony screening. The plates were incubated overnight at 37^{o}C.

2.2.2.4 DNA extraction from colonies

Two to three white colonies were picked per plate so that around ten white colonies were picked for each gene amplified. The picked white colonies were incubated overnight in a shaking incubator at 37^{o}C in 2ml of LB broth containing 100µg/ml ampicillin in 10ml snap top tubes. Plasmid DNA was purified using the plasmid buffer set P1, P2, and P3 solutions (Qiagen, Mississauga, ON) according to the manufacturer's instructions. The reactions were scaled down to a third of the protocol volume: 300µl of each solution were added and the extractions were carried out in 1.5ml microfuge tubes. The DNA was precipitated in 800µl of isopropanol (Fisher Scientific, Ottawa, ON) and the pellet washed in 500µl of 70% ethanol. After drying, the plasmid DNA was dissolved in 30µl of water and the concentration of DNA was measured by

spectrophotometry (Eppendorf Canada, Mississauga, ON). Typically the concentration of the plasmid DNA ranged from 1.0 to 2.5µg/µl with a 260/280 ratio between 1.7 and 1.9.

2.2.2.5 Restriction digest

The presence of the correct insert was confirmed by restriction digest. 10µg of plasmid DNA were incubated overnight at 37^{o}C in a 20µl reaction containing 20U of EcoRI (New England Biolabs Ltd., Pickering, ON), 1X EcoRI buffer and 0.1µg/µl acetylated bovine serum albumin (Promega, Madison, WI). The digests were run on a 1% agarose gel (Invitrogen Canada Inc., Burlington, ON) containing 2µl ethidium bromide (Sigma-Aldrich Canada Ltd, Oakville, ON) and the products were visualized on a gel trans-illuminator. Plasmid samples containing the correct insert DNA were submitted for sequencing.

2.2.2.6 Sequencing

Between 3µg and 5µg of sample in a total volume of 10µl in water were submitted for sequencing to the McGill University and Génome Québec Innovation Centre (Montreal, QC). Products were sequenced with the SP6 sequencing primer (5'-tatttaggtgacactatag-3') using the Applied Biosystems 3730xl technology (Applied Biosystems Inc., Foster City, CA). Once the sequencing results were ready, the files were downloaded from the McGill University and Génome Québec Innovation Centre Nanuq web application (Montreal, QC). The sequences were downloaded as FASTA text files either in the forward or reverse complement.

2.2.2.7 Alignment and analysis of sequences

FASTA files were aligned using ClustalW2, an online program for multiple DNA sequence alignment (Larkin et al., 2007). The FASTA test files were aligned against the corresponding non-modified genomic sequence (Table 2.1). Sequences were manually analyzed for differences in methylation at the CpG sites as well as for any single nucleotide changes such as SNPs and single base changes. These differences were used to determine the unique clone status of each strand. Unique clones originate from different sperm cells and may be more representative of the methylation status of different sperm cells and the sample. The amplification and cloning of multiple sequences for the same gene per sample also allowed for the analysis of unique clones. Products from each reaction would have originated from different

starting DNA or sperm cells. Small quantities of starting material may be particularly sensitive to preferential amplification (Walsh et al., 1992; Findlay et al., 1995) especially when amplified using nested PCR where more than the usual number (usually 35) of cycles is used.

FASTA sequences were converted into diagrams representing methylated (black beads) and unmethylated (white beads) CpG sites within a sequence using the online tool for analysis of bisulphite sequencing results QUantification tool for Methylation Analysis (QUMA) (Kumaki et al., 2008). Only the unique clones for each gene were displayed. The proper alignment and presence of single nucleotide differences among the sequences were confirmed using this online tool.

2.2.3 Data analysis

The methylation level for each sample was calculated based on the number of methylated CpGs in proportion to the total number of CpGs analyzed at unique clones within each DMR analyzed. This analysis provided a percent methylation value. The methylation level was used to calculate the median and mean methylation for each group analyzed and the standard deviation of the mean. Differences in the methylation level between the Control group and the Oligo group were determined using the non-parametric Mann-Whitney test. Differences in gene methylation level between the Control group and the two sub-groups were determined using the Kruskal-Wallis test with Dunn's multiple comparison post hoc test. One-tailed p-values <0.05 were considered significant.

The number of individuals with abnormal methylation at imprinted genes was determined and compared between groups. An individual was designated as having abnormal methylation at an imprinted gene based on the presence of at least one improperly methylated unique clone. Improperly methylated clones were defined as being either fully unmethylated or hypomethylated: identified as having less than 50% of unmethylated CpGs, at the *H19* DMR and the *IG-GTL2* DMR. Improperly methylated clones were also defined as being either fully methylated or hypermethylated: identified as having more than 50% of methylated CpGs, at the *MEST* DMR. Differences in the number of individuals affected per group were determined using Fisher's exact test. One-sided p-values <0.05 were considered significant. . Bonferroni corrected p values were also shown.

The frequency of improper methylation at each CpG site within an analyzed sequence was also determined. This was defined as the number of improper methylation at each CpG site analyzed within a sequence in proportion to the total number of CpG sites analyzed at that site in all unique clones. Differences in methylation at each CpG among groups were determined using Fisher's exact test. Two-tailed p-values <0.05 were considered significant. Bonferroni corrected p values were also shown.

Other statistical tests were performed as indicated. All statistical analysis was done using GraphPad Prism (version 5.02) for Windows (GraphPad Software, San Diego, CA).

2.3 RESULTS

2.3.1 Patient clinical information

The mean age of the men in the control group was 31.6 years. Age was available for 18 patients and the mean age of the Oligo group was 35.8±4.9 years. The mean age of the Oligo-I group was significantly increased compared to the Oligo-II groups (38.1±4.6 vs. 33.0±3.8, respectively, p=0.023). Four patients were affected by varicocele (P02, P09, P18 and P20; two from each group), one patient had an AZFc deletion (P16) and one patient had an inv 8 (P23). All other patients had a normal 46, XY karyotype and did not have Y chromosome microdeletions (Table 2.3).

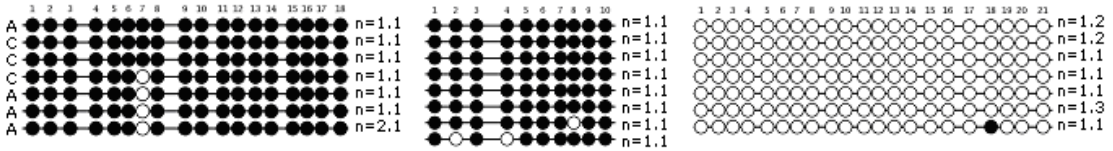
Table 2.3. Clinical information for oligozoospermic men.

Population	N	Sample ID	Sperm concentration (10^6/ml)	Age (mean±SD)	Abnormalities found
Oligo	26	P01-P26	<5	35.8±4.9	-
Oligo-I	15	P01-P15	1-5	38.1±4.6	varicocele (P02-P09)
Oligo-II	11	P016-P26	<1	33.0±3.8	varicocele (P18, P20), AZFc deletion (P16), 46,XY, inv 8 (P23)

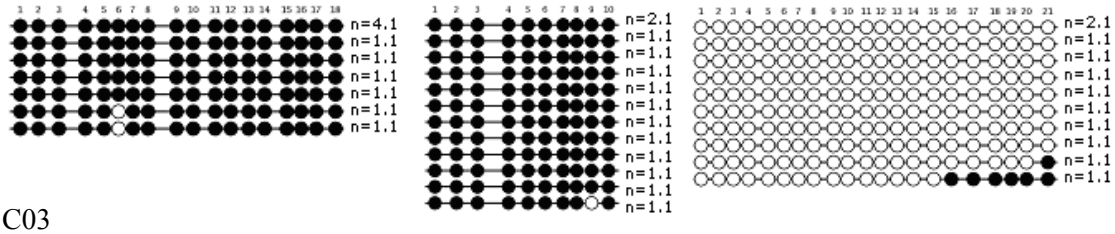
[a] abnormalities found include chromosome abnormalties, Y chromosome microdeletions and presence of varicocele.
[b] age was available for 18 patients

A. CONTROL MEN

C01



C02



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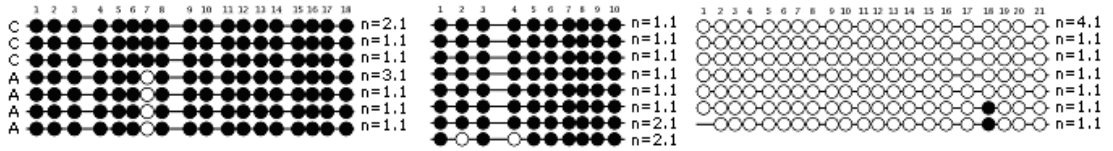
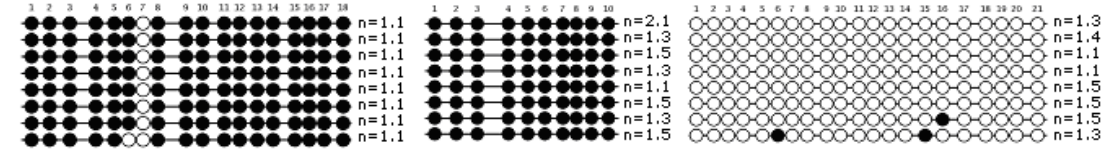
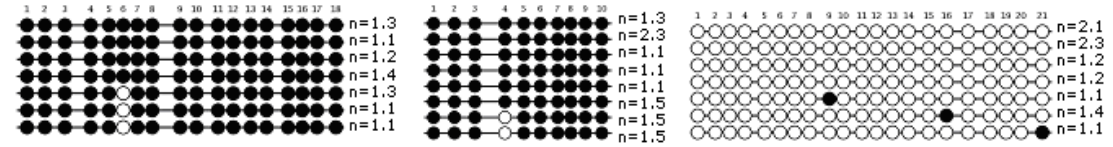


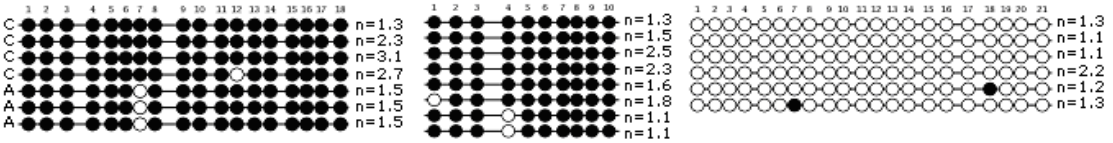
C04



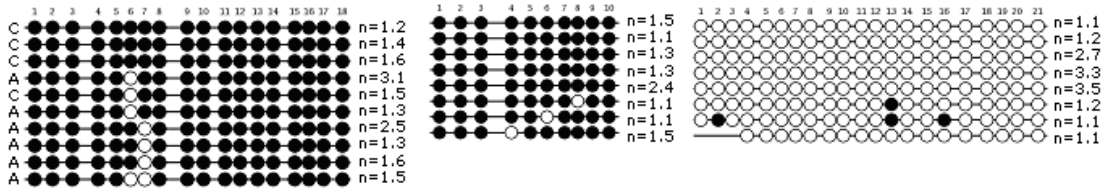
C05



C06



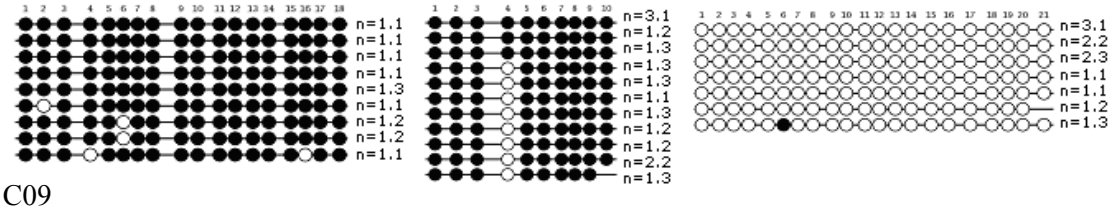
C07



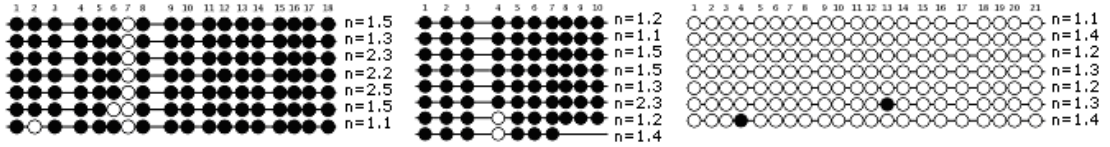
H19 DMR *IG-GTL2* DMR *MEST* DMR

A. CONTROL MEN (continued)

C08

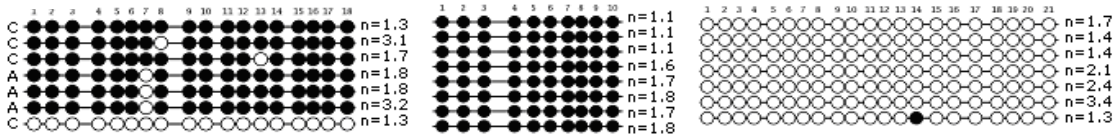


C09

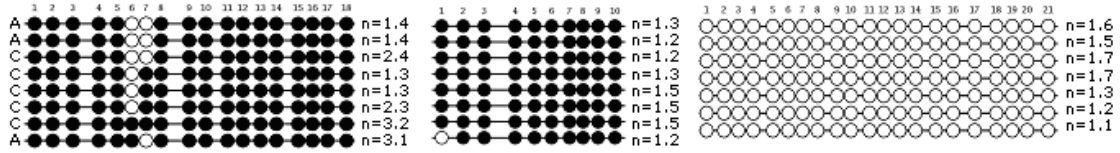


(severe oligozoospermia)

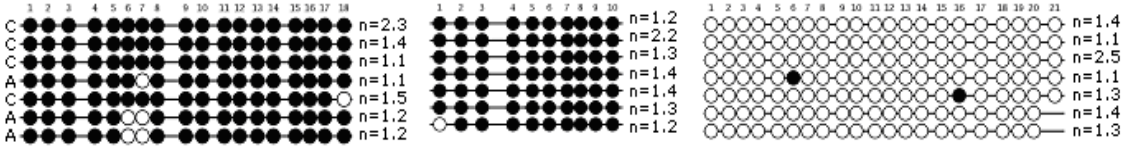
P01



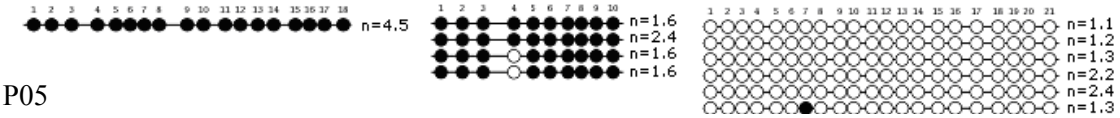
P02



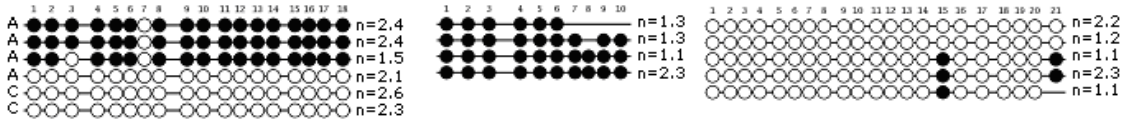
P03



P04



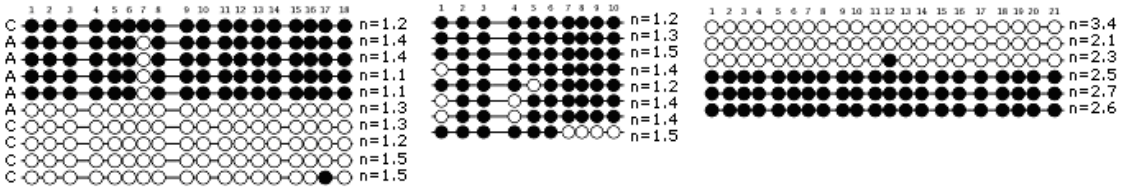
P05



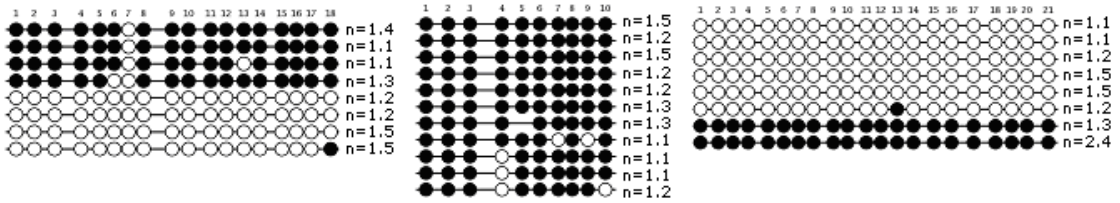
H19 DMR *IG-GTL2* DMR *MEST* DMR

B. OLIGOZOOSPERMIC MEN (continued)

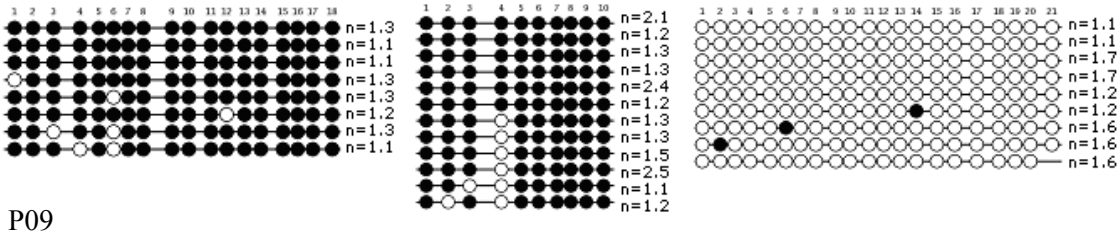
P06



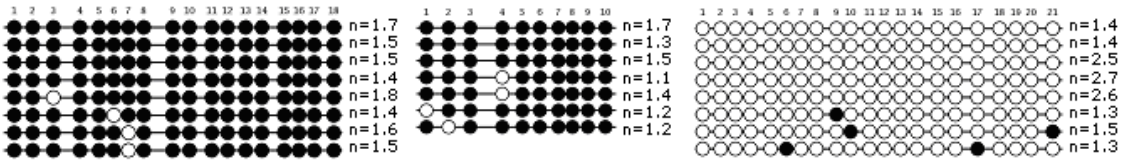
P07



P08



P09



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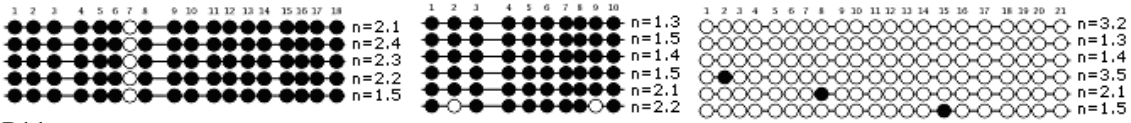

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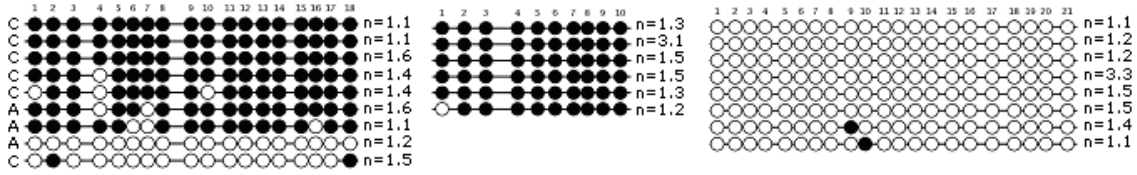

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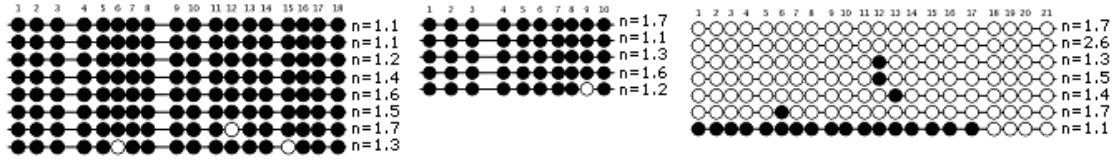
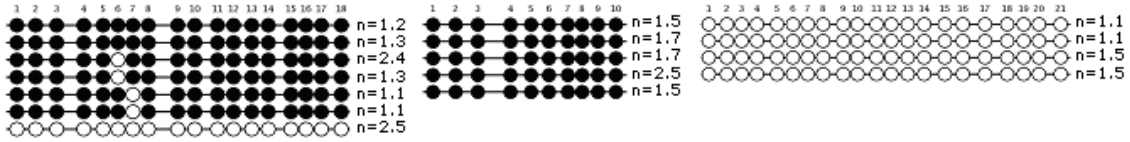


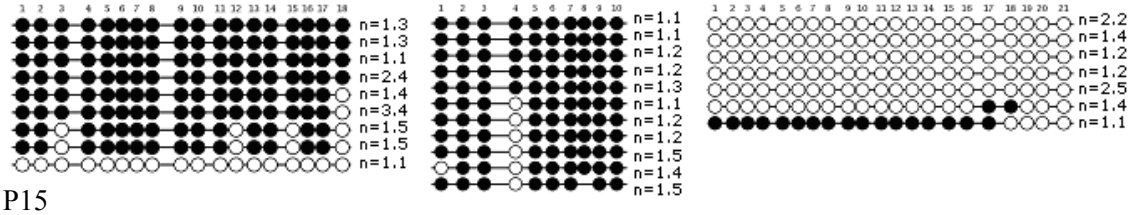
H19 DMR *IG-GTL2* DMR *MEST* DMR

B. OLIGOZOOSPERMIC MEN (continued)

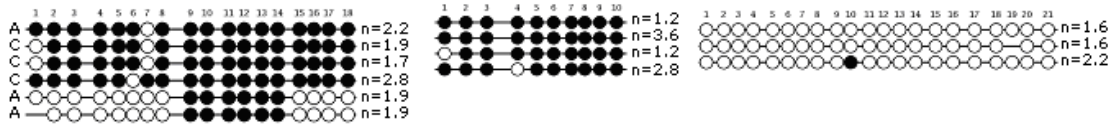
P13



P14

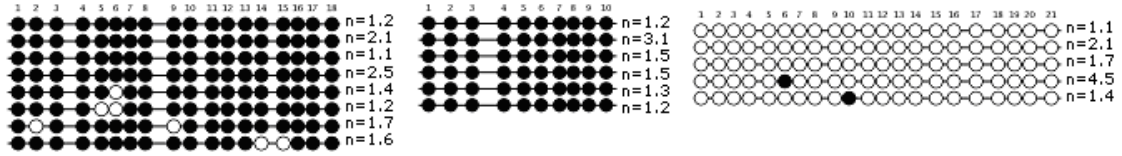


P15

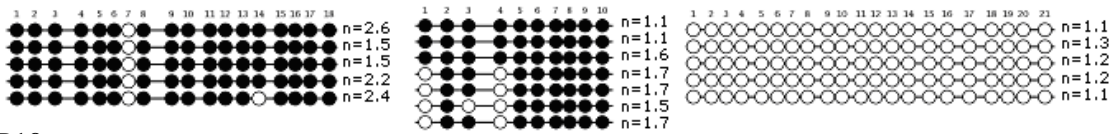


(very severe oligozoospermia)

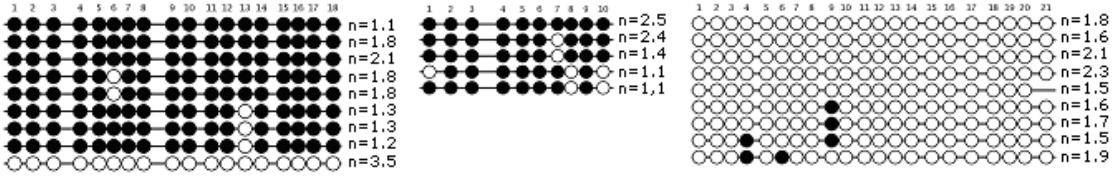
P16



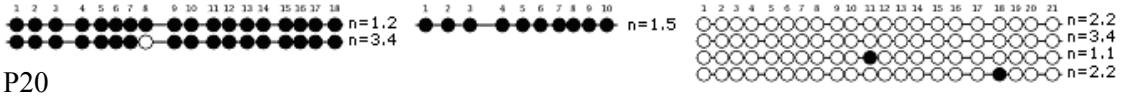
P17



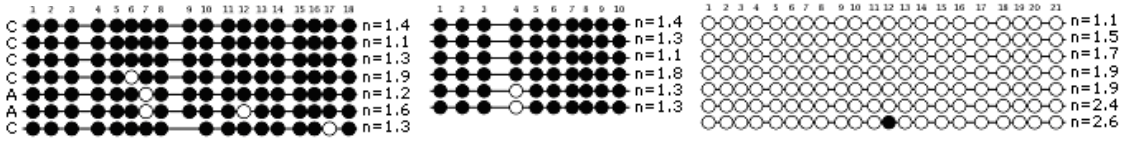
P18



P19



P20



H19 DMR *IG-GTL2* DMR *MEST* DMR

B. OLIGOZOOSPERMIC MEN (continued)

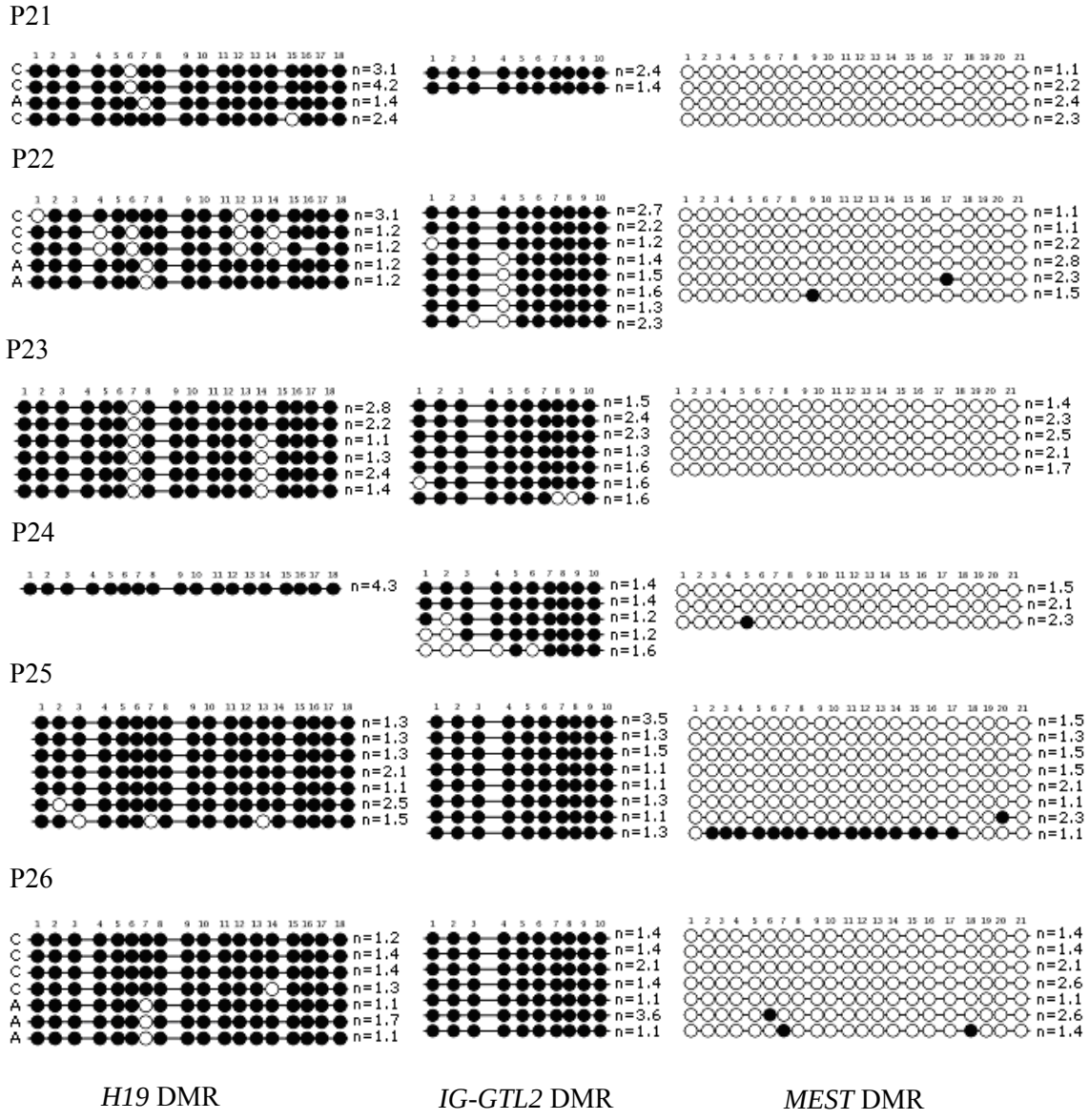


Figure 2.2. **Bead diagrams representing methylation at CpG sites studied at the *H19* DMR, *IG-GTL2* DMR and *MEST* DMR in the control and oligozoospermia groups.** Methylated (black bead) and unmethylated (open bead) status of each CpG site is indicated within the studied sequences. Missing beads represent CpG sites that could not be analyzed. Unique clones analyzed at each DMR are shown directly in the diagram, and are coded on the right-hand side: the first number indicates the number of non-unique clones that were analyzed for each sequence followed by the amplification reaction the clones came from. The amplification reactions are not necessarily labeled in consecutive order. In samples containing an informative SNP, the allele is indicated on the left-hand side of each clone. In this data set, two SNPs were informative: C/T at nucleotide 67 (at CpG #7) and C/A at nucleotide 109 (indicated on the left-hand side), both within the *H19* DMR sequence.

2.3.2 Analysis of methylation at imprinted genes

2.3.2.1 Analysis of sequencing data

Eighteen CpGs were analyzed at the *H19* DMR, ten CpGs were analyzed at the *IG-GTL2* DMR and 21 CpGs were analyzed at the *MEST* DMR. Figure 2.2 shows bead diagrams representing methylation at CpGs studied at the *H19*, *IG-GTL2* and *MEST* DMRs. Unique clones analyzed at each DMR are shown directly in the diagram, and are coded on the right-hand side with the first number designating the number of non-unique clones that were analyzed for each sequence followed by the amplification reaction each clones came from. The amplification reactions are not necessarily labeled in consecutive order. In samples containing an informative SNP, the allele is indicated on the left-hand side of each clone. In this data set, two SNPs were informative: C/T at nucleotide 67 and C/A at nucleotide 109 both in the *H19* sequence. The C/T SNP locates to CpG number 7 and methylation at that CpG implies the presence of the C allele, while a lack of methylation implies the presence of either allele.

Unique clones were identified based on single nucleotide differences among clones. An example of this analysis is shown in Figure 2.3. In total 894 clones were analyzed, 260 in the Control group and 498 in the Oligo group. Of the 894 clones analyzed, 713 were unique. On average, eight unique clones were analyzed per gene for the control group and six to seven unique clones were analyzed for the Oligo group: with seven unique clones being analyzed for the Oligo-I sub-group and five to six unique clones for the Oligo-II sub-group. In some cases, multiple amplification reactions failed and due to a limited amount of sample available fewer clones could be analyzed. Table 2.4. shows the general trend for a decrease in the proportion of unique clones in study groups with decreasing sperm concentrations. For example, 80.7% of clones analyzed for the *MEST* DMR were unique in the Control group, compared to 76.7% in the Oligo-I sub-group and 69.2% in the Oligo-II sub-group. A similar trend was noticed for *H19* and *IG-GTL2* DMRs but to a lesser extent. This analysis suggests that samples with a smaller amount of starting material, such as those with a lower sperm concentration, may be more prone to preferential amplification resulting in clones having originated from the same strand of DNA. On average, 79.8% of sequenced clones were unique: 85.8% of analyzed clones for *IG-GTL2* were unique compared to 78.2% for *H19* and 75.6% for *MEST* (Table 2.4). This difference is likely related to the number of non-CpG cytosines within these sequences that allowed

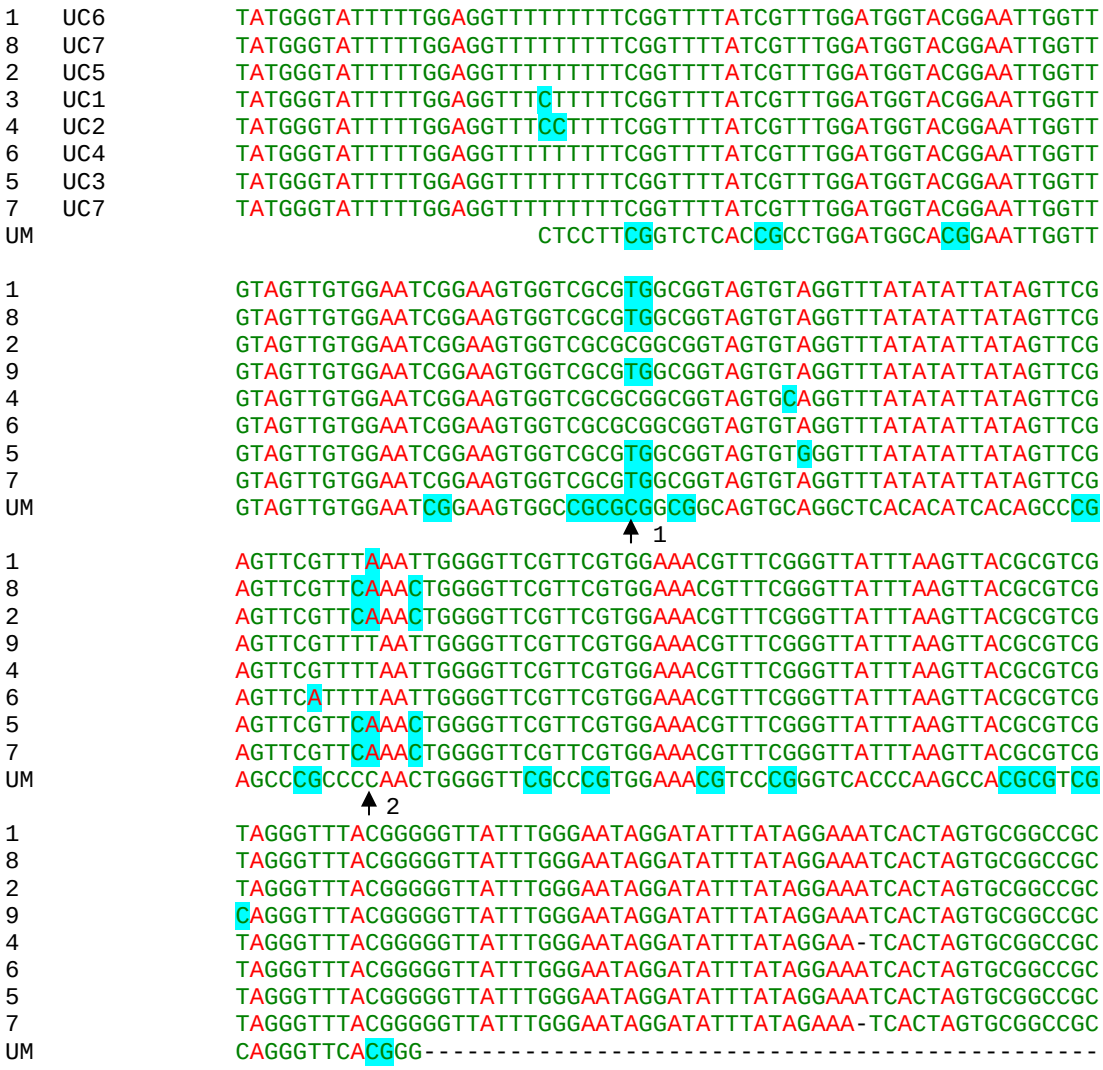


Figure 2.3. **Analysis of sequenced clones**. An example of analysis of the sequenced clones for the *H19* DMR is shown for the control sample C01. The sequenced clones, labeled from 1 to 8 on the left hand side, were aligned against the unmodified (UM) *H19* sequence. Seven unique clones, labeled as UC 1 to 7, were identified based on single nucleotide differences among clones, such as unmodified cytosines outside of CpGs and in this case A/G changes. The presence of informative SNPs, C/T at nucleotide 67 and C/A at nucleotide 109 is indicated by arrowheads 1 and 2, respectively. The SNPs were also used to mark differences between clones. There were no differences between clones 7 and 8; therefore, these two clones were counted as one unique clone. In this example all clones originated from the same amplification reaction. Differences in the sequences are highlighted.

Table 2.4. Proportion of clones analyzed in control and oligozoospermic men.

Study Group	*H19*	*GTL2*	*MEST*	Group total
	unique clones/ all clones (%)			
Control	69/86 (80.2)	79/91 (86.8)	67/83 (80.7)	215/260 (82.7)
Oligo-I	107/133 (80.5)	106/120 (88.3)	99/129 (76.7)	312/382 (81.7)
Oligo-II	61/84 (72.6)	62/77 (80.5)	63/91 (69.2)	186/252 (73.8)
Oligo	168/217 (77.4)	168/197 (85.3)	162/220 (73.6)	498/634 (78.5)
Gene total	237/303 (78.2)	247/288 (85.8)	229/303 (75.6)	713/894 (79.8)

distinguishing between unique and non-unique clones. In the *IG-GTL2* sequence there are 60 cytosines, while there are 39 and 27 cytosines outside of CG dinucleotides in the *H19* and *MEST* sequences, respectively. These numbers correspond to the proportion of unique clones obtained for each gene.

As it can be seen in Figure 2.2 multiple amplification reactions were performed for each gene per sample. Between one and eight amplification reactions were set up per gene, with more reactions being set up for samples in the Oligo group as it was more difficult to obtain unique clones from these samples. For example, six amplification reactions were needed to obtain six unique clones for analysis of *MEST* in patient P06 (Figure 2.2). The six unique clones were obtained from the analysis of a total of thirteen clones. All clones originating from the same amplification reaction were identical.

Based on the presence of the C/A SNP at nucleotide 109 in the *H19* sequence in fifteen samples analyzed it was possible to determine whether there was an amplification bias toward one of the alleles. In total, one hundred unique clones containing the SNP were analyzed: 53 clones had the C allele and 47 clones had the A allele. The difference was not statistically significant (Fisher's exact test, p=0.78), confirming a lack of amplification bias towards one of the alleles.

2.3.2.2 Analysis of methylation at DMRs of imprinted genes

Methylation level for each sample was calculated based on the proportion of methylated CpG sites to the total number of CpG sites analyzed in unique clones at each DMR. Mean and median methylation for each group were also calculated. These results are presented in Table 2.5 and in Figure 2.4. Presence of hypomethylated or fully unmethylated clones found in

Table 2.5. Methylation level at each DMR analyzed in control and oligozoospermia groups.

	H19 DMR		*IG-GTL2* DMR		*MEST* DMR	
Control men	**methylation (%)**	**# hypome clones**	**methylation (%)**	**# hypome clones**	**methylation (%)**	**# hyperme clones**
C01	100.00	0	96.25	0	0.68	0
C02	98.32	0	99.17	0	0.95	0
C03	100.00	0	97.50	0	1.37	0
C04	99.26	0	100.00	0	1.79	0
C05	97.48	0	97.50	0	2.04	0
C06	99.16	0	96.25	0	1.59	0
C07	97.65	0	96.25	0	2.42	0
C08	97.39	0	92.66	0	0.68	0
C09	98.32	0	97.40	0	1.37	0
mean ± SD	98.62±1.03		97.00±2.09		1.43±0.60	
median	98.32		97.40		1.37	
Oligo-I						
P01	84.03	1	100.00	0	0.68	0
P02	95.59	0	98.75	0	0.00	0
P03	97.48	0	98.75	0	1.38	0
P04	100.00	0	95.00	0	1.36	0
P05	49.02	3	100.00	0	4.81	0
P06	50.59	5	87.50	0	50.79	3
P07	49.26	4	94.50	0	25.60	2
P08	94.85	0	93.33	0	1.60	0
P09	97.06	0	94.29	0	2.98	0
P10	100.00	0	96.67	0	2.38	0
P11	74.51	2	98.33	0	1.19	0
P12	97.79	0	98.00	0	14.29	1
P13	84.03	1	100.00	0	0.00	0
P14	82.35	1	93.58	0	12.93	1
P15	76.24	2	95.00	0	1.61	0
mean ± SD	82.19±18.82		96.25±3.43		8.11±13.81	
median	84.03*		96.67		1.61	
Oligo-II						
P16	94.85	0	100.00	0	1.90	0
P17	98.82	0	87.14	0	0.00	0
P18	85.62	1	86.00	0	3.19	0
P19	97.06	0	100.00	0	2.38	0
P20	98.32	0	96.67	0	0.68	0
P21	95.59	0	100.00	0	0.00	0
P22	88.24	0	91.25	0	1.59	0
P23	96.08	0	95.71	0	0.00	0
P24	100.00	0	84.00	0	1.59	0
P25	97.48	0	100.00	0	10.71	1
P26	99.16	0	100.00	0	2.04	0
Mean ± SD	95.57±4.58		94.62±6.36		2.19±3.02	
median	97.06		96.67		1.59	
Oligo group						
mean ± SD	87.85±15.88		95.56±4.84		5.60±10.92	
median	95.59*		96.67		1.61	

* significant difference between control group

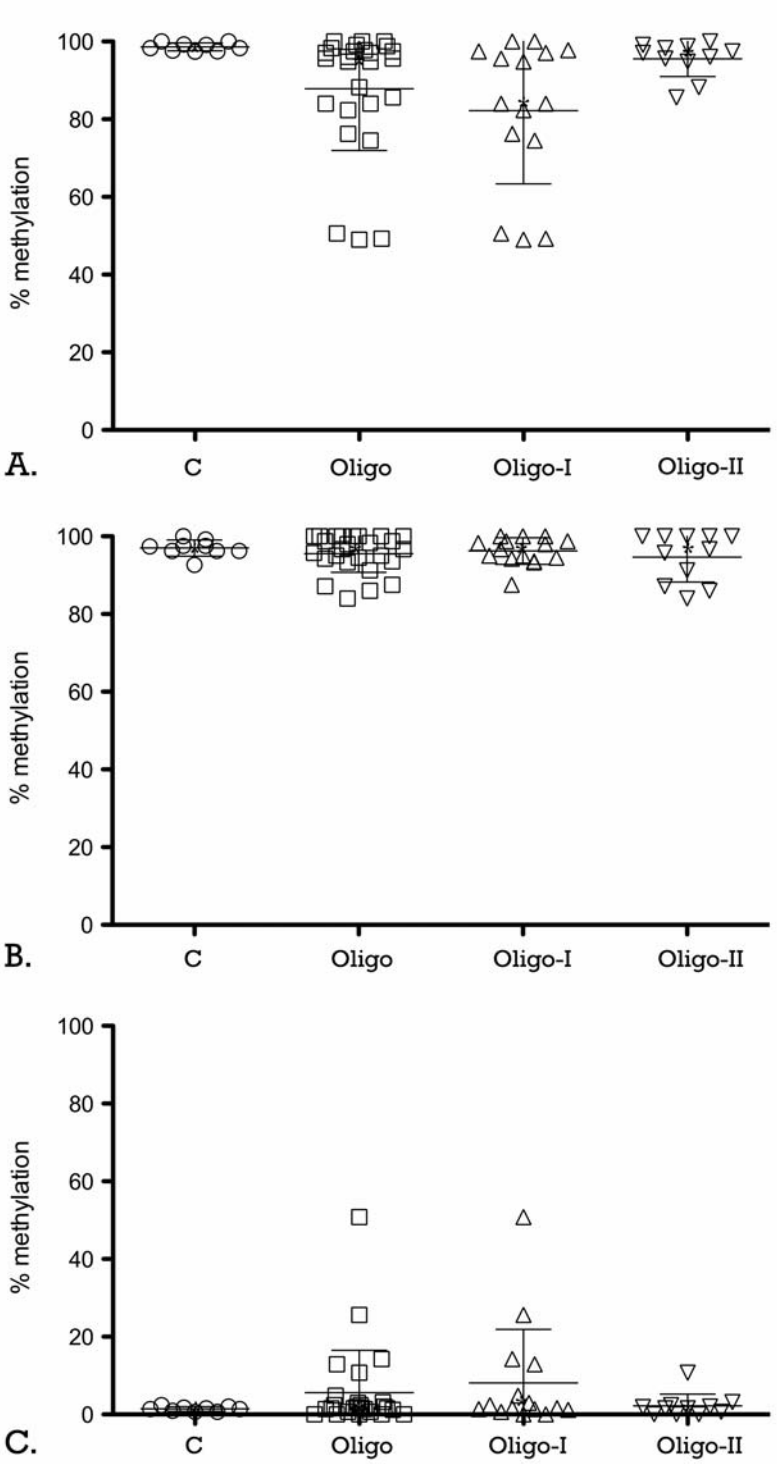


Figure 2.4. Methylation level imprinted genes in oligozoospermic men. The methylation level is shown for each sample analyzed within (A) *H19* DMR, (B) *IG-GTL2* DMR and (C) *MEST* DMR. Methylation level was analyzed in control men (C) (n=9), in oligozoospermic men (Oligo) (n=26). The Oligo group was further subdivided into two sub-groups: Oligo-I (n=15) and Oligo-II (n=11). Most men with abnormal methylation were from the Oligo-I sub-group. The horizontal lines indicate the group mean and the whiskers indicate standard deviation of the group mean. * indicates the median.

samples at the *H19* and *IG-GTL2* DMRs and presence of hypermethylated or fully methylated clones found in samples at the *MEST* DMR is also indicated, including the number of such clones found (Table 2.5).

2.3.2.2.1 Methylation at the H19 DMR

Methylation at the *H19* DMR ranged between 97.39% and 100% in control samples (Table 2.5). None of the clones analyzed in the control samples showed hypomethylation or a complete lack of methylation. At most methylation was lost at two CpGs in the same clone (for example CpG #4 and #16 in sample C08, Figure 2.2) or at four or five CpGs in one sample either at the same or different CpG, respectively (for example C07 and C08; Figure 2.2). In the Oligo group, mean methylation ranged between 49.02% and 100% (Table 2.5). Hypomethylated or completely non-methylated clones were found in nine of the twenty-six oligo samples analyzed: in eight samples from the Oligo-I sub-group and in one sample from the Oligo-II sub-group (Figure 2.4).

There was a significant decrease in methylation at the *H19* DMR in the Oligo group compared to the Control group (MW, $p=0.0032$). We also found a significant decrease in methylation at the *H19* DMR in the Oligo-I sub-group compared to the Control group (KW, $p<0.01$). The difference in methylation between the Oligo-II sub-group and the Control group was not significant (KW, $p>0.05$). Although an overall decrease in DNA methylation was found at the *H19* DMR in the Oligo-I sub-group compared to the Oligo-II sub-group, the difference in methylation between the two sub-groups was not significant (KW, $p>0.05$; Table 2.5). Up to 50% of improperly methylated clones were identified in samples from the Oligo-I sub-group, while only one improperly methylated clone was found in the one sample from the Oligo-II sub-group (Figure 2.2; Table 2.5). Demethylation was also found at randomly distributed CpGs within the *H19* DMR in the Oligo samples, affecting anywhere from one to ten CpGs; however, in most samples demethylation affected three CpGs in the Oligo-I sub-group and two CpGs in the Oligo-II sub-group (Table 2.6).

Table 2.6. **Number of demethylated CpG sites found at the *H19* DMR outside of hypomethylated or unmethylated clones in oligozoospermia.**

Number of de-methylated CpGs	Control group (N)	Oligo group (N)	Oligo-I sub-group (N)	Oligo-II sub-group (N)
0	2	4	3	1
1	2	4	1	3
2	2	4	4	
3	1	6	3	3
4	1	1		1
5	1	1		1
6		1	1	
7		3	2	1
10		2	1	1

The CTCF binding protein regulates gene expression of *H19* and IGF2 through binding to the 6th CTCF binding region located within CpGs 4 to 8 in the *H19* DMR (Takai et al., 2001). Presumably methylation at CpG 7 does not affect the binding since this site is a polymorphism. Methylation of these sequences on the paternal allele prevents the CTCF protein from binding to the *H19* DMR allowing expression of IGF2. Demethylation of these CpGs could enable CTCF binding and potentially reduce or inhibit expression of IGF2 from the paternal allele. Demethylation of at least one CpG within CpG 4 to 8 was found in six control samples and in twenty Oligo samples. In the control samples, in all but C08, only one CpG was demethylated that was always CpG 6. Among the Oligo samples with hypomethylated or completely demethylated clones, CpGs 4 to 8 were also demethylated. However, the demethylation was limited to just one (for example sample P01, P07, P18) or two CpGs (only sample P11). Eleven samples, in which hypomethylated or completely demethylated clones were not found, had demethylated CpGs within CpG 4 to 8 either at one CpG or at four CpGs in sample P22. It is not known whether demethylation of only one CpG within the binding site would affect binding of the CTCF binding protein. The CpG that was most often demethylated within the binding site was CpG 6.

2.3.2.2.2 Methylation at the IG-GTL2 DMR

Methylation at the *IG-GTL2* DMR ranged between 92.66% and 100% in control samples (Table 2.5). No hypomethylation or complete lack of methylation was found at any of the clones analyzed in the control samples. Most samples showed a loss of methylation at two to three CpGs, with the exception of sample C08 that showed the loss of methylation at eight CpGs affecting CpG 4 in eight unique clones (Table 2.7; Figure 2.2). In the Oligo group, methylation

ranged between 84.0% and 100%, but hypomethylated or fully demethylated clones were not found (Table 2.5). Eight of the 26 oligo samples showed demethylation at more than five CpGs primarily affecting CpG 1 or 4 in multiple clones. In sample P24, half of the CpGs within one clone were demethylated (Figure 2.2). The methylation at the *IG-GTL2* DMR was not significantly different between the Control group and the Oligo group (MW, p=0.43). We also did not find a significant difference in methylation at the *IG-GTL2* DMR between the Oligo-I or Oligo-II sub-group and the Control group, or between the two sub-groups (KW, p>0.05) (Table 2.5).

Table 2.7. Number of demethylated CpG sites found at the *IG-GTL2* DMR in oligozoospermia.

Number of de-methylated CpGs	Control group (N)	Oligo group (N)	Oligo-I sub-group (N)	Oliog-II sub-group (N)
0	1	8	3	5
1	1	4	4	
2	3	4	3	1
3	3	1		1
4		1	1	
6		1	1	
7		3	1	2
8	1	2	1	1
9		1		1
10		1	1	

2.3.2.2.3 Methylation at the MEST DMR

Methylation at the *MEST* DMR ranged between 0.68% and 2.42% in the control samples, and between 0% and 50.79% in the Oligo group, with lower methylation seen in the Oligo-I sub-group (Table 2.5; Figure 2.4C). Hypermethylated or fully methylated clones were not observed in any of the control samples, but were observed in five samples from the Oligo group: in four samples in the Oligo-I sub-group and in one sample in the Oligo-II sub-group. Demethylation was also found at CpGs outside of the hypermethylated or methylated clones. In the control samples, between one and four methylated CpGs were found in most samples, while one sample had seven methylated CpGs. Among the oligo samples, between one and four methylated CpGs were most commonly observed, with a maximum of six methylated CpGs observed in one patient (Table 2.8). Methylation at the *MEST* DMR was not significantly different between the Control group and the Oligo group (MW, p=0.21). We also did not find a

Table 2.8. Number of methylated CpG sites found at the *MEST* DMR outside of hypermethylated or methylated clones in oligozoospermia.

Number of methylated CpGs	Control group (N)	Oligo group (N)	Oligo-I sub-group (N)	Oliog-II sub-group (N)
0	0	5	2	3
1	2	7	4	3
2	3	7	4	3
3	2	3	2	1
4	1	1	1	
5		2	2	
6		1		1
7	1			

significant difference in methylation at the *MEST* DMR between the Oligo-I or Oligo-II sub-groups and the Control group, or between the two sub-groups (KW, $p>0.05$) (Table 2.5).

2.3.2.3 *Analysis of methylation at individual CpG sites*

Percentage of abnormal methylation at all CpG sites within the *H19* DMR was significantly increased in the Oligo group compared to the Control group (Fisher's exact test, $p<0.05$), with the exception of CpG 6 (Table 2.9). This was also true for the Oligo-I sub-group compared to the Control group. Six and fourteen of the CpG sites retained the significance after the Bonferroni correction in the comparison between the Oligo and Control group, and the Oligo-I and Control group, respectively (Table 2.9). A significant increase in the percentage of abnormal methylation at the *H19* DMR was only significant at two CpG sites between the Control group and the Oligo-II sub-group; CpG 13 and 14, and at fourteen CpG sites between the Oligo-I and Oligo-II sub-groups (Fisher's exact test, $p<0.05$). However, none of these CpG sites retained significance after the Bonferroni correction. CpG 6 was most often demethylated in the *H19* DMR. In the *IG-GTL2* DMR the CpG that was most often demethylated was CpG 4, this was seen in the two groups and sub-groups analyzed (Table 2.10). Percentage of abnormal methylation at the *IG-GTL2* DMR was significantly increased only at CpG 1 in the Oligo group compared to the Control group, in the Oligo-I group compared to the Control group and in the Oligo-II group compared to the Control group (Fisher's exact test, $p>0.05$). However, significance at CpG 1 was not retained after the Bonferroni correction in any of the comparisons. At the *MEST* DMR no single CpG site was most often methylated (Table 2.11). Percentage of abnormal methylation was significantly increased at CpG 10 and 12 in the *MEST*

Table 2.9. Percentage of unmethylated CpG sites analyzed within the *H19* DMR in oligozoospermic men.

CpG	Percent (%) unmethylated				P value			
	Control (n=69)	Oligo-I (n=107)	Oligo-II (n=61)	Oligo (n=168)	C vs. O	C vs. O-I	C vs. O-II	O-I vs. O-II
1	0	20.8	3.3	14.4	0.0002*	0.0001*	NS	0.0013
2	2.9	16.8	4.9	12.5	0.028	0.0035	NS	0.029
3	0	22.4	3.3	15.5	0.0001*	0.0001*	NS	0.0007
4	1.4	21.5	4.9	15.5	0.0012	0.0001*	NS	0.0037
5	0	17.8	3.3	12.5	0.0007	0.0001*	NS	0.0066
6	18.8	32.7	16.4	26.8	NS	NS	NS	0.029
7	-	-	-	-	-	-	-	-
8	0	18.7	3.3	13.1	0.0004	0.0001*	NS	0.0038
9	0	15.9	3.3	11.4	0.0013	0.0001*	NS	0.011
10	0	16.8	1.6	11.3	0.0013	0.0001*	NS	0.0019
11	0	15.9	1.6	10.7	0.0022	0.0001*	NS	0.0034
12	1.4	19.6	8.2	15.5	0.0012	0.0003	NS	NS
13	0	17.8	8.2	14.3	0.0002*	0.0001*	0.021	NS
14	0	15.9	16.4	16.1	0.0001*	0.0001*	0.0003	NS
15	0	20.6	4.9	14.9	0.0001*	0.0001*	NS	0.0062
16	1.4	18.7	1.7	12.6	0.0058	0.0001*	NS	0.0011
17	0	16.8	3.3	11.9	0.0013	0.0001*	NS	0.012
18	0	20.6	1.6	13.7	0.0002*	0.0001*	NS	0.0003

Uncorrected significant P values (<0.05) are indicated, Fisher's exact

*Bonferroni corrected P value considered significant <0.00026 (0.05/192) for this data set (H19, IG-GTL2 and MEST)

Table 2.10. P**ercentage of unmethylated CpG sites analyzed within the *IG-GTL2* DMR in oligozoospermic men.**

CpG	Percent (%) unmethylated				P value			
	Control (n=79)	Oligo-I (n=107)	Oligo-II (n=62)	Oligo (n=168)	C vs. O	C vs. O-I	C vs. O-II	O-I vs. O-II
1	1.3	8.5	14.5	11.1	0.0088	0.045	0.0051	NS
2	2.5	2.8	4.8	3.6	NS	NS	NS	NS
3	0	0.9	4.8	2.4	NS	NS	NS	NS
4	21.5	10.8	19.3	20.2	NS	NS	NS	NS
5	0	0.9	0	0.6	NS	NS	NS	NS
6	1.3	0	1.6	0.6	NS	NS	NS	NS
7	0	1.9	3.2	2.4	NS	NS	NS	NS
8	2.5	1.0	4.8	0.2	NS	NS	NS	NS
9	0	3.8	1.6	3.0	NS	NS	NS	NS
10	1.3	1.9	3.3	2.4	NS	NS	NS	NS

Uncorrected significant P values (<0.05) are indicated, Fisher's exact

*Bonferroni corrected P value considered significant <0.00026 (0.05/192) for this data set (H19, IG-GTL2 and MEST)

DMR in the Oligo group compared to the Control group, at nine CpG sites in the Oligo-I sub-group compared to the Control group, and at CpG 1 and 21 in the Oligo-I group compared to the Oligo-II group (Fisher's exact test, $p>0.05$). However, none of the CpG sites retained significance after the Bonferroni correction. The data suggest that analysis of methylation at CpG 6 within the *H19* DMR, CpG 1 and 4 within the *IG-GTL2* DMR may not be representative of methylation at neighboring CpG sites. There were no single CpG sites within the *MEST* DMR that seemed to be preferentially methylated.

Table 2.11. **Percentage of methylated CpG sites analyzed within the *MEST* DMR in oligozoospermic men.**

CpG	Percent (%) methylated				P value			
	Control (n=67)	Oligo-I (n=99)	Oligo-II (n=63)	Oligo (n=162)	C vs. O	C vs. O-I	C vs. O-II	O-I vs. O-II
1	0	7.1	0	4.3	NS	0.043	NS	0.044
2	1.5	9.1	1.6	6.1	NS	NS	NS	NS
3	0	7.1	1.6	4.9	NS	0.042	NS	NS
4	1.5	7.1	4.8	6.2	NS	NS	NS	NS
5	0	7.1	3.2	5.6	NS	0.042	NS	NS
6	3.0	11.1	6.3	9.3	NS	NS	NS	NS
7	1.5	8.1	3.2	6.2	NS	NS	NS	NS
8	0	8.1	1.6	5.6	NS	0.022	NS	NS
9	1.5	9.1	7.9	8.6	NS	NS	NS	NS
10	0	10.1	3.2	8.0	0.012	0.006	NS	NS
11	0	7.1	3.2	5.6	NS	0.042	NS	NS
12	0	10.1	3.2	7.4	0.021	0.006	NS	NS
13	4.5	9.1	1.6	6.2	NS	NS	NS	NS
14	0	9.1	1.6	6.2	NS	0.011	NS	NS
15	1.5	11.1	1.6	7.4	NS	0.029	NS	NS
16	5.6	8.1	1.6	5.6	NS	NS	NS	NS
17	1.5	9.1	3.2	6.8	NS	NS	NS	NS
18	7.5	6.1	3.2	4.9	NS	NS	NS	NS
19	1.5	5.1	0	3.1	NS	NS	NS	NS
20	1.5	6.1	1.6	4.3	NS	NS	NS	NS
21	4.6	8.4	0	4.9	NS	NS	NS	0.022

Uncorrected significant P values (<0.05) are indicated, Fisher's exact

*Bonferroni corrected P value considered significant <0.00026 (0.05/192) for this data set (H19, IG-GTL2 and MEST)

2.3.2.4 Incidence of abnormal methylation at imprinted genes in oligozoospermic men

The number of individuals with abnormal methylation at imprinted genes was determined and compared between groups. An individual was designated as having abnormal methylation at an imprinted gene based on the presence of at least one improperly methylated unique clone. 34.6% of patients in the Oligo group had abnormal methylation at the *H19* DMR (Table 2.12), this was significant compared to the Control group (Fisher's exact test, p=0.044). Most of the abnormalities observed affected patients in the Oligo-I sub-group (53.5%), and the difference was significant compared to the Control group (Fisher's exact test, p=0.0087). 9.1% of Oligo-II patients had abnormal methylation at the *H19* DMR, and this was not significantly different from the Control group (Fisher's exact test, p=0.55), but the difference was significant between the Oligo-II subgroup and the Oligo-I sub-group (Fisher's exact test, p=0.024). Post Bonferroni correction for multiple testing p values <0.0042 (0.05/12) were considered significant. None of the comparisons for methylation at the H19 DMR passed the correction. None of the patients or controls analyzed had abnormal methylation at the *IG-GTL2* DMR (Fisher's exact test, p>0.05). Abnormal methylation at the *MEST* DMR was found in 19.2% of patients in the Oligo group; however, this difference was not significant compared to the Control group (Fisher's exact test, p=0.20). Similar to the distribution of abnormal methylation at the *H19* DMR, most of the abnormalities seen at the *MEST* DMR affected patients in the Oligo-I sub-group (26.7%); however, the difference between the Oligo-I sub-group and the Control group was not significant (Fisher's exact test, p=0.13). The difference in methylation between the Control group and the Oligo-II group, or between the Oligo-I group and Oligo-II group was not significantly different (Fisher's exact test, p=0.36). Three of the nine patients (P06, P07, and P14) with abnormal methylation at the *H19* DMR also had abnormal methylation at the *MEST* DMR. All other patients had abnormalities at only one gene.

Table 2.12. **Incidence of imprinting errors in the sperm of oligozoospermic men.**

	H19	***IG-GTL2***	***MEST***
Control	0/9	0/9	0/9
Oligo	9/26 (34.6)*	0/26	5/26 (19.2)
Oligo-I	8/15 (53.3)*	0/15	4/15 (26.7)
Oligo-II	1/11 (9.1)**	0/11	1/11 (9.1)

* significant compared to the Control group (Fisher's exact test, $p<0.05$)
** significant compared to the Oligo-I group (Fisher's exact test, $p<0.05$)
The significance did not pass the Bonferroni correction.
exact P values are indicated in the text
Percentages shown in brackets

2.4 DISCUSSION

2.4.1 Methylation at imprinted genes and incidence of abnormal methylation at imprinted genes in the sperm of men with severe oligozoospermia

Here we report a significant decrease in methylation at the *H19* DMR in the sperm of oligozoospermic patients compared to control men. The observed decrease in methylation at the *H19* DMR in oligozoospermic patients primarily affected patients with severe oligozoospermia and we found a significant decrease in the methylation in this sub-group compared to control men. These conclusions were also supported by the significant increase in methylation observed at individual CpG sites within the *H19* DMR between the Oligo and Control groups and the Oligo-I and Control groups. Furthermore, we identified abnormal methylation at the *H19* DMR in nine oligozoospermic patients (34.6%): in eight patients affected by severe oligozoospermia (53.3%) and in one patient affected by very severe oligozoospermia (9.1%). Abnormal methylation was not found in the sperm of control men. The higher rates of abnormal methylation found at the *H19* DMR in the oligozoospermic men and in the patients affected by severe oligozoospermia were statistically significant compared to the rate found in the control men. However, the significance was lost post the Bonferroni correction. The conclusions are supported by comparisons of methylation levels between groups and analysis of differences in methylation at individual CpGs between the control and Oligo-II groups that retained significance after being corrected for multiple testing. To date four studies have reported on DNA methylation in the sperm of men affected by severe oligozoospermia (Table 2.13). The published studies listed in Table 2.13 did not perform corrections for multiple testing; therefore the results discussed are based on uncorrected statistical analysis. Marques et al. (2004) defined abnormal methylation as the presence of at least one improperly methylated CpG site within the

H19 DMR and in most patients only one unmethylated CpG site was found among the seventeen CpG sites analyzed; however, it is likely that the samples had normal methylation. It is not clear whether the demethylation of one CpG site is biologically relevant. Another study reported uncharacteristically low methylation at the *H19* DMR affecting all patients analyzed (Boissonnas et al., 2010). Methylation at the *H19* DMR was below 42% (12%-42%) in eight patients, and was 63% and 90% in two other patients (Boissonnas et al., 2010). In the two remaining studies abnormal methylation at the *H19* DMR was found in around 30% of men with severe oligozoospermia (Kobayashi et al., 2007; Marques et al., 2008; Table 2.13), which was similar to that found in the current study (34.6%).

We identified abnormal methylation at the *MEST* DMR in the sperm of five oligozoospermic patients (19.2%); four of whom were affected by severe oligozoospermia (26.7%) and one was affected by very severe oligozoospermia (9.1%). Abnormal methylation at the *MEST* DMR was not observed in control men. The rate of abnormal methylation at the *MEST* DMR was around 30% in two previous studies (Kobayashi et al., 2007; Marques et al., 2008). Our rate of 19.2% is lower compared with previously published reports (Marques et al., 2008; Kobayashi et al.,

Table 2.13. **Abnormal DNA methylation at imprinted genes in the sperm of men affected by severe and very severe oligozoospermia.**

Study	Population	*H19*		*GTL2*		*MEST*	
		Mean me (%)	rate	Mean me (%)	rate	Mean me (%)	rate
Marques et al. 2004	Control	100	0/27			0	0/27
	Oligo <5	97.4	15/50 (30)			0	0/50
Marques et al., 2008	Control	94.8	0/5			0.5	0/5
	Oligo 1-5	90.1	2/5 (40)			3.86	1/5 (20)
	Oligo <1	95.6	1/5 (20)			7.6	2/5 (40)
	All oligo	92.9	3/10 (30)			5.5	3/10 (30)
Kobayashi et al., 2007	Control	99.7	0/79 (0)	97.3	5/79 (6.3)	2.08	7/79 (8.9)
	Oligo <5	82.9	3/9 (33.3)	88.7	4/9 (44.4)	14.4	3/9 (33.3)
Boissonnais et al., 2010	Control	83.7	0/17				
	Oligo 1-5	41.4	6/6 (100)				
	Oligo <1	31.6	6/6 (100)				
	All oligo	36.2	12/12 (100)				
This study	Control	98.6±1.0	0/9	97.0±2.1	0/9	1.4±0.6	0/9
	Oligo 1-5	82.2±18.8	8/15 (53.3)	96.3±3.4	0/15	8.1±13.8	4/15 (26.7)
	Oligo <1	95.6±4.6	1/11 (9.1)	94.6±6.4	0/11	2.2±3.0	1/11 (9.1)
	All oligo	87.9±15.9	9/26 (34.6)	95.6±4.8	0/26	5.6±10.9	5/26 (19.2)

Mean me; mean methylation
Percentages indicated in brackets

2007). The lower rate may be explained by the lower rate of abnormal methylation we found in the sperm of men affected by very severe oligozoospermia compared to the Marques et al. (2008) study. The rate of abnormal methylation and the methylation levels at the *MEST* DMR were not statistically significant between patients and controls, which is likely due to a small sample size. At most, a significant difference in methylation was observed at nine CpG sites at the *MEST* DMR between men affected by severe oligozoospermia compared to control men, but the difference lost significance after the Bonferroni correction.

We found a higher rate of abnormal methylation at the *H19* and *MEST* DMRs among men affected by severe oligozoospermia compared to men affected by very severe oligozoospermia, but this difference was only significant at the *H19* DMR. The methylation level was also lower in men affected by severe oligozoospermia (Table 2.5). This finding was unexpected as published results suggest a correlation between increased abnormal methylation and reduced sperm count (Marques et al., 2008; Boissonnas et al., 2010). The men affected by severe oligozoospermia were on average older (38.1 vs. 33.0, p=0.023) and perhaps with age these men may have increased their exposure to environmental factors affecting DNA methylation. Also, in the men affected by very severe oligozoospermia infertility may be primarily associated with clinical or genetic factors. The two men with genetic abnormalities had normal imprinting in their sperm, while abnormal methylation at the *H19* DMR was found in one of the four men with varicocele. The number of patients analyzed with genetic abnormalities and varicocele is too small to draw any conclusions. However, DNA methylation at imprinted genes in such patients has not been previously reported and further studies should be done to determine whether aberrant imprinting in the sperm of these patients is also present, potentially increasing the severity of the patient's infertility. In this study three men had abnormal methylation at both *H19* and *MEST* DMRs. Abnormal methylation at multiple DMRs in the same patient has been reported before (Kobayashi et al., 2007; Marques et al., 2008) and suggests that improper imprint erasure or re-establishment may not be gene specific.

In this study we did not find abnormal methylation at the *IG-GTL2* DMR in patients or controls. The difference in the methylation level at the *IG-GTL2* DMR was not significant between oligozoospermic men and control men, or between the two sub-groups and control

men. A significant difference in methylation was only found at one CpG site between control men and men affected by oligozoospermia and between control men and men affected by severe oligozoospermia. Only one study to date has examined the methylation at the *IG-GTL2* DMR in sperm from oligozoospermic men and identified abnormal methylation at the *IG-GTL2* DMR in 44.4% of men affected by severe oligozoospermia, but also in 6.3% of control men. However, the mean methylation for most of the patients and controls was relatively high (Kobayashi et al., 2007; Table 2.13). Also, methylation at only one site was analyzed (Kobayashi et al., 2007) (CpG 4 in the original untruncated *IG-GTL2* sequence analyzed in this study). Lack of methylation at this CpG site was identified in three patients in this study (results not shown); two from the Oligo-I sub-group and one from the Oligo-II sub-group. Analysis of methylation at this single site in this study would not have been representative of the methylation results at the sperm level as the lack of methylation at this site in the three patients was limited to CpG 4. Methylation at CpG sites surrounding CpG 4 was not analyzed by Kobayashi et al. (2007). Methylation at CpG 4 may not have been representative of the methylation at surrounding CpG sites, emphasizing the importance of analyzing multiple CpG sites within a DMR. We identified two CpG sites within the *H19* DMR and one site within the *IG-GTL2* DMR at which methylation was not representative of the methylation at surrounding sites.

2.4.2 Sensitivity of *H19* and *MEST* to abnormal methylation.

In the present study, abnormal methylation only at the *H19* and *MEST* DMRs was identified in the sperm of oligozoospermic men, but was not identified at the *IG-GTL2* DMR. The results suggest that the *H19* and *MEST* DMRs may be more prone to improper methylation compared to the *IG-GTL2* DMR. There are many examples in the literature describing improper methylation at the *H19* DMR induced either by culture conditions (Doherty et al., 2000; Mann et al., 2004), superovulation (Sato et al., 2006; Fortier et al., 2008) or *in vitro* maturation of oocytes (Borghol et al., 2006), resulting in abnormal *H19* or *IGF2* expression. Abnormal methylation at the *H19* DMR has also been described in patients with BWS (Steenman et al., 1994) and in abortuses following ART (Kobayashi et al., 2009). Loss of methylation at the *H19* DMR has also been consistently described in infertile men (Marques et al., 2008; Kobayashi et al., 2007). A gain of methylation at the *H19* DMR was also seen after environmental exposure to toxins (Wu et al., 2004). Changes in methylation at the *MEST* DMR remain not as well

studied but have been shown. Abnormal methylation at the *MEST* DMR has been observed after superovulation (Sato et al., 2006), in infertile men (Marques et al., 2008; Kobayashi et al., 2007) and in abortuses following ART (Kobayashi et al., 2009). It may be that *H19* is particularly prone to a loss or a gain of methylation from the paternal or maternal alleles, respectively. The *IG-GTL2* DMR has not been well studied, but loss of methylation from this DMR was found in abortuses following ART and in the father's sperm (Kobayashi et al., 2009). Abnormal methylation at the *IG-GTL2* may occur but may be more rare compared to the *H19* and *MEST* DMRs. This difference may be related to the molecular structure of the DMRs or of surrounding sequences and may explain the absence of abnormal methylation in the sperm of oligozoospermic men observed in this study.

The *DLK1/GTL2* region is highly repetitive. 35.8% of the *DLK1/GTL2* region is made up of interspersed repeats, compared to 12.3% of the IGF2/*H19* region (Paulsen et al., 2001). The *DLK1/GTL2* region contains a highly conserved tandem repeat located within the IG area. In humans it contains nine 18 base pair repeats (Paulsen et al., 2001). Li et al. (2004) found that methylation at *Gtl2* was still present at a time when it was erased from *H19* and *Rasgrf1*. The authors suggested that higher methylation at *Gtl2* may be related to the presence of repetitive stretches of DNA in the gene, which the cell may recognize and suppress as it similarly does to repetitive sequences (Li et al., 2004). The highly repetitive IG area may also be associated with amplification of a shortened sequence due to potential mispriming resulting from binding to a repetitive sequence of similar homology (Geuns et al., 2007). The repetitive area around the primers would explain the generation of truncated products as well as the difficulty associated with sequencing of the IG area.

2.4.3 Examining unique clones and multiple PCR reactions

In this study unique clones were analyzed. This approach was chosen to avoid preferential amplification of few stands of DNA that may occur when small quantities of starting material are used (Walsh et al., 1992; Findlay et al., 1995). For the majority of samples analyzed around three hundred sperm cells were isolated and used for the amplification of three genes. In some samples a high proportion of clones that originated from the same amplification reaction were identical, therefore multiple reactions were set up for each gene. Most of the

unique clones analyzed were unique because they originated from a different amplification reaction, this was particularly observed in patient samples. Analysis of all clones would not have affected the rate of abnormal methylation in infertile men as this was defined as the presence of at least one hypomethylated or hypermethylated clone in a sample, but could have affected the results when determining the mechanism that may have given rise to the abnormality. For example, presence of methylation at the *MEST* DMR in 50% of clones may suggest a failure of methylation erasure from the maternal allele at the primordial germ cell stage. Analysis of non-representative results may obstruct such information. Also, analysis of all clones would have increased the mean methylation for each patient and group. This effect was suggested by a study that found a high rate of abnormalities and an un-proportionately high methylation levels (Marques et al., 2008). Up to twenty clones were analyzed and in patients with the lowest sperm counts most clones were identical (Marques et al., 2008).

2.4.4 Mechanisms associated with a loss or a gain of methylation

There are three mechanisms that are associated with abnormal methylation at imprinted genes and include improper erasure, establishment or maintenance. The presence of an informative SNP within the sequence and knowing the parental origin of the two alleles could be used to identify the mechanism responsible for the abnormality. Methylation of only the maternal allele within the *MEST* DMR would imply improper erasure while methylation of both parental alleles would imply improper establishment. In the case of the *H19* and *IG-GTL2* DMRs, presence of SNPs would help to determine the parental alleles on which methylation is not being properly reset. Errors in maintenance of methylation could also result in the presence of improper methylation in the sperm, explaining the loss or gain of methylation at either clones or random CpG sites at the *H19* and *IG-GTL2* DMRs or the *MEST* DMR, respectively, as observed in many of the samples analyzed (Figure 2.2). Five of the nine patients with abnormal methylation affecting whole clones at the *H19* DMR were informative for a SNP (Figure 2.2). In three of these samples (P05, P06 and P11), both parental alleles were unmethylated, while in one sample (P15) only one parental allele was unmethylated. The fifth informative sample (P01) had only one unmethylated clone. None of the samples analyzed had an informative SNP within the *MEST* DMR.

In animal studies abnormal methylation at imprinted genes in the sperm has been associated with male infertility and mutations in *Dnmt3a* and *Dnmt3l.* Loss of methylation at the *H19* DMR and at the *IG- Gtl2* DMR were observed in the infertile mutant males (Kaneda et al., 2004; Yaman and Grandjean, 2006; Webster et al., 2005). However, no clear mutations could be identified in *DNMT3A* and *DNMT3L* in infertile men with abnormal methylation at the *H19*, *GTL2* and *MEST* DMRs (Kobayashi et al., 2009). DNMT1 is the primary enzyme responsible for the maintenance of DNA methylation (Li et al., 1992), and inactivation of DNMT1 is associated with the loss of methylation at imprinted genes, among other sequences (Walsh and Bestor, 1999). However, loss of DNA methylation due to errors in maintenance of methylation would have to be moderate in infertile men and only affect certain sequences, as a more pronounced loss of DNA methylation affecting different types of DNA sequences is often associated with cancer (Mossman and Scott, 2006). Mutations or sequence variations at the mentioned DNMTs are possible mechanisms for abnormal methylation at imprinted genes in infertile men.

2.4.5 Possible causes of abnormal methylation at imprinted genes in infertile men

Abnormal methylation at imprinted genes in the sperm of infertile men may originate during *in utero* development or may be acquired after birth. Factors such as maternal diet have been shown to affect DNA methylation in the fetus (Waterland and Jirtle, 2003; Dolinoy et al., 2006), and stressful *in utero* development has been associated with adult onset disease (Lawlor et al., 2005; Rich-Edwards et al., 2005; Gortner, 2007). Furthermore, *in utero* exposure to endocrine disruptors has been associated with a reduction in sperm numbers, motility and increased germ cell apoptosis, in addition to a trans-generational effect that was passed on through the male germ line (Anway et al., 2005). With ageing, the males also had a higher risk for cancer, prostate and kidney disease and immune abnormalities (Anway et al., 2006). Gametes may be particularly vulnerable to perturbations of methylation during *in utero* development as it is during this time that genomic imprinting is established. Exposure to environmental factors after birth may also affect spermatogenesis. For example, higher levels of methyl donors in males correlated with improved testicular histology, increased sperm numbers and fertility in male mice (Kelly et al., 2005), and increased sperm concentration and decreased sperm DNA damage in humans (Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002).

Although the evidence is not direct, it does however suggest the possibility that methylation acquired through environmental exposure may affect spermatogenesis and fertility. Genetic factors may also play a role and include mutations in enzymes responsible for imprint establishment (Kobayashi et al., 2009) or folate metabolism (Kelly et al., 2005).

2.4.6 Consequences associated with abnormal methylation at imprinted genes

Imprinted genes are important regulators of fetal and placental growth. CTCF binding to the *H19* DMR controls expression of *H19* and *IGF2* in a parental specific manner. Methylation at the DMR prevents the CTCF protein from binding thus allowing *IGF2* expression from the paternal allele, while lack of methylation at the DMR allows CTCF binding and expression of *H19* from the maternal allele (Bell and Felsenfeld, 2000; Hark et al., 2000; Hark et al., 1998). The 6th CTCF binding site locates to CpGs 4 to 8 within the *H19* DMR (Takai et al., 2001), and loss of methylation at this site may be particularly important for the regulation of expression of *IGF2* and *H19*. Loss of methylation of at least one CpG within CpGs 4 to 8 was found in six control samples and in twenty men affected by oligozoospermia, while the loss of methylation at all these CpGs was identified in nine men affected by oligozoospermia. Although the consequences related to the loss of methylation at a single CpG are unknown, the loss of methylation at multiple CpGs within the *H19* DMR has been associated with small for gestational age placentae in humans (Guo et al., 2008). Lack of *Igf2* expression, likely through the loss methylation at the *H19* DMR, has been associated with fetal (De Chiara et al., 1990) and placental growth retardation (Constancia et al., 2002). With respect to *GTL2/DLK1* and *MEST*, UPD for the respective chromosomes has been associated with growth retardation in mice and humans (Georgiades et al., 2000; Georgiades et al., 1998; Kaneko-Ishino et al., 1995). Decreased methylation at the *H19* DMR in sperm has been associated with decreased fertilization rates (Boissonnas et al., 2010), but more severe consequences have also been described.

Consequences associated with abnormal methylation at imprinted genes are primarily related to abnormal methylation at the single sperm level and not at randomly distributed CpGs. For example, decreased methylation at the *H19* DMR and the *IG-GTL2* DMR was found in abortuses after ART and these abnormalities were traced back to the sperm of men with

oligozoospermia as well as normozoospermia (Kobayashi et al., 2009). Some abortuses and sperm samples analyzed showed an almost complete loss of methylation at either one or both DMRs (Kobayashi et al., 2009). The loss of methylation at the *H19* DMR and the gain of methylation at the *MEST* DMR have also been described in children born through IVF and ICSI affected with SRS (Bliek et al., 2006; Kagami et al., 2007; Kanber et al., 2009). These abnormalities would have affected the paternal allele and could have originated in the sperm, although this was not investigated by any of the studies. This is particularly important as low birth weight after IVF and ICSI has been reported by many studies (Sutcliffe et al., 2001; Sutcliffe et al., 2003; Katalinic et al., 2004; Merlob et al, 2005; Bonduelle et al., 2004; Tan et al., 1992; Wang et al., 1994), and abnormal methylation at imprinted genes originating in the sperm may be a mechanism for the association. Low birth weight, or in some cases growth restriction, has been associated with an increased risk for cardiovascular disease (Lawlor et al., 2005; Rich-Edwards et al., 2005), stroke (Lawlor et al., 2005; Rich-Edwards et al., 2005) and hypertension (Gortner, 2007) and it remains to be determined whether children affected by low birth weight born through IVF and ICSI are at a greater risk for developing adult onset disease. These studies show that abnormal methylation in the gametes may be passed on to progeny through ART and affect the outcome of the fertility treatment and of the pregnancy. Furthermore, with the possibility of trans-generational inheritance more than one generation may be affected (Anway et al., 2005).

2.5 CONCLUSION

In this study DNA methylation in the sperm of men affected by oligozoospermia, severe and very severe, was investigated at three imprinted genes, and compared to methylation in the sperm of control men of proven fertility. We found a higher rate of imprinting abnormalities in the sperm of oligozoospermic men compared to control men; the difference was significant at the *H19* DMR, but not at the *MEST* DMR. We did not find imprinting abnormalities at the *IG-GTL2* DMR. We also found that imprinting abnormalities mainly affected men with severe oligozoospermia compared to men with very severe oligozoospermia. Based on results from previous studies a correlation between an increase in abnormal methylation and reduced sperm count was anticipated. The observed increase in abnormal methylation in patients affected by severe oligozoospermia may be associated with the increased average age of the patients and the

accumulated effects of exposure to environmental factors that may affect methylation. Our study of DNA methylation at three imprinted DMRs in human sperm shows that the *H19* DMR is particularly prone and the *IG-GTL2* DMR resistant to imprinting errors. The differences in susceptibility of DMRs may be related to the genetic makeup of the DMRs or the sequences around them. The analysis of unique clones may have provided a more representative measurement of DNA methylation at imprinted genes in patients with low sperm counts. Abnormal methylation in the sperm can be passed on to the offspring and have detrimental effects on the development and well being of the child. The relatively high rate of abnormal methylation in the sperm of infertile men should be a factor to consider during clinical counseling of couples wanting to seek treatment of infertility.

CHAPTER 3: EVALUATION OF DNA METHYLATION AT NON-IMPRINTED GENES IN MEN AFFECTED BY SEVERE OLIGOZOOSPERMIA

3.1 INTRODUCTION

Data from the literature suggest that abnormalities in DNA methylation may be associated with spermatogenesis failure seen in male factor infertility. Abnormal DNA methylation at imprinted genes in the sperm has been reported primarily in men affected by moderate oligozoospermia (Marques et al., 2008; Kobayashi et al., 2007; Boissonnais et al., 2010; Houshdaran et al., 2007), while methylation at repetitive DNA sequences, such as LINEs and Alus, appears to be normal in infertile men (Marques et al., 2008; Kobayashi et al., 2007), information regarding methylation at non-imprinted genes in the sperm of infertile men remains limited. Therefore it is currently not known whether abnormal methylation in the sperm of infertile men is specific to imprinted genes or whether non-imprinted genes are also affected.

One study to date has evaluated DNA methylation at non-imprinted genes in the sperm of infertile men (Houshdaran et al., 2007). A general trend for gain of methylation was found for three non-imprinted genes *NTF3, MT1A, PAX8* and one imprinted gene, *PLAGL1*, in samples with decreasing sperm parameters. In addition lower sperm concentrations were associated with a decrease in methylation at two additional non-imprinted genes *HRAS* and *SFN* and at two imprinted genes *MEST* and *DIRAS3*, as well as at the satellite 2 repetitive sequences (Houshdaran et al., 2007). Seven sperm samples obtained from men with normozoospermia were also subjected to methylation analysis using high throughput bead array technology (Houshdaran et al., 2007). The most genes with abnormal DNA methylation were found in a sperm sample with a concentration of twenty million sperm per milliliter (Houshdaran et al., 2007); however, there was no comparative analysis performed on the data and the results were not confirmed using gene-specific methodology.

Currently little information is available regarding methylation at non-imprinted genes in the sperm of infertile men. A high throughput array based approach was selected for this study with the aim of identifying sequences that may be of interest for further evaluation. Array based methodology provides the advantage of simultaneous analysis of methylation at multiple CpG

sites when insufficient information is available regarding potentially informative targets. The Illumina GoldenGate methylation Cancer Panel I was used to study DNA methylation at 1,505 CpG sites selected from 807 genes. For each CpG analyzed there are two pairs of probes, an allele specific oligonucleotide (ASO) and a locus-specific oligonucleotide (LSO), where each ASO-LSO pair is specific to either the methylated or unmethylated CpG site. The ASO anneals either to the modified T or the non-modified C within bisulphite modified DNA, while the LSO binds next to the allele. Following extension and ligation of the ASO and LSO sequences, analyzable products are generated through amplification using fluorescently labeled universal primers complementary to the ASO and LSO sequences. The LSO also contains a unique address sequence that is complementary to a sequence on the bead array. The amplified products are then hybridized to a bead array where products bind to beads complementary to the address sequence on the LSO. Fluorescent signals that are proportionate to the C and T allele at each CpG site are quantified and reported as a beta value that is representative of methylation at the CpG site (Biblikova et al., 2006; Biblikova and Fan et al., 2009). Methylation analysis by Illumina is sensitive enough to detect a 17% difference in methylation between samples and is reproducible (Biblikova et al., 2006). The Illumina GoldenGate methylation Cancer Panel I array evaluates DNA methylation at CpG sites specific to tumor suppressor genes, oncogenes, genes involved in DNA repair, cell cycle control, differentiation, apoptosis, X-linked, and imprinted genes. These genes may also be relevant to infertility. Analysis of methylation by Illumina is intended as a high throughput methodology used to identify targets that may be of interest, but the results should be confirmed using a CpG site or gene specific methodology. Confirmation of results may also be one way to control the false discovery rate associated with simultaneous multiple hypothesis testing (Benjamini and Hochber, 1995). Pyrosequencing is a methodology that allows quantitative assessment of methylation at a single or multiple CpG sites within a sequence. A sequence of interest is first amplified using conventional PCR where one of the primers is biotin labeled, followed by the pyrosequencing process. Pyrosequencing is a DNA sequencing method that relies on the detection of pyrophosphate release upon the incorporation of nucleotides. The release of pyrophosphate is proportional to the light signal that is released and measured by a camera, and analyzed by a computer program that calculates the percent methylation at each CpG site analyzed (Tost and Gut, 2007).

Due to the relatively high rate of methylation abnormalities at imprinted genes identified in men affected by severe oligozoospermia (Marques et al., 2008; Kobayashi et al., 2007; Boissonnais et al., 2010) and limited data suggesting that infertile men may have abnormal methylation at non-imprinted genes in their sperm (Houshdaran et al., 2007), DNA methylation at non-imprinted genes was analyzed in the sperm of men affected by severe and very severe oligozoospermia and compared to methylation in the sperm of control men of proven fertility. A genome wide approach was selected using the Illumina GoldenGate methylation Cancer Panel I array and pyrosequencing for confirmation. Based on limited data available, a higher incidence of abnormal methylation at non-imprinted genes was hypothesized in the sperm of men affected by severe oligozoospermia and very severe oligozoospermia compared to control men.

3.2 MATERIAL AND METHODS

3.2.1 Sample Preparation

3.2.1.1 Sample collection

DNA samples used for this study were left-over sperm DNA samples from those used for the analysis of DNA methylation at imprinted genes in Chapter 2. Sufficient quantity of DNA was available from seven control men (Control group; C01, C02, C03, C05, C06, C07, C09) and three infertile men, two affected by severe oligozoospermia and one affected by very severe oligozoospermia (Oligo group; P08, P09, P26). The sperm in these samples were isolated by swim-up. The controls and patients included in this study did not have Y chromosome microdeletions and had a normal 46,XY karyotype. Patient P09 had varicocele. Abnormal methylation at the *H19*, *IG-GTL2* and *MEST* DMRs was not found in the control or infertile men included in this study, as analyzed at multiple CpG sites in Chapter 2. Ethical approval was obtained from the University of British Columbia Ethics Committee before initiating this study.

3.2.1.2 Bisulphite modification

Bisulphite modification was carried out on 20 µl samples containing 700ng of sperm DNA using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). The

modification was carried out according to the manufacturer's instructions. Samples were eluted in water. The bisulphite modified DNA was stored short term for up to four days at –20^{o}C or long-term for up two months at –80^{o}C.

3.2.2 Analysis of DNA methylation

3.2.2.1 Illumina

700ng of bisulphite modified DNA were submitted for analysis by Illumina to the Genetic Analysis Facility at The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON). The Illumina GoldenGate methylation Cancer Panel I was used for the analysis of methylation at multiple CpGs (Illumina, Inc., San Diego, CA). The panel contains 1,505 CpG sites selected from 807 genes where 28.6% of sites contain one CpG site per gene, 57.3% contain two CpG sites, and 14.1% have three or more sites. Genes present on the array include tumor suppressor genes, oncogenes, genes involved in DNA repair, cell cycle control, differentiation, apoptosis, X-linked, and imprinted genes.

Methylation data for each of the CpG sites on the array was reported as a beta value. The beta value represents the ratio of fluorescent signal read from the methylated allele to the fluorescent signal read from the methylated and unmethylated alleles. The beta value ranges from 0 to 1 representing a complete lack of methylation to complete methylation, respectively (Biblikova et al., 2006; Biblikova and Fan et al., 2009). CpG sites with a significant difference in mean methylation between control sperm samples and patient sperm samples were selected for confirmation by pyrosequencing.

3.2.2.2 Pyrosequencing

3.2.2.2.1 Assay design

The gene sequence containing the CpG sites selected for confirmation was obtained by performing a Blast search for the corresponding Illumina input sequence (sequence containing the CG of interest). Assays for pyrosequencing were designed to contain the CG of interest as well as any other neighboring CpGs located within a stretch of 100 base pairs. A pyrosequencing assay is limited to the analysis of short stretches of DNA, 80 to 100 base pairs, at a time. Primers and assays were designed using PSQ Assay Design software (Qiagen,

Mississauga, ON). Optimal primers for each assay were selected based on the design criteria built into the program and were designed to exclude SNPs. Three different primers are needed for methylation analysis by pyrosequencing; two primers for the amplification step and one primer for the sequencing step. One of the two primers for the amplification step is biotin labeled at the 5' end so that the amplified product, after being annealed to streptavidin sepharose beads, can be aspirated using the vacuum preparation tool. The specificity of the primers designed was evaluated using BiSearch with the ePCR option for bisulphite modified DNA (NCBI web site). Primers were purchased from Sigma-Genosys (Oakville, ON).

3.2.2.2.2 Amplification

Amplification was carried out in a 25µl volume containing 0.625U HotStarTaq DNA, Polymerase, 1X PCR Buffer (Qiagen, Mississauga, ON), 0.2mM dNTPs (Invitrogen Canada Inc., Burlington, ON), 0.5µM of each primer (Sigma-Genosys, Oakville, ON) and 0.5-1µl of bisulphite modified DNA was added to each reaction. Amplification was performed using the following conditions: initial denaturation at 95^{o}C for 15 min, followed by 40 cycles of 95^{o}C for 1 min, 55-57 ^{o}C for 1 min, 72 ^{o}C for 45 sec, and a final extension step at 72 ^{o}C for 10 min. The annealing temperature was 55^{o}C for RIPK3_P24, RASSF1_P244, AXL_P223, JAK3_P156, PTPRO_P371 and 57 ^{o}C for PGR_P790, MMP19_P306, TNK1_P221, COL1A2_P48, RASSF1_E116, PI3_P1394. A reagent control was included with each amplification reaction. Presence of the correct-size product was verified before sequencing by running 5µl of PCR product on a one percent agarose gel (Invitrogen Canada Inc., Burlington, ON) containing 2µl ethidium bromide (Sigma-Aldrich Canada Ltd, Oakville, ON). 3µl of bench top 100bp DNA ladder (Promega, Madison, WI) were also loaded. The products were visualized on a gel trans-illuminator.

3.2.2.2.3 Sequencing

Sequencing was carried out using the PyroMark MD Q96 System (Biotage, Foxboro, MA) following the manufacturer's protocol. In short, 7-12µl (depending on the target) of the PCR product was mixed with binding buffer (Qiagen, Mississauga, ON), streptavidin sepharose HP beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and water in a semi skirted 96 well plate (Diamed Lab Supplies Inc., Mississauga, ON). 0.15µM of sequencing primer (Sigma-

Genosys, Oakville, ON) and annealing buffer (Qiagen, Mississauga, ON) were mixed in a pyrosequencing plate (Biotage, Foxboro, MA). After shaking to ensure proper binding of the biotin labeled PCR product to the streptavidin beads, the products were aspirated with the vacuum preparation tool and, under vacuum, treated with 70% ethanol, denaturation and wash buffers (Qiagen, Mississauga, ON). After breaking the vacuum seal, the products were released into the sequencing plate containing the sequencing primer. The products and primer were then denatured and allowed to anneal before being transferred to the PyroMark Q96 MD System for analysis. Enzyme, substrate and nucleotides (Qiagen, Mississauga, ON) were dispensed into the reagent and nucleotide tips according to the volumes calculated by the PyroMark Q96 MD system (Qiagen, Mississauga, ON). Tips were tested for proper dispensation and the assays were run. Each sample was run at least in duplicate, including reagent controls from the amplification step. Methylation was analyzed using the Pyro Q-CpG Software (Qiagen, Mississauga, ON). Assays and samples that passed the built-in software checkpoints were reported. Reports were generated for each run containing accurate methylation measurements at each CpG site analyzed. Methylation values were entered into an Excel spreadsheet (Microsoft) and used to calculate mean methylation and standard deviation at each CpG site and sequence analyzed in each sample and in the two groups.

3.2.3 Data Analysis

3.2.3.1 Analysis of data generated through the Illumina array

Illumina data were analyzed in two ways: initially by a statistician when specialized software was not available and by using the Illumina BeadStudio software (version 3.2.2) (Illumina, Inc., San Diego, CA). To determine significant differences in mean methylation between the Control group and the Oligo group, the statistician analyzed the data using the Mann-Whitney and LIMMA statistics. P values <0.05 were considered significant. In addition, Illumina is able to detect a difference in mean methylation of at least 17%, therefore a cut off of 30% was selected, meaning that CpG sites showing at least an absolute 30% difference in methylation between control and patient samples would be considered.

Illumina data were also analyzed using the BeadStudio software. Data were normalized using background normalization and the Illumina custom statistic was used (with the false

discovery rate option) to determine significance. Targets showing an absolute difference in mean methylation of at least 35% and an absolute diff score of at least 33 were considered for confirmation. The diff score is a proprietary Illumina statistic that corresponds to a P value of 0.001. CpG sites were selected for confirmation if they showed an absolute difference in mean methylation of at least 35% between the Control and Oligo group, and if significance was determined for at least two CpG sites for the same gene. These selection criteria were used to increase the chance of finding positive results using pyrosequencing.

In addition to a significant difference in mean methylation between the Control and Oligo groups, CpG sites were selected based on their location within a gene and within a CpG rich area. CpG sites located within promoter sequences were selected over CpG sites located within exons. Abnormal methylation within promoter sequences may affect the expression of the gene and may therefore be more clinically relevant compared to methylation within an exon. Also CpG sites located within CpG islands were selected over sites located in CpG poor areas so that multiple neighboring CpG sites could be analyzed.

3.2.3.2 Analysis of data generated through pyrosequencing

Significant differences between the Control and Oligo groups for the mean methylation at individual CpG sites and for the mean methylation of all CpG sites analyzed at each sequence were determined using the unpaired two-tailed t-test. A P value <0.05 was considered significant. The Bonferroni correction was applied to control for multiple testing.

3.3 RESULTS

3.3.1 DNA methylation at CpG sites analyzed by Illumina

At the 0.05 significance level, differences in methylation between the Control and Oligo groups were significant for seventy-three CpG sites using the Mann-Whitney test and for forty-one CpG sites using the LIMMA statistic. Eleven CpG sites were significant for both tests. CpG sites showing at least an absolute 30% difference in methylation between control and patient samples were selected and nine of the eleven sequences met this criterion (Table 3.1). Out of the nine CpG sites, the following six sites were selected for confirmation by pyrosequencing: PGR_P790_F, MMP19_P306_F, RASSF1_E116_F, RIPK3_P24_F, PI3_P1394_R, and

RASSF1_P244_F. The letter following the first underscore indicates whether the CpG site is located within the gene promoter or within the exon: P for promoter and E for exon.

Analysis of the Illumina data using the BeadStudio software identified seventy-five CpG sites showing a significant difference in mean methylation between the Control group and the Oligo group (Figure 3.1). On average, these sequences showed an overall loss of DNA methylation in the Oligo group compared to the Control group (41.8% vs. 53.0%, respectively, Figure 3.2). The identity and measured methylation at each CpG site is presented as a heat map where color intensity correlates with the degree of methylation at each CpG site analyzed (Figure3.1). The absolute difference in mean methylation of at least 35% and the selection of CpG sites for confirmation where at least two CpG sites from the same gene showed a significant difference in mean methylation between the Control groups and the Oligo group were used as selection criteria to increase the chance of confirming the Illumina results using pyrosequencing. Of the seventy-five CpG sites identified, there were twelve pairs of CpG sites (Table 3.2), of which seven sites each from a different pair, were selected for confirmation by pyrosequencing: AXL_P223_R, COL1A2_P48_R, JAK3_P156_R, PRSS1_P1249_R, PTPRO_P371_F, TNFSF8_P184_F and TNK1_P221_F. The IGFBP1_P12_R and GSTM1_P363_F (Table 3.2) are single nucleotide polymorphisms and were excluded from further analysis. The six CpG sites selected using the Mann- Whitney and LIMMA analysis were also significant using the BeadStudio software for analysis.

Table 3.1. Mean difference in DNA methylation between control and test samples at CpG sites significant using the Mann-Whitney test and the LIMMA statistic.

Target ID	CpG island	Mean methylation in test samples	Mean Methylation in control samples	Mean methylation difference
PGR_P790_F*	N	0.81	0.33	0.48
PLXDC1_P236_F	Y	0.05	0.48	-0.43
MMP19_P306_F*	N	0.89	0.36	0.53
MC2R_P1025_F	N	0.64	0.15	0.49
RASSF1_E116_F*	Y	0.13	0.70	-0.57
RIPK3_P24_F*	N	0.06	0.50	-0.44
PI3_P1394_R*	N	0.77	0.24	0.53
GABRA5_P1016_F	N	0.89	0.43	0.46
RASSF1_P244_F*	Y	0.12	0.65	-0.53

*samples selected for confirmation by pyrosequencing
Y (yes)/N (no) designates whether CpG site is located within a CpG island

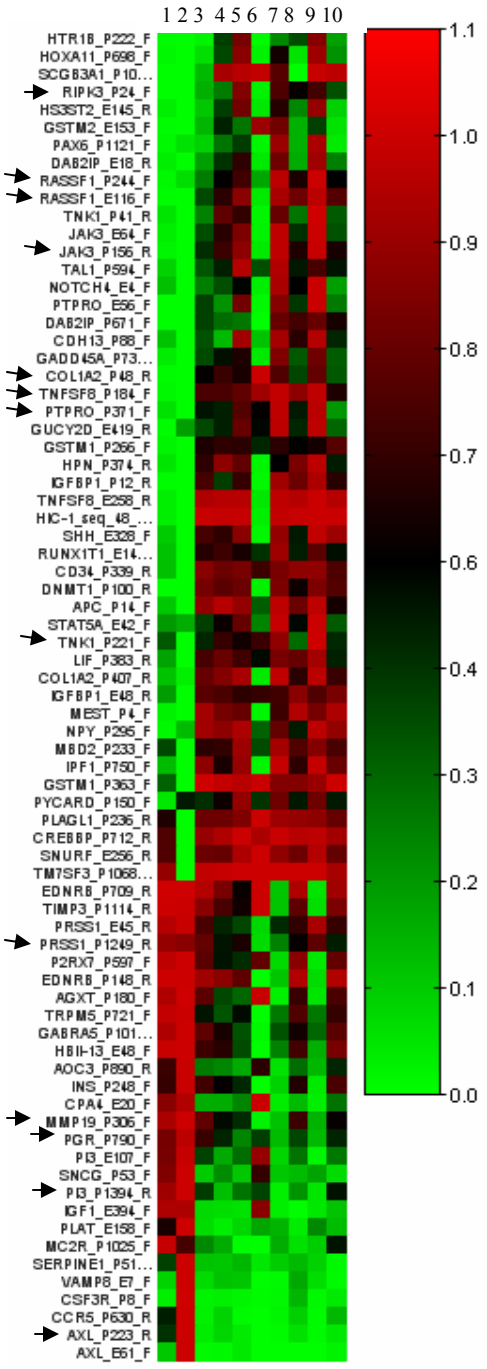


Figure 3.1. A heat map representing methylation at significant CpG sites in patient and control samples assayed by Illumina. Seventy-five CpG sites showed a significant difference in methylation between patient and control samples: the sites showed an absolute difference in mean methylation of at least 35% and an absolute diff score of at least 33. CpG sites selected for confirmation by pyrosequencing are indicated by arrows. Methylation at each CpG site is represented by a gradient from red to green, corresponding to a methylated to unmethylated state of a CpG site. Sample ID: (1) P26, (2) P08, (3) P09, (4) C01, (5) C02, (6) C03, (7) C05, (8) C06, (9) C07, (10) C09.

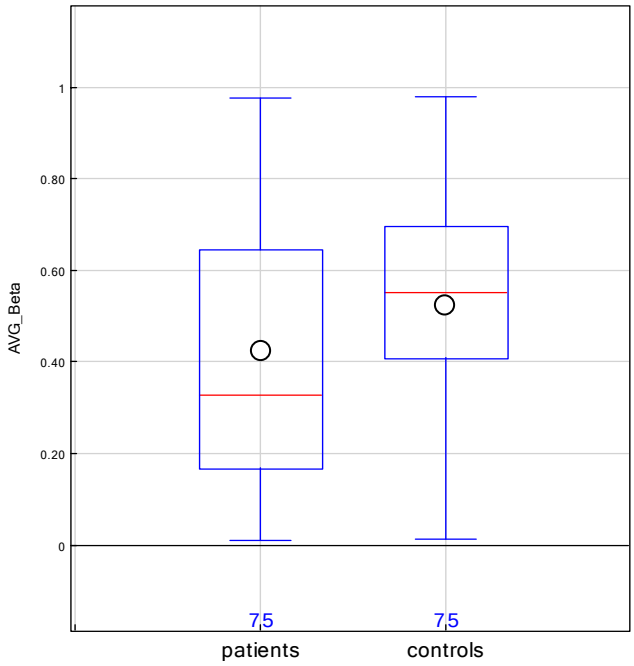


Figure 3.2. **Mean methylation at significant CpG sites in patients and controls**. The significant CpG sites showed a lower mean methylation in the patients compared to the controls. The range of methylation for the seventy-five significant CpG sites is shown in the box plots, where the upper and lower box limits represent the 75th and 25th percentiles, respectively. The whiskers represent the higher and lower range of methylation; 100% and 0%, respectively. The red horizontal bar represents the median and the circle represents the mean methylation.

Table 3.2. **List of pairs of CpG sites showing a significant difference in DNA methylation between patients and controls.**

Target ID	CpG island	Mean methylation Oligo group	Mean methylation Control group	Mean methylation difference
AXL_E61_F	N	0.37	0.01	0.36
AXL_P223_R*	Y	0.49	0.04	0.45
COL1A2_P407_R	N	0.31	0.72	-0.41
COL1A2_P48_R*	Y	0.19	0.64	-0.45
EDNRB_P148_R	N	0.96	0.52	0.44
EDNRB_P709_R	N	0.98	0.63	0.35
GSTM1_P266_F	Y	0.21	0.61	-0.40
GSTM1_P363_F	Y	0.44	0.92	-0.48
IGFBP1_E48_R	Y	0.32	0.73	-0.41
IGFBP1_P12_R	Y	0.24	0.62	-0.38
JAK3_E64_F	Y	0.14	0.61	-0.47
JAK3_P156_R*	N	0.16	0.68	-0.52
PI3_E107_F	N	0.73	0.30	0.43
PI3_P1394_R**	N	0.77	0.24	0.53
PRSS1_E45_R	N	0.88	0.52	0.36
PRSS1_P1249_R*	N	0.83	0.47	0.36
PTPRO_E56_F	Y	0.13	0.51	-0.37
PTPRO_P371_F*	N	0.19	0.63	-0.44
RASSF1_E116_F**	Y	0.13	0.70	-0.57
RASSF1_P244_F**	Y	0.12	0.65	-0.53
TNFSF8_E258_R	N	0.32	0.82	-0.51
TNFSF8_P184_F*	Y	0.24	0.82	-0.58
TNK1_P221_F*	Y	0.27	0.65	-0.38
TNK1_P41_R	Y	0.11	0.55	-0.44

Y (yes)/N (no) designates whether CpG site is located within a CpG island
*CpG sites selected for confirmation by pyrosequencing
**CpG sites selected for confirmation by pyrosequencing based on statistical analysis by Mann-Whitney and the LIMMA statistic.

Based on the analysis of results obtained through Illumina thirteen CpG sites were selected for confirmation by pyrosequencing: PGR_P790_F, MMP19_P306_F, RASSF1_E116_F, RIPK3_P24_F, PI3_P1394_R, RASSF1_P244_F. AXL_P223_R, COL1A2_P48_R, JAK3_P156_R, PRSS1_P1249_R, PTPRO_P371_F, TNFSF8_P184_F and TNK1_P221_F.

3.3.2 Confirmation of DNA methylation by pyrosequencing

A total of thirteen CpG sites were selected for confirmation by pyrosequencing. Primers used for the amplification and sequencing of the site of interest as well as the DNA sequence analyzed in each pyrosequencing assay are shown in Table 3.3. Two assays, for CpG sites

Table 3.3. Primers and sequences analyzed for each CpG site assayed by pyrosequencing

CpG sites		Primers used	Sequence analyzed
MMP19_P306_F	For	TTTTTGGAGAGATTAGAGATAGGG	TTTTAGGGATTTTGGGTTAGTAAATTAT
	Rev B	CCTTCACCTAAAACCCAAACTAC	TTYGTTTTTATTTTTGAGTTTTTTTAGA
	SeqF	TTTGGAATTAGATATTAATGTG	ATAA
PGR_P790_F	For B	TGTTTTATTGAGGTGTAATTTTAG	AAATACAAAAAACATATTAATAACTAC
	Rev	CCTTAATCCAAAATAACCAAATC	TAAAAAATTTAAACRAAAATATAAAAT
	SeqR	CCAAAATAACCAAATCAC	AACTAC TAATAAAT
RASSF1_E116_F	For	AGYGGGTGTTAGTTTTYGTAGT	GTTYGGTTGGGTTYGTGTTTYGTTGGTT
	Rev B	TCCCTACACCCAAATTTCCAT	TTGGGYGTTAGTAAGYGYGGGTYGGG
	SeqF	TTTAGGTTTTTTYGATATG	YGGGGTTATAGGGYGGGTTTYGATTTT AGYGTTTTT TTTAGGATTTA
RASSF1_P244_F	For	AGATATTYGTGTTTTTGGAGGT	TTYGGTTTTGGTTTTTTTGGTTYGGTTT
	Rev B	ATAAAACTACTAACYGATCTCCCT	GTTGAAGTAATARTATTTGGTTTATTTA
	SeqF	GAGGAAGAGGGTTTTTATA	TTGGGTGGGGTAGGAAGTTTYGAGTTT TTATTTGGGGTGAGGAGGAG
PI3_P1394_R	For B	TTTGTGTAAGGTAGTTAAAGGTTTT	CRATTTAAACTCTAACCACATACCCAC
	Rev	AACTAATAAAATCCCCACCC	CAAACCTAAAACTAAAAAAAAACATAA
	SeqR	ATCCCCACCCCTTAT	ACRAATATCAAACTATA AAAACCTTTAA
RIPK3_P24_F	For	GGAAGGGGTTTGTTTGTGTAG	AYGGTGAGTTTATTTTTYGGGTTGTTAT
	Rev B	AAAAACACTAACTTTYGCTCTACC	TTTTTTTTYGAGTGATTGAATAATTTTT
	SeqF	GGGGTTAGTTTTTAGATTAAG	TTTATAGGYGTTTTTATAGTTTYGTTTT TGGGGTGGGGTAGGGGGTAGA
AXL_P223_R	For	TGTTTTTGAGGTTTTTTTAGGAA	YGAGTTTTTGGTTTGGTTTGTTTGAGTG
	Rev B	CACACACACTCTTAAACRTCACTA	TGTTTGTGGGTTAGTAGTATGTTTTTGT
	Seq	TTGAGGTTTTTTTAGGAAT	TYGTTTGGGTTTTTTTGYGTGTTTTTGT TTGTTTTAGTTTGTGT
JAK3_P156_R	For	GTGGGTTTTTTGGTTTTTTGAG	ATYGAAAGTTAGGGTTTTGYGGGAGTT
	Rev B	CCTAATATAACCCAAACCCTTCTC	GGGGGYGGGGAGGYGGGTAAGGAGGG
	Seq	GTTTTTTGAGTTATTGTTATTT	GTAGAAAGTTTYGGAAGTTTTTGTATT AGTYGTTTYGTTTAGATAGGTTGTTGG AGATTTTT
COL1A2_P48_R	For	GTGGTTTATAGGGTATAGGTGAGG	YGGGATTGGATAGTTTTTGTTTTGATYG
	Rev B	CCCCRACATAAACAAAATTTACA	TYGGAGATTTGTAAATTTTGTT TATGTT
	Seq	TTTATAGGGTATAGGTGAGG	
PTPRO_P371_F	For B	GTAGGAAGATTGAGAGGGAATTG	CCRCCTCAACCCCTCCCTCAATCTCTAA
	Rev	ACTAAACAACCCCCATTACAAACT	ATCTCAAAAATTCTAAAAAATTTCCCT
	Seq	AACCCCCATTACAAACT	AACCTCTCTTAAACAATCCAAACRCCA AATCCCAATTCCCTCTCAATCT
TNK1_P221_F	For	GGGGGATAGAGATAGAGGAGTGAA	YGGAGAAGGTTGGAAAGAYGTGAAGG
	Rev B	CTCCCTCCTCCAAACCAAAT	AAGAYGAGTAGAGGAGAAGGGAAGGG
	Seq	AGGGTTGGGAAGTTATT	GGTTTTTTATAGTYGGGGYGATTTGGTT TGGAGGAGGGAGGGGTT

F/R refer to the forward and reverse sequence complement
For/Rev refer to the forward and reverse primer
Seq refers to the sequencing primer
B refers to the biotin label
Y refers to the degenerate nucleotide; either C or T
R refers to the degenerate nucleotide; either A or G: an R not within a CG context represents an A/G SNP.

PRSS1_P1249_R and TNFSF8_P184_F, did not produce satisfactory results and were excluded from analysis. Information for these two assays is not presented in Table 3.3. The forward or reverse strand was sequenced. CpG sites analyzed within the DNA sequence are indicated as YG on the forward strand or the complementary CR on the reverse strand. Between one and ten CpG sites were analyzed in each assay (Table 3.3). In three of the eleven usable assays not all

the CpG sites could be analyzed; the sequences were not fully sequenced and methylation could not be measured at the distal CpG sites. Methylation was measured at eight of the ten CpG sites within the RASSF1_E116_F sequence, at four of the five CpG sites within the TNK1_P221_F sequence and at one of the two CpG sites within the PTPRO_P371_F sequence. The CpG site that could not be analyzed within the PTPRO_P371_F sequence was the site analyzed in the Illumina array and therefore methylation at the nearby CpG site is presented. The sequences became truncated likely due to their length as all three of them were in the upper recommended size limit for the pyrosequencing assay and ranged in size from 98 to 104 base pairs.

Mean methylation at each CpG site and at the eleven sequences analyzed is presented in Table 3.4. The CpG sites analyzed in the Illumina array are indicated in bold text. A significant difference in methylation between the Control group and the Oligo group was found at three CpG sites originally analyzed by the Illumina array; RASSF1_P244_F (CpG 2; 4.73±0.62 vs. 6.46±1.13, p=0.0123), JAK3_P156_R (CpG 6; 2.66±0.54 vs. 1.49±1.08, p=0.045) and COL1A2_P48_R (CpG 3; 1.51±0.26 vs. 1.03±0.37, p=0.0441). Although, these differences in methylation are statistically significant, they do not correspond to the methylation measured by the Illumina assay (Table 3.2). Neighboring CpG sites to the ones listed above also showed a significant difference in methylation between the Control group and the Oligo group These included site CpG 3 within the RASSF1_P244_F sequence (1.55±0.42 vs. 3.17±1.61, p=0.0289), site CpG 3 and 5 within the JAK3_P156 sequence (1.77±0.53 vs. 2.86±1.01, p=0.0493; 10.66±2.60 vs. 5.32±3.17, p=0.0228) and site CpG 1 within the COL1A2_P48_R sequence (0.81±0.24 vs. 0.35±0.40, p=0.0496). Site CpG 5 within the RASSF1_E116_F sequence also showed a significant difference in methylation between the Control group and the Oligo group (1.49±0.46 vs. 0.79±0.10, p=0.0354). None of the CpG sites listed retained significance after the Bonferroni correction. A significant difference in methylation was detected at between one and three CpG sites within each sequence analyzed and at three of the four sequences a consistent loss or gain of methylation was seen across the significant CpG sites. A loss of methylation in the sperm of oligozoospermic men was detected at CpG sites within JAK3 and COL1A2. The loss of methylation at these two CpG sites was initially detected by the Illumina assay, while the gain of methylation within the promoter of RASSF1

Table 3.4. DNA methylation at selected CpG sites analyzed by pyrosequencing.

Target ID	Study Group	Methylation at Studied CpG Sites								
		Sequence Mean (%) ± SD	CpG Analyzed Mean (%) ± SD							
			1	2	3	4	5	6	7	8
RASSF1_E116_F	control	1.16±0.52	1.81±0.63	**2.49±0.74**	1.68±0.56	2.02±0.50	1.49±0.46	0.88±0.45	1.82±0.95	0.93±0.49
	oligo	1.17±0.22	2.42±0.84	**2.36±0.59**	2.77±1.68	2.61±0.20	0.79±0.10*	0.67±0.26	1.61±0.13	0.61±0.09
P value							0.0354			
Corrected P value							1.73			
RASSF1_P244_F	control	1.86±0.46	1.79±0.60	**4.73±0.62**	1.55±0.42					
	oligo	3.73±2.50	1.57±0.75	**6.46±1.13***	3.17±1.61*					
P value				0.0123	0.0289					
Corrected P value				0.467	1.42					
RIPK3_P24_F	control	2.68±0.42	1.19±0.16	2.34±0.46	**3.83±0.68**	2.43±0.29	3.62±0.72			
	oligo	3.04±0.57	1.40±0.64	2.95±0.43	**4.44±0.81**	2.87±0.33	4.02±0.92			
PI3_P1394_R	control	82.97±2.97	84.43±2.44	**81.52±3.99**						
	oligo	83.78±2.82	86.10±3.88	**81.46±2.36**						
PGR_P790_F	control	74.48±2.05	**74.48±2.05**							
	oligo	73.63±11.74	**73.63±11.74**							
MMP19_P306_F	control	89.77±2.33	**89.77±2.33**							
	oligo	89.39±1.39	**89.39±1.39**							
AXL_P223_R	control	89.43±0.84	95.23±1.51	**92.90±3.22**	80.38±1.77					
	oligo	90.73±1.71	94.71±2.85	**97.73±3.37**	79.74±5.63					
JAK3_P156_R	control	3.17±3.35	1.55±0.64	2.01±0.44	1.77±0.53	1.05±0.38	10.66±2.60	**2.66±0.54**	2.52±0.51	
	oligo	2.85±1.48	2.18±1.15	2.63±0.60	2.86±1.01*	1.09±0.38	5.32±3.17*	**1.49±1.08***	4.16±2.54	
P value					0.0493		0.0228	0.0450		
Corrected P value					2.42		1.12	2.21		
COL1A2_P48_R	control	1.93±1.37	0.81±0.24	3.45±0.38	**1.51±0.26**					
	oligo	1.71±1.80	0.35±0.40*	3.75±1.09	**1.03±0.37***					
P value			0.0496		0.0441					
Corrected P value			2.43		2.16					
PTPRO_P371_F	control	1.17±1.15	1.17±1.15**							
P value	oligo	0.17±0.29	0.17±0.29							
TNK1_P221_F	control	1.96±0.46	2.93±0.10	1.93±0.71	**1.86±1.21**	1.14±0.61				
	oligo	4.60±4.17	10.50±11.30	2.22±2.06	**4.50±3.50**	1.17±0.29				

* significant compared to controls, unpaired two-tailed p value <0.05, corrected p values were obtained by multiplying p values by 49, the number of tests performed in this data set, SD standard deviation

values indicated in bold text correspond to CpGs analyzed by Illumina

**the CpG site that was analyzed by the Illumina GoldenGate assay is located next to this site

did not correspond with the loss of methylation within the exon. Loss of DNA methylation at both of these CpG sites was initially detected using the Illumina assay. We found a standard deviation of 11.30 for the mean methylation at CpG 1 within the TNK1_P221 sequence for the Oligo group (Table 3.4). An average DNA methylation of 23.5% was detected in sample P26, while an average methylation of 3% and 5% was detected in the other two samples, P08 and P09, respectively, accounting for the group standard deviation. The average methylation at CpG 3 within the TNK1_P221 sequence in sample P26 was 8% and was also higher compared to samples P08 and P09, 1% and 4.5%, respectively. The mean methylation at CpG sites 1 and 3 did not differ significantly between the control and oligo groups. There were no significant differences in methylation observed between the Control group and the Oligo group for the mean methylation of all the CpG sites analyzed at each sequence (Table 3.4).

3.4 DISCUSSION

Abnormal DNA methylation at imprinted genes has been associated with spermatogenesis failure in infertile men affected by oligozoospermia (Marques et al., 2008; Kobayashi et al., 2007; Boissonnais et al., 2010), while DNA methylation at repetitive gene sequences appears to be normal (Marques et al., 2008; Kobayashi et al., 2007). There is little information available regarding DNA methylation at non-imprinted genes in the sperm of infertile men. The results presented in this study obtained from a small number of samples suggest that abnormal methylation in the sperm of infertile men may also affect non-imprinted genes. Small, but significant differences in methylation at multiple CpG sites were found between patient and control men at three genes: *RASSF1, JAK3* and *COL1A2* (Table 3.4). The significant difference in methylation at the multiple CpG sites was lost in the genes mentioned after the Bonferroni correction. However, the detection of significant changes in methylation at multiple CpGs sites within *RASSF1, JAK3* and *COL1A2* does warrant further study of methylation at these genes in relation to infertility. In this study only three sperm samples obtained from men affected by severe and very severe oligozoospermia were studied. The analysis should be extended to a larger number of samples to confirm the findings. Analysis of DNA methylation at *RASSF1, JAK3* and *COL1A2* was not extended to additional samples obtained from men with oligozoospermia studied in Chapter 2 because sufficient amounts of DNA were not available to perform pyrosequencing analysis. For the majority of samples

analyzed in Chapter 2 sperm were isolated by micromanipulation, providing insufficient numbers of sperm from which a sufficient quantity of DNA could be extracted for analysis either through the GoldenGate Illumina or the pyrosequencing assays. There has been one report to date of abnormal methylation at non-imprinted genes in the sperm of infertile men (Houshdaran et al., 2007). Five non-imprinted genes, *HRAS, NTF3, MT1A, PAX8* and *SFN*, showed hypermethylation in patients with severe oligozoospermia (Houshdaran et al., 2007). An overall increase in DNA methylation, also affecting non-imprinted genes, was found in cord blood samples collected from ART children (Katari et al., 2009). Although, the abnormal methylation reported may be acquired through the procedures involved in ART, the abnormality may be passed on through the use of ART with fertilization using a sperm carrying the methylation abnormality.

DNA methylation at 1,505 CpG sites was investigated by a high-throughput array approach in the sperm of men affected by severe oligozoospermia. Comparison of the results to fertile control men identified up to seventy-five CpG sites that showed a significant difference in methylation between the control and patient samples. An average loss of methylation was observed in the sperm of men affected by severe oligozoospermia compared to control men (Figure 3.2). The overall reduction in DNA methylation in the sperm of infertile men is consistent with previous reports that have been associated with reduced pregnancy rates in couples undergoing infertility treatment (Benchaib et al., 2003; Benchaib et al., 2005). Of the seventy-five CpG sites showing a significant difference in methylation between patient and control samples, eleven were selected for confirmation using a sequence-specific approach enabling the analysis of methylation at the CpG sites of interest and, when possible, at neighboring CpG sites. DNA methylation was measured at between one and eight CpG sites located near the CpG site of interest within the promoter of most genes analyzed. A number of CpG sites within four of eleven sequences analyzed, specific to three genes showed a small, but significant, difference in methylation between patient and control sperm samples. Genes with CpG sites showing a significant difference in methylation included *RASSF1, JAK3*, and *COL1A2*. A significant difference in methylation was found at three of the CpG sites selected for confirmation. Although a significant difference in methylation between the sperm of oligozoospermic and control men was observed at these CpG sites, the magnitude of

methylation did not correspond to the methylation measured by the Illumina array. This could have resulted from an error introduced by the analysis of a small number of samples, or the quality of DNA samples obtained from sperm. Furthermore, the average methylation in patients seems to correlate more between the two assays than the average methylation in the controls. The lack of expected results may also be related to the high rate of false positives associated with the simultaneous analysis of many hypotheses, 1,505 CpG sites, and a small sample size (Pawitan et al., 2005). Therefore a high rate of false positive results would have been expected. On the other hand, the strict selection criteria used to select CpG sites for confirmation could have also eliminated false negative sites from being investigated. The pyrosequencing and Illumina results did, however, show a consistent decrease in methylation at two of the three CpG sites. Furthermore, the changes in methylation observed are small, ranging from 0.5% to 5.28%. It is not known whether these small changes would affect gene expression and therefore, the changes may not be significant at the biological level. The study may have; however, identified genes that warrant further study in a larger number of samples namely *RASSF1, JAK3* and *COL1A2*. *RASSF1* is a tumor suppressor gene whose inactivation through promoter hypermethylation has been observed in several tumors including retinoblastoma (Harada et al., 2002). Children born though ART are at a higher risk for developing this kind of tumor (Marees et al., 2009). Whether hypermethylation at *RASSF1* is a contributing factor in these cases is currently unknown. Abnormal expression of *COLIA2* and *JAK2* has been associated with bone density and immunodeficiency disorders in humans, respectively (Korkko et al., 1998; Cornejo et al., 2009).

This is the first genome-wide analysis of DNA methylation in the sperm of men with oligozoospermia. Four of the eleven genes analyzed by pyrosequencing (PI3, *PGR, MMP19* and *AXL*) showed hypermethylation, while the remaining seven genes were hypomethylated in the sperm (Table 3.4). The methylation values obtained through pyrosequencing are in accordance with the methylation values reported for the CpG sites in the sperm of normozoospermic men undergoing evaluation for infertility (Houshdaran et al., 2007). The analysis was performed using the GoldenGate Illumina assay. CpG site-specific methylation was observed at the *JAK3* and *PGR* genes analyzed: *JAK3* was hypomethylated at two CpG sites and hypermethylated at one CpG site and *PGR* was hypomethylated at one CpG site and hypermethylated at two CpG

sites (Houshdaran et al., 2007). The Illumina analysis was also performed on DNA extracted from peripheral blood (Houshdaran et al., 2007). The level of DNA methylation was similar between sperm and blood at seven of the eleven genes evaluated by pyrosequencing in this study: *PTPRO, RASSF1, JAK3, PGR, PI3, MMP19* and *RPK3*. However, the reported level of DNA methylation at *PGR* in the blood differed between two published studies. Houshdaran et al. (2007) reported hypermethylation while Kroeger et al. (2008) reported hypomethylation at *PGR*. The discordance in methylation is likely related to the CpG sites analyzed by the two studies as different levels of methylation have been reported at different CpG sites within the *PGR* gene (Houshdaran et al., 2007). A lack of tissue specific methylation at most of the genes analyzed suggests two things. First, gene methylation is associated with suppression of gene expression (Jones et al., 1998; Nan et al., 1998), therefore the methylated genes may not be expressed in the adult tissues analyzed and may show development stage-specific expression by being unmethylated and expressed at a different stage in development. Second, a lack of methylation is associated with gene expression (Jones et al., 1998; Nan et al., 1998), therefore the unmethylated genes may be expressed in the tissues analyzed and their expression in different cell types suggests that these genes may be associated with cell function that would be seen in diverse cell types. DNA methylation at *COL1A2, TNK1* and *AXL* showed tissue specificity: methylation at *COL1A2* and *TNK1* was higher in blood compared (methylation at both genes in blood was below 50%) to sperm (unmethylated) and methylation at *AXL* was lower in blood (methylation in the blood was at 30%) compared to sperm (methylated) (Houshdaran et al., 2007). The tissue specific methylation at the three genes suggests that these genes are associated with specialized cell function that is specific to each cell type.

A lack of methylation was reported at *COL1A2* in normal melanocytes and hepatocytes, while methylation was present in melanomas and hepatomas (Koga et al., 2009; Chiba et al., 2005). The lack of methylation at *COL1A2* found in normal tissues (Koga et al., 2009; Chiba et al., 2005) corresponds with methylation found in sperm in this study. Methylation at some of the genes analyzed by pyrosequencing (Table 3.4) has been analyzed in cancers and the corresponding healthy tissues. Lack of methylation was found at *RASSF1* in healthy lung and breast tissue (Fukasawa et al., 2006; Feng et al., 2008; Shukla et al., 2006), at *RIPK3* in lung tissue (Fukasawa et al., 2006), at *PGR* in lymphocytes (Kroeger et al., 2008) and at *PTPRO* in

lymphocytes (Motiwala et al., 2007; Motiwala et al., 2004) and hepatocytes (Motiwala et al., 2003). The genes were methylated in cancers involving the corresponding healthy tissues (Feng et al., 2008; Fukasawa et al., 2006; Shukla et al., 2006; Kroeger et al., 2008; Motiwala et al., 2004; Motiwala et al., 2003). DNA methylation seen in cancer has been proposed to present one mechanism associated with the repression of gene expression observed in some cancers (Motiwala et al., 2007; Motiwala et al., 2003). The association of modified DNA methylation at non-imprinted genes with cancer may be one reason why only small changes in DNA methylation at non-imprinted genes were identified in the sperm of men affected by severe oligozoospermia in this study. Significant changes in methylation at the genes studied, such as changes in methylation from hypomethylation to hypermethylation and from hypermethylation to hypomethylation, may be associated with cancer and not male infertility.

Houshdaran et al. (2007) reported significant changes in DNA methylation at five non-imprinted genes (*HRAS, NFT3, MT1A, PAX8* and *SFN*) and at three imprinted genes (*MEST, PLAGL1* and *DIRAS3*) in the sperm of men affected by severe oligozoospermia. The analysis was performed using MethyLight assays on thirty-five pre-selected CpG sites from analyses on donor sperm samples. Depending on primer design, MethyLight assays can evaluate DNA methylation at multiple CpG sites within each gene analyzed (Eads et al., 2000). Of the eight genes that showed significant changes in DNA methylation, five are included in the GoldenGate Illumina array used in this study: *MT1A, SFN, PLAGL1, MEST* and *DIRAS3*. Differences in DNA methylation at *MT1A, SFN* and *DIRAS3* were not identified as significant between control and patient samples by the GoldenGate Illumina assay in this study. Differences in DNA methylation at *PLAGL1* and *MEST* were identified as significant between control and patient samples. However, the significant changes in methylation were identified at one of three CpG sites included in the array for both genes. CpG sites for these genes were not selected for confirmation by pyrosequencing. It should be mentioned that DNA methylation at *MEST* was analyzed in Chapter 2 for all samples included in this study and was normal for all samples tested at multiple CpG sites within the gene.

DNMTs are responsible for proper establishment and maintenance of DNA methylation in the genome. Methylation at imprinted genes is re-established in a sex-specific manner in germ cells and is maintained through the second phase of genome-wide demethylation and

remethylation when methylation at non-imprinted genes is modified (Kafri et al., 1992; Tremblay et al., 1997; Olek and Walter, 1997). Gene mutations in Dnmt3l and Dnmt3a in male mice have been associated with loss of DNA methylation at imprinted genes (Bourch'his and Bestor, 2004; Yaman and Grandjean, 2006) without affecting DNA methylation at non-imprinted sequences such as tandem repeats of satellite DNA (Bourch'his and Bestor, 2004) and *IAP*s and *LINE1* elements (Yaman and Grandjean, 2006). These results show that mutations in DNMTs may be associated with abnormal methylation at imprinted genes without affecting methylation at other sequences. The second phase of genome reprogramming occurs after fertilization during preimplantation development and while DNA methylation at imprinted genes in the sperm is protected from the second phase of genome reprogramming (Olek and Walter 1997; Tremblay et al., 1997). DNA methylation at non-imprinted genes may be established according to the cell-specific needs of differentiating cells. Abnormal DNA methylation at non-imprinted genes seen in sperm may be associated with spermatogenesis failure but is unlikely to be passed on to the progeny or affect pregnancy outcome.

Of further interest may be delineation of mechanisms that can affect methylation at non-imprinted genes, without implementing changes at imprinted genes. Patients in this study had normal methylation at multiple CpG sites tested within imprinted genes *H19, GTL2* and *MEST* (results shown in Chapter 2). However, changes in methylation at non-imprinted genes were detected in the same patients. One confounding factor associated with the study of methylation of non-imprinted genes in sperm may be the finding that most variability in methylation in sperm was detected at promoter-specific CpG sites located within CpG islands and centromeric satellite DNA (Flanagan et al., 2006). Ten of the eleven sequences selected for confirmation were promoter specific. Further research is needed to determine whether the changes in methylation at non-imprinted genes identified in this study and the Housharan et al. (2007) study represent variation in the sperm epigenome or may be associated with male infertility.

Previous studies (Kobayashi et al., 2007; Marques et al., 2008, Boissonnais et al., 2010) as well as results presented in Chapter 2, have demonstrated abnormal methylation at imprinted genes in the sperm of infertile men. However, the results presented in this study and the Houshdaran et al. (2007) study suggest that abnormal methylation in the sperm of infertile men

may be associated with spermatogenesis failure. Due to the low statistical power observed for the sequences studied, a larger sample size is needed to confirm the findings presented.

3.5 CONCLUSION

The current study on a limited number of samples suggests that abnormal methylation at non-imprinted genes may be associated with spermatogenesis failure. Of particular interest may be genes *RASSF1*, *JAK3* and *COL1A2*, for which small, but significant differences in methylation at multiple CpG sites were found between patient and control sperm samples. It is uncertain whether the small changes in methylation would affect gene expression and contribute to a negative clinical outcome. The results do; however, warrant further study of DNA methylation at non-imprinted genes in the sperm of infertile men as well as the analysis of a larger number of samples to increase the statistical power of the analysis. Spermatogenesis failure may not only be associated with aberrant imprinting but also with changes in DNA methylation at non-imprinted genes.

CHAPTER 4: EVALUATION OF DNA METHYLATION AT IMPRINTED GENES IN TESTICULAR SPERM RETRIEVED FROM MEN AFFECTED BY AZOOSPERMIA

4.1 INTRODUCTION

Male factor infertility contributes to the infertility experienced by a couple in up to 50% of cases. It is estimated that in 10% to 15% of cases of male factor infertility the man is affected by azoospermia (Jarow et al., 1989). Azoospermia is defined as the absence of sperm from the ejaculate. It results from obstruction in 40% of cases (Jarow et al., 1989), while NOA due to spermatogenesis failure is seen in the remaining cases. Although, in these men sperm is absent from the ejaculate, it may be retrieved from the testes and used to achieve pregnancy through the use of ICSI.

Recent reports have associated male factor infertility with an increased risk for abnormal DNA methylation at imprinted genes in the sperm (Marques et al., 2008; Kobayashi et al., 2007; Poplinski et al., 2010). However, most of the published studies have only evaluated DNA methylation at imprinted genes in the sperm of men affected by oligozoospermia, with little information available on the status of DNA methylation in the sperm of men affected by azoospermia (Hartmann et al., 2006; Marques et al., 2009; Manning et al., 2001). Two imprinted genes, *H19* and the *GTL2*, show sperm specific DNA methylation in man (Kerjean et al., 2000; Geuns et al., 2007). Most imprinted genes are methylated in the oocyte, including *MEST*. Abnormal methylation at imprinted genes in the sperm may not only be associated with male factor infertility but may also be passed on to the progeny through the use of ICSI. Aberrant imprinting has been associated with imprinting syndromes observed in children (Kanber et al., 2009; Orstavik et al., 2003) and abortuses achieved through the use of ICSI (Kobayashi et al., 2009). As pregnancy can now be achieved with sperm extracted from the testes through the use of ICSI, it is important to be aware of the risks of aberrant imprinting in the sperm of these men.

While DNA methylation should not differ between sperm retrieved from the testes or the ejaculate, as it is established before germ cells enter meiosis (Kerjean et al., 2000), the knowledge of the status of DNA methylation at imprinted genes in the sperm of men affected by OA and NOA is limited. The few number of samples analyzed suggest a much lower rate of

abnormal methylation in the sperm of men affected by azoospermia, from 0% to 5.3% (Marques et al., 2009; Hartmann et al., 2006) compared to the sperm of men affected by oligozoospermia, from 20% to 68% (Marques et al., 2008; Kobayashi et al., 2007; Boissonnas et al., 2010). Furthermore, analysis of DNA methylation at imprinted genes in the sperm retrieved from men with different etiologies, NOA and OA, would help in the understanding of factors that may disrupt DNA methylation such as spermatogenesis failure in NOA patients or obstruction in OA patients. Currently, the available results do not show a clear association between aberrant imprinting and NOA or OA (Marques et al., 2009).

The three studies published evaluating DNA methylation at imprinted genes in testicular sperm of men affected by azoospermia did not include controls in their data sets (Manning et al., 2001a; Marques et al., 2009; Hartmann et al., 2006). Appropriate controls may include testicular sperm retrieved from men undergoing a vasectomy reversal or ejaculate sperm retrieved from fertile men. However, there is evidence to suggest that the testicular environment may be compromised post vasectomy and that this environment may be associated with abnormal DNA methylation (Weitzman et al., 1994; Turk et al., 1995; Hepburn et al., 1991; Tan et al., 1990; Tunc et al., 2009). ROS induced DNA damage may present one mechanism that is associated with changes in DNA methylation in the testis (Weitzman et al., 1994; Turk et al., 1995; Hepburn et al., 1991; Tan et al., 1990; Tunc et al., 2009). Because of the possibility that methylation abnormalities may occur in the sperm of men undergoing vasectomy reversal, ejaculate sperm samples obtained from men of proven fertility were used as controls in this study. Furthermore, evaluation of DNA methylation in sperm retrieved from men undergoing a vasectomy reversal would help to determine whether changes in DNA methylation are associated with obstruction.

In this study DNA methylation at the DMRs of three imprinted genes, *H19, GTL2* and *MEST,* was evaluated in the testicular sperm retrieved from men affected by azoospermia, NOA and OA, and compared to DNA methylation at imprinted genes in the sperm retrieved from men undergoing vasectomy reversal and ejaculate sperm from control men. DNA methylation was carried out using bisulphite sequencing so that DNA methylation at multiple CpG sites could be simultaneously analyzed and DNA methylation could be visualized at the single sperm level. We hypothesized that a higher incidence of abnormal DNA methylation at imprinted genes

would be identified in testicular sperm of azoospermic men and men undergoing vasectomy reversal compared to ejaculate control men. Based on the limited data available, we also hypothesized that sperm obtained from men affected by OA would be more prone to methylation abnormalities at imprinted genes compared to sperm retrieved from men affected by NOA. In addition, where possible based on the presence of polymorphisms within the DMR, the origin of an error was evaluated, to determine whether the error occurred due the lack of erasure or improper establishment.

4.2 MATERIALS AND METHODS

4.2.1 Sample preparation

4.2.1.1 Sample collection

Ethical approval was obtained from the University of British Columbia Ethics Committee before initiating this study. Testicular spermatozoa were isolated from testicular biopsy samples. Testicular biopsy samples were obtained from men undergoing vasectomy reversal and from infertile men presenting absence of spermatozoa in the ejaculate. Men undergoing vasectomy reversal were of proven fertility having had at least one child prior to vasectomy; however, we did not know the time interval from vasectomy to reversal. Spermatozoa were isolated from leftover testicular samples. Testicular samples obtained from infertile men underwent pathological evaluation to determine the type of spermatogenesis failure present. Based on the pathology results samples were subdivided into two categories: OA, determined based on the presence of normal spermatogenesis and NOA, determined based on the finding of spermatogenesis failure.

In total 35 testicular biopsy samples were obtained: 17 testicular biopsy samples were obtained from men undergoing a vasectomy reversal (VR01-VR17) and 18 samples were obtained from infertile men presenting with azoospermia based on the absence of spermatozoa in the ejaculate (AZO; TP01-18). The infertile study group was further subdivided into two sub-groups; patients with OA (OA; TP01-10) and NOA (NOA; TP11-15). The pathology results were not available for three patients (TP16-18), and these patients were not assigned to the OA or NOA sub-group. Patients were assigned to the OA sub-group based on the presence of normal spermatogenesis upon pathological evaluation (TP03-TP05, TP07, TP10), or the

presence of mutations within the CFTR gene associated with obstruction due to CBAVD (TP01, TP02, TP08; had Δ508 mutation). Patient TP06 was assigned to the OA sub-group based on the diagnosis of normal spermatogenesis and CBAVD due to the 5T allele. Patient TP09 was assigned to the OA sub-group based on the presence of epididymal head calcification. Patients were assigned to the NOA sub-group based on the pathological evaluation of spermatogenesis failure due to hypospermtogenesis or partial maturation arrest.

4.2.1.2 Karyotyping and screening for Y chromosome microdeletions

The results for chromosome analysis and Y chromosome microdeletion testing were obtained from patient charts. When the information was not available, peripheral blood was collected and processed for chromosome and Y chromosome microdeletion analysis as outlined in section 2.2.1.2 of Chapter 2.

4.2.1.3 Purification of sperm

Testicular sperm were purified by micromanipulation as outlined in section 2.2.1.3 of Chapter 2.

4.2.1.4 DNA isolation

DNA isolation from testicular sperm was performed using the alkaline lysis and neutralization buffers as outlined in section 2.2.1.4 of Chapter 2.

4.2.1.5 Sodium bisulphite modification

Samples were bisulphite modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). Each sample was split into two aliquots and bisulphite modified as outlined in section 2.2.1.5 of Chapter 2. Clones showing a bisulphite modification conversion rate of or above 95% were analyzed.

4.2.2 Analysis of DNA methylation

4.2.2.1 Sequences analyzed

DNA methylation was analyzed at three DMRs: *H19, IG-GTL2* and *MEST*. The sequences analyzed are the same as those outlined in section 2.2.2.1 of Chapter 2. The genomic

sequences analyzed are presented in Table 2.1. The SNPs present in the sequences analyzed are shown in Figure 2.1.

4.2.2.2 DNA amplification

The primers selected to amplify the sequences of interest within the *H19, IG-GTL2* and *MEST* DMRs were described in section 2.2.2.2 of Chapter 2. The samples were amplified using a semi-nested approach outlined in Chapter 2.

4.2.2.3 Cloning

Amplification products were run on an agarose gel and bands of the correct size were isolated, as outlined in section 2.2.2.3 of Chapter 2. Cloning of the isolated bands was carried out as outlined in section 2.2.2.3 of Chapter 2.

4.2.2.4 DNA extraction from colonies

Between two to three white colonies were picked from each plate. An average of ten colonies were picked for each gene amplified and set up for DNA extraction. DNA extraction from colonies was set up and carried out as described in section 2.2.2.4 of Chapter 2.

4.2.2.5 Restriction digest

Prior to submitting clones for sequencing, the presence of the appropriate insert was assessed by restriction digestion as outlined in section 2.2.2.5 of Chapter 2. Plasmids containing inserts of the appropriate size were submitted for sequencing.

4.2.2.6 Sequencing

Between 0.3ug and 0.5ug of extracted DNA was submitted for sequencing to the McGill University and Génome Québec Innovation Centre as outlined in section 2.2.2.6 of Chapter 2.

4.2.2.7 Alignment and analysis of sequences

An online tool, ClustalW2, was used to align FASTA files containing the sequences of the submitted clones. The analysis of the clones was performed as outlined in section 2.2.2.7 of

Chapter 2. Differences among clones, described in section 2.2.2.7 of Chapter 2, were used to determine if clones were unique. Bead diagrams representing methylation at the CpG sites analyzed at each clone were created using QUantification tool for Methylation Analysis (QUMA) (Kamuki et al., 2008). The QUMA software was also used to confirm the proper alignment of sequences and differences among sequences.

4.2.3 Data analysis

The methylation level for each sample was calculated as outlined in Chapter 2 in section 2.2.3. Differences in gene methylation level between groups were determined using the Kruskal-Wallis test with Dunn's multiple comparison post hoc test or the non-parametric Mann-Whitney test. One-tailed p-values <0.05 were considered significant.

The number of individuals with abnormal methylation at imprinted genes was also determined and compared between groups. Individuals with abnormal methylation were defined as having at least one improperly methylated unique clone. Improperly methylated clones were defined as in Chapter 2 in section 2.2.3. Differences in the number of individuals affected per group were determined using Fisher's exact test. One-sided p-values <0.05 were considered significant. The Bonferroni correction was used to correct for multiple testing.

The frequency of improper methylation at each CpG site within an analyzed sequence was also determined. This was defined as the number of improper methylation at each CpG site analyzed within a sequence in proportion to the total number of CpG sites analyzed at that site in all unique clones. Differences in methylation at each CpG site among groups were determined using Fisher's exact test. Two-tailed p-values <0.05 were considered significant. The Bonferroni correction was used to correct for multiple testing.

Other statistical tests were performed as indicated. All statistical analysis was done using GraphPad Prism (version 5.02) for Windows (GraphPad Software, San Diego, CA).

4.3 RESULTS

4.3.1 Patient clinical information

Age was available for 13 of the 17 vasectomy reversal cases and 12 of the 18 azoospermic patients (9 of the 10 OA, 3 of the 5 NOA). The mean age (±SD) for the vasectomy reversal patients was 46.2±4.0 (41-53). The mean age (±SD) for the azoospermic patients was 37.8±6.9 (28-51): 37.0±5.3 (28-46) for the OA group and 40.3±11.6 (28-51) for the NOA group (Table 4.1). All men who underwent vasectomy reversal and infertile men had a normal 46, XY karyotype and did not have Y chromosome microdeletions.

Table 4.1. Clinical information for men undergoing vasectomy reversal and affected by azoospermia.

Population	N	Sample ID	Age (mean±SD)	Pathology
VR	17	VR01-VR17	46.2±4.0	-
AZO	18	TP01-TP18	37.8±6.9	-
OA	10	TP1-TP10	37.0±5.3	CFTR mutations (TP01, TP02, TP06, TP08) Normal spermatogenesis (TP03-TP07, TP10) Epididymal head calcification (TP09)
NOA	5	TP11-TP15	40.3±11.6	Spermatogenesis failure
UP	3	TP16-TP18	-	-

4.3.2 Analysis of methylation at imprinted genes

4.3.2.1 Analysis of sequencing data

Eighteen CpGs were analyzed at the *H19* DMR, ten CpGs were analyzed at the *IG-GTL2* DMR and 21 CpGs were analyzed at the *MEST* DMR. Figure 4.1 shows bead diagrams representing methylation at CpGs studied at the *H19* DMR, *IG-GTL2* DMR and *MEST* DMR. Unique clones analyzed at each DMR are shown directly in the diagram, and are coded on the right-hand side with the first number designating the number of non-unique clones that were analyzed for each sequence followed by the amplification reaction each clone came from. The amplification reactions are not necessarily labeled in consecutive order. In samples containing an informative SNP, the allele is indicated on the left-hand side of each clone. In this data set, three SNPs were informative. Two SNPs within the *H19* DMR sequence were informative: C/T

at nucleotide 67 and C/A at nucleotide 109. The C/T SNP locates to CpG number 7 and methylation at that CpG implies the presence of the C allele, while a lack of methylation implies the presence of either allele. One SNP was identified within the *IG-GTL2* DMR sequence: G/A at nucleotide 34 (Table 2.1).

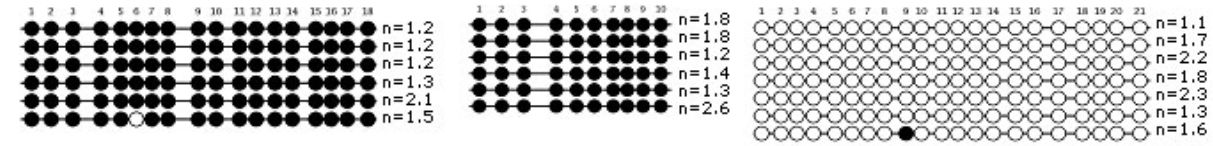
Unique clones were identified based on single nucleotide differences among clones. In total 907 clones were analyzed; 455 in the vasectomy reversal group and 452 in the azoospermia study group. Of the 907 clones analyzed, 687 were unique. Between 6 and 7 unique clones were analyzed per gene in the vasectomy reversal cases and azoospermic cases. In some cases, multiple amplification reactions failed and due to a limited amount of sample available fewer clones could be analyzed. On average, 69.9%, 83.0% and 74.8% of clones analyzed were unique for the *H19* DMR, *IG-GTL2* DMR and *MEST* DMR, respectively (Table 4.2), suggesting that samples with a small amount of starting material may be prone to preferential amplification resulting in clones having originated from the same strand of DNA. As it can be seen in Figure 4.1 multiple amplification reactions were performed for each gene per sample. Between one and seven amplification reactions were set up per gene with an average of 4.3 amplification reactions being performed per gene per sample.

Table 4.2. Proportion of unique clones analyzed in the sperm of vasectomy reversal and azoospermic men

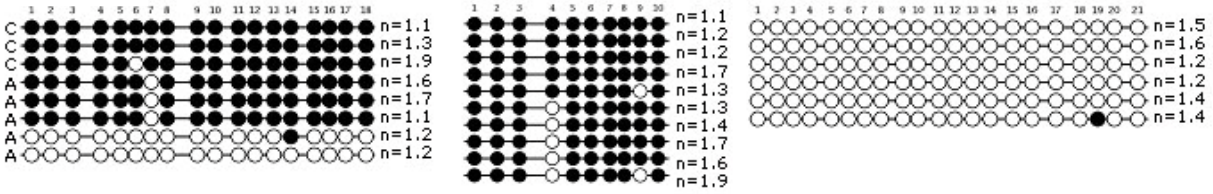
Study Group	*H19*	*IG-GTL2*	*MEST*	Group total
	unique clones/ all clones (%)			
VR	112/164 (68.3)	124/149 (83.2)	107/142 (75.4)	343/455 (75.4)
AZO	106/148 (71.6)	120/145 (82.8)	118/159 (74.2)	344/452 (76.1)
OA	60/91 (65.9)	67/82 (81.7)	66/90 (73.3)	193/263 (73.4)
NOA	35/42 (83.3)	34/39 (87.2)	33/42 (78.6)	102/123 (82.9)
Gene total	218/312 (69.9)	244/294 (83.0)	225/301 (74.8)	687/907 (75.7)

A. VASECTOMY REVERSAL MEN

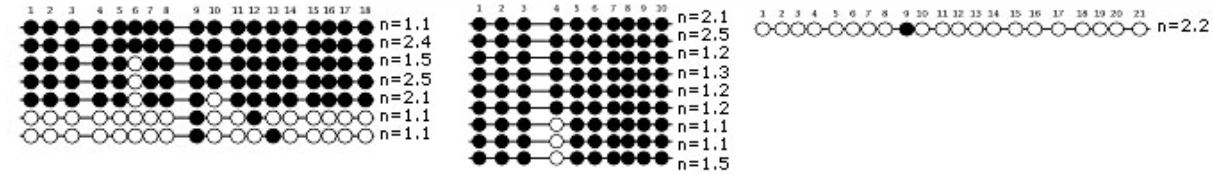
VR01



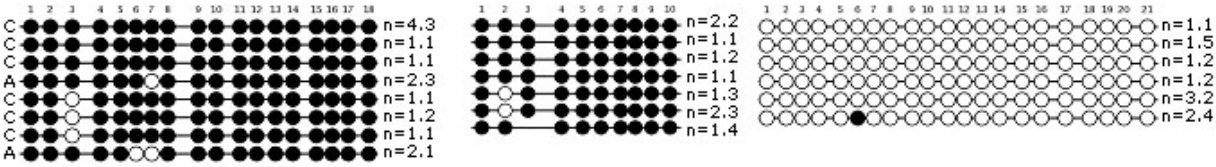
VR02



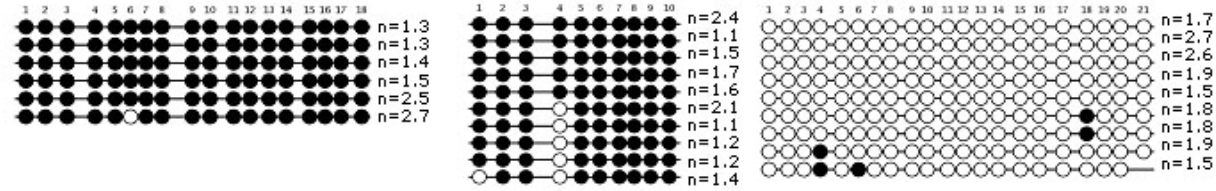
VR03



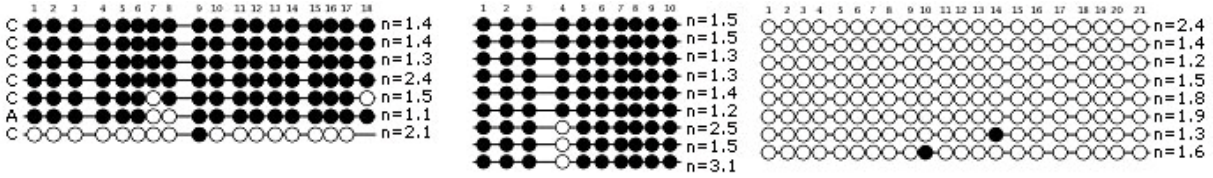
VR04



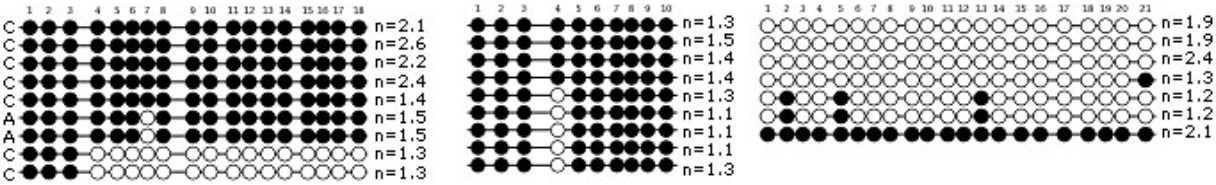
VR05



VR06



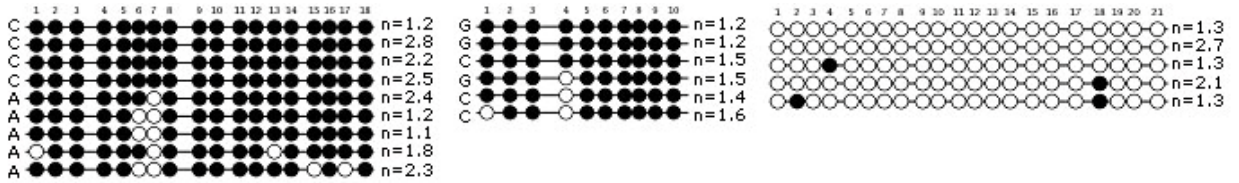
VR07



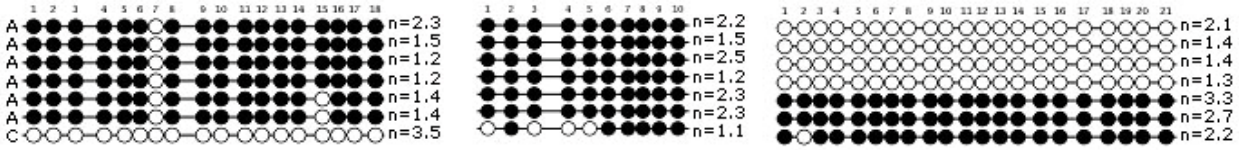
H19 DMR *IG-GTL2* DMR *MEST* DMR

A. VASECTOMY REVERSAL MEN (continued)

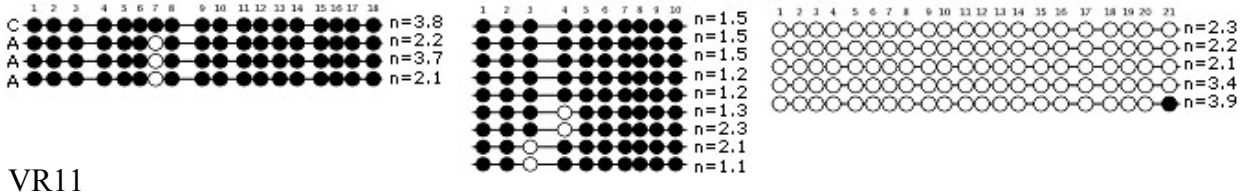
VR08



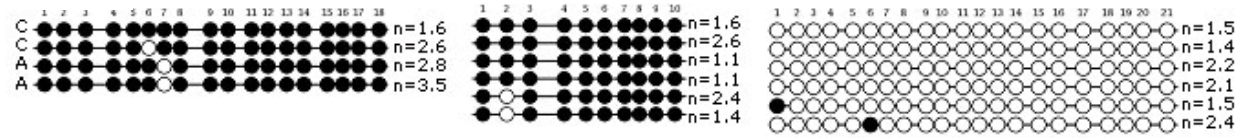
VR09



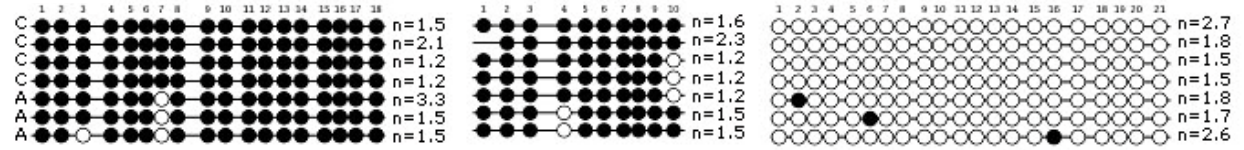
VR10



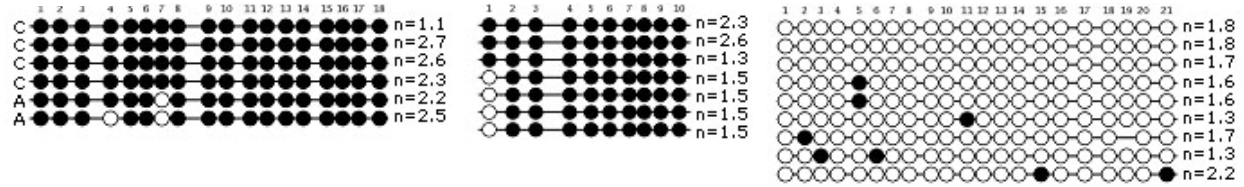
VR11



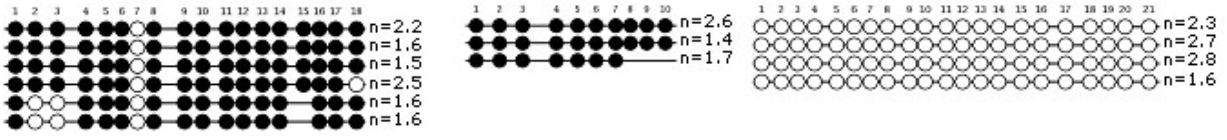
VR12



VR13



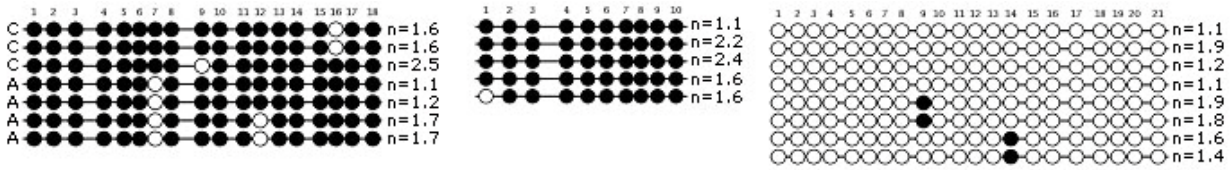
VR14



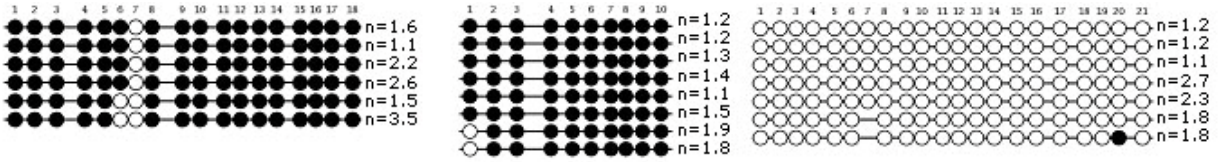
H19 DMR *IG-GTL2* DMR *MEST* DMR

A. VASECTOMY REVERSAL MEN (continued)

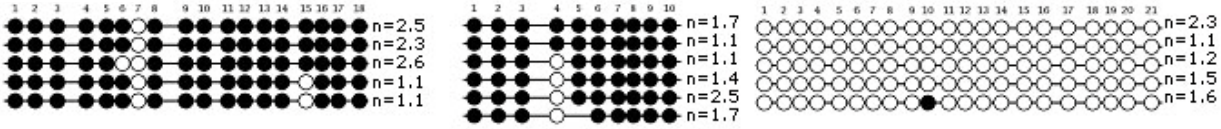
VR15



VR16



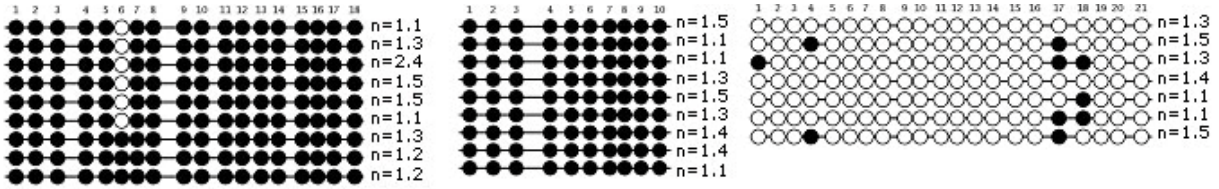
VR17



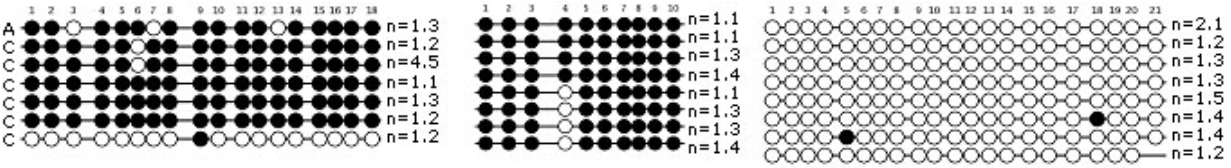
B. AZOOSPERMIC MEN

(obstructive azoospermia)

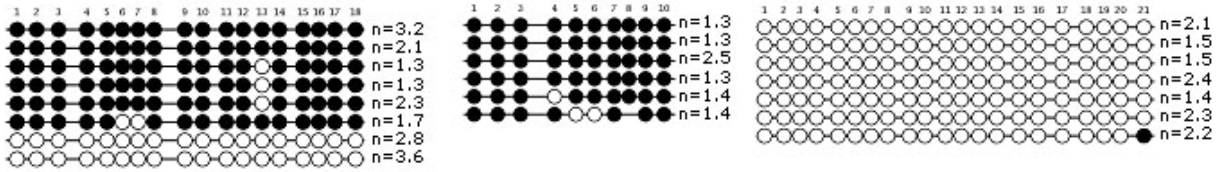
TP01



TP02



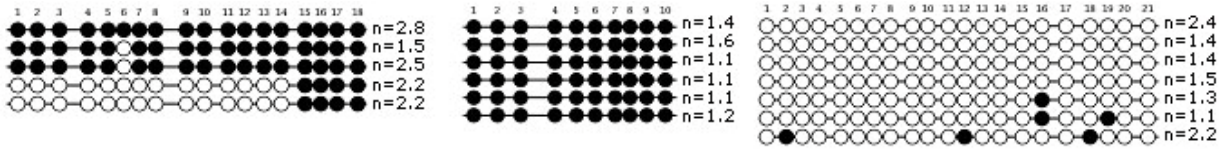
TP03



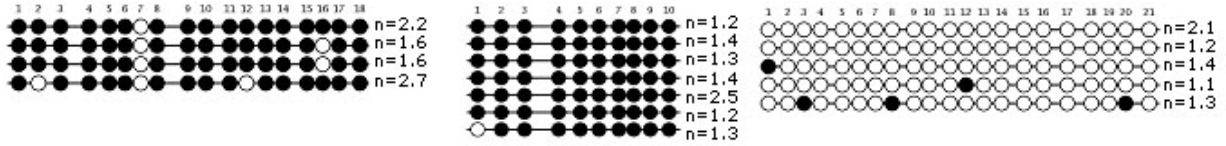
H19 DMR *IG-GTL2* DMR *MEST* DMR

B. AZOOSPERMIC MEN (continued)

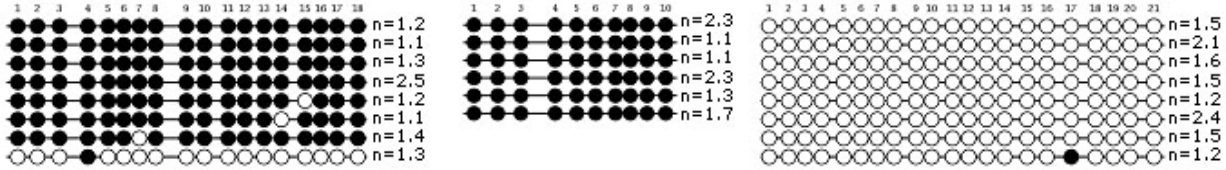
TP04



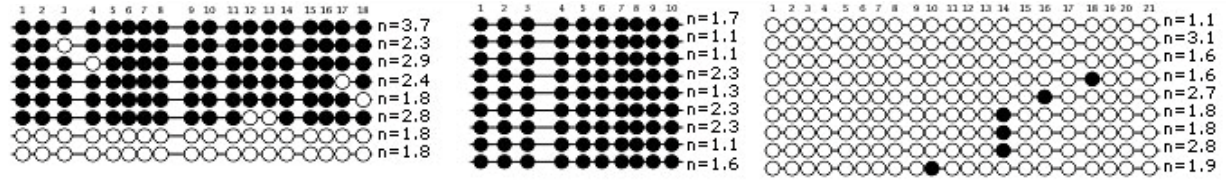
TP05



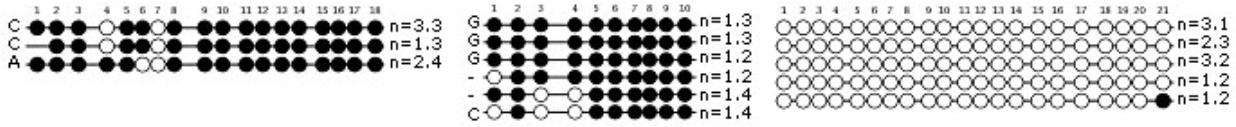
TP06



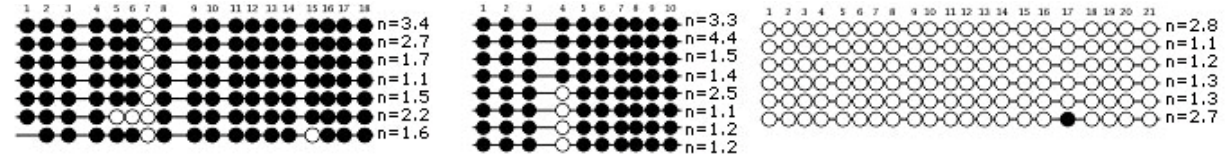
TP07



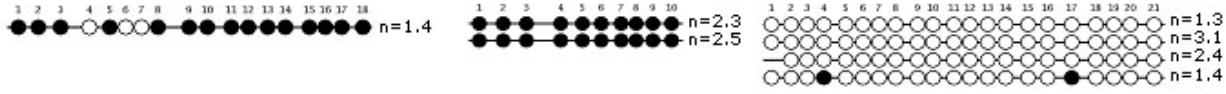
TP08



TP09



TP10

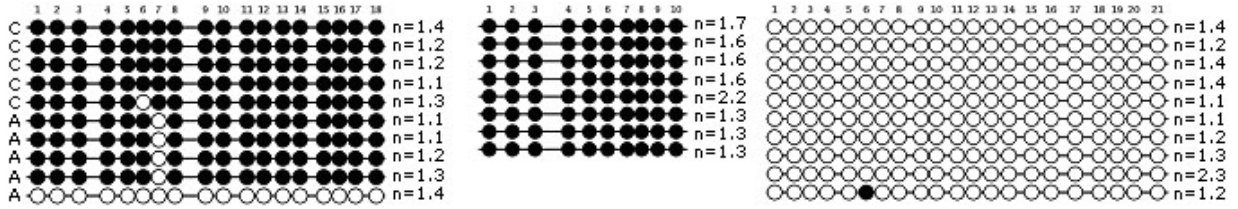


H19 DMR *IG-GTL2* DMR *MEST* DMR

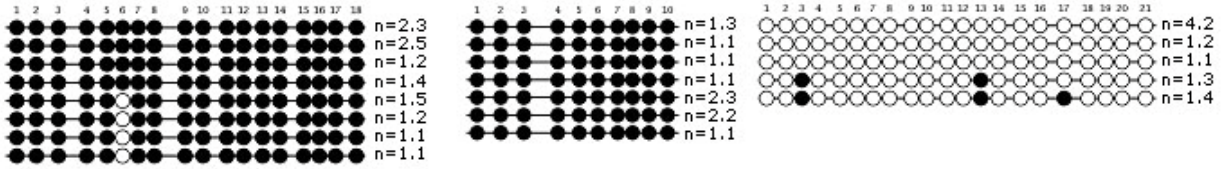
B. AZOOSPERMIC MEN (continued)

(non-obstructive azoospermia)

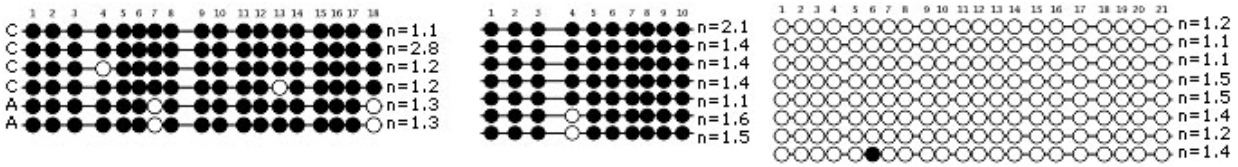
TP11



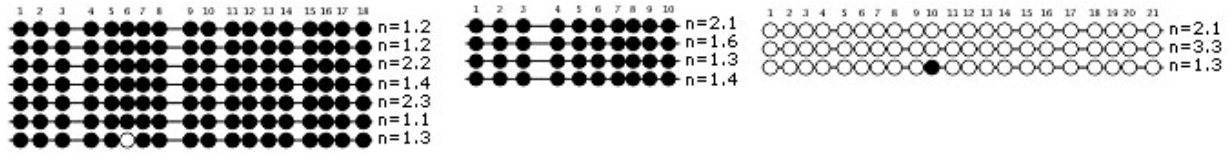
TP12



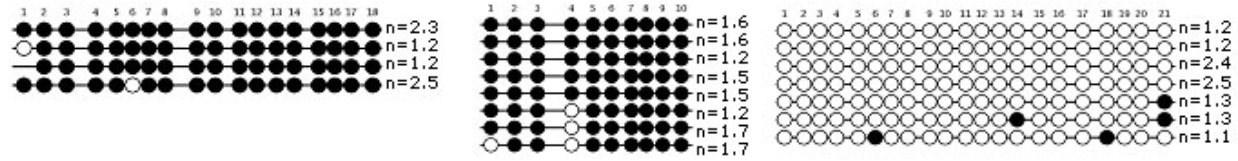
TP13



TP14



TP15



H19 DMR *IG-GTL2* DMR *MEST* DMR

B. AZOOSPERMIC MEN (continued)

(unknown pathology)

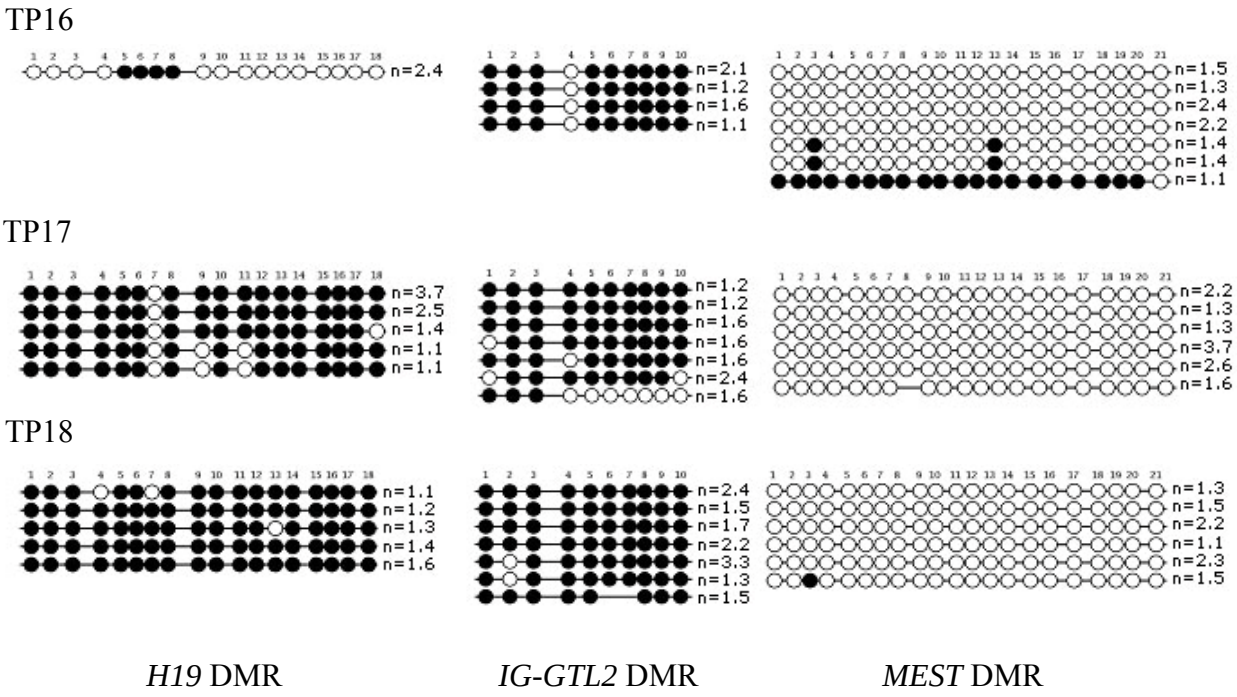


Figure 4.1. **Bead diagrams representing methylation at CpG sites studied at the *H19* DMR, *IG-GTL2* DMR and *MEST* DMR in vasectomy reversal and azoospermia groups.** Methylated (black bead) and unmethylated (open bead) status of each CpG site is indicated within the studied sequences. Missing beads represent CpG sites that could not be analyzed. Unique clones analyzed at each DMR are shown directly in the diagram, and are coded on the right-hand side with the first number designating the number of non-unique clones that were analyzed for each sequence followed by the amplification reaction each clone came from. The amplification reactions are not necessarily labeled in consecutive order. In samples containing an informative SNP, the allele is indicated on the left-hand side of each clone. In this data set, three SNPs were informative: C/T at nucleotide 67 (at CpG 7) and C/A at nucleotide 109 both in the *H19* sequence, and G/A at nucleotide 34 in the *IG-GTL2* sequence.

Based on the presence of the C/A SNP at nucleotide 109 in the *H19* DMR sequence in fifteen samples and the presence of the G/C SNP at nucleotide 34 in the *IG-GTL2* DMR sequence in two samples it was possible to determine whether there was an amplification bias toward one of the alleles at both DMRs analyzed. At the *H19* DMR, one hundred and two unique clones containing the SNP were analyzed: 58 clones had the C allele and 44 clones had the A allele. The difference was not statistically significant (Fisher's exact test, p=0.40). At the *IG-GTL2* DMR, ten unique clones containing the SNP were analyzed: 6 clones had the G allele and 4 clones had the C allele. The difference was not statistically significant (Fisher's exact test, P=1.00). The lack of statistical significance for the difference in the number of clones analyzed with each allele at the *H19* and *IG-GTL2* DMRs confirms a lack of amplification bias toward one of the alleles.

4.3.2.2 Analysis of methylation at DMRs of imprinted genes

The methylation level for each sample was calculated based on the proportion of methylated CpGs to the total number of CpGs analyzed in unique clones at each DMR. The median and mean methylation levels for each group were also calculated. These results are presented in Table 4.3. The number of hypomethylated or fully unmethylated clones found in samples at the *H19* and *IG-GTL2* DMRs and presence of hypermethylated or fully methylated clones found in samples at the *MEST* DMR is also indicated (Table 4.3).

Methylation at imprinted genes in azoospermic patients was compared to the vasectomy cases and to the control ejaculate samples analyzed in Chapter 2 (C01-C09). The methylation results obtained for the vasectomy reversal cases, azoospermic men and ejaculate control samples are presented in Figure 4.2. Genomic imprinting is already fully set in the male germ cells before cells enter meiosis (Kerjean et al., 2000), therefore testicular sperm analyzed from testicular tissue and spermatozoa analyzed from the ejaculate would have already acquired methylation at imprinted genes.

Table 4.3 DNA methylation level at each DMR analyzed in sperm of vasectomy reversal and azoospermic men

	H19 DMR		IG-GTL2 DMR		MEST DMR	
Vasectomy reversal men	**methylation (%)**	**# hypome clones**	**methylation (%)**	**# hypome clones**	**methylation (%)**	**# hyperme clones**
VR01	99.02	0	100	0	0.68	0
VR02	75.00	2	93.0	0	0.79	0
VR03	71.43	2	96.67	0	4.76	0
VR04	97.06	0	97.10	0	0.79	0
VR05	99.02	0	94.00	0	2.66	0
VR06	85.59	1	96.67	0	1.19	0
VR07	81.70	2	94.44	0	19.05	1
VR08	95.42	0	93.33	0	3.81	0
VR09	84.03	1	94.29	0	42.86	3
VR10	100	0	95.56	0	0.95	0
VR11	98.53	0	96.67	0	1.59	0
VR12	99.16	0	92.75	0	2.06	0
VR13	99.02	0	94.29	0	4.25	0
VR14	95.00	0	100	0	0.00	0
VR15	95.80	0	98.00	0	2.38	0
VR16	98.04	0	97.5	0	0.69	0
VR17	96.47	0	93.22	0	0.95	0
Mean±SD	92.37±9.16		95.73±2.33		5.26±10.63	
Median	96.47		95.56		1.59	
OA						
TP01	96.08	0	100	0	6.8	0
TP02	83.19	1	95.0	0	1.20	0
TP03	72.06	2	94.92	0	0.68	0
TP04	67.06	2	100	0	4.08	0
TP05	94.12	0	98.57	0	3.81	0
TP06	86.76	1	100	0	0.59	0
TP07	70.59	2	100	0	3.17	0
TP08	94.0	0	88.0	0	0.95	0
TP09	97.46	0	93.33	0	0.79	0
TP10	88.24	0	100	0	2.41	0
Mean±SD	84.96±11.33		96.98±4.09		2.45±2.03	
Median	87.50		99.29		1.81	
NOA						
TP11	89.41	1	100	0	0.48	0
TP12	97.06	0	100	0	4.76	0
TP13	96.08	0	97.14	0	0.59	0
TP14	99.16	0	100	0	1.59	0
TP15	97.01	0	95.00	0	3.40	0
Mean±SD	95.74±3.72		98.43±2.28		2.16±1.86	
Median	97.01		100		1.59	
Unknown pathology						
TP16	17.65	1	90.00	0	16.33	1
TP17	94.12	0	84.29	1	0.00	0
TP18	97.65	0	97.06	0	0.79	0
Mean±SD	69.81±45.20		90.45±6.40		5.71±9.21	
Median	94.12		90.00		0.79	
AZO Mean±SD	85.43±19.65		96.30±4.75		2.91±3.83	
AZO Median	94.06		97.86		1.40	

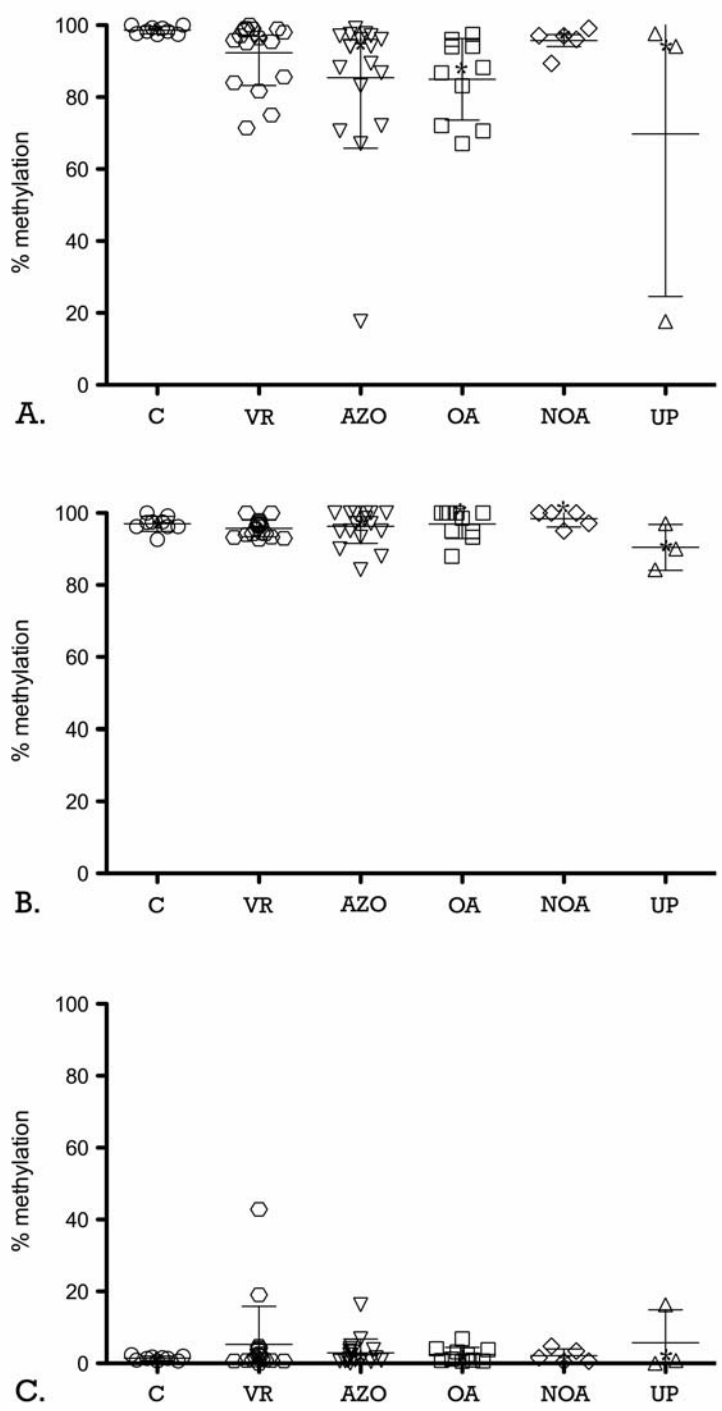


Figure 4.2. DNA methylation level at imprinted genes in azoospermia and vasectomy reversal groups. The methylation level is shown for each sample analyzed within the (A) *H19* DMR, (B) *IG-GTL2* DMR and (C) *MEST* DMR. Methylation level was analyzed in control men (C) (n=9), in men undergoing vasectomy reversal (VR) (n=17), and in men affected by azoospermia (AZO) (n=18). The AZO group was further subdivided into three sub-groups: obstructive and non-obstructive azoospermia (OA (n=10) and NOA (n=5), respectively) and unknown pathology (UP) (n=3). The horizontal lines indicate the group mean and the whiskers indicate standard deviation of the group mean. * indicates the median.

4.3.2.2.1 Methylation at the H19 DMR

The methylation level at the *H19* DMR was first compared between the vasectomy reversal group and the azoospermic patient group. The difference in methylation between the two groups was not significant (KW, $p>0.05$). The difference in methylation between the vasectomy reversal group and the OA or NOA sub-groups, as well as between the OA and NOA sub-groups was not significant (KW, $p>0.05$ for each comparison). There was a significant decrease in methylation at the *H19* DMR in the vasectomy reversal group compared to the ejaculate control group (MW, $p=0.0165$). However, the significance was lost after the post hoc Dunn's correction following ANOVA (KW, $p>0.05$). We also found a significant decrease in methylation at the *H19* DMR in the azoospermic patient group compared to the ejaculate control group (KW, $p<0.01$). Furthermore, there was a significant decrease in methylation at the *H19* DMR between the OA group and the ejaculate control group (KW, $p<0.01$). Methylation at the *H19* DMR was not significantly different between the ejaculate control group and the NOA group (KW, $p>0.05$) or between the OA and the NOA groups (KW, $p> 0.05$) (Table 4.3).

The methylation level at the *H19* DMR for each sample analyzed ranged between 71.43% and 100% in the vasectomy reversal group (Table 4.3). Hypomethylated or completely unmethylated unique clones were found in 5 of the 17 vasectomy reversal samples (VR02, VR03, VR06, VR07 and VR09; Figure 4.1). One or two unique clones were hypomethylated or completely unmethylated in the five samples (Table 4.3; Figure 4.1). In the azoospermic patient group the methylation level at the *H19* DMR for each sample analyzed ranged between 17.65% and 99.16%. In the sample in which a methylation level of 17.65% was found (TP16) at the *H19* DMR, only one unique and two non-unique clones could be analyzed, and these may not be representative of the overall methylation in that sample. The pathology result for this sample was not available and the sample was not assigned to the OA or the NOA group. The methylation level at the *H19* DMR ranged between 67.06% and 97.46% in the OA group and between 89.41% and 99.16% in the NOA group (Table 4.3). In total, hypomethylated or completely unmethylated unique clones were found in 7 of the 18 azoospermic patient samples: in 5 of the 10 OA samples (TP02, TP03, TP04, TP06, TP07), in 1 of the NOA samples (TP11) and in one sample of unknown pathology (TP16). One or two unique clones were hypomethylated or completely unmethylated in the seven samples (Table 4.3, Figure 4.1). A

lack of methylation was also found at randomly distributed CpG sites within the *H19* DMR in the samples analyzed. Up to seven unmethylated CpG sites were found outside of the hypomethylated or unmethylated clones within the vasectomy reversal samples; however, in most samples only one unmethylated CpG site was found (Table 4.4). In the azoospermia group up to six unmethylated CpG sites were found, and in most samples between two and four unmethylated CpG sites were found (Table 4.4).

Table 4.4. **Number of unmethylated CpG sites found at the *H19* DMR outside of hypomethylated or unmethylated clones of azoospermia and vasectomy reversal groups**

Number of de-methylated CpGs	Vasectomy reversal group (N)	Azoospermia group (N)	OA sub-group (N)	NOA sub-group (N)
0	2			
1	6	2		2
2	3	5	3	1
3	1	3	2	
4	2	5	3	2
5	2	1		
6		2	2	
7	1			

As previously discussed, expression of *H19* and *IGF2* is regulated by the CTCF binding protein that has a binding site located within CpGs 4 to 8 in the *H19* DMR (Takai et al., 2001). At least one unmethylated CpG site within CpG 4 to 8 was found in thirteen vasectomy reversal samples and in sixteen azoospermic samples: in nine samples in the OA sub-group, in all samples in the NOA sub-group and in two samples with unknown pathology. Among the samples with hypomethylated or completely demethylated clones, CpGs 4 to 8 were also demethylated, with the exception of sample TP16. Unmethylated CpG sites were also found in thirteen samples in which hypomethylated or completely demethylated clones were not found: in eight vasectomy reversal samples, four OA samples, four NOA samples, and in one sample with unknown pathology. In most cases, only one unmethylated CpG within the 6th CTCF binding site was found and it is not known whether improper methylation at one CpG site within the binding site would affect binding of the CTCF binding protein. The CpG that was most often demethylated within the binding site was CpG 6.

Sample TP16 showed an interesting pattern of methylation at the *H19* DMR. All CpG sites located outside of the 6th CTCF binding region were unmethylated; however, only one of

the CpG sites located within the 6th CTCF binding region was unmethylated with the remaining sites showing proper methylation. This pattern of methylation may not affect CTCF binding and proper regulation of gene expression may be maintained regardless of the loss of methylation at the CpG sites located outside of the 6th CTCF binding region.

4.3.2.2.2 Methylation at the IG-GTL2 DMR

The difference in the level of methylation at the *IG-GTL2* DMR between the vasectomy reversal group and the azoospermic patient group was not significant (KW, $p>0.05$). There was also no significant difference in methylation between the vasectomy reversal group and the OA or NOA sub-groups, as well as between the OA and NOA (KW, $p>0.05$ for each comparison). A significant difference in methylation between the ejaculate control group and the vasectomy reversal group was also not found (KW, $p>0.05$). The difference in methylation between the ejaculate control group and the azoospermic patient group was not significant (KW, $p>0.05$). There was also no significant difference in methylation between the ejaculate control group and the OA or NOA sub-groups, as well as between the OA and NOA (KW, $p>0.05$ for each comparison).

The methylation level at the *IG-GTL2* DMR ranged between 92.75% and 100% in the vasectomy reversal samples and between 84.29% and 100% in the azoospermic patient group: 88.00% and 100% in the OA sub-group and 95.00% and 100% in the NOA sub-group (Table 4.3). Hypomethylation at the *IG-GTL2* DMR affecting one of seven unique clones analyzed was found in one azoospermic patient sample of unknown pathology (TP17) (Table 4.3). A lack of methylation was also found at randomly distributed CpG sites within the *IG-GTL2* DMR in the samples analyzed. Up to seven unmethylated CpG sites were found in clones within the vasectomy reversal samples. In most cases four unmethylated CpG sites were found in both the vasectomy reversal group and the azoospermia patient group (Table 4.5). Most often methylation at CpG 4 was absent.

Table 4.5. Number of unmethylated CpG sites found at the *IG-GTL2* DMR outside of hypomethylated or unmethylated clones in azoospermia and vasectomy reversal groups.

Number of de-methylated CpGs	Vasectomy reversal group (N)	Azoospermia group (N)	OA sub-group (N)	NOA sub-group (N)
0	2	8	5	3
1	1	1	1	
2	3	2		1
3	2	1	1	
4	5	5	2	1
5	2			
6	1	1	1	
7	1			

4.3.2.2.3 Methylation at the MEST DMR

The difference in the level of methylation at the *MEST* DMR between the vasectomy reversal group and the azoospermic patient group was not significant (KW, $p>0.05$). There was also no significant difference in methylation between the vasectomy reversal group and the OA or NOA sub-groups, as well as between the OA and NOA (KW, $p>0.05$ for each comparison). A significant difference in methylation between the ejaculate control group and the vasectomy reversal group was also not found (KW, $p>0.05$). The difference in methylation between the ejaculate control group and the azoospermic patient group was not significant (KW, $p>0.05$). There was also no significant difference in methylation between the ejaculate control group and the OA or NOA sub-groups, as well as between the OA and NOA (KW, $p>0.05$ for each comparison).

The methylation level at the *MEST* DMR ranged between 0% and 42.86% in the vasectomy reversal samples and between 0% and 16.33% in the azoospermic patient group: 0.59% to 6.8% in the OA sub-group and 0.48% to 4.76% in the NOA sub-group (Table 4.3). Hypermethylated or fully methylated clones were found in three samples analyzed: in two samples from the vasectomy reversal group (VR07 and VR09) and in one sample from the azoospermic patient group in a sample with unknown pathology (TP16) (Table 4.3). Gain of methylation was found in one of seven unique clones analyzed in sample VR07, in the only unique clone analyzed in sample TP16 and in three of seven unique clones analyzed in sample VR09. A gain of methylation was also found at randomly distributed CpG sites located outside of the hypermethylated or fully methylated unique clones in samples analyzed (Table 4.6); however, in most samples one CpG site was methylated. The gain of methylation was observed

Table 4.6. Number of methylated CpG sites found at the *MEST* DMR outside of hypermethylated or methylated clones in azoospermia and vasectomy reversal groups.

Number of methylated CpGs	Vasectomy reversal group (N)	Azoospermia group (N)	OA sub-group (N)	NOA sub-group (N)
0	2	1		
1	7	8	4	3
2	2	2	2	
3	1			
4	2	1		
5	1	3	1	2
6		2	2	
7	1			
8	1			
10		1	1	

in up to ten CpG sites in one sample (Table 4.6).

4.3.2.3 *Analysis of methylation at individual CpGs*

A significant difference in methylation was found at 11 CpG sites at the *H19* DMR between the ejaculate Control samples and the vasectomy reversal samples, at 14 CpG sites between the ejaculate Control samples and the azoospermic patient samples, and at all 17 CpG sites analyzed between the ejaculate Control samples and the OA (Fisher's exact test, $p<0.05$; Table 4.7). Ten of the CpG sites showing a significant difference in methylation were the same in the three groups. There was no significant difference in methylation at any of the CpG sites analyzed between the ejaculate Control samples and the NOA samples (Fisher's exact test, $p>0.05$). Methylation at three CpG sites, CpG 3, CpG 12 and CpG 14, was significantly different between the OA and the NOA group. Methylation at two CpG sites, CpG 13 and CpG 14, was significantly different between the vasectomy reversal cases and the OA group (Fisher's exact test, $p<0.05$). No other differences were observed at the CpG sites located within the *H19* DMR. However, after the Bonferroni correction for multiple testing, significance was retained at only one CpG site, CpG 13, between the Control and OA groups (Table 4.7). The CpG that was most often unmethylated within the *H19* DMR was CpG 6 in all groups analyzed (Table 4.7). Within the *IG-GTL2* DMR the CpG that was most often demethylated was CpG 4 (Table 4.8). No differences in methylation at individual CpG sites within the *IG-GTL2* DMR were found.

Table 4.7. **Percentage of unmethylated CpG sites analyzed within the *H19* DMR in azoospermia and vasectomy reversal groups.**

CpG	Percent (%) methylation					P value				
	Control (n=69)	VR (n=112)	OA (n=60)	NOA (n=35)	AZO (n=106)	C vs. VR	C vs. AZO	C vs. OA	OA vs. NOA	VR vs. OA
1	0	6.3	13.8	5.9	2.9	0.0425	0.0034	0.0014	NS	NS
2	2.9	7.1	15.0	2.9	10.4	NS	NS	0.0024	NS	NS
3	0	10.7	16.7	2.9	11.3	0.0038	0.0037	0.0003	0.05	NS
4	1.4	8.0	18.3	5.7	14.2	NS	0.0055	0.0013	NS	NS
5	0	7.1	15.0	2.9	9.4	0.0248	0.0067	0.0007	NS	NS
6	18.8	19.6	36.7	22.9	28.3	NS	NS	0.0292	NS	NS
7	-	-	-	-	-	-	-	-	-	-
8	0	8.0	13.3	2.9	8.5	0.0138	0.0123	0.0017	NS	NS
9	0	5.4	11.7	2.9	10.4	NS	0.0037	0.0039	NS	NS
10	0	8.0	13.3	2.9	9.4	0.0138	0.0067	0.0017	NS	NS
11	0	7.1	13.3	2.9	11.3	0.0248	0.0037	0.0017	NS	NS
12	1.4	8.0	16.7	2.9	11.3	NS	0.0166	0.0028	0.05	NS
13	0	7.1	20.0	5.7	15.1	0.0248	0.0003	0.0001*	NS	0.022
14	0	6.3	16.7	2.9	11.3	0.0452	0.0037	0.0003	0.05	0.035
15	0	11.7	13.3	2.9	9.4	0.0020	0.0067	0.0017	NS	NS
16	1.4	8.9	13.3	2.9	9.4	NS	NS	0.0006	NS	NS
17	0	8.0	11.7	2.9	8.5	0.0138	0.0123	0.0039	NS	NS
18	0	8.1	11.7	8.6	11.3	0.0138	0.0037	0.0039	NS	NS

Comparisons are shown between groups for which significant differences were found
Uncorrected significant P values (<0.05) are indicated, Fisher's exact
*Bonferroni corrected P value considered significant <0.00013 (0.05/384) for this data set (*H19, IG-GTL2* and *MEST*).
Data for the control group were shown in Chapter 2.
C; control ejaculate, VR; vasectomy reversal, OA; obstructive azoospermia, NOA; non-obstructive azoospermia, AZO; azoospermia

Table 4.8. **Percentage of unmethylated cytosines at each CpG site analyzed within the *IG-GTL2* DMR in azoospermia and vasectomy reversal groups**

CpG	Percent (%) methylation				
	Control (n=79)	VR (n=124)	OA (n=67)	NOA (n=36)	AZO (n=119)
1	1.3	8.1	4.5	2.8	5.0
2	2.5	3.2	0	0	1.7
3	0	2.4	3.0	0	1.7
4	21.5	26.6	16.4	13.9	22.0
5	0	0.8	1.5	0	1.7
6	1.3	0	1.5	0	1.8
7	0	0	0	0	0.8
8	2.5	0	0	0	0.8
9	0	1.6	0	0	1.0
10	1.3	2.4	0	0	1.7

Difference in methylation at individual CpG sites did not reach significance between any groups (Fisher's exact test, $p<0.05$). Data for the control group were shown in Chapter 2.
C; control ejaculate, VR; vasectomy reversal, OA; obstructive azoospermia, NOA; non-obstructive azoospermia, AZO; azoospermia

At the *MEST* DMR no single CpG was most often methylated in the study groups (Table 4.9). A significant difference in methylation was found at two CpG sites, CpG 5 and CpG 14, within the *MEST* DMR between the ejaculate Control samples and the vasectomy reversal samples (Fisher's exact test, p=0.026 and p=0.047, respectively, Table 4.9). Methylation was significantly different at only one CpG site between the ejaculate Control samples and the OA samples (CpG 17, Fisher's exact test, p=0.029), as well as between the ejaculate Control samples and the azoospermia patient samples (CpG 3, Fisher's exact test, p=0.032). A difference in methylation at one CpG site was significant between the OA and the NOA groups (CpG 6, Fisher's exact test, p=0.0348), and between the vasectomy reversal samples and the azoospermia patient samples (CpG 9, Fisher's exact test, p=0.017). Two CpG sites showed a significant difference in methylation between the vasectomy reversal group and the OA

Table 4.9. **Percentage of methylated cytosines at each CpG site analyzed within the *MEST* DMR in azoospermia and vasectomy reversal groups**

	Percent (%) methylation					P value					
CpG	Control (n=67)	VR (n=113)	OA (n=66)	NOA (n=33)	AZO (n=118)	C vs. VR	C vs. OA	C vs. AZO	AZO vs. VR	VR vs. OA	NOA vs. OA
1	0	4.4	3.1	0	2.6	NS	NS	NS	NS	NS	NS
2	1.5	7.1	1.5	0	1.7	NS	NS	NS	NS	NS	NS
3	0	4.4	1.5	6.1	5.9	NS	NS	0.032	NS	NS	NS
4	1.5	6.2	4.5	0	3.4	NS	NS	NS	NS	NS	NS
5	0	7.1	1.5	0	1.7	0.026	NS	NS	NS	NS	NS
6	3.0	8.0	0	9.1	3.4	NS	NS	NS	NS	0.027	0.0348
7	1.5	3.6	0	0	0.8	NS	NS	NS	NS	NS	NS
8	0	3.5	1.5	0	1.7	NS	NS	NS	NS	NS	NS
9	1.5	7.1	0	0	0.8	NS	NS	NS	0.017	0.027	NS
10	0	5.3	1.5	1	2.5	NS	NS	NS	NS	NS	NS
11	0	4.4	0	0	0.8	NS	NS	NS	NS	NS	NS
12	0	3.5	3.0	0	2.5	NS	NS	NS	NS	NS	NS
13	4.5	5.3	0	6.1	4.2	NS	NS	NS	NS	NS	NS
14	0	6.2	4.5	3.0	4.2	0.047	NS	NS	NS	NS	NS
15	1.5	4.4	0	0	0.8	NS	NS	NS	NS	NS	NS
16	5.6	4.4	4.5	0	3.4	NS	NS	NS	NS	NS	NS
17	1.5	3.5	10.6	3.0	7.6	NS	0.0329	NS	NS	NS	NS
18	7.5	7.1	9.1	3.0	6.8	NS	NS	NS	NS	NS	NS
19	1.5	4.5	1.5	0	1.7	NS	NS	NS	NS	NS	NS
20	1.5	4.4	1.5	0	1.7	NS	NS	NS	NS	NS	NS
21	4.6	6.3	3.0	6.1	3.4	NS	NS	NS	NS	NS	NS

Comparisons are shown between groups for which significant differences were found.
Uncorrected significant P values (<0.05) are indicated, Fisher's exact
*Bonferroni corrected P value considered significant <0.00013 (0.05/384for this data set (H19, IG-GTL2 and MEST). Data for the control group were shown in Chapter 2.
C; control ejaculate, VR; vasectomy reversal, OA; obstructive azoospermia, NOA; non-obstructive azoospermia, AZO; azoospermia

samples: CpG 6 and CpG 9 (Fisher's exact test, p=0.027 and p=0.027, respectively). No other differences were found. However, none of the CpG sites retained significance following the Bonferroni correction. The results suggest that analysis of methylation at CpG 6 within the *H19* DMR, at CpG4 within the *IG-GTL2* DMR may not be representative of the methylation at neighboring CpG sites. There were no single CpG within the *MEST* DMR that seemed to be preferentially methylated.

4.3.2.4 Incidence of abnormal methylation at imprinted genes in azoospermic men

The number of individuals with abnormal methylation at imprinted genes was determined and compared among groups. An individual was designated as having abnormal methylation at an imprinted gene based on the presence of at least one improperly methylated unique clone. Abnormal methylation within the *H19* DMR in sperm was found in 29.4% of vasectomy reversal samples and in 38.9% of the azoospermia patient samples: in 50% of OA samples and in 20% of NOA samples (Table 4.10). The incidence of abnormal methylation at the *H19* DMR was significantly different between the ejaculate control group and the azoospermia patient group (0/9 vs. 7/18, Fisher's exact test, p=0.036). The incidence was also significant between the ejaculate control group and the OA group (0/9 vs. 5/10, Fisher's exact test, p=0.022). No other significant differences were found between groups at the *H19* DMR. Post Bonferroni correction for multiple testing, p values <0.0021 (0.05/24) were considered significant. None of the comparisons for methylation at the *H19* DMR passed the correction. Abnormal methylation at the *IG-GTL2* DMR was found only in one sample from the azoospermia patient group (5.5%); however, the pathology for this patient was not known. There were no significant differences in the incidence of abnormal methylation at the *IG-GTL2* DMR between any of the groups studied. Abnormal methylation at the *MEST* DMR was found in 11.8% (2/17) of vasectomy reversal samples and in 5.5% (1/18) of the azoospermia patient group (Table 4.10). The one sample showing abnormal methylation at the *MEST* DMR in the azoospermia group was from a patient with unknown pathology. Three of the twelve samples with abnormal methylation at the *H19* DMR (VR07, VR09, TP16) also had abnormal methylation at the *MEST* DMR. The one patient with abnormal methylation at the *IG-GTL2* DMR (TP17) had normal methylation at the *H19* and the *MEST* DMRs. All other patients had abnormal methylation only at the *H19* DMR.

Table 4.10. Incidence of imprinting errors in the sperm of men with azoospermia and undergoing vasectomy reversal.

Study Group	DMR analyzed		
	H19	***IG-GTL2***	***MEST***
Control	0/9[a]	0/9[a]	0/9[a]
Vasectomy reversal	5/17 (29.4)	0/17	2/17 (11.8)
AZO	7/18 (38.9)*	1/18 (5.5)	1/18 (5.5)
OA	5/10 (50)*	0/10	0/10
NOA	1/5 (20)	0/5	0/5
Unknown pathology	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)

*statistically significant compared to the Control group (Fisher's exact test, $p<0.05$), the significance did not pass the Bonferroni correction. Exact P values are indicated in the text

[a] data reported in Chapter 2

Percentages shown in brackets

4.4 DISCUSSION

4.4.1 Methylation at imprinted genes and incidence of abnormal methylation at imprinted genes in the sperm of men with azoospermia and of men undergoing vasectomy reversal.

This is the first study to evaluate DNA methylation at the *IG-GTL2* DMR in testicular sperm isolated from men affected by azoospermia. This is also the first study to evaluate DNA methylation at imprinted genes in the testicular sperm isolated from men undergoing a vasectomy reversal. Abnormal DNA methylation at the three studied DMRs of imprinted genes was identified in samples obtained from men undergoing a vasectomy reversal and in samples obtained from men affected by azoospermia. In most samples showing aberrant methylation at an imprinted gene, the abnormality affected the *H19* DMR; 12 of 35 samples analyzed had abnormal methylation at the *H19* DMR, while abnormal methylation was identified in 3 of 35 samples at the *MEST* DMR and in 1 of 35 samples at the *IG-GTL2* DMR.

In this study a significant decrease in methylation at the *H19* DMR was found in the vasectomy reversal group compared to the ejaculate control group (MW, $p=0.0165$), but the significance was lost after correction for multiple testing using Dunn's post hoc test. Abnormal methylation at the *H19* DMR was found in 5 of 17 (29.5%) vasectomy reversal samples; however, this incidence was not significantly different from the incidence in the ejaculate control group. This is the first study to analyze DNA methylation at imprinted genes in the sperm of men having undergone a vasectomy reversal, and to report on the presence of abnormal methylation in such samples. A significant decrease in methylation at the *H19* DMR was also found in the azoospermia patient group compared to the ejaculate control group (KW,

$p<0.01$). The decrease in methylation was also significant in the OA sub-group compared to the ejaculate control group (KW, $p<0.01$). Abnormal methylation at the *H19* DMR was found in 7 of 18 (38.9%) azoospermia patient samples: in 5 of 10 (50%) OA samples, in 1 of 5 (20%) NOA samples, and in 1 of 3 (33.3%) samples with unknown pathology. The rate of abnormal methylation at the *H19* DMR was significantly higher in the azoospermia patient group compared to the ejaculate control group (Fisher's exact test, $p=0.036$) as well as in the OA sub-group compared to the ejaculate control group (Fisher's exact test, $p=0.022$). These results were in accordance with the analysis of methylation at individual CpG sites (Table 4.7). However, significance was lost following the Bonferroni correction for the tests performed. Although the significance was lost post the Bonferroni correction, comparison of methylation differences at individual CpG sites and of abnormal methylation among groups show a decrease in methylation at the *H19* DMR at the uncorrected significance level in the sperm of men with azoospermia and in those affected by OA compared to control men. Comparison of methylation levels at the *H19* DMR among the groups mentioned also supports these conclusions.

Methylation at the *H19* DMR has been previously reported in testicular germ cells of infertile men by two studies (Table 4.11). Hartmann et al. (2006) assessed methylation at CpG 10 within the *H19* DMR and found normal methylation in nine cases of NOA due to spermatogenesis arrest: in three cases of arrest at the spermatogonia stage and in six cases of arrest at the spermatocyte stage. Marques et al. (2009) reported abnormal methylation in testicular sperm at the *H19* DMR in 1 of 19 (5.3%) men affected by azoospermia; abnormal methylation was identified in 1 of 9 studied men affected by NOA. Abnormal methylation at *H19* DMR was not found in OA samples or in the anejaculatory samples (Marques et al., 2009). The results from the literature do confirm our observation of a low rate of abnormal methylation in NOA samples; however, the rate of abnormal methylation found in OA samples does vary considerably between the literature reports and this study. Marques et al. (2009) did not find abnormal methylation at the *H19* DMR in ten OA samples studied. In this study abnormal methylation at the *H19* DMR was identified in 50% of OA samples (5/10) (Table 4.11). Even though the two studies analyzed methylation by bisulphite sequencing, the difference in methylation observed between the two studies may be explained by a difference in how the data were acquired. Marques et al. (2009) set up only one amplification reaction per gene for each

Table 4.11. Abnormal methylation at imprinted genes in the sperm of men affected by azoospermia.

Study	population	*H19*		*IG-GTL2*		*MEST*	
		Mean me (%)	rate	Mean me (%)	rate	Mean me (%)	rate
Hartmann et al., 2006[1]	NOA	100	0/9				
	3 spermatogonia	100	0/3				
	6 spermatocyte	100	0/6				
Marques et al., 2009	ANJ	97.6	0/5			2.1	0/5
	AZO	94.6	1/19 (5.3)			1.4	1/19 (5.3)
	OA	97.2	0/10			1.8	1/10 (10)
	- 2^o	98.1	0/5			2.4	1/5 (20)
	- CBAVD	96.3	0/5			1.2	0/5
	NOA	91.3	1/9 (11.1)			0.9	0/9
This study	Ejaculate control	98.6±1.0	0/9	97.0±2.1	0/9	1.4±0.6	0/9
	VR	92.4±9.2	5/17 (29.4)	95.7±2.3	0/17	5.3±10.6	2/17 (11.8)
	AZO	85.4±19.7	7/18* (38.9)	90.5±6.4	1/18 (5.6)	5.7±9.2	1/18 (5.6)
	- OA	85.0±11.3	5/10* (50)	97.0±4.1	0/10	2.2±1.9	0/10
	- NOA	95.7±3.7	1/5 (20)	98.4±2.3	0/5	5.7±9.2	0/5
	- No path	69.8±45.2	1/3 (33.3)	90.5±6.4	1/3 (33.3)	2.9±3.8	1/3 (33.3)

Mean me; mean methylation, ±SD; standard deviation
Percentages indicated in brackets
VR; vasectomy reversal, no path; unknown pathology
ANJ; no ejaculation due to spinal cord injury
2^o; OA due to inflammatory epididymal disease
[1] study reported methylation results based on the presence or absence of a methylated or unmethylated H19 product. In this table, 100% mean methylation indicates the reported presence of a methylated H19 product.
* statistically significant compared to ejaculate control, Fisher's exact test, $p<0.05$

sample analyzed, while in this study an average of 4.3 independent amplification reactions were performed for each sample per gene. Marques et al. (2009) analyzed between seventeen and twenty-four non-unique clones for each patient at the *H19* DMR with very little variation among the clones, suggesting that most clones may have originated from the same cell. For example, one unique clone was observed after amplification of 19 and 24 clones and two to three unique clones were observed after amplification of 17 to 24 clones (Marques et al., 2009). Cells carrying the normal imprint could have been preferentially amplified and the results may not be representative. Our analysis of unique clones having originated from multiple amplification reactions may present results that are more representative of methylation when starting with limited material.

We also found abnormal methylation at the *MEST* DMR in 2 of 17 (11.8%) samples from the vasectomy reversal group and in 1 of 18 (5.6%) samples from the azoospermia patient group: in a patient with unknown pathology. However, there was no significant difference in the

rate of abnormal methylation at the *MEST* DMR between any of the groups and sub-groups analyzed. There was also no statistical difference in methylation level between any of the groups and sub-groups analyzed. A significant difference in the methylation level between the groups and the sub-groups studied was limited to only one or two CpG sites within the *MEST* DMR. One other study has evaluated DNA methylation at the *MEST* DMR in azoospermic patients; however, no information is available regarding DNA methylation in samples obtained at vasectomy reversal. Marques et al. (2009) reported abnormal methylation at the *MEST* DMR in 1 of 10 samples obtained from men affected by azoospermia: the patient was affected by OA. A relatively low rate of abnormal methylation at the *MEST* DMR was identified by our and the Marques et al. (2009) study in samples obtained from azoospermic patients. However, as previously mentioned regarding methylation at the *H19* DMR, methylation assessed by Marques et al. (2009) may not be representative. Marques et al. (2009) analyzed between 11 and 27 clones at the *MEST* DMR, and in 13 of 19 azoospermic samples analyzed between one and three clones were unique. The results are suggestive of preferential amplification that may occur when amplifying small quantities of starting material (Walsh et al., 1992; Findlay et al., 1995). DNA methylation at one other imprinted gene has been studied in azoospermic patients. The expected lack of methylation at the *SNRPN* DMR, unmethylated in male sperm, was found in four OA and two NOA samples (Manning et al., 2001).

This is the first study to report on DNA methylation at the *IG-GTL2* DMR in sperm obtained from testicular tissue; in vasectomy reversal cases and in men affected by azoospermia. Abnormal methylation was found in one sample obtained from a patient affected by azoospermia with unknown pathology. The rate of abnormal methylation was not significant different between the azoospermia patient group and the vasectomy reversal group or the ejaculate control group. There were no significant differences in methylation at the *IG-GTL2* DMR between any of the groups analyzed. The analysis shows that DNA methylation at the *IG-GTL2* DMR is resistant to methylation abnormalities in sperm retrieved from testicular tissue.

In this study three men had abnormal methylation at the *H19* and *MEST* DMRs. Abnormal methylation at multiple DMRs in the same patient has been reported before in oligozoospermic patients (Kobayashi et al., 2007; Marques et al., 2008) and suggests that improper imprint erasure or re-establishment may not be gene specific. Abnormal methylation

identified in one sample obtained from an azoospermic man at the *IG-GTL2* DMR was limited to this one gene. Of the three imprinted genes analyzed, abnormal methylation was primarily observed at the *H19* DMR suggesting that methylation at the *H19* DMR may be particularly prone to aberrant methylation. Abnormal methylation primarily affecting the H19 DMR was also observed in the sperm of men with oligozoospermia analyzed in Chapter 2. The propensity of the *H19* DMR to disturbances of DNA methylation may be related to molecular structure of the DMR or of surrounding sequences. The *H19* DMR is less repetitive compared to the *IG-GTL2* DMR (Paulsen et al., 2001) and it has been suggested that DNA methylation at more repetitive regions is more strictly conserved compared to regions that are less repetitive in nature, such as the *H19* DMR (Li et al., 2004). The repetitive nature of the *IG-GTL2* DMR may explain the generation of truncated *IG-GTL2* cloning products.

As previously discussed in Chapter 2, methylation at the 6th CTCF binding region at the *H19* DMR, CpG 4 to 8, prevents the CTCF protein from binding thus allowing *IGF2* expression from the paternal allele and preventing *H19* expression from the maternal allele (Bell and Felsenfeld, 2000; Hark et al., 2000; Hark et al., 1998). In this set of samples, at least one unmethylated CpG site within CpG 4 to 8 was found in thirteen vasectomy reversal samples and in sixteen azoospermic samples. However, in most cases, only one unmethylated CpG within the 6th CTCF binding region and it is not known whether improper methylation at one CpG site within the binding site would affect binding of the CTCF binding protein. The consequences associated with changes in methylation within the *H19, IG-GTL2* and *MEST* DMRs have been discussed previously in Chapter 2 and may include abortion (Kobayashi et al., 2009), and SRS in children born through IVF and ICSI (Bliek et al., 2006; Kagami et al., 2007; Kanber et al., 2009). Abnormal methylation in testicular sperm, passed on through the use of ICSI, may be a source of abnormalities seen in abortuses and children born through ART.

4.4.2 Etiology of abnormal DNA methylation in sperm.

An informative SNP within the sequence can be used to determine how the abnormal methylation observed in some of the sperm cell analyzed arose. As discussed in Chapter 2, changes in methylation can occur due to errors in erasure, establishment or maintenance. Methylation of only the maternal allele within the *MEST* DMR would imply improper erasure

while methylation of both parental alleles would imply improper establishment. In the case of the *H19* and *IG-GTL2* DMRs, presence of SNPs would help to determine the parental alleles on which methylation is not being properly reset. Errors in maintenance of methylation could also result in the presence of improper methylation in the sperm. Six of the twelve samples with abnormal methylation at the *H19* DMR were informative for a SNP: four samples from the vasectomy reversal group and two samples from the azoospermia patient group (Figure 4.1). In two samples (VR02, VR07) only one parental allele was unmethylated, while in the other four samples only one clone was unmethylated (VR06, VR09, TP02, TP11). Based on the limited number of clones showing abnormal methylation, it is difficult to determine whether the abnormality resulted from improper establishment or improper maintenance. The sample with abnormal methylation at the *IG-GTL2* DMR (TP17) did not have an informative SNP and only one clone was hypomethylated. None of the samples analyzed had an informative SNP within the *MEST* DMR. The presence of methylation in 42.86% of clones in sample VR09 possibly suggests a lack of erasure of methylation from the maternal allele.

As discussed in Chapter 2, mutations in *Dnmt3a* and *Dnmt3l* have been associated with loss of methylation at imprinted genes in the sperm of infertile mouse males (Kaneda et al., 2004; Yaman and Grandjean, 2006; Webster et al., 2005). However, with the reported absence of mutations in these genes in infertile men with abnormal methylation at imprinted genes (Kobayashi et al., 2009), gene mutations in other enzymes involved in methylation are possible, including *DNMT1* (Li et al., 1992) and *MTHFR* (Kelly et al., 2005). Factors such as maternal diet have been shown to affect DNA methylation in the fetus (Waterland and Jirtle, 2003; Dolinoy et al., 2006) and *in utero* exposure to endocrine disruptors has been associated with male infertility in animals (Anway et al., 2005). Furthermore, exposure to environmental factors in adulthood may also affect spermatogenesis. Higher levels of methyl donors in males correlated with improved sperm concentration and decreased sperm DNA damage in humans (Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). However, environmental factors that can affect spermatogenesis after birth have been found in seminal plasma (Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). It is not clear whether factors found in seminal plasma can affect sperm found in the testis or epididymis because in these locations sperm have not yet been mixed with seminal plasma (Tremellen, 2008). Factors limited to the

testicular environment may be responsible for abnormal methylation particularly in patients with obstructive azoospermia or those after vasectomy as sperm in these patients are limited to the testis and the epididymis and have not been exposed to seminal plasma.

4.4.3 Methylation abnormalities in testicular sperm retrieved from men affected by obstructive azoospermia or undergoing vasectomy reversal

Ten of the eighteen men affected by azoospermia in this study were diagnosed with OA. Four of the ten men were diagnosed with OA based on the presence of gene mutations within the CFTR gene. Mutations in this gene are associated with CBAVD (Chillon et al., 1995; Meng et al., 2001). The vas deferens is a conduit that connects the epididymis to the ejaculatory duct, where sperm are mixed with fluids from the seminal vesicle and the prostate gland to form semen (Tremllen 2008). Men with CBAVD show azoospermia because the sperm they produce cannot reach the ejaculate. Spermatogenesis is normal in most of these men (Meng et al., 2001). The remaining six men diagnosed with OA had normal spermatogenesis upon pathological examination. It is assumed that since these men are affected by azoospermia, but have normal spermatogenesis, undetected obstruction must be present preventing sperm from reaching the ejaculate. OA patients have normal spermatogenesis but the sperm cannot reach the ejaculate. One of the OA patients had obstruction due to epididymal head calcification.

A vasectomy is a surgical procedure where the vas deferens is severed bilaterally. In these men spermatogenesis occurs but similar to OA patients, the sperm cannot reach the ejaculate, but in this case due to the created break in the vas deferens. The sperm from active spermatogenesis accumulate in the vas deferens duct causing swelling and bursting of the duct, forming sperm granulomas in some cases (Jones, 2004). Due to the inflammatory reaction, sperm antibodies can be found in over 50% of men within nine months after vasectomy (Tung, 1975). Alternatively, the sperm may be resorbed by epithelial cells through spermiophagy. Spermiophagy has been reported to occur in the epididymal epithelium after vasectomy and in cases of CBAVD (Jones, 2004). However, it has been suggested that over time the build up of pressure in the epididymis will reach the testis disrupting and reducing spermatogenesis (Jones, 2004). A significant reduction in sperm yield per gram of testis has been reported in vasectomized men and in OA men compared to fertile men undergoing vasectomy (McVicar et al., 2005). Reduction of spermatogenesis may be associated with testicular tissue destruction

related to an increase in ROS (Aydos et al., 1998) or increased apoptosis (Shiraishi et al., 2001; O'Neill et al., 2007). ROS are molecules with unpaired electrons that seek to get rid of them by participating in chemical reactions (Oschsendorf, 1999). Excessive presence of ROS has been associated with DNA damage, including DNA strand breaks, as well as the generation of DNA base adducts (Franco et al., 2008). Excessive ROS production has been associated with DNA double strand breaks and H2AX phosphorylation in sperm (Li et al., 2006) and with abnormal chromatin condensation (Henkel et al., 2010), suggesting that the presence of ROS can affect the sperm epigenome. Furthermore, the DNA base adducts affecting guanines, including 8-hydroxyl-2'-deoxyguanosine and O^6-methylguanine, have been shown to impede DNA methylation of neighboring cytosine nucleotides by interfering with proper function of DNMTs (Weitzman et al., 1994; Turk et al., 1995; Hepburn et al., 1991; Tan et al., 1990). The blockage of DNMT function has been associated with DNA hypomethylation (Weitzman et al., 1994; Turk et al., 1995; Hepburn et al., 1991; Tan et al., 1990). It has also been suggested that DNA base adducts, such as 8-oxoguanine and 5-hydroxymethylcytosine, may interfere with the binding of the MBD located in the MeCP2 to methylated DNA (Valinluck et al., 2004). Binding of the MeCP2 to methylated DNA recruits the necessary proteins, including cytosine methyltransferases and histone deacetylases, involved in chromatin remodeling. The inability of the MeCP2 to bind could affect chromatin remodeling (Valinluck et al., 2004). A negative correlation has been suggested between sperm global DNA methylation and seminal ROS production in ejaculate samples of infertile men (Tunc et al., 2009). However, it is currently unknown whether DNA methylation at imprinted genes can also be affected by ROS induced DNA damage. The same mechanisms may affect methylation at imprinted genes in sperm retrieved from OA men and men undergoing vasectomy reversal. However, with the additional effect of infertility, a higher, although not significant, rate of abnormal methylation was observed in OA patients compared to men undergoing vasectomy reversal. A vasectomy is normally performed to prevent pregnancy, but it can be reversed. Vasectomy reversal is a surgical procedure that rejoins the severed vas deferens. The success with which fertility is regained has been related to the duration of the vasectomy (Silber, 1977; Belker et al., 1991). Better pregnancy rates have been achieved with a shorter time interval from the time of vasectomy to the reversal; up to 76% within three years and just 30% after fifteen years since vasectomy (Belker et al., 1991); further suggesting that sperm from vasectomized men are

subjected to greater damage with increased exposure to a testicular environment created by obstruction.

4.5 CONCLUSION

In this study DNA methylation at the DMRs of three imprinted genes, *H19, IG-GTL2* and *MEST*, was analyzed in sperm retrieved from testicular tissue of men affected by azoospermia, OA and NOA, and from men undergoing vasectomy reversal. We found aberrant imprinting primarily in azoospermic men affected by OA and in vasectomy reversal cases. The OA pathology is similar to that of vasectomy reversal cases in that both types of samples came from men with normal spermatogenesis where the sperm cannot reach the ejaculate due to obstruction. Our results suggest that an altered testicular environment may disrupt DNA methylation at imprinted genes. Therefore, aberrant imprinting may not only be related to spermatogenesis failure, as previously believed, but may also be disrupted by environmental factors. Furthermore, our results also show that DNA methylation at the *H19* DMR is particularly prone to methylation abnormalities in vasectomy reversal and azoospermic patients. Men undergoing vasectomy reversal and men affected by azoospermia, particularly those affected by OA, should be counseled regarding their risk of having sperm affected by methylation abnormalities at imprinted genes.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTION

5.1 STUDY SUMMARY AND CONCLUSIONS

Infertility affects an estimated 15% of couples. Male factor infertility contributes to the inability to conceive in 50% of couples and remains idiopathic in 50% of cases. Recent reports have suggested that aberrant DNA methylation at imprinted genes may be associated with spermatogenesis failure seen in male factor infertility. Imprinted genes undergo a process of genomic reprogramming. The genome undergoes demethylation at the primordial germ cell stage (Szabo et al., 2002; Davis et al., 2000). Re-establishment of DNA methylation in the sperm at imprinted genes is initiated at the prospermatogonia stage (Li et al., 2004) and is fully set in premeiotic germ cells (Kerjean et al., 2000). Data in the literature show that DNA methylation is important for proper spermatogenesis and male fertility (Doerksen and Trasler, 1996, Doerksen et al., 2000; Oakes et al., 2007; Kaneda et al., 2004; Yaman and Granjean, 2006; Webster et al., 2005). It is also suggested that abnormal DNA methylation may be acquired during *in utero* development or environmental exposure to certain factors (Anway et al., 2005; Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). Furthermore, sperm carrying aberrant DNA methylation can contribute to pregnancy through the use of ART. Abnormal DNA methylation has been reported in pregnancies conceived through ART: in abortuses (Kobayashi et al., 2009) and in children (Kanber et al., 2009; Orstavid et al., 2003).

Most studies show an association between infertility and abnormal DNA methylation at imprinted genes in the sperm of men affected by mild to moderate oligozoospermia (Kobayashi et al., 2007; Marques et al., 2008; Poplinski et al., 2009; Hoummond et al., 2009). Limited information is currently available on the status of methylation at imprinted genes in the sperm of men affected by severe male factor infertility. The studies presented in this thesis evaluated DNA methylation at imprinted and non-imprinted genes in the ejaculate sperm of men affected by oligozoospermia, severe and very severe, and at imprinted genes in the testicular sperm of men affected by azoospermia, OA and NOA, and men undergoing vasectomy reversal.

In Chapter 2 and Chapter 4 DNA methylation was investigated at the DMRs of three imprinted genes in the sperm of men affected by severe male factor infertility and in men undergoing vasectomy reversal. DNA methylation was studied at two imprinted genes that are

methylated at the DMR in sperm, *H19* and *IG-GTL2* (Kerjean et al, 2000; Geuns et al., 2007), and in one imprinted gene that is unmethylated in the sperm, *MEST* (Kerjean et al., 2000). Summary of the results from the two chapters is presented in Tables 5.1 and 5.2. DNA methylation was studied by the bisulphite sequencing method and multiple unique clones were analyzed for each gene in each sample. The bisulphite sequencing method allowed the simultaneous study of methylation at multiple CpG sites that can be visualized and measured in single sperm cells. Small quantities of starting material may be prone to preferential amplification (Walsh et al., 1992; Findlay et al., 1995), possibly providing non-representative results. Therefore, analysis of unique clones, originating from different amplification reactions provided a representative measure of DNA methylation at imprinted genes in the small quantities of sperm analyzed.

In Chapter 2 DNA methylation in the sperm of men affected by oligozoospermia, severe and very severe, was investigated at the DMRs of three imprinted genes, and compared to methylation in the sperm of control men of proven fertility. The working hypothesis was that men affected by oligozoospermia would be more prone to methylation abnormalities at imprinted genes compared to control men. Our results supported this hypothesis. A significant decrease in methylation was observed at the *H19* DMR in the sperm of men affected by oligozoospermia compared to sperm of control men. An increase in methylation was observed at the *MEST* DMR although the difference was not significant compared to control men. The

Table 5.1 Methylation level at each DMR analyzed in sperm retrieved from the ejaculate and testis

	***H19* DMR**		***IG-GTL2* DMR**		***MEST* DMR**	
Study group	mean ± SD	median	mean ± SD	median	mean ± SD	median
Control men	98.62±1.03	98.32	97.00±2.09	97.40	1.43±0.60	1.37
Oligo	87.85±15.88	84.03[a]	95.56±4.84	96.67	5.60±10.92	1.61
Oligo-I	82.19±18.82	97.06[b]	96.25±3.43	96.67	8.11±13.81	1.59
Oligo-II	95.57±4.58	95.59	94.62±6.36	96.67	2.19±3.02	1.61
Vasectomy reversal	92.37±9.16	96.47[c]	95.73±2.33	95.56	5.26±10.63	1.59
AZO	85.43±19.65	94.06[d]	96.30±4.75	97.86	2.91±3.83	1.40
OA	84.96±11.33	87.50[e]	96.98±4.09	99.29	2.45±2.03	1.81
NOA	95.74±3.72	97.01	98.43±2.28	100	2.16±1.86	1.59
Unknown pathology	69.81±45.20	94.12	90.45±6.40	90.00	5.71±9.21	0.79

statistically significant compared to control men: [a] MW, p=0.0032; [b] KW, p<0.01; [c] MW, p=0.0165; [d] KW, p<0.01; [e] KW, p<0.01

Table 5.2. Incidence of imprinting errors in the sperm of men affected by severe male factor infertility

Study Group	DMR analyzed		
	H19	***IG-GTL2***	***MEST***
Control	0/9	0/9	0/9
Oligo	9/26 (34.6)[a]	0/26	5/26 (19.2)
Oligo-I	8/15 (53.3)[b]	0/15	4/15 (26.7)
Oligo-II	1/11 (9.1)	0/11	1/11 (9.1)
Vasectomy reversal	5/17 (29.4)	0/17	2/17 (11.8)
AZO	7/18 (38.9)[c]	1/18 (5.5)	1/18 (5.5)
OA	5/10 (50)[d]	0/10	0/10
NOA	1/5 (20)	0/5	0/5
Unknown pathology	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)

*statistically significant compared to the Control group (Fisher's exact test: [a] p=0.044; [b] p=0.0087; [c] p=0.036; [d] p=0.022). Significance was lost post Bonferroni correction.
Percentages shown in brackets

changes in the methylation level observed at the two DMRs were associated with an increased rate of abnormal methylation found in men affected by oligozoospermia, although this was significant only for the *H19* DMR. Significance post the Bonferroni correction for multiple testing was lost for the comparison of the rates of abnormal methylation at the *H19* DMR between control men and men affected by oligozoospermia and severe oligozoospermia. Significance post the Bonferroni correction was maintained for comparisons of methylation at individual CpG sites at the *H19* DMR between the control and severe oligozoospermia groups, but was lost for the analyses performed between the control and the oligozoospermia groups. However, collectively the evidence provided from the comparison of methylation levels, methylation at individual CpG sites and the rate of abnormal methylation at the *H19* DMR among the groups analyzed supports the conclusion of decreased DNA methylation at the *H19* DMR in men affected by oligozoospermia and severe oligozoospermia compared to control men. The published studies that will be discussed reported results that were uncorrected for multiple testing. With the exception of one study that reported abnormal methylation at the *H19* DMR in 100% of sperm samples obtained from men affected by severe and very severe oligozoospermia (Boissonnais et al., 2010), our finding of abnormal methylation at the *H19* DMR is in accordance with other studies that have also detected abnormal methylation in around 30% of samples obtained from men with severe and very severe oligozoospermia (Marques et al., 2004; Marques et al., 2008; Kobayashi et al., 2007). The 19.2% rate of abnormal methylation at the *MEST* DMR in the sperm of men affected by severe and very

severe oligozoospermia found in this study is a lower compared with previously published reports (Marques et al., 2008; Kobayashi et al., 2007). This may be due to the lower rate of abnormal methylation at the *MEST* DMR found in this study in the sperm of men affected by very severe oligozoospermia compared to other studies. Methylation at the *IG-GTL2* DMR was not significantly different among the groups studied. Abnormal DNA methylation was not found at the *IG-GTL2* DMR in control or oligozoospermic men. One previous study has evaluated DNA methylation at the *IG-GTL2* DMR in the sperm of men affected by severe oligozoospermia. Kobayashi et al. (2007) reported abnormal DNA methylation at the *IG-GTL2* DMR in the sperm of 44.4% men affected by severe oligozoospermia, but also in the sperm of 6.3% of normozoospermic men. Although the findings of Kobayashi et al. (2007) differ from results obtained in this study, methylation at the *IG-GTL2* DMR was evaluated at one CpG site (Kobayashi et al., 2007) and may not have been representative of methylation at neighboring CpG sites.

In addition, we also hypothesized that men affected by very severe oligozoospermia would be affected by a higher rate of methylation abnormalities compared to men affected by severe oligozoospermia. The opposite was found. Abnormal methylation at the *H19* and *MEST* DMRs tended to be more prevalent in men affected by severe oligozoospermia compared to men with very severe oligozoospermia. Methylation abnormalities at imprinted genes may be related to spermatogenesis failure primarily in men affected by severe oligozoospermia; however, other factors may be associated with spermatogenesis failure in men with very severe oligozoospermia. Based on published literature reports a correlation between an increase in abnormal methylation and reduced sperm concentration was expected (Kobayashi et al., 2007; Marques et al., 2008; Boissonnais et al., 2010), therefore our finding of methylation abnormalities primarily in the samples obtained from men affected by severe oligozoospermia was unexpected. In our study men affected by severe oligozoospermia were on average older compared to men affected by very severe oligozoospermia. The increase in methylation abnormalities at imprinted genes seen in the sperm of men affected by severe oligozoospermia may have been related to accumulated effects of exposure to environmental factors, some of which have been shown to affect DNA methylation in sperm (Anway et al., 2005). It may also be that the underlying factors associated with very severe oligozoospermia may be related to

undetermined clinical or genetic factors. In our group of men affected by very severe oligozoospermia we found one case with a Y chromosome microdeletion, one case with a chromosome abnormality and four cases of varicocele; implying that factors other than aberrant imprinting may be associated with spermatogenesis failure in very severe oligozoospermia.

In Chapter 4 DNA methylation in the sperm isolated from testicular tissue of men affected by azoospermia, OA and NOA, and of men undergoing vasectomy reversal was investigated at the DMRs of three imprinted genes, and compared to methylation in the ejaculate sperm of control men of proven fertility. The working hypothesis was that imprinting abnormalities would be more prevalent in the sperm of men affected by azoospermia and in men undergoing vasectomy reversal compared to fertile control men analyzed in Chapter 2. We also hypothesized that imprinting abnormalities would be more prevalent in the sperm of men affected by OA compared to sperm retrieved from men affected by NOA. We found abnormal methylation in 29.4% (5/17) at the *H19* DMR and in 11.8% (2/17) at the *MEST* DMR in men undergoing a vasectomy reversal, while DNA methylation at the *IG-GTL2* DMR was normal in all seventeen samples analyzed. We found a statistically significant decrease in DNA methylation at the *H19* DMR in the sperm of men undergoing vasectomy reversal compared to the methylation in the ejaculate sperm of control men. However, the significant difference in methylation at the *H19* DMR between the two groups was found for analysis uncorrected with the Dunn's post hoc test. There have been reports of changes in the testicular environment after vasectomy affecting spermatogenesis (Jones, 2004; McVicar et al., 2005; Aydos et al., 1998; Shiraishi et al., 2001). Our results suggest that the changes in testicular environment that occur as a result of vasectomy may also affect DNA methylation at imprinted genes. DNA methylation at imprinted genes in testicular sperm retrieved from men undergoing vasectomy reversal has not been previously reported. We also analyzed DNA methylation at the three imprinted genes in testicular sperm retrieved from men affected by azoospermia, OA and NOA. Where possible, the azoospermia cases were grouped into the OA or NOA sub-groups based on the pathological examination of testicular tissue. The pathology was unknown in three cases. We found abnormal methylation in 38.9% (7/18) at the *H19* DMR in the sperm of azoospermic men. The rate of abnormal methylation at the *H19* DMR in azoospermic men was significantly higher compared to control men. A significant decrease in methylation in the sperm of

azoospermic men compared to ejaculate sperm of control men was also found. We found a significant decrease in methylation at the *H19* DMR between OA samples, but not NOA samples, compared to ejaculate control samples. We also found abnormal methylation at the *IG-GTL2* and *MEST* DMRs in one sample each, in azoospermic samples of unknown pathology. This was the only sample in which abnormal methylation at the *IG-GTL2* DMR was found in all of the samples analyzed in the presented experiments. However, significance was lost following the Bonferroni correction for the comparison of rates of abnormal methylation between the ejaculate control men and the azoospermic men, and between the ejaculate control men and the OA men at the *H19* DMR. Despite the loss of significance, the evidence from comparisons of methylation levels at the *H19* DMR do support the conclusion of higher prevalence of methylation abnormalities at the *H19* DMR in men affected by azoospermia and OA compared to control men. Collectively the uncorrected analyses also support these conclusions. The observed evidence showing a decrease in methylation at the *H19* DMR in vasectomy reversal cases compared to control men is weaker as it lost significance when the Dunn's post hoc test was applied.

The OA pathology is similar to that of vasectomy reversal cases in that both types of samples came from men with normal spermatogenesis where the sperm cannot reach the ejaculate due to obstruction. In vasectomy reversal and most OA cases the obstruction is at the level of vas deferens. Studies have shown a decrease of spermatogenesis as a result of the blockage (McVicar et al., 2005) suggesting that changes in the testicular environment may be associated with the decrease in spermatogenesis to help relieve pressure generated through the accumulation of spermatozoa in the vas deferens (Jones et al., 2004). Our results suggest that an altered testicular environment may affect the DNA methylation at imprinted genes. Our results also show that DNA methylation at the *H19* DMR is particularly prone to methylation abnormalities in vasectomy reversal and azoospermic samples.

The study of DNA methylation at three imprinted DMRs in human sperm shows that imprinted genes are not equally affected by epigenetic abnormalities; of the seventy samples analyzed in Chapter 2 and Chapter 4 we identified abnormal methylation in twenty-one, one and eight samples at the *H19*, *IG-GTL2* and *MEST* DMRs, respectively. The two imprinted genes methylated at the DMR in the sperm showed different susceptibility to methylation

abnormalities despite being exposed to the same factors that may presumably disrupt methylation in men affected by severe factor infertility and undergoing vasectomy reversal. We found that the *H19* DMR was particularly prone to methylation abnormalities in men affected by oligozoospermia, azoospermia as well as in men undergoing vasectomy reversal. Furthermore, we found the *IG-GTL2* DMR resistant to imprinting errors in all samples analyzed. Of the seventy samples analyzed, abnormal methylation at the *IG-GTL2* was identified in one sample from a man with azoospermia of unknown pathology. The differences in susceptibility of DMRs to methylation abnormalities may be related to the genetic makeup of the DMRs or the sequences around them. The *DLK1/GTL2* region is highly repetitive compared to the *H19* DMR. 35.8% of the *DLK1/GTL2* region is made up of interspersed repeats, compared to 12.3% of the IGF2/*H19* region (Paulsen et al., 2001). In humans, there are nine 18 base pair repeats within the IG area (Paulsen et al., 2001). It has been suggested that methylation at the *IG-GTL2* DMR is more conservatively maintained as a result of the presence of repetitive stretches of DNA. The presence of repetitive stretches of DNA may be recognized and suppressed by the cell (Li et al., 2004). The highly repetitive nature of the IG area may also be related to amplification of shortened products and difficulty with sequencing that was experienced for this region. One other study has evaluated DNA methylation at the *IG-GTL2* DMR in the sperm of men affected by severe oligozoospermia (Kobayashi et al., 2007). However, methylation at this DMR was only evaluated at one CpG site (Kobayashi et al., 2007) and may not have been representative of methylation at neighboring CpG sites. Our study is the first to report DNA methylation at the *IG-GTL2* DMR in testicular sperm obtained from men affected by azoospermia and men undergoing vasectomy reversal.

In Chapter 3 a limited number of sperm samples obtained from men affected by severe and very severe oligozoospermia were subjected to the study of DNA methylation at non-imprinted genes. Based on the limited data available, we hypothesized that significant differences in DNA methylation at non-imprinted genes would be present in the sperm of men affected by severe and very severe oligozoospermia compared to sperm retrieved from control men. DNA methylation was analyzed using a high throughput analysis of 1,505 CpG sites selected from 807 genes using the Illumina GoldenGate methylation Cancer Panel I. CpG sites showing a significant difference in methylation between oligozoospermic and control samples

were selected for confirmation using pyrosequencing. DNA methylation was evaluated at the CpG sites studied by the Illumina assay and, where possible, at neighboring CpG sites to the ones selected. Our study on a limited number of samples suggests that abnormal methylation in the sperm of infertile men may be present at non-imprinted genes. Of particular interest may be genes *RASSF1*, *JAK3* and *COL1A2*, for which small, but significant differences in methylation at multiple CpG sites were found between patient and control sperm samples. Although the significant difference in methylation was lost at the multiple CpG sites in the genes mentioned after the Bonferroni correction, the detection of significant changes in methylation at multiple CpGs within *RASSF1, JAK 3* and *COL1A2* does warrant further study of methylation at these genes in relation to infertility. It is uncertain whether the small changes in methylation would affect gene expression and contribute to a negative clinical outcome. One report has evaluated DNA methylation at non-imprinted genes in the sperm of men affected by severe oligozoospermia. The limited data suggest the presence of abnormal methylation at non-imprinted genes in sperm of oligozoospermic men (Houshdaran et al., 2007); however, only a trend for significance was found in the study for a number of genes and the differences in methylation were not clear. Our results, although obtained from a limited number of samples, warrant further study of DNA methylation at non-imprinted genes in the sperm of infertile men.

Collectively, our results demonstrate that sperm retrieved from men affected by severe oligozoospermia and obstructive azoospermia are prone to methylation abnormalities at imprinted genes. Furthermore, sperm retrieved from men undergoing vasectomy reversal are also prone to methylation abnormalities at imprinted genes. We also show that DNA methylation at the *H19* DMR is particularly affected by abnormal methylation in the sperm of these men, while DNA methylation at the *IG-GTL2* DMR seems more robust. The results suggest that different mechanisms may be responsible for the methylation abnormalities seen in men affected by severe male factor infertility, depending on the type of the infertility. Presence of abnormal methylation at imprinted genes in men affected by severe oligozoospermia and obstructive azoospermia suggests that abnormal methylation at imprinted genes may be associated with spermatogenesis failure. However, the presence of abnormal methylation at imprinted genes in men undergoing vasectomy reversal suggests that defective imprinting may not only be associated with spermatogenesis failure but also with an altered testicular

environment induced by blockage. Changes in testicular environment have been reported in patients after vasectomy due to the induced blockage. The OA pathology may be similar to vasectomies as it also is involves blockage. Therefore, defective imprinting seen in obstructive azoospermia may not only be associated with infertility, but also with the exposure of the sperm to a testicular environment that may disrupt DNA methylation at imprinted genes. This observation suggests that defective imprinting during spermatogenesis or transfer of sperm from the testes to the ejaculate may arise as a result of an unfavorable environment and may not be just a function of spermatogenesis failure. Factors associated with severe oligozoospermia, or the reduction in spermatogenesis seen in obstructive azoospermia and after vasectomy are currently unknown (McVicar et al., 2005), but the presence of defective imprinting in all three pathologies suggests that these three seemingly unrelated pathologies may be subjected to similar factors that disrupt methylation at imprinted genes. In addition, our analysis of methylation at non-imprinted genes in the sperm of men affected by severe and very severe oligozoospermia suggests that methylation abnormalities in the sperm of these men may not be limited to imprinted genes and warrants the analysis of a larger number of sperm samples obtained from infertile men. We also found that abnormal methylation at the *H19* DMR is relatively common among patients affected by male factor infertility and methylation abnormalities at imprinted genes may be a contributing factor to spermatogenesis failure.

5.2 STRENGTHS AND WEAKNESSES OF THE THESIS RESEARCH

The strengths of the study include the fact that methylation analysis was carried out on data generated from multiple amplification reactions for each gene per patients. Multiple amplification reactions allowed for the analysis of unique clones generated from the amplification of different cells. Bisulphite sequencing also allowed the analysis of methylation at the single sperm level instead of limiting analysis to changes in the overall methylation level in each sperm sample studied. Abnormal DNA methylation at imprinted genes at the sperm level has been associated with negative outcomes such as abortion (Kobayashi et al., 2009), imprinting syndromes in children born through ART (Bliek et al., 2006; Kagami et al., 2007; Kanber et al., 2009) and male infertility in mice (Kaneda et al., 2004; Yaman and Grandjean, 2006). Abnormal DNA methylation at the sperm level may therefore be a more relevant indicator of methylation abnormalities in patients. The analysis of unique clones, originating

from multiple amplification reactions, allowed us to more accurately study methylation at imprinted genes in sperm samples obtained from cases where only a small quantity of sperm is available such as severe oligozoospermia, very severe oligozoospermia, azoospermia and vasectomy reversal cases. It has been reported that small quantities of starting material may be prone to preferential amplification (Walsh et al., 1992; Findlay et al., 1995), possibly providing non-representative results. However, bisulphite sequencing may have also provided limited information on the status of methylation at imprinted genes in the sperm because few clones were analyzed and raises the question of whether the results are representative of all sperm cells present in the samples analyzed. However, other studies evaluating methylation in sperm face the same limitation. A better approach may have been to first analyze the overall methylation at an imprinted gene by pyrosequencing followed by bisulphite sequencing to study methylation at the sperm level. Analysis of methylation by pyrosequencing in the sperm should also be performed on multiple amplification reactions to avoid preferential amplification. Analysis of DNA methylation in this study was extended to both imprinted genes with methylated DMRs in human sperm, *H19* and *GLT2.* We found that these two genes had very different susceptibility to imprinting defects despite originating from the same patient sample. Another strength of this study was that men of proven fertility were chosen as controls. Other studies have used sperm from normozoospermic men of unknown fertility status as control samples and a number of studies identified abnormal methylation in the sperm of these men (Kobayashi et al., 2007; Manning et al., 2001).

In the presented studies we report abnormal methylation at imprinted genes in men affected by severe male factor infertility and in men undergoing vasectomy reversal; however, the cause of these abnormalities was not addressed. Abnormal methylation at imprinted genes may be associated with errors in erasure, establishment or maintenance of the imprints. Although the origin of clones showing abnormal DNA methylation, whether the abnormal methylation affected the maternal or paternal allele, was addressed based on the presence of an informative SNP within the sequence, only limited information was obtained. The SNPs within the sequences were not informative for most samples analyzed. Regions should be selected to incorporate more heterogeneous SNPs within the *IG-GTL2* and *MEST* DMRs so that more information regarding the origin of the clones can be obtained. The SNPs within the two regions

were informative in only two samples at the *IG-GTL2* DMR and for none of the samples analyzed at the *MEST* DMR.

Other weaknesses of the study include the selection of control samples for the azoospermia study group. Vasectomy reversal samples were initially believed to represent appropriate controls for the azoospermia study group; however, abnormal methylation in vasectomy reversal samples was also identified at the *H19* DMR and the *MEST* DMR. As a result data for the azoospermia study group were compared to both the vasectomy reversal samples and the ejaculate control samples. DNA methylation at imprinted genes should not differ between mature sperm in the ejaculate and testicular sperm in the testes since imprints are set before male germ cells enter meiosis (Kerjean et al., 2000). Better controls would have been testicular sperm obtained from men undergoing vasectomy. The testicular environment changes in patients after vasectomy (Jones, 2004; McVicar et al., 2005); therefore, sperm obtained during a vasectomy procedure would not have been exposed to any of the changes that occur. It is uncertain whether ethical approval would be granted for obtaining testicular biopsies from men undergoing a vasectomy as a testicular biopsy would be a clinically unnecessary procedure in such cases and the risks involved with the procedure may not be justifiable for research. Another drawback of the study is that few NOA samples were analyzed. More samples should be analyzed to determine whether NOA patients are truly less prone to abnormal methylation at imprinted genes compared to OA patients. Furthermore, methylation at non-imprinted genes should be analyzed in more samples to determine whether methylation at non-imprinted genes is also disrupted in association with spermatogenesis failure.

Multiple testing was performed to analyze the data presented. To correct for the error introduced by multiple testing, the analyses were corrected using the Dunn's post hoc test, the Bonferroni correction or the FDR, depending on the statistical test performed. While the corrections decrease the rate of false positives, their use may increase the rate of false negatives. Statistical significance of some tests performed was lost following the correction.

5.3 FUTURE DIRECTION

Studies presented in this thesis and previous studies have demonstrated defective imprinting in the sperm of men affected by severe and very severe oligozoospermia (Marques et

al., 2004; Marques et al., 2008; Kobayashi et al., 2007; Boissonnais et al., 2010) and in men affected by azoospermia (Marques et al., 2009). In addition, we demonstrated defective imprinting in the sperm of men undergoing vasectomy reversal. What remains unknown are the mechanisms involved that give rise to the methylation abnormalities at imprinted genes in the studied cases. Errors in erasure, establishment or maintenance are mechanisms associated with changes in DNA methylation. Also, relatively little information is available on environmental factors that can disrupt DNA methylation. During early development, maternal and paternal imprints are erased in the primordial germ cells (Kafri et al., 1992; Davis et al., 2000) and are re-established in a sex specific manner. The imprints are then maintained throughout development. During spermatogenesis, the re-establishment of methylation is almost complete in spermatogonia (Li et al., 2004; Kerjean et al., 2000) and is fully set in post-meiotic male germ cells, such as sperm isolated from the ejaculate or testicular tissue. Furthermore, abnormal methylation at imprinted genes in the sperm could be associated with genetic mutations in enzymes involved in imprint establishment. However, a recent study failed to identify mutations in *DNMT3A* and *DNMT3*L in oligozoospermic men showing abnormal DNA methylation at imprinted genes in their sperm (Kobayashi et al., 2009). One way to determine when methylation abnormalities arise during spermatogenesis would be to evaluate methylation at imprinted genes in germ cells other than mature spermatozoa or testicular sperm, as analyzed in this study. In the samples in which abnormal methylation was found, methylation in spermatocytes or in spermatogonia isolated from testicular tissue could be evaluated. Presence of abnormal methylation in these cells would imply that the abnormal methylation arose early on in spermatogenesis or that it may have been present from birth, having arisen during *in utero* development. Presence of normal methylation in early germ cells would imply that abnormal methylation was acquired at the sperm stage, perhaps during spermiogenesis. During spermiogenesis, the sperm chromatin undergoes compaction through the exchange of histones to protamines (Gusse et al., 1986; Vu et al., 2004; Delaval et al., 2007). A recent study has identified higher rates of imprinting errors in sperm samples obtained from men with an abnormal Protamine 1 to Protamine 2 ratio compared to men affected by oligozoospermia (Hammoud et al., 2009). An abnormal Protamine 1 to Protamine 2 ratio is suggestive of abnormal histone to protamine exchange and therefore abnormal chromatin compaction. Abnormally packaged sperm may be more prone to DNA damage, potentially affecting the

epigenome. DNA damage can be evaluated by studying DNA fragmentation. Increased DNA fragmentation was seen in sperm after vasectomy (O'Neill et al., 2007). We found aberrant imprinting in sperm retrieved from men after vasectomy. DNA fragmentation should be studied in men with aberrant imprinting to determine whether damaged sperm DNA is prone to methylation abnormalities.

More research is also required on environmental factors, either toxins or dietary, which may affect gene methylation. Factors such as maternal diet have been shown to affect DNA methylation in the fetus (Waterland and Jirtle, 2003; Dolinoy et al., 2006; Anway et al., 2005). However, only one of these studies analyzed methylation in the germ cells of the progeny (Anway et al., 2005) to determine whether environmental perturbations during *in utero* development can affect methylation in germ cells. The study found that abnormal methylation in male germ cells could be passed on to the next generation (Anway et al., 2005), showing that DNA methylation can be heritable. Male gametes may be particularly vulnerable to perturbations of methylation during *in utero* development as it is during this time that genomic imprinting is established. Exposure to environmental factors after birth may also affect spermatogenesis. For example, higher levels of methyl donors in males correlated with improved testicular histology, increased sperm numbers and fertility in male mice (Kelly et al., 2005), and increased sperm concentration and decreased sperm DNA damage in humans (Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). Although these studies suggest a link between methyl donors and infertility, their effects on DNA methylation in sperm has not been studied. Future studies should identify environmental factors that may affect methylation in gametes. One of the first factors that should be studied is folate since it is a methyl donor that has been associated with increased sperm concentration and decreased sperm damage (Boxmeer et al., 2007; Boxmeer et al., 2009; Wong et al., 2002). Of interest may also be worthwhile to look for gene mutations in enzymes responsible for folate synthesis, specifically in patients with abnormal DNA methylation in their sperm.

Currently, little information is also available on the consequences of abnormal methylation at imprinted genes identified in infertile men. To date, only one study has demonstrated a direct association between the presence of abnormal methylation at imprinted genes in the sperm and spontaneous abortion following ART treatment (Kobayashi et al., 2009).

Other studies have identified imprinting abnormalities in children born through ART that could have been of a paternal origin (Bliek et al., 2006; Kagami et al., 2007; Kanber et al., 2009), but failed to analyze the sperm. Patients with abnormal methylation at imprinted genes in the sperm should be followed up to determine whether the outcome of fertility treatment is affected by methylation abnormalities in the sperm. This information was not yet available to us. DNA methylation could be analyzed in products of conception in cases of spontaneous abortion, fetal and placental tissues, and in the child and placenta after birth to determine if the abnormality present in sperm was passed on and whether it affected the pregnancy outcome. The analysis of SNPs in the sperm could be used to determine whether abnormal methylation found in the offspring originated from an improperly methylated sperm. This approach has been successfully used before (Kobayashi et al., 2009).

Furthermore, vasectomy reversal cases showing abnormal methylation at imprinted genes in the sperm should be retested once normal spermatogenesis returns and sperm are present in the ejaculate of these men. Our explanation of the presence of abnormal methylation in vasectomy reversal cases stated that the altered testicular environment, as a result of the vasectomy, was associated with abnormal methylation. Therefore once the testicular environment returns to normal the methylation should also return to a normal state. Such a study would directly demonstrate that methylation at imprinted genes responds to environmental cues.

Lastly, there is an obvious lack of data on the status of DNA methylation at non-imprinted genes in the sperm of infertile men. It is still not known whether methylation abnormalities present in the sperm are limited to imprinted genes or whether methylation at non-imprinted genes is also affected. With high throughput and more sensitive technologies becoming available for the study of DNA methylation hopefully more information will become available on this topic.

5.4 SIGNIFICANCE AND CONCLUSION

Our analysis of DNA methylation at the two methylated DMRs in sperm, *H19* and *IG-GTL2*, showed different susceptibility of the two DMRs to abnormal DNA methylation. This finding may be related to the genetic make up of the DMR or of the sequences surrounding the DMR, suggesting that the genetic structure may influence the susceptibility of a DMR to

changes in DNA methylation. In the set of samples analyzed we found that abnormal DNA methylation at imprinted genes may be related to spermatogenesis failure in cases of severe oligozoospermia, while genetic or clinical factors may be associated with very severe oligozoospermia. Our analysis of DNA methylation at imprinted genes in the sperm of men affected by azoospermia showed that most abnormalities found were seen in the sperm of men affected by obstructive azoospermia. Furthermore, we identified aberrant imprinting in the sperm of men undergoing vasectomy reversal. The OA pathology is similar to that of vasectomy reversal cases in that both types of samples came from men with normal spermatogenesis where the sperm cannot reach the ejaculate due to obstruction. There have been reports of changes in the testicular environment after vasectomy affecting spermatogenesis (Jones, 2004; McVicar et al., 2005; Aydos et al., 1998). Our results suggest that the changes in testicular environment that occur as a result of blockage may also affect DNA methylation at imprinted genes. This finding suggests that abnormal methylation at imprinted genes may not only be related to spermatogenesis failure, as seen in patients affected by severe oligozoospermia, but also to changes in the environment. Lastly, our analysis of a limited number of samples suggests that abnormal methylation in the sperm of men affected by severe oligozoospermia may also affect non-imprinted genes. However, due to the small sample size and low statistical power, the findings should be confirmed in a larger samples size.

Patients should be informed during clinical counseling prior to fertility treatment. Patients at greatest risk, such as those affected by severe oligozoospermia and obstructive azoospermia may choose to get tested prior to attempting fertility treatment. In addition, vasectomy is a common form of contraception. Our results demonstrate that sperm from these men may carry defective imprints. These men should also be informed of the potential risk of having sperm affected by abnormal methylation at imprinted genes.

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APPENDIX I: ETHICS APPROVAL CERTIFICATES

The original certificate of full board approval and the certificate of amendment approval obtained from the UBC Clinical Research Ethics Board are included. Ethics certificate of minimal risk approval received from the UBC C&W Research Ethics Board is also included.

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF FULL BOARD APPROVAL

PRINCIPAL INVESTIGATOR:	INSTITUTION / DEPARTMENT:	UBC CREB NUMBER:
Sai Ma	UBC/Medicine, Faculty of/Obstetrics & Gynaecology	H06-03547

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Vancouver Coastal Health (VCHRI/VCHA)	Vancouver General Hospital

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
Victor Chow
Mark K. Nigro

SPONSORING AGENCIES:
Canadian Institutes of Health Research

PROJECT TITLE:
Epigenetic modifications in severe male infertility (Version 1.0 November 2006)

THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES: March 12, 2008

The full UBC Clinical Research Ethics Board has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.

REB FULL BOARD MEETING REVIEW DATE:
March 12, 2007

DOCUMENTS INCLUDED IN THIS APPROVAL:

Document Name	Version	Date	DATE DOCUMENTS APPROVED:
Protocol:			
Epigenetic modifiction in severe male infertility	1.0	January 10, 2007	
Consent Forms:			
Control Group: testicular sperm	1.3	February 6, 2007	
Control Group: Ejaculate sperm	1.3	February 6, 2007	**March 12, 2007**
consent form	1.0	February 6, 2007	
Advertisements:			
Recruitment poster	1.0	January 11, 2007	

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The documentation included for the above-named project has been reviewed by the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.

Approval of the Clinical Research Ethics Board by one of:

Dr. Gail Bellward, Chair

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: AMENDMENT

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC CREB NUMBER:
Sai Ma	UBC/Medicine, Faculty of/Obstetrics & Gynaecology	H06-03547

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Children's and Women's Health Centre of BC (incl. Sunny Hill)	Children's and Women's Health Centre of BC (incl. Sunny Hill)

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
Victor Chow
Mark K. Nigro

SPONSORING AGENCIES:
Canadian Institutes of Health Research (CIHR)

PROJECT TITLE:
Epigenetic modifications in severe male infertility (Version 1.0 November 2006)

REMINDER: The current UBC CREB approval for this study expires: March 12, 2008

AMENDMENTS:

Document Name	Version	Date
Protocol:		
protocol	1.0	August 19, 2007
Assent Forms:		
study group	1.4	August 19, 2007
Control Group: testicular sperm	1.4	August 19, 2007
Control Group: Ejaculate sperm	1.1	August 19, 2007
Advertisements:		
Recruitment Poster	1.1	August 1, 2007

AMENDMENT APPROVAL DATE:
August 31, 2007

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The amendment(s) for the above-named project has been reviewed by the Chair of the University of British Columbia Clinical Research Ethics Board and the accompanying documentation was found to be acceptable on ethical grounds for research involving human subjects.

Approval of the Clinical Research Ethics Board by one of:

Dr. James McCormack, Associate Chair

CHILDREN'S & WOMEN'S HEALTH CENTRE OF BRITISH COLUMBIA
AN AGENCY OF THE PROVINCIAL HEALTH SERVICES AUTHORITY

UBC C&W Research Ethics Board
A2-136, 950 West 28th Avenue
Vancouver, BC V5Z 4H4
Tel: (604) 875-3103 Fax: (604) 875-2496
Email: cwreb@cw.bc.ca
Website: http://www.cfri.ca/research_support > Research Ethics

ETHICS CERTIFICATE OF MINIMAL RISK APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC C&W NUMBER:
Sai Ma	UBC/Medicine, Faculty of/Obstetrics & Gynaecology	H06-03547

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Children's and Women's Health Centre of BC (incl. Sunny Hill)	Child & Family Research Institute

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
Victor Chow
Mark K. Nigro

SPONSORING AGENCIES:
- Canadian Institutes of Health Research (CIHR)

PROJECT TITLE:
Epigenetic modifications in severe male infertility (Version 1.0 November 2006)

REMINDER: The current UBC Children's and Women's approval for this study expires: March 19, 2011

APPROVAL DATE: March 19, 2010

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Children's and Women's Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Children's and Women's Research Ethics Board.

Approved by one of:

Dr. Marc Levine, Chair Dr. Caron Strahlendorf, Associate Chair