MEIOTIC DEFECTS IN INFERTILE MEN

by

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ABSTRACT

While the introduction of intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of male infertility, concerns have been raised regarding the risk of chromosomal abnormalities in pregnancies derived from ICSI. Studies on sperm from infertile men have suggested that this population may produce higher rates of aneuploid sperm. Thus, we hypothesized that defects in early meiotic events may contribute to both male infertility and the production of aneuploid sperm.

We used immunofluorescent techniques to observe the synapsis and recombination of chromosomes during meiosis, and fluorescent in-situ hybridization (FISH) to assess sperm aneuploidy. We analyzed testicular tissue from thirty-one men (10 fertile and 21 infertile men). We observed that ~36% (5/14) of men with impaired spermatogenesis displayed reduced genome-wide recombination. When all men were pooled, we observed an inverse correlation between the frequency of sex chromosome recombination and XY disomy in the sperm. We combined immunofluorescent and FISH techniques to study recombination patterns on chromosomes 13, 18 and 21 in fifteen men (5 fertile and 10 infertile men). Four of the infertile men displayed altered recombination distributions on at least one of the chromosome arms studied. Finally, we examined early meiotic events in two biopsies from an azoospermic t(8;13) carrier. While global recombination rates were not altered, recombination frequencies were reduced specifically on the rearranged chromosomes. Asynapsed quadrivalents were observed in 90% and 87% of pachytene nuclei from the first and second biopsies, respectively, and were frequently associated with the sex chromosomes. BRCA1 and γH2AX, two proteins implicated in meiotic sex chromosome inactivation, localized along asynapsed regions regardless of whether or not they were associated with the sex chromosomes, suggesting that regions of autosomal chromosomes that fail to synapse undergo transcriptional silencing in humans.

In summary, we observed that a subset of infertile men display alterations in the number and position of meiotic crossovers, which may contribute to both infertility and an increased risk of sperm aneuploidy. The fidelity of synapsis is also a critical factor in determining the outcome of gametogenesis in humans, as the transcriptional inactivation of asynapsed regions may silence meiotic genes, leading to meiotic arrest and infertility.

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Rules established by the Human Genome Organization (HUGO) for naming genes are followed in this thesis. Human genes are reported as all capital letters, while those in mice have only the first letter capitalized. Italicized letters indicate the gene or RNA, while non-italicized indicate the protein.

γH2AX	Phosporylated H2AX
2n	Diploid
1n	Haploid
Ad	Dark type A (spermatogonia)
Ap	Pale type A (spermatogonia)
ADB	Antibody diluting buffer
AMCA	Aminomethyl coumarin acetic acid
ART	Assisted reproductive technologies
ATR	Ataxia telangiectasia and Rad3 related
AZF	Azoospermic factor
BPY2	Basic protein Y2
BRCA1	Breast cancer 1, early onset
c	Centromere
CBAVD	Congenital absence of the vas deferens
cenM-FISH	centromere-specific mulicolour fluorescent in-situ hybridization
CEP	Centromeric probe
CF	Cystic fibrosis
CFTR	Cystic fibrosis trans-membrane conductance receptor
CI	Confidence interval
CREST	Calcinosis/Raynaud's phenomenom/esophageal/dysmotility/ sclerodactyly/telangiectasia
DAPI	4,6-diamidino-2 phenylindole
DAZ	Deleted in azoospermia
der	derivative chromosome
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DTT	Dithiothreitol
f	Frequency
FISH	Fluorecent in-situ hybridization
FITC	Fluorescein isothiocyanate
Fkbp6	FK506 binding protein
FSH	Follicle stimulating hormone
GCA	Germ cell arrest
GnRH	Gonadotropin-releasing hormone

H2AX	H2A histone family, member X
Hypo	Hypospermatogenesis
ICSI	Intracytoplasmic sperm injection
IgG	Immunoglobulin G
IVF	In vitro fertilization
LH	Luteinizing Hormone
LSI	Locus-specific identifier
MI	Meiosis I
MII	Meiosis II
MA	Maturation arrest
MESA	Microsurgical epididymal sperm aspiration
min	Minute
MLH1	Mut-L homolog 1
MLH3	Mut-L homolog 3
Mre11a	Meiotic recombination 11 homolog A
mRNA	Messenger ribonucleic acid
MSCI	Meiotic sex chromosome inactivation
MSH4	MutS homolog 4
MSH5	MutS homolog 5
MSUC	Meiotic silencing of unsynapsed chromatin
MYC	v-myc myelocytomatosis viral oncogene homolog
No	Number
NP-40	Nonyl phenoxylpolyethoxylethanol-40
NOA	Non-obstructuve azoospemria
OA	Obstructive-azoospermia
OAT	Oligoasthenoteratozoospermia
p	Short chromosome arm
PBD	Phosphate-buffered detergent
PBS	Phosphate-buffered saline
PESA	Percutaneous sperm aspiration
PMA	Partial maturation arrest
PMSF	Phenylmethylsulphonyl fluoride
PSCR	Post-meiotic sex chromosome repression
PZD	Partial zona dissection
q	Long chromosome arm
RAD51	RAD51 homolog (RecA homolog E. coli)
RNA	Ribonucleic acid
RNA Pol II	Ribonuclei acid polymerase II
RPA	Replication protein A
SAC	Spindle assembly checkpoint
SC	Synaptonemal complex
SCC	Saline sodium citrate
SCOS	Sertoli cell only syndrome
SPO11	SPO11 meiotic protein covalently bound to DSB homolog
SRY	Sex determining region Y
SUZI	Subzonal sperm injection

SYCP1	Synaptonemal complex protein 1
SYCP2	Synaptonemal complex protein 2
SYCP3	Synaptonemal complex protein 3
TESE	Testicular sperm extraction
TRITC	Tetramethyl rhodamine isothiocyanate
TTY2	Testis transcript Y2
WHO	World Health Organization
XIST	X-inactive specific transcript

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CO-AUTHORSHIP STATEMENTS

CHAPTER II:

The experiments were conceived of by my supervisor, Dr. Sai Ma. The majority of the experiments and data analysis were performed by Kyle Ferguson. Edgar Chan Wong assisted with the analysis of fluorescent in-situ hybridization on testicular sperm from two cases. Drs. Victor Chow and Mark Nigro (collaborators of Dr. Sai Ma) of the UBC Department of Urological Sciences performed the testicular biopsies. Dr. Peter Moens of York Univerisity, Toronto, provided the SYCP3 and SYCP1 primary antibodies. With the permission of Dr. Sai Ma, I have included previously published data (five infertile men and two control men; Ma *et al*., 2006a and Ma *et al*., 2006b) in my thesis in order to examine the association between meiotic defects, male infertility and sperm aneuploidy. All figures were prepared by Kyle Ferguson and the manuscript was written by Kyle Ferguson and Dr. Sai Ma.

CHAPTER III:

The experiments were conceived of by Dr. Sai Ma and Kyle Ferguson. The MicroMeasure analysis was performed by Kyle Ferguson, with assistance from Stefanie Leung and Dennis Jiang. Data analysis and figure preparation was performed by Kyle Ferguson. The manuscript was written by Kyle Ferguson and Dr. Sai Ma.

CHAPTER IV:

The experiments were conceived of by Dr. Sai Ma and Kyle Ferguson. Dr. Victor Chow perfomed both biopsies on the t(8;13) carrier. All experiments, data analysis and figure preparation were performed by Kyle Ferguson. The manuscript was written by Kyle Ferguson and Dr. Sai Ma.

CHAPTER 1: INTRODUCTION

Sexual reproduction is composed of two main processes: (1) gametogenesis, in which gametes are produced containing half the number of chromosomes as the parent cell, and (2) fertilization, in which two gametes fuse to restore the original number of chromosomes. During these processes cells undergo two types of cell division, known as mitosis and meiosis. Mitosis gives rise to daughter cells with the same number of chromosomes as the parent cell, and are responsible for somatic cell divisions and the proliferation of germinal stem cells. Meiosis is unique to gametogenesis, and is essential for producing gametes with half the number of chromosomes as somatic cells, thus ensuring a full chromosome complement upon fertilization. The work presented in this thesis will try to address the role that meiosis plays in male factor infertility and the production of chromosomally abnormal sperm. To begin, I will address the basic processes of spermatogenesis and discuss the defining events that make meiosis such a unique and important cell division.

1.1 SPERMATOGENESIS AND MEIOSIS

During early embryo development the primordial germ cells migrate to the site of the developing gonads, known as the genital ridge. Upon reaching the genital ridge, the *sex-determining region Y* (*SRY*) gene on the Y chromosome induces testis differentiation (Koopman *et al*., 1991). The two primary functions of the testes are the secretion of testosterone by the Leydig cells and the production of spermatozoa in the seminiferous tubules through spermatogenesis (Sherwood, 2004). Spermatogenesis is the process whereby a population of self-renewing spermatogonial stem cells differentiate into mature spermatozoa. In humans, each cycle of spermatogenesis takes approximately 64 days to complete (Heller and Clermont, 1963). Spermatogenesis can be subdivided into three phases: 1) mitotic division of spermatogonia, 2) meiosis and 3) cellular differentiation (spermiogenesis) (Figure 1.1).

A pool of replenishing spermatogenic stem cells in the testes allows spermatogenesis to proceed continuously throughout life, from puberty onwards. Along the outermost layer of the seminiferous tubules are the spermatogonia, which can be classified as: dark type A (Ad), pale

type A (Ap) and type B spermatogonia (Clermont 1966). The Ad spermatogonia undergo mitotic divisions to give rise to Ap spermatogonia, while also replenishing the undifferentiated Ad spermatogonia population. After a series of mitotic divisions, the Ap spermatogonia give rise to the type B spermatogonia. These type B spermatogonia develop into primary spermatocytes for entry into meiosis. Primary spermatocytes enter meiosis I, where the cells undergo a reductional division to reduce the chromosome number by half (discussed in detail in the following sections). The resulting secondary spermatocytes then undergo meiosis II to segregate sister chromatids. During the final stage of spermatogenesis the spermatids differentiate into the highly specialized spermatozoa through a process known as spermiogenesis. During spermiogenesis the Golgi apparatus develops into the acrosome cap, the flagellum develops, and DNA is repackaged to one tenth the volume of an immature spermatid (Holstein *et al*., 2003). The spermatozoa are released from the Sertoli cells into the lumen of the seminiferous tubule through a process called spermiation, and are then transported to the epididymis. The spermatozoa remain in the epididymis for 1-2 weeks, where they obtain their motility and ability to fertilize an oocyte (Jones, 1999).

At puberty, testosterone secretion and spermatogenesis in the testis is regulated by two hormones secreted by the anterior pituitary: luteinizing hormone (LH) and follicle stimulating hormone (FSH). Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus, stimulating LH and FSH secretion by the anterior pituitary. LH acts on the Leydig cells to enhance testosterone secretion; whereas FSH acts on the Sertoli cells to initiate and maintain spermatogenesis (Sherwood, 2004). The Leydig cells secrete testosterone which acts on the Sertoli cells, providing the high levels of testosterone required for spermatogenesis. Testicular function is further regulated by two negative feedback loops: 1) testosterone released by the Leydig cells acts on the hypothalamus to inhibit GnRH release, and 2) inhibin released by the Sertoli cells acts on the anterior pituitary to inhibit FSH secretion. Measuring serum FSH levels is part of the routine diagnostic work-up for male infertility, as elevated FSH levels are a cause of impaired spermatogenesis (Novero *et al*., 1997).

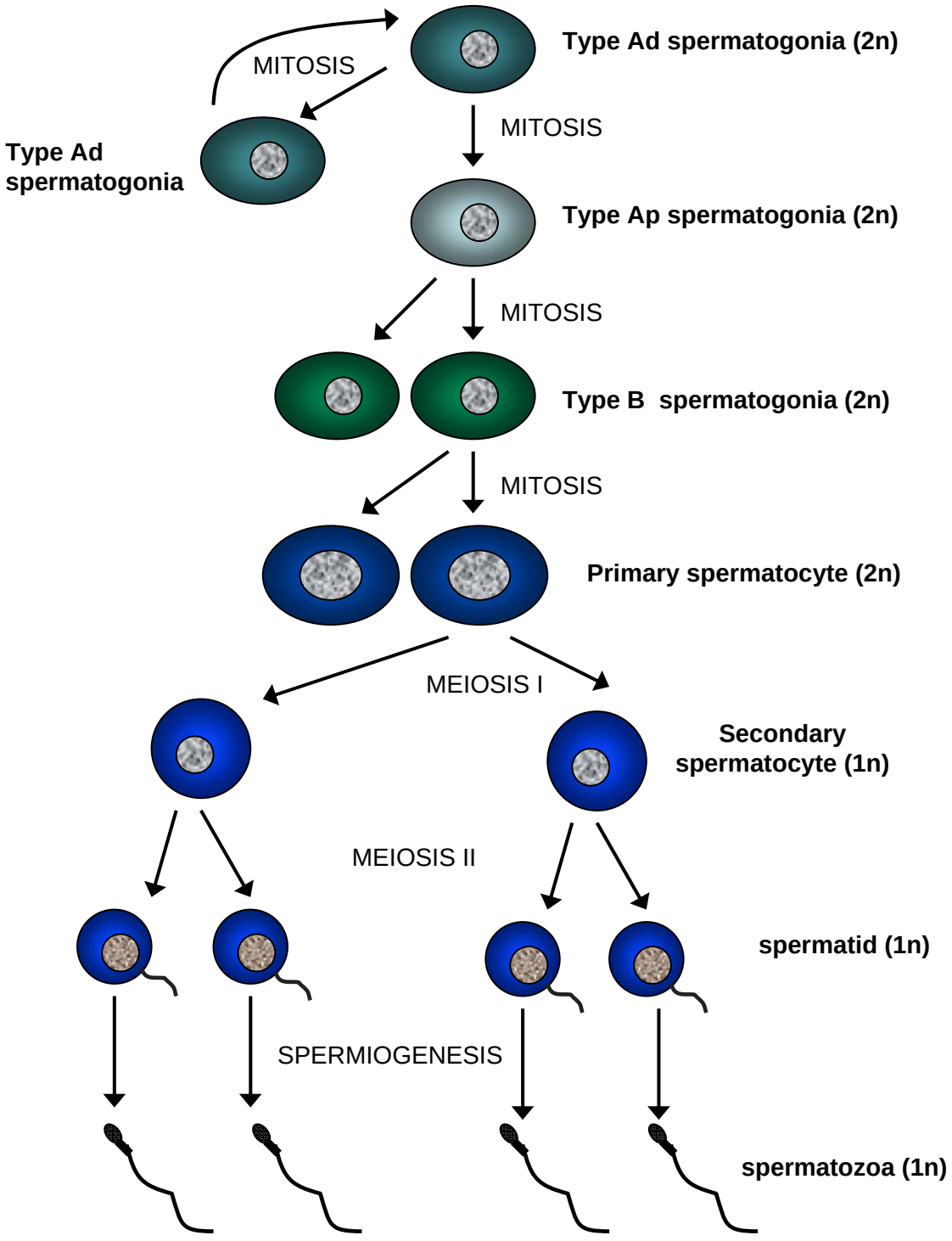

Figure 1.1 Spermatogenesis. Spermatogenesis consists of mitotic differentiation of the spermatogonial stem cells, meiosis I (MI) and meiosis II (MII) to reduced the chromosome content from diploid (2n) to haploid (1n), and spermiogenesis, in which the spermatid differentiate into the highly specialized spermatozoa.

1.1.1 Introduction to meiosis

Meiosis consists of a round of DNA replication, followed by two successive cellular divisions, meiosis I (MI) and meiosis II (MII). The first meiotic division, MI, is a reductional division in which the homologous chromosomes segregate to separate daughter cells, reducing the genetic content of the cell by half from diploid (2n) to haploid (1n). MI is characterized by three unique events: 1) the pairing of homologous chromosomes, 2) synapsis, in which a protein structure known as the synaptonemal complex (SC) forms between the two chromosomes, and 3) meiotic recombination, in which the homologous chromosomes exchange genetic material (Roeder, 1997). During the second meiotic division, MII, sister chromatids are segregated in a cellular division that is similar to mitosis.

Both MI and MII can be subdivided into four stages: prophase, metaphase, anaphase and telophase. Prophase of MI (prophase I) can be further divided into five substages, leptotene, zygotene, pachytene, diplotene and diakinesis, based on the development of the SC (see Page and Hawley, 2003; Champion and Hawley, 2002 for review; Figure 1.2). During leptotene, the first stage, homologous chromosomes condense, pairing between homologs is initiated, and short fragments of the SC begin to form the axial elements along sister chromatids. During zygotene, the second stage, lateral elements are formed from the axial elements, and synapsis is initiated when transverse elements of the SC begin to appear between homologs. The pachytene stage is characterized by the completion of homologous synapsis. During diplotene and diakinesis chromosomes undergo desynapsis and homologs remain connected only at chiasmata, which result from homologous recombination during zyogotene and pachytene. Prophase I is perhaps the most critical stage in the formation of a haploid gamete, as it is during this stage that the pairing, synapsis and recombination of homologous chromosomes occur.

1.1.2 Chromosome pairing

Although both the pairing and synapsis of chromosomes requires close associations between homologous chromosomes, they are distinct processes. The pairing of chromosomes during MI requires homologous chromosomes to overcome spatial separations within the nucleus

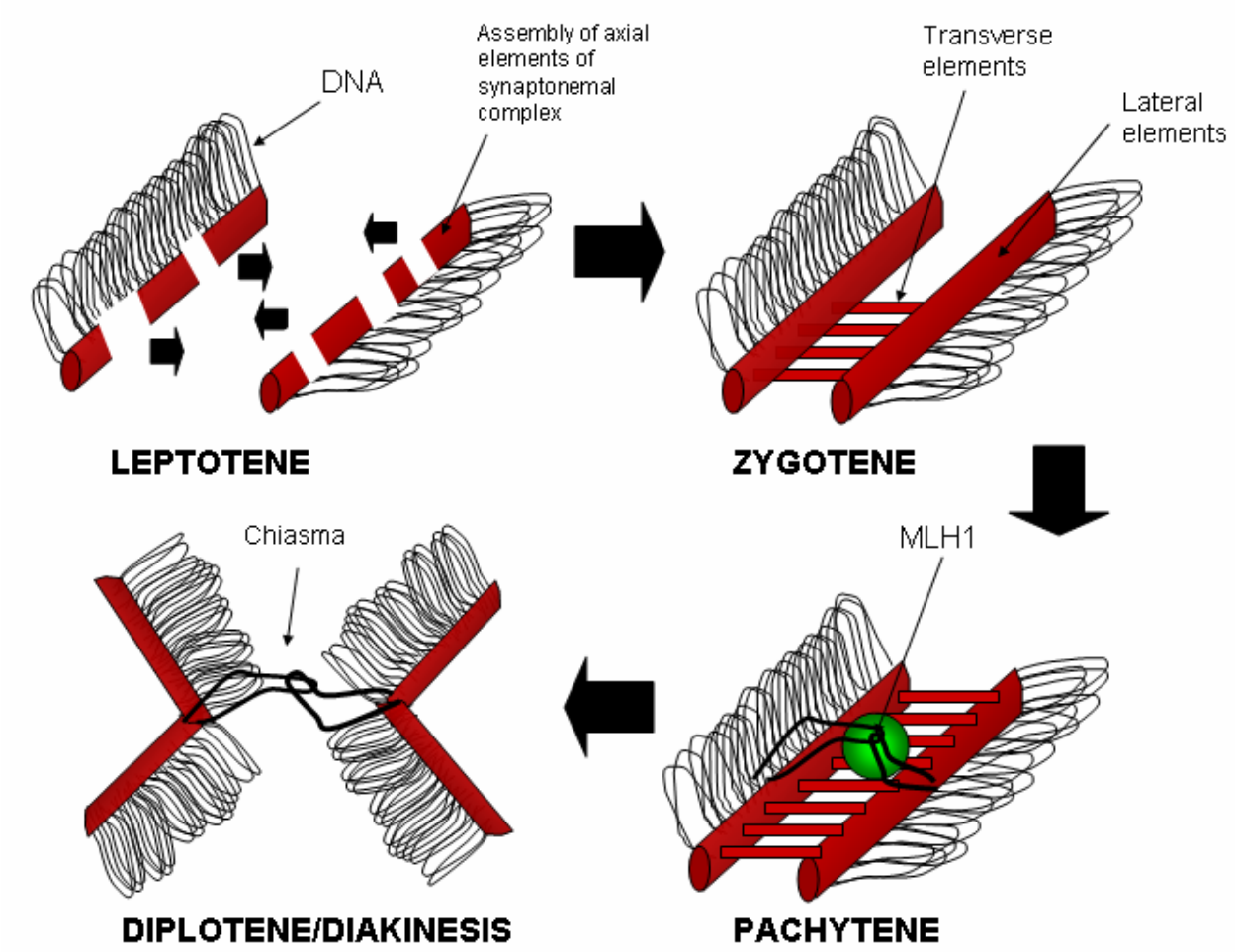

Figure 1.2: Stages of prophase of meiosis I. During leptotene chromosomes undergo a search for homology and axial elements of the synaptonemal complex (SC) form; during zygotene the homologous chromosomes undergo synapsis with the formation of the transverse elements of the SC; at the pachytene stage synapsis is completed, followed by the completion of meiotic recombination which is characterized by the localization of the DNA mismatch repair protein MLH1; finally, during diplotene/diakinesis the chromosomes desynapse and begin to segregate, forming chiasmata at sites of recombination.

to pair with its partner chromosomes based on sequence homology, and remains one of the least understood aspects of meiosis. In the yeast *Saccharomyces cerevisiae*, plants and mammals the DNA double strand breaks (DSBs) that are required for recombination are initiated prior to the pairing of homologous chromosomes, while in other organisms such as the fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans* chromosomes pair before recombination is initiated (Barzel and Kupiec, 2008). However, pairing in nearly all organisms is initiated through the formation of a telomere bouquet during the leptotene-zygotene transition, which is characterized by the tight clustering of telomeres at the nuclear envelope (Page and Hawley, 2003). There may also be specific pairing elements on the chromosomes that are required for meiotic pairing. For example, in *C. elegans* homolog recognition regions have been identified at the end of each chromosome that facilitate pairing (McKim *et al*., 1988).

1.1.3 Synapsis and the synaptonemal complex

Following pairing of the homologous chromosomes, the chromosomes undergo synapsis in which the SC arises and acts as a bridge between the homologous chromosomes. The SC was first described in 1956 (Moses), and subsequently named the *synaptinemal complex*, once it was confirmed to be "exclusively of synaptic occurrence" (Moses, 1958). The SC is a tripartite protein structure composed of two axial/lateral elements and a transverse element (Figure 1.3). The two lateral elements run along homologous chromosomes, and in mammals the known components are the synaptonemal complex proteins 2 (SYCP2) and 3 (SYCP3) (Heyting *et al*., 1985). DNA-binding domains have been identified in both SYCP2 and SYCP3, which allow the proteins to interact with the chromosomes (Lammers *et al*., 1995; Offenberg *et al*., 1998). The lateral elements are joined by the transverse filaments, of which SYCP1 is a known component (Meuwissen *et al*., 1992). The protein structure of SYCP1 is characterized by a coiled-coil segment in the center of the protein, with two globular domains on each end. The coiled-coil

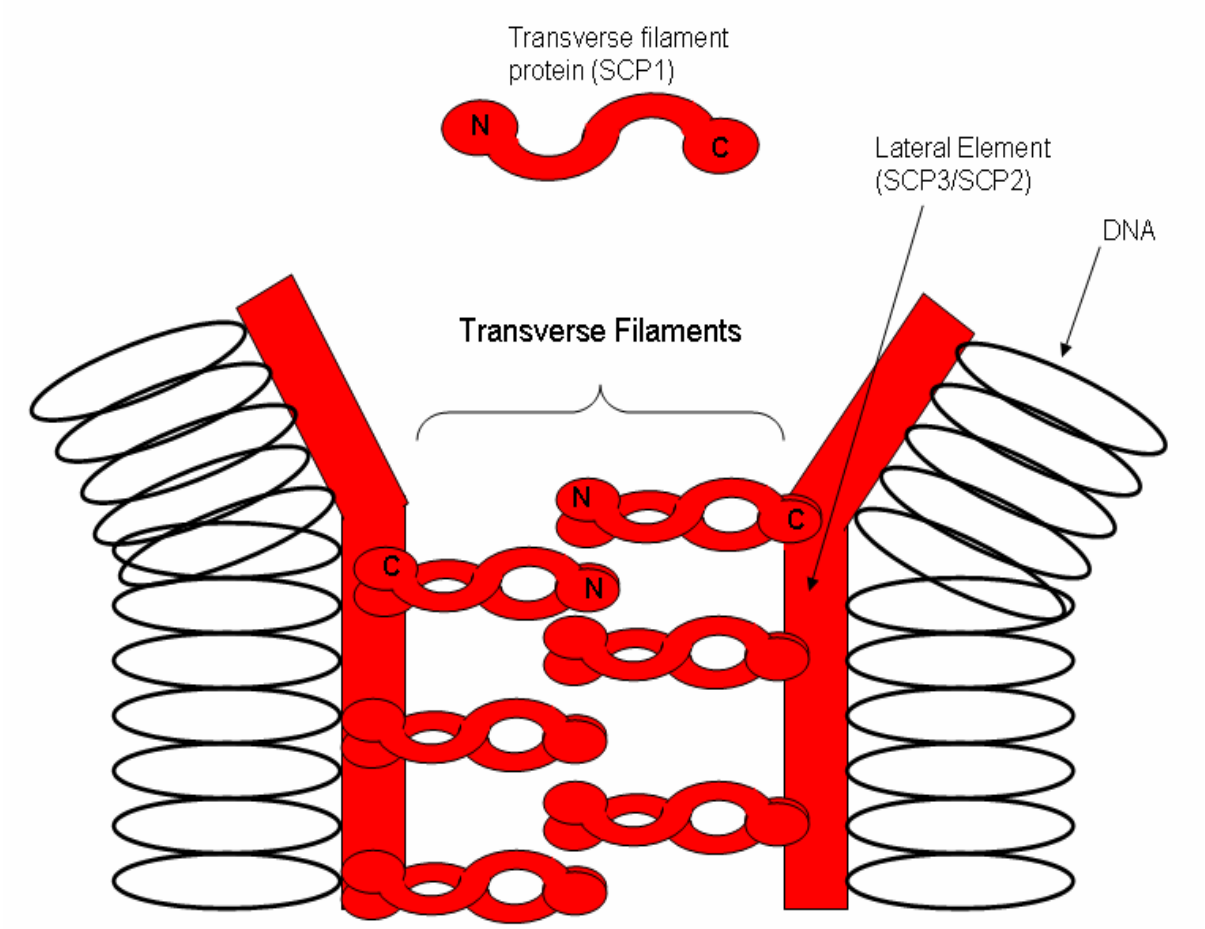

Figure 1.3: Structure of the synaptonemal complex. The transverse filament protein in humans, SYCP1, has a central region rich in coiled-coils flanked by a globular C termini which embed in the transverse elements, and a globular N termini which forms the central of the synaptonemal complex. The lateral elements form along homologous chromosomes and are composed of SYCP3 in humans.

region allows for the formation of parallel SYCP1 dimers, while the globular C-termini embed in the lateral elements, and the N-termini form the center of the SC (reviewed in Page and Hawley, 2003; Figure 1.3). Other than the SC proteins 1, 2 and 3, few structural components of the SC have been identified. Recently, the FK506 binding protein (Fkbp6) was identified in mice as a component of the SC, binding to SYCP1 and co-localizing to sites of synapsis (Crackower *et al*., 2003).

The SC is highly conserved among eukaryotes, present in yeast, flys, worms and mammals; however, the temporal relationship between recombination and synapsis appears to vary among species (Brown *et al*., 2005). In some eukaryotes, such as *Drosophila* and *C. elegans* the DNA DSBs that are required for meiotic recombination appear only after a mature SC has formed between homologous chromosomes (McKim *et al*., 1998; Dernburg *et al*., 1998). However, in other eukaryotes, such as the yeast *S. cerevisiae*, the DSBs that initiate recombination appear prior to synapsis (Padmore *et al*., 1991). Similar to the pairing of chromosomes, humans and other mammals seem to follow the single-celled yeast model, with recombination initiating prior to the formation of the SC (Mahadevaiah *et al*., 2001; Brown *et al*, 2005; Lenzi *et al*., 2005).

1.1.4 Meiotic recombination

Meiotic recombination, or crossing-over, involves the reciprocal exchange of genetic material between homologous chromosomes during prophase I. Crossing-over during meiosis serves two functions: 1) the generation of genetic diversity, and 2) the generation of chiasmata which ensure the proper segregation of chromosomes. Chiasmata form at the sites of recombination and maintain the physical connection between the homologous chromosomes until they segregate into separate daughter cells. The process of recombination is complex; while many of the proteins involved in recombination have now been identified, their exact functions are only beginning to be understood.

1.1.4.1 Mechanism of recombination

During meiotic recombination chromosomes are broken by DSBs and rejoined with the homologous chromosome to result in a recombinant product. The number of DSBs far exceeds the number of crossovers, with many of the breaks being resolved without recombination. Recombination is initiated by the nuclease SPO11, which generates DSBs within one of the DNA molecules (Keeney *et al*., 1997; Figure 1.4, step 1). In mammals, failure to induce the DSBs results in a lack of recombination and synapsis, leading to a failure of meiosis and infertility (Baudat *et al*., 2000; Romanienko and Camerini-Otero, 2000). Once the DSBs are generated, 3' overhangs are created in the DNA through the action of an unknown exonuclease (Figure 1.4, step 2). These 3' overhangs undergo homology search and strand invasion into the homologous chromosome (Figure 1.4, step 3-4), through a process catalyzed by the proteins RAD51, DMC1 (Shinohara and Shinohara, 2004) and replication protein A (RPA) (Sung *et al*., 2003). This process is known as single end invasion and results in the formation of a D-loop structure which anneals to the single-stranded tail of the other side of the break (Figure 1.4, step 5). Further DNA synthesis results in the homologous chromosomes being connected by two DNA junctions, known as a double Holliday junction (Figure 1.4, step 6). The double Holliday junctions are resolved, becoming either a crossover or non-crossover (Figure 1.4, step 7), by several DNA-mismatch repair proteins, including the mutS homologs MSH4, MSH5 (de Vries *et al*., 1999; Kneitz *et al*., 2000) and the mutL homologues MLH1 and MLH3 (Hunter and Borts, 1997).

1.1.4.2 Distribution of crossovers

Crossovers are distributed non-randomly across chromosomes, and there appear to be regions of the genome in which recombination is more likely to occur. Early cytological studies revealed several trends in the distribution of meiotic recombination in humans: 1) most chromosomes show at least one crossover per chromosome arm; 2) crossovers are very rare around the centromeres of chromosomes; 3) crossovers are preferentially located distally; 4) when two crossovers are present on the same chromosome arm they do not occur close to one another (interference); and 5) there are significant differences in the frequency and distribution

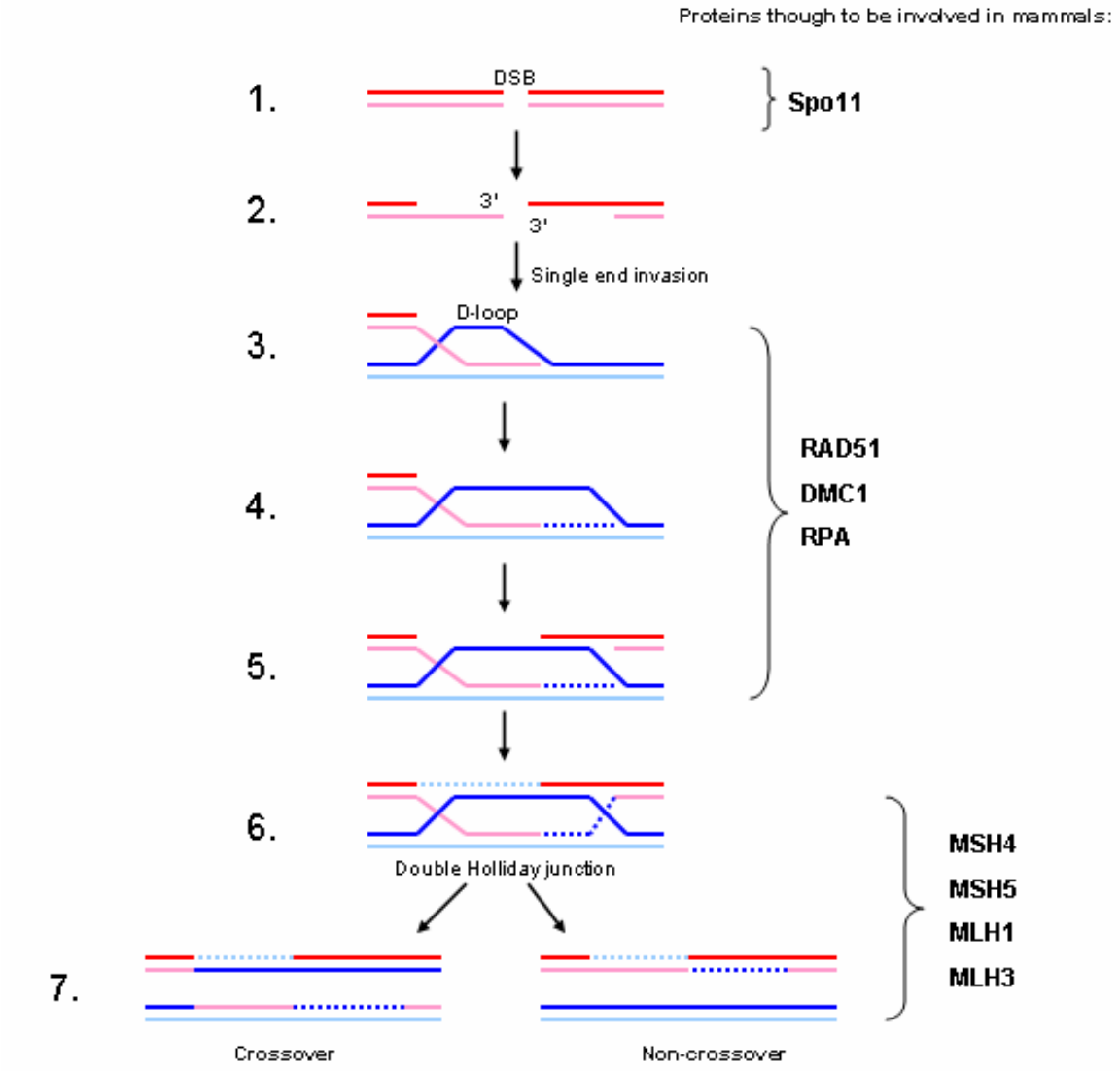

Figure 1.3: Mechanism of meiotic recombination through repair of a double strand breaks (DSB). Double strand breaks (DSBs) are initiated (step 1) and an exonuclease generates 3' overhangs (step 2) which undergo homology search, single end invasion and formation of the D-loop (steps 3-5). A double Holliday junction is formed (step 6) and resolved as either a crossover or non-crossover (step 7).

of crossovers between the sexes (Laurie and Hulten, 1985a; Laurie and Hulten, 1985b; Tease *et al*., 2002).

The position of crossovers appears to be influenced by several chromosomal factors, including the centromeres and telomeres of chromosomes and the SC (reviewed in Lynn *et al*., 2004). Crossovers are rarely observed near the centromeres in males (Lynn *et al*., 2002) and females (Tease *et al*., 2002), which may be due to the high level of centromeric heterochromatin (Choo, 1998). In males, crossovers occur frequently in the subtelomeric regions of chromsomes, whereas in females such crossovers are less frequent (Blouin *et al*., 1995; Badge *et al*., 2000). The differences in telomeric recombination between the sexes may be due to differences in sites of synaptic initiation. In males synapsis is intitated at subtelomeric regions (Brown *et al*., 2005), whereas in females interstitial synaptic intitation sites are common (Bojko *et al*., 1983). Thus, it has been hypothesized that a subset of synaptic initiation sites may be converted into crossovers (Brown *et al*., 2005). The structure of the SC itself has also been implicated in the distribution of recombination events. Chromosomes displaying SCs with discontinuities (gaps) or incomplete synapsis (splits) appear to have more distally located crossovers, which become more distal as the length of the gap/split increases (Sun *et al*., 2005a). Recent experiments have also used high resolution techniques to identify small regions of the genome that have a high probability of displaying crossovers, known as recombination hotspots. In yeast, recombination hotspots are often found near the promoter regions of genes (Wu and Lichten, 1994). However, studies on human sperm (Jeffreys *et al*., 1998; Jeffreys *et al*., 2001; Schneider *et al*., 2002) have suggested that recombination hotspots are clustered within small (1-2 kilobase) regions, and can be located throughout the genome with no obvious sequence similarities (reviewed in Kauppi *et al*., 2004).

1.1.5 Inactivation of meiotic chromosomes

In mammals, sex chromosome inactivation occurs in female somatic cells where it plays an essential role in achieving dosage compensation between the sexes through the random inactivation of one of the X chromosomes. However, another form of sex chromosome inactivation also occurs during male gametogenesis. This process, known as meiotic sex chromosome inactivation (MSCI), results in the X and Y chromosome being transcriptionally silenced upon entering prophase I. Unlike the autosomal chromosomes, homologous pairing of

the sex chromosomes during prophase I is limited to small regions at the distal ends of the chromosomes, known as the pseudoautosomal regions (PAR). The rest of the X and Y chromosome remain unpaired, and are converted into a visually distinct domain of heterochromatin, known as the XY- or sex-body. This XY-body is characterized by the recruitment of numerous chromatin remodeling proteins and a lack of RNA synthesis.

1.1.5.1 Mechanism and timing of MSCI

The mechanism of MSCI appears to be distinct from that of X chromosome inactivation (XCI) in female somatic cells. In females, XCI is initiated by a functional RNA known as the *inactive X specific transcript* (*XIST*) which is transcribed from and localizes along the inactivated X chromosome. *XIST* recruits numerous chromatin remodeling proteins, which induce heterochromatinizaton of the X chromosome through changes in methylation, ubiquitylation and deacetylation of histones (reviewed in Chow *et al.,* 2005). In male mice, *Xist* is expressed exclusively in the testis (McCarrey and Dilworth, 1992) and localizes specifically along the sex chromosomes (Ayoub *et al*., 1997), suggesting that it too plays a role in MSCI. However, studies on mice have shown that MSCI proceeds even in the absence of a functional *Xist*, suggesting that MSCI is regulated by a different mechanism than that which regulates XCI in females (McCarrey *et al*., 2002).

During the leptotene and zygotene stages of prophase I the sex chromosomes remain transcriptionally active (Turner *et al*, 2004). At leptotene, H2AX (histone family, member X) is rapidly phosphorylated to form γH2AX in response to the DNA DSBs that are initiated along all chromosomes for meiotic recombination (Mahadevaiah *et al*., 2001). At the zygotene-pachytene transition the breast cancer 1, early onset (BRCA1) protein coats the sex chromosomes which recruits the ataxia telangiectasia and Rad3 relative (ATR) protein (Turner *et al*., 2004). ATR localizes along the sex chromosomes, where it phosphoryates H2AX, leading to a second round of H2AX phosphorylation that is located exclusively on the sex chromosomes and initiates MSCI (Turner *et al*., 2004). Following the phosphorylation of H2AX, several further chromatin modifications are initiated, including: ubiquitylation of H2A (Baarends *et al*., 1999), deacetylation of histones H3 and H4 (Khallil *et al*., 2004), dimethylation of histone H3 (Khallil

et al., 2004), and sumoylation of an unidentified target (Vigodner and Morris, 2005). At the diplotene stage BRCA1, ATR and γH2AX are lost from the sex chromosomes, but the other chromatin modifications persist and the sex chromosomes remain inactive through diplotene and the two successive meiotic division (MI and MII). Recent studies have suggested that transcriptional inactivity persists beyond meiosis into the spermatids and mature sperm in a process called post-meiotic sex chromosome repression (PSCR) (Namekawa *et al*., 2006). This persisted inactivation may also explain the presence of an imprinted paternal X chromosome in the early embryo (Huynh and Lee, 2003). However, there is now evidence that some genes on the X and Y chromosome are reactivated in post-meiotic cells (Mueller *et al*., 2008). Interestingly, Mueller *et al*. (2008) found that only a small percentage of single-copy X-linked genes are reactivated in spermatids, whereas multi-copy X-linked genes exhibit similar expression patterns as autosomal genes. Thus, Mueller *et al*. (2008) have suggested that the amplification of X-linked genes may have evolved in order to compensate for the repressive chromatin environment from PSCR.

1.1.5.2 Meiotic silencing of unsynapsed chromatin

There is now accumulating evidence that the transcriptional silencing of unpaired meiotic chromosomes may not be limited to the sex chromosomes. Studies on mice that carry reciprocal translocations have shown that unpaired regions of the autosomal chromosomes are transcriptionally silenced during meiosis and display the hallmarks of MSCI, including BRCA1, ATR and γH2AX localization (Baarends *et al*., 2005; Turner *et al*., 2005). Thus, MSCI may be the result of a more generalized mechanism known as meiotic silencing of unsynapsed chromatin (MSUC) that is not specific to the sex chromosomes. This process does not appear to be limited to mammals, as similar processes were previously described in the yeast *Neurosporra crassa* (Shiu *et al*., 2001) and the worm *C. elegans* (Bean *et al*., 2004). Similarly, while autosomal chromosomes may be inactivated if unpaired, it also appears that the sex chromosomes can remain transcriptionally active during meiosis if they undergo homologous synapsis. For example, studies on XYY mice have shown that if the two Y chromosomes form a bivalent they will be negative for γH2AX and the genes on the Y chromosomes will escape MSCI (Turner *et al*., 2006). However, the expression of genes on the Y chromosome during meiosis may be

lethal to cells, as these configurations do not reach late pachytene. Rather, in XYY mice only configurations that lead to all three sex chromosomes being inactivated are able to proceed through meiosis (Turner *et al*., 2006). Furthermore, studies on XO female mice have suggested that MSCI may not be limited to males. In XO female mice the single X chromosome has no homolog to pair with and is subsequently silenced, leading to meiotic arrest and infertility (Baarends *et al*., 2005; Turner *et al*., 2005). However, it is has been suggested that the single X chromosome can fold over onto itself and undergo non-homologous self-synapsis, which prevents the X chromosome from being transcriptionally silenced (Turner *et al*., 2005). Together, these results suggest that asynapsis is the driving force behind the silencing of chromosome regions during meiosis, and may not be limited to males or to the sex chromosomes.

While the mechanisms of MSCI and MSUC are beginning to be understood, the functional and evolutionary importance of these processes is unclear. Silencing of the sex chromosomes may be necessary to prevent recombination between non-homologous regions of the sex chromosomes (McKee and Handel, 1993), to prevent detection of the unpaired regions of the sex chromosomes by meiotic checkpoints (Odorisio *et al*., 1998), or to prevent the transcription of sex-linked genes that may be lethal to meiotic cells (Lifschytz and Lindsley, 1972). However, given that meiotic silencing appears to be an ancient process, predating multicellular organisms, it is important to consider its role in genome evolution. It has been suggested that meiotic silencing may have originated as a mechanism to prevent the expression of novel insertions, such as transposable elements, during meiosis without affecting the expression of adjacent genes that may be necessary for meiosis (Kelly and Aramayo, 2007). Furthermore, if a genome were to evolve significant differences in genes required for meiotic progression, these genes would be silenced, leading to meiotic arrest and infertility. Thus, meiotic silencing may also play an important role in speciation by preventing species that are too genetically different from producing fertile offspring (Kelly and Aramayo, 2007). Furthermore, if differences were to arise in genes not required for meiosis, silencing of these genes may not be fatal to meiotic cells. Thus, such a mechanism would still have allowed for the duplication of genes required for development, which is though to have played an important role in generating evolutionary complexity.

1.1.6 After prophase I: segregation of chromosomes

MI is characterized by the segregation of homologous chromosomes, while MII is characterized by the segregation of sister chromatids. While chiastmata are necessary for tethering homologous chromosomes, cohesins are necessary for joining sister chromatids and preventing the premature separation of sister chromatids, which will result in aneuploidy (Hoque and Ishikawa, 2002). During metaphase I the homologous chromosomes align along the equatorial plate where microtubles from the centrioles attach the kinetochores on the homologous chromosomes. Sister kinetochores must attach to microtubules arising from the same spindle poles, known as monopolar attachment, to ensure that homologous chromosomes segregate to separate daughter cells (Watanabe, 2004). During anaphase I there is a breakdown in chromatid cohesion along chromosome arms, while centromeric cohesion persists, preventing the separation of sister chromatids (Riedel *et al*., 2006). The microtubes then shorten, pulling the homologous chromosomes to separate daughter cells. Finally, during telophase I the microtubules disappear and new nuclear membranes form around each haploid set of chromosomes. Cytokinesis occurs in which the cell membranes are separated, forming two haploid daughter cells. Each cell now contains half the number of chromosomes, with each chromosome consisting of two chromatids. At metaphase II the chromosomes line up along the equatorial plate, and during anaphase II the centromeric cohesion between sister chromatids is released, leading to the segregation of sister chromatids to separate daughter cells.

1.1.7 Meiotic checkpoints

At least two checkpoints that monitor the integrity of key meiotic events have been proposed to exist. The first proposed checkpoint, known as the pachytene checkpoint, or meiotic recombination checkpoint, is thought to prevent the exit of cells from the pachytene stage if synapsis or meiotic recombination is incomplete (Odorisio *et al*., 1998; Roeder and Bailis, 2000). This pachytene checkpoint was proposed after meiotic arrest at the pachytene stage and apoptosis was observed in mice with mutations in genes involved in recombination and synapsis (reviewed in Hunt and Hassold, 2002). The pachytene checkpoint is thought to be analogous to the DNA damage checkpoint that operates in somatic cells as the two checkpoints involve many

of the same proteins (Roeder and Bailis, 2000). A second checkpoint, known as the spindle assembly checkpoint (SAC), has been proposed to operate at metaphase I. The SAC is thought to block the transition from metaphase I to anaphase I when a defective spindle is detected or if chromosomes are misaligned on the equatorial plate (Eaker *et al*., 2001). The SAC in meiosis is thought to be analogous to the well characterized SAC that monitors the tension exerted on the kinetochores by microtubles during mitosis (Zhou *et al*., 2002).

1.1.8 Sex differences in meiosis

In addition to the silencing of the sex chromosomes during male meiosis, which was previously discussed, there are several other differences between male and female meioses, the most obvious of which is the timing. In males, meiosis begins after puberty and proceeds from start to finish without interruption. In females, meiosis is initiated during fetal development; oocytes then enter meiotic arrest part-way through MI, and finally complete the first division upon ovulation. Cells enter a second meiotic arrest part-way through MII, and the second division is only completed upon fertilization of the oocyte by a sperm (see Figure 1.5). There are also significant differences in recombination rates between the sexes. Male spermatocytes contain approximately 50 crossovers per cell, while in comparison the female oocytes contain approximately 70 crossovers per cell (Tease and Hulten, 2004). Recombination in XY sex-reversed female mice mimics that of an XX female, suggesting that rates of recombination are not dictated by sex-chromosome genotypes, but rather the environment in which the germ cells develop (Lynn *et al*., 2005). There appears to be a correlation between the length of the SC and the overall number of meiotic exchanges, with longer SCs associated with more crossovers (Lynn *et al*., 2002). Indeed, the SCs of females appear to be significantly longer than those of the male (Tease *et al*., 2004). It is possible that SC length may influence rates of recombination; however, the lower rates of recombination and shorter SCs in the male may be a reflection of the more condensed male chromosomes, which are less accessible for the DNA DSBs required to initiate recombination. Nevertheless, the exact mechanisms that contribute to the differences in recombination between the sexes remain unknown.

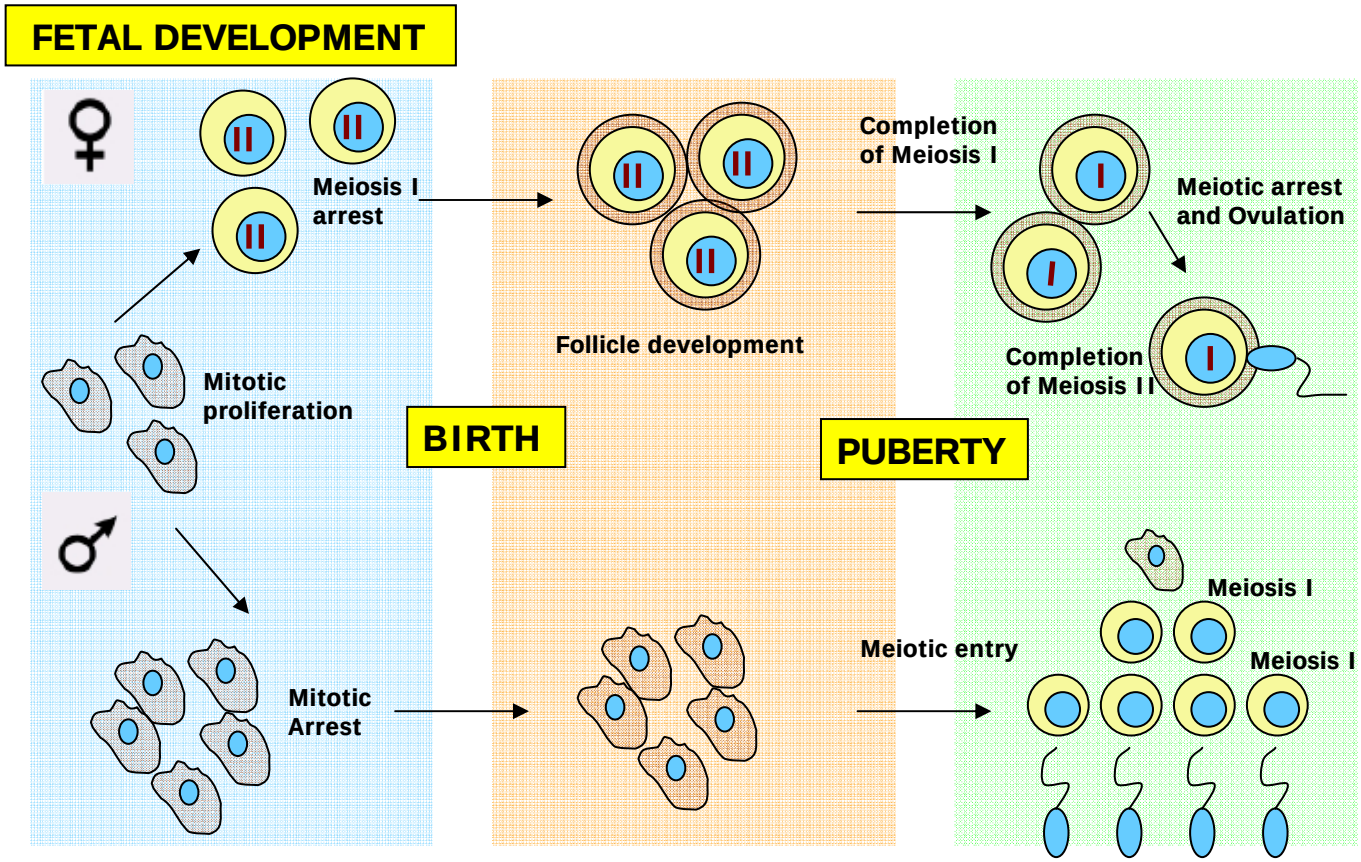

Figure 1.5: Sex difference in the progression of meiosis. During fetal development the germ cells in both males and females undergo mitotic divisions. In males, these mitotic divions are followed by a long period of mitotic arrest. At puberty, the spermatogonia resume miotitic divions, and proceed through spermatogenesis, consisting of meiosis and spermiogenesis. In males spermatogenesis continues throughout life. In females, after a short period of mitotic divisions the germ cells enter meiosis I. Prior to birth the germ cells enter a period of meiotic arrest at the diplotene stage, which persists until just prior to the individual oocyte is ovulated. Ovulated oocytes enter a second round of meiotic arrest, which persists until fertilization when meiosis II in the oocyte is completed.

There is now mounting evidence that there are differences between the sexes in the checkpoints that monitor meiosis, with the female meiosis being subject to less stringent checkpoints (Hunt and Hassold, 2002). Gene knock-out mice carrying mutations in meiotic genes have shown that the same mutations will often cause infertility in the male, while oocytes continue to progress through meiosis and the females remain fertile. For example, deletion of the gene *Scp3* results in prophase arrest and infertility in the male mouse (Yuan *et al*., 2000). The same *Scp3* mutation in the female mice does not cause infertility, although the mice show reduced litter sizes and an increase in chromosomal abnormalities in the offspring (Yuan *et al*., 2002). Likewise, mutations in the SC-associated protein Fkbp6 results in prophase arrest in the spermatocyte, whereas the females are fertile (Crackower *et al*., 2003). Thus, mutations in meiotic genes seem to lead to spermatogenic arrest and infertility in male mice; whereas when females are faced with the same mutation meiosis proceeds further, but may result in an increase in aneuploid oocytes.

1.1.9 Environmental effects on meiosis

There is little information on the environmental influences on mammalian meiosis. However, studies by Hunt *et al*. (2003) on the effects of low does of bisphenol A (BPA), a component of polycarbonate plastics, has provided the first compelling evidence that a chemical exposure can negatively affect meiosis in mammals. In 1998, Hunt and colleagues were performing meiotic studies on mouse oocytes when they observed a sudden spike in the frequency of aneuploid oocytes. Further analysis showed that these changes coincided with the use of a harsh alkaline detergent to clean the caging materials and water bottles, which were both composed of polycarbonate plastics. When the damaged caging material was removed they observed a subsequent decrease in aneuploid oocytes, suggesting that exposure to BPA may contribute to meiotic defects and an increased risk of aneuploidy (Hunt *et al*., 2003). In a further study, they treated pregnant mice with BPA during mid-gestation to investigate the effect of BPA on the developing ovary (Susiarjo *et al*., 2007). Fetal oocytes that were exposed to BPA displayed synaptic abnormalities, as well as an increase in meiotic crossovers and an altered distribution of crossovers. Furthermore, when mice exposed to BPA in utero grew-up they displayed an increase in aneuploid oocytes and embryos (Susiarjo *et al*., 2007). Thus, BPA

provides a compelling example of a chemical exposure that can have detrimental effects on meiosis. Further studies will be necessary to investigate the effects of other chemicals and to determine if these chemical have gender-specific effects on meiosis.

1.1.10 Origins of aneuploidy

Aneuploidy refers to the condition in which the number of chromosomes in a cell is not a multiple of the haploid set; in other words, it is a chromosomal state in which there are either extra or missing chromosomes. Aneuploidy can arise from errors in chromosome or chromatid segregation during MI, MII or mitotic divisions, known as nondisjunciton (Figure 1.6). Nondisjunction is relatively common in human meiosis, with an estimated 5% of pregnancies being aneuploid (Hassold and Hunt, 2001). The vast majority of these pregnancies will not survive to term. Aneuploidy is detected in approximately 35% of spontaneous abortions and 4% of stillbirths, making it the leading genetic cause of pregnancy loss (Hassold and Hunt, 2001). Aneuploidy can occur with any of the chromosomes; however, only a few of the trisomies (gain of a whole chromosome) and a single monosomy (loss of a whole chromosome) are compatible with survival. These include: trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome), trisomy 21 (Down syndrome) and sex chromosome aneuploidies such as XXY (Klinefelter syndrome), XYY and monosomy X (Turner syndrome). Aneuploidy occurs in approximately 0.3% of liveborns, making it a leading cause of congenital malformations and the most common known genetic cause of mental retardation (Hassold *et al*., 1996).

The introduction of techniques using polymorphic DNA markers to identify the parental origin of chromosomal abnormalities has provided a wealth of information on the origins of human aneuploidies (Table 1.1). These studies have shown that the vast majority of aneuploidies are the result of errors during the maternal MI (Hassold *et al*., 1996). However, there also appear to be chromosome-specific patterns of non-disjunction: some trisomies, such as those involving chromosomes 16 and 22 almost always originate during MI, whereas others such as trisomy 18 originate more frequently during MII (Bugge *et al*., 1998). Certain chromosomes, such as chromosomes 7 and 8, appear to be at a high risk for nondisjunction during post-zygotic mitotic (PZM) divisions (Table 1.1). While autosomal trisomies rarely originate during the paternal

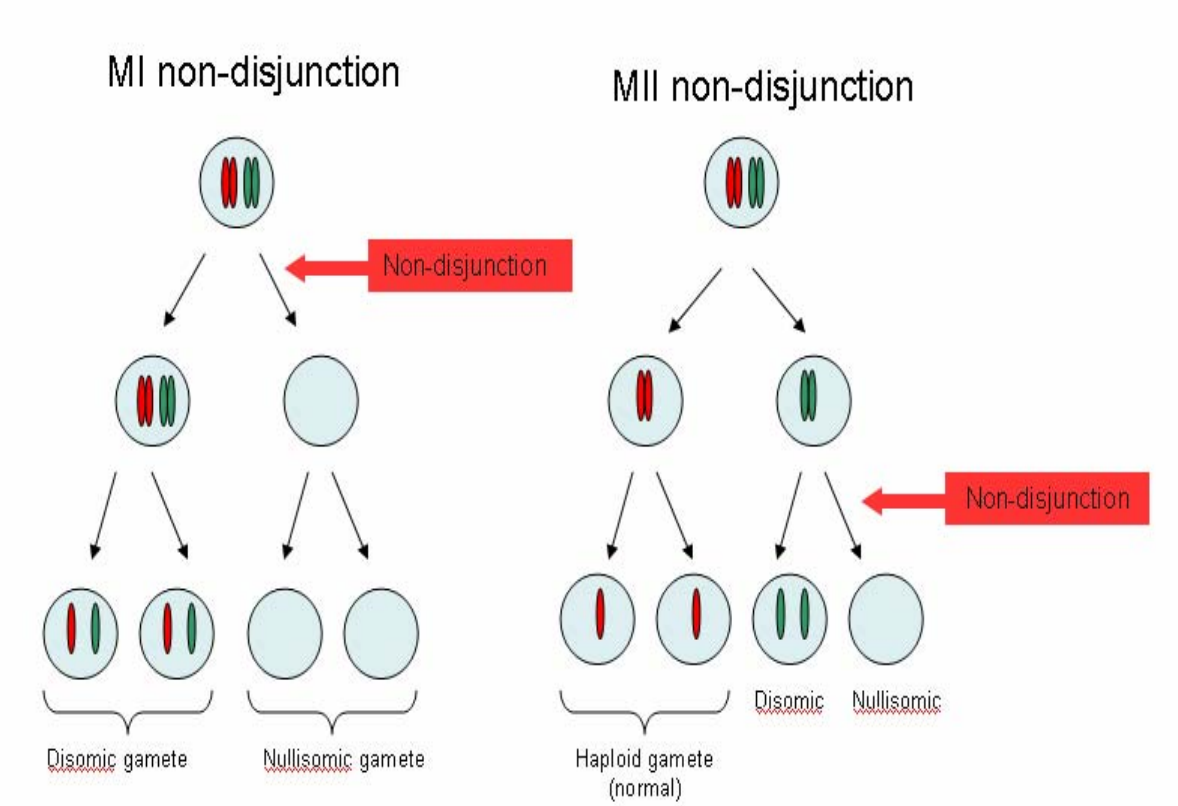

Figure 1.6: Production of aneuploid gametes through meiotic non-disjunction. Meiosis I (MI) non-disjunction produces disomic (extra chromosome) and nullisomic (missing a chromosome) gametes, whereas MII non-disjunction produces normal haploid, disomic and nullisomic gametes.

meiosis, sex chromosome aneuploidies are frequently of paternal origin. Paternal errors account for approximately 50% of XXY males (MacDonald *et al*., 1994) and 70-80% of 45,X females (Jacobs *et al*., 1997). Increasing maternal age has been linked with an increased risk of meiotic nondisjunction. This increased risk seems to affect most chromosomes (Hassold *et al*., 1984) and may be caused by deterioration in the chiasmata or spindles that tether the chromosomes during the period of meiotic arrest that occurs in between fetal development and the ovulation of each oocyte (Lamb *et al*., 1996). Whether errors in the paternal meiosis increase with age remains unclear (Buwe *et al*., 2005).

Table 1.1: Origins of human aneuploidies[a]

		Maternal		Paternal			
Aneuploidy	*n*	MI (%)	MII (%)	MI (%)	MII (%)	PZM (%)	References
+2	18	53.4	13.3	27.8	0	5.6	Zaragoza *et al*., 1998
+7	14	17.2	25.7	0	0	57.1	Zaragoza *et al*., 1998
+8	12	50.0[b]		0	0	50.0	James and Jacobs, 1996 ; Karadima *et al*., 1998
+13	74	56.6	33.9	2.7	5.4	1.4	Hall *et al*., 2007a
+14	26	36.5	36.5	0	19.2	7.7	Hall *et al*., 2007b
+15	34	76.3	9.0	0	14.7	0	Zaragoza *et al*., 1998
+16	104	100.0	0	0	0	0	Hassold and Hunt, 2001
+18	150	33.3	58.7	0	0	8.0	Bugge *et al*., 1998
+21	671	67.5	22.1	3.9	0	19.2	Hassold and Sherman. 2000
+22	130	86.4	10.0	1.8	0	1.8	Hall *et al*., 2007b
XXX	46	63.0	17.4	0	0	19.6	MacDonald *et al*., 1994
XXY	224	23.7	14.2	50.9	0	7.7	Thomas and Hassold, 2003
XYY	19	0	0	0	84.2	15.8	Robinson *et al*, 1999
XO[c]	93	25.8		74.2			Jacobs *et al*., 1997

[a]Table adapted from Hall *et al*., 2007b

[b]did not distinguish between MI and MII

[c]cannot distinguish between MI and MII in XO females; parental origin only was determined

1.2 MALE INFERTILITY AND INTRACYTOPLASMIC SPERM INJECTION

1.2.1 Overview of male infertility

Infertility, which is defined as the inability to conceive after one year of regular unprotected intercourse, is a major health concern, affecting approximately 10-15% of couples. The causes of infertility can be divided into four catergories: 1) ~35% female factor; 2) ~30% male factor; 3) ~20% combined factors; 4) ~15% unexplained infertility (Crosignani and Rubin, 1996). Male infertility can be further divided into four categories: 1) obstruction or blockage of the reproductive tract; 2) inflammation or immunological dysfunction; 3) sexual disorders, such as impotence; and the most common cause, 4) defective sperm production (Namiki, 2000). Semen analyses and testicular biopsies are routinely performed to assess the cause of infertility and treatment options.

1.2.1.1 Semen parameters

Semen analyses can provide important information on the cause of infertility, the likelihood of conceiving a natural pregnancy, and the treatment options of infertile men. The World Health Organization (WHO) has developed a set of criteria for evaluating the semen of infertile men, based on three characteristics: sperm concentration, motility and morphology (WHO, 1999). Concentration is reported as the number of sperm per milliliter of seminal fluid. In assessing motility, individual sperm are graded as displaying either: a) rapid progressive motility ($\geq$25 μm/s at 37^{o}C); b) slow progressive motility; c) nonprogressive motility (<5 μm/s); or d) immotility (WHO, 1999). Sperm morphology classifies sperm into four categories of defects: a) head defects; b) neck and midpiece defects; c) tail defects; and d) the presence of cytoplasmic droplets in the midpiece (WHO, 1999). Based on semen parameters, sperm quality can be diagnosed as: *oligozoospermia*, low sperm concentration; *asthenozoospermia*, low sperm motility; or *teratozoospermia*, abnormal sperm morphology (Table 1.2). These abnormal sperm parameters can be present in isolation or in a combination, such as *oligoasthenoteratozoospermia (OAT)*, which is a combination of low concentration, low motility and abnormal sperm morphology. A complete absence of sperm in the ejaculate is classified as *azoospermia*, which

can be further subdivided into non-obstructive azoospermia (NOA) or obstructive azoospermia (OA).

Table 1.2 World Health Organization (WHO) diagnoses of semen parameters (WHO, 1999)

Type of male infertilty	Sperm parameters		
	Concentration (10^6/ml)	Motility (%)	Normal morphology (%)
Oligozoospermia	<20	Normal	Normal
Asthenozoospermia	Normal	<50%	Normal
Teratozoospermia	Normal	Normal	<30%
Oligoastenoteratozoospermia (OAT)	<20	<50%	<30%
Azoospermia	No sperm in the ejaculate		
Aspermia	No ejaculate		
Normal sperm parameters	≥20	≥50%	≥30%

1.2.1.2 Histological evaluation of the testes

There are four commonly used descriptive classifications for spermatogenesis in infertile men: normal spermatogenesis, hypospermatogenesis, germ cell arrest (GCA), and Sertoli-cell only syndrome (SCOS) (McLachlan *et al*., 2007). Hypospermatogenesis is diagnosed when all stages of spermatogenesis are present but reduced to a varying degree. GCA, or maturation arrest, is diagnosed when there is a total arrest at a particular stage of spermatogenesis, most frequently at the level of the spermatogonia or primary spermtocyte stages. SCOS is diagnosed when there are no seminiferous tubules containing germ cells. When a semen analysis reveals azoospermia, a testicular biopsy is often required to distinguish NOA from OA and predict the recovery of sperm for assisted reproductive therapies. The clinical features of OA include normal testicular volume, serum FSH and inhibin; however, sometimes a distinction between OA and NOA cannot be made based on anatomy or endocrine factors. Therefore confirming normal spermatogenesis in OA patients is often performed prior to undertaking expensive and invasive therapies such as reconstruction surgery to repair the blockage (McLachlan *et al*., 2007). Histological evaluations of the testes are also strong predictors of sperm retrieval for assisted reproduction. Diagnoses of SCOS and GCA are associated with poor outlooks for successful

testicular sperm extraction (TESE), whereas a diagnosis of hypospermatogenesis is a good indicator for a successful TESE (McLachlan *et al*., 2007). Nevertheless, even with a diagnosis of GCA focal spermatogenesis may be present; in which case, performing a testicular microdissection may allow for sperm retrieval from the testes.

1.2.2 Genetic causes of male infertility

Defective sperm production is the most common cause of male infertility, with more than 50% of infertile men suffering from idiopathic spermatogenic defects (Namiki, 2000). Genetic factors have been suspected to play a role in impaired spermatogenesis, however only a few of these factors have been identified. Currently the known genetic causes of male infertility are limited to somatic chromosomal abnormalities, cystic fibrosis, Y chromosome microdeletions, and possibly mutations in critical meiotic genes.

1.2.2.1 Chromosomal abnormalities

Chromosomal abnormalities are closely linked with infertility, with an incidence of 5.8% in the azoospermic and oligozoospermic population, compared to 0.5% in the general population (Johnson, 1998). Sex chromosome abnormalities are most frequent in the azoospermic and oligozoospermic population with an incidence of 4.2% (vs. 0.14% in the general population), while autosomal abnormalities are found in 1.5% of the infertile population (vs. 0.25% in the general population) (Johnson, 1998). There are two main classes of chromosomal abnormalities: numerical and structural. Numerical sex chromosome abnormalities such as the gain or loss of an entire chromosome (aneuploidy) are associated with infertility. Structural abnormalities such as translocations, which involve the rearrangement of chromosome parts between nonhomologous chromosomes, as well as inversions and deletions of chromosomal regions, are associated with infertility.

Numerical sex chromosome abnormalities, such as Klinefelter syndrome (47,XXY) and 47,XYY are associated with male infertility. Men with Klinefelter syndrome typically display testicular atrophy, a depletion of germ cells and are usually sterile. Germ cell death may be the

result of a lethal gene dosage due to the presence of an extra X chromosome (Burgoyne, 1978). Thus, Klinefelter men who do display sperm production are likely to have 46,XY/47,XXY gonadal mosaicism, in which the viable XY germ cells allow for the production of some sperm (Steinberger, 1965). The risk of infertility among 47,XYY men is slightly increased, although the majority of these men are fertile (Nakamura *et al*., 2001). Structural abnormalities, such as reciprocal and Robertsonian translocations also occur more frequently in the infertile population (Jaffe and Oates, 1996). Robertsonian translocations are chromosomal rearrangements in which two acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) fuse; whereas, reciprocal translocations involve the mutual exchange of chromosome parts between two nonhomologous chromosomes. A recent review of the chromosomal constitution of males undergoing intracytoplasmic sperm injection, found that 0.98% were carriers of reciprocal translocations (11.5 times greater than the general population), and 0.95% were carriers of Robertsonian translocations (10.3 times greater than the general population) (Morel *et al*., 2004). Carriers of structural abnormalities produce higher levels of chromosomally abnormal gametes, and thus are at a higher risk of miscarriage (Benet *et al*., 2005); however translocation can also directly impair spermatogenesis by disrupting the fidelity of chromosome pairing during meiosis (Oliver-Bonet *et al*., 2005).

1.2.2.2 *Cystic fibrosis transmembrane regulator* (*CFTR*) mutations

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the *cystic fibrosis transmembrane regulator* (*CFTR*) gene. The clinical manifestations of CF include progressive lung disease and pancreatic insufficiency, and the median life expectancy is in the mid-30's (Gazvani and Lewis-Jones, 2006). Almost all men with CF display a congenital bilateral absence of the vas deferens (CBAVD), which leads to OA and an absence of sperm in the ejaculate. However, carrying only one mutated copy of the *CFTR* gene can result in infertility without the clinical phenotype of CF, as 60% of men with CBAVD are found to be carriers of only one *CFTR* mutation (de Braekeller and Ferec, 1996). Genetic screening of couples is strongly recommended prior to seeking assisted reproduction, as *CFTR* mutations can be transmitted to the offspring (Gazvani and Lewis-Jones, 2006).

1.2.2.3 Y chromosome microdeletions

The Y chromosome is essential for both sex determination and normal spermatogenesis. In 1976 it was reported that deletions of the distal Yq11 region resulted in infertility, providing the first evidence for the existence of a spermatogenesis-controlling locus on Yq11, known as the azoospermic factor (AZF) (Tiepolo and Zuffardi, 1976). Three non-overlapping distinct intervals have now been identified in the AZF region, and are associated with varying degrees of infertility: AZFa, AZFb and AZFc (Vogt *et al*., 1996). An AZFd interval has been suggested to lie between AZFb and AZFc (Kent-First *et al*., 1999). Microdeletions in AZFa are associated with an absence of germ cells, or SCOS (Vogt *et al*., 1996). The candidate genes in the AZFa regions that cause infertility are the *dead box on the Y* (*DBY*) and *USP9Y* genes, as deletions of both of the genes results in SCOS (Foresta *et al*., 2000). Microdeletions in AZFb are associated with meiotic arrest at the spermatocyte stage, whereas deletions in AZFc are associated with impaired maturation of postmeiotic germ cells (Vogt *et al*., 1996). *DAZ* (*deleted in azoospermia*) was the first gene identified in the AZFc region that was thought to play a role in spermatogenesis (Reijo *et al*., 1995). Other genes in the AZFc that may be critical for spermatogenesis include the *testis transcript Y2* (*TTY2*) and *basic protein Y2* (*BPY2*) (Lahn and Page, 1997). Y chromosome microdeletions are the most frequent known genetic cause of severe oligozoospermia and azoospermia, present in 10-18% of these men (Krausz *et al*., 2003). The most frequently deleted region is AZFc, which accounts for approximately 60% of all AZF deletions. Deletions in AZFb, AZFb+AZFc, or AZFa + AZFb + AZFc account for 35% of cases, while deletions in AZFa account for only 5% of AZF deletions (Krausz *et al*., 2003).

1.2.2.4 Mutations in meiotic genes

The importance of meiotic genes for fertility has been displayed through gene knock-out mice, which display infertility when meiotic genes are mutated (reviewed in Hunt and Hassold, 2002). However, little is known about the prevalence of meiotic mutations in humans, and few genes have been studied. Miyamoto *et al*. (2003) screened 19 men with NOA and 75 normal fertile controls for mutations in the gene *SYCP3*, and identified two patients with a 1 basepair deletion that resulted in a premature stop codon and truncation of the C-terminal of the SYCP3

protein. However, screening for mutations in other meiotic genes has been less successful. A screening of 51 men with NOA failed to identify any mutations in the *FKBP6* gene, which is a component of the SC (Westerveld *et al*., 2005). Furthermore, the gene *SPO11*, which is involved in initiating meiotic recombination, was screened for mutations in 192 infertile men, and identified only 2 men who were heterozygous for missense mutations (a change in an amino acid of the protein product) (Christensen *et al*., 2005). Similarly, Zhang *et al*. (2008) observed no mutations in the *H2AX* gene in 302 patients with either azoospermia or severe oligozoospermia. Thus, while mutations in meiotic genes are a possible cause of infertility, further studies will be required to identify the mutations that have the most clinical relevance to male infertility.

1.2.3 Development of assisted reproductive technologies (ART)

The introduction of assisted reproductive technologies (ARTs) has revolutionized the treatment of infertility. The first *in vitro* fertilization (IVF) of an oocyte followed by embryo transfer was performed in 1978, and resulted in the birth of Louise Brown (Steptoe and Edwards, 1978). Since then, over two million children have been conceived world-wide through ART (Anderson *et al*., 2004). The first pregnancy achieved using ART in an infertile man was reported in 1985 when epididymal sperm was used for IVF from a man who had previously undergone a vasectomy (Temple-Smith *et al*., 1985). However, IVF proved to be of limited use for men with severe oligospermia (<5 million sperm/ml of semen) (Yovich and Stanger, 1984), and men with abnormal sperm morphology or motility (Tournaye, *et al*., 1992). Thus, at the end of the 1980's new techniques, such as partial zona dissection (PZD) and subzonal sperm injection (SUZI), were introduced to improve outcomes for infertile men. PZD was performed in conjunction with IVF, and involved creating a small hole in the zona pelucida of the oocyte to facilitate fertilization by the sperm. The SUZI technique involved the direct injection of one or several sperm into the perivitrilline space between the zona pelucida and the oocyte membrane. While both PZD and SUZI were able to improve outcomes relative to IVF, fertilization rates did not exceed 20-25% (Cohen *et al*., 1988; Ng *et al*., 1988). In 1992 the first successful application of intracytoplasmic sperm injection (ICSI) was performed. ICSI involves the microinjection of a single sperm directly into the cytoplasm of an oocyte. The introduction of ICSI was a

breakthrough in the treatment of male factor infertility, allowing men with severely compromised sperm parameters to achieve biological paternity (Palermo *et al*., 1992).

1.2.4 Use of ICSI to treat male factor infertility

ICSI can be applied with sperm retrieved from the epididymis and testes, making it useful for the treatment of obstructive azoospermia (OA). ICSI can also be used to treat non-obstructive azoospermia (NOA) if a sufficient number of sperm can be retrieved from testicular tissue. Epididymal sperm is obtained through microsurgical epididymal sperm aspiration (MESA), or percutaneous sperm aspiration (PESA), while testicular sperm is retrieved through a TESE. Ovarian stimulation and oocyte retrieval is the same as conventional IVF. After oocyte retrieval, only MII oocytes that display a polar body can be used for ICSI. Sperm are immobilized by crushing the tail with the injection pipette and then injected in an oocyte (Ma and Ho Yuen, 2001). The resulting embryos are cultured in vitro. Embryos are most often transferred to the uterus at day 3 after fertilization, but can be transferred up to the blastocyst stage (day 5 after fertilization). In Canada, usually 2-3 embryos are transferred, although this number often goes up as maternal age increases (Gunby *et al*., 2006).

1.2.5 Chromosomal abnormalities after ICSI

With the introduction of ICSI, several concerns were raised about the safety of the technique because it bypasses many of the physiological steps required for natural fertilization (Martin, 1996). Thus, there have been several studies that have used prenatal diagnosis to examine the chromosomal constitution of fetuses derived from ICSI (Table 1.3). The incidence of chromosomal abnormalities in pregnancies derived from ICSI is significantly higher than the incidence reported in the general population (~3.0% vs. ~0.9% in natural pregnancies; Table 1.3). A significant increase in both numerical autosomal (0.85% vs. 0.14% in natural pregnancies) and sex chromosome abnormalities (0.76% vs. 0.20% in natural pregnancies) have been reported after ICSI. While there does not appear to be an increase in *de novo* structural abnormalities, there is a significant increase in inherited structural chromosomal abnormalities (0.95% vs. 0.21% in natural pregnancies) after ICSI.

Table 1.3 Prenatal and postnatal diagnoses of chromosomal abnormalities in ICSI pregnancies

References		De novo abnormalities *f*% (*n*)			Inherited	
		Gonosomal	Autosomal		Structural	Total
	n	(numerical)	(numerical)	Structural	*f*% (*n*)	*f*% (*n*)
ICSI pregnancies						
Van Opstal *et al*., 1997	71	8.45 (6)	4.23 (3)	0	0	12.7 (9)
Loft *et al*., 1999	209	0	2.39 (5)	0.48 (1)	0.48 (1)	3.35 (8)
Wennerholm *et al*., 2000	149	0	0.67 (1)	0.67 (1)	1.34 (2)	2.68 (4)
Aboulghar *et al*., 2001	430	1.40 (6)	0.47 (2)	1.40 (6)[a]		3.26 (14)
Bonduelle *et al*., 2002	1586	0.63 (10)	0.50 (8)	0.44 (7)	1.39 (22)	2.96 (47)
Vernaeve *et al*., 2003	85	0	1.18 (1)	1.18 (1)	1.18 (1)	3.53 (3)
Samli *et al*., 2003	142	2.81 (4)	1.41 (2)	0	0	4.22 (6)
Jozwiak *et al*., 2004	1136	0.62 (7)	0.26 (3)	0.35 (4)	0.26 (3)	1.50 (17)
Gjeris *et al*, 2008	536	0.19 (1)	1.87 (10)	0.93 (5)	1.50 (8)	4.48 (24)
Ma *et al*., unpublished	256	0.30 (1)	1.60 (4)	1.2 (3)[a]		3.10 (8)
TOTAL	**4600**	**0.76 (35)**	**0.85 (39)**	**0.49 (19)[b]**	**0.95 (37)[b]**	**3.04 (140)**
Natural pregnancies						
Jacob *et al*., 1992[a]	56952	0.20 (116)	0.14 (82)	0.61 (149)		0.92 (347)
Jacob *et al*., 1992[c]	14677	n/a	n/a	0.46 (68)	0.21 (31)	n/a

[a]did not distinguish between inherited and *de novo* structural abnormalities
[b]total does not include studies that did not distinguish between inherited and *de novo* structural abnormalities
[b]only the frequencies of structural chromosomal abnormalities were reported

Several factors such as advanced maternal age of the mothers, increased chromosomal abnormalities in the sperm of the infertile fathers, and the invasiveness of the technique could increase the risk of chromosomal abnormalities in pregnancies after ICSI. It is well established that increasing maternal age is correlated with an increased risk of an aneuploid pregnancy. Some studies have found that an increased proportion of abnormalities resulted from mothers over 35 years-old (Loft *et al*., 1999), while others found no increase (Jozwiak *et al*., 2004). The injection procedure during ICSI could also damage the spindles and cytoskeleton, leading to MII or mitotic segregation errors (Terada *et al*., 2000). Finally, the increase in chromosomal abnormalities after ICSI could reflect an increase in chromosomal abnormalities in the sperm of the infertile fathers (Burrello *et al*., 2005). The developments in our understanding of the chromosomal content of human sperm will be discussed in the following section.

1.3 CYTOGENETICS OF MALE GERM CELLS

1.3.1 Sperm aneuploidy

1.3.1.1 Incidence of chromosomal abnormalities in sperm

Studying the chromosomal constitution of human sperm was not possible until the introduction of the sperm karyotyping technique developed by Rudak *et al.* (1978). This technique involves the fertilization of zona-free hamster oocytes with human sperm, followed by treatment with colchicine to arrest the cells at the metaphase stage. The oocytes are then fixed onto slides and the chromosomes are stained to allow for karyotyping of the sperm chromosomes. Sperm karyotyping allows for the identification of both structural and numerical chromosomal abnormalities within a human sperm. Early work on sperm karyotyping from normal men estimated that 1-4% of sperm carry numerical chromosomal abnormalities, while 4-9% of sperm carry structural chromosomal abnormalities, such as translocations, inversions, insertions and deletions that may have originated during spermatogenesis (Guttenbach *et al*., 1997). Sperm karyotyping studies found that chromosome 21 and the sex chromosomes were most at risk of aneuploidy (Martin and Rademaker, 1990; Templado *et al*., 1996). Because sperm karyptyping is an extremely time consuming and labour intensive technique, only 20,000 sperm from normal men have been karyotyped to date. Furthermore, in order for sperm karyptyping to be successful, a sperm must be capable of fertilizing an oocyte and must be present in sufficient numbers in the ejaculate. Thus, sperm karyotyping is of limited use for men with severe infertility who show either very low levels of sperm in the ejaculate or sperm that are incapable of naturally fertilizing an oocyte, such as immotile sperm.

More recently, a molecular cytogenetic technique called fluorescent in-situ hybridization (FISH) has been used for studying aneuploidy in human gametes and embyros. FISH involves the denaturation and subsequent hybridization of fluorochrome-labeled DNA probes to the target DNA. This technique allows for the identification of numerical chromosomal abnormalities in a cell, but cannot identify structural abnormalities. FISH on decondensed sperm nuclei was introduced as a method for studying numerical chromosomal abnormalities in sperm in much

larger sample sizes than were capable using sperm karyotyping (Williams *et al*., 1993). Furthermore, FISH is particularly useful for studying sperm from infertile men, as it does not require the sperm to fertilize an oocyte. The initial FISH studies examined sperm from normal men, and confirmed the initial studies using sperm karyotyping. Two main trends were observed from FISH analysis of sperm: 1) there is significant inter-individual variation in rates of sperm aneuploidy among men, and 2) chromosome 21, 22 and the sex chromosomes are more susceptible to errors during meiosis than the rest of the chromosomes (reviewed in Templado *et al*., 2005).

1.3.1.2 Sperm aneuploidy in infertile men

Constitutional chromosomal abnormalities, such as translocations and inversions, are a major cause of male infertility and it is well established that carriers face an increased risk of producing chromosomally unbalanced sperm (Balkan and Martin, 1983). However, there is now evidence that infertile men with normal karyoptypes may also produce elevated rates of chromosomally abnormal sperm. Sperm from infertile men were first studied by Moosani *et al*. (1995) using both sperm karyotyping and FISH on men with severe oligozoospermia. Using both methods, Moosani *et al* (1995) found an increase in numerical chromosomal abnormalities in the sperm of the infertile men when compared to fertile controls. This increase in aneuploidy in the sperm of men with severe oligozoospermia is further supported by observations of those in other laboratories (Pang *et al*., 1999; Nishikawa *et al*., 2000; Tang *et al*., 2004; Kirkpatrick *et al*., 2008), and has also been observed in men with asthenozoospermia (Hristova *et al*., 2002) and teratozoospermia (Templado *et al*., 2002). The reported increases in sperm aneuploidy in infertile men have been two to ten times that observed in the control populations. Several studies have also used FISH to examine aneuploidy in sperm retrieved from the testes in men with azoospermia. While several of these studies have found that azoospermic men produced higher rates of sperm aneuploidy (Bernardini *et al*., 2000; Burrello *et al*., 2002; Rodrigo *et al*., 2004), others have found no significant difference between azoospermic and control men (Martin *et al*., 2000). Nevertheless, the overall trend appears to be that men with NOA produce higher rates of sperm aneuploidy than men with OA and men with normal semen parameters (reviewed in Burrello *et al*., 2005). Furthermore, there appears to be high variability in sperm aneuploidy

among azoospermic men, possibly due to multiple causes of spermatogenic failure and the grouping of different testicular pathologies.

The increased rates of sperm aneuploidy among azoospermic men and men with severely impaired semen parameters may contribute to an increased risk of chromosomal abnormalities in the offspring after ICSI. Several case reports have identified the transmission of aneuploidy from the sperm to the fetus in men undergoing ICSI. Moosani *et al*. (1999) reported a 47,XXY pregnancy that resulted from a man with an elevated frequency of 24,XY sperm. Tang *et al*. (2004) reported a 45,X abortus of paternal origin that was fathered by a man with extremely elevated rates of sex chromosome aneuploidy in the sperm (~40% vs. 0.4% in controls). Similarly, Carrell *et al.* (2001) reported a case of trisomy 15 of paternal origin, conceived using sperm that had elevated rates of disomy 15 (4.03% vs. 0.4% in controls). Calogero *et al*. (2001) studied sperm aneuploidy in 18 men undergoing ICSI, and they found that a lack of pregnancy was associated with a tendency towards increased sperm aneuploidy. These reports suggest that some infertile men produce higher rates of sperm aneuploidy, and transmission of these chromosomal abnormalities from the sperm to the embryo may be facilitated by ICSI. Nevertheless, few studies have examined the parental origin of chromosomal abnormalities after ICSI to determine if maternal or paternal factors are the major cause of the increase in chromosomal abnormalities after ICSI.

1.3.2 Meiotic recombination

While studies on human gametes and aneuploid conceptions have provided considerable data on the frequency and parental origin of numerical chromosomal abnormalities, little is known about the underlying mechanisms that lead to nondisjunction during meiosis. Henderson and Edwards (1968) were the first to propose a link between recombination and nondisjunction, suggesting that declining levels of recombination may explain the increase in trisomic pregnancies as a mother ages. Subsequent studies on model organisms have shown that the number and position of recombination events is important in chromosome segregation during meiosis. Recombination itself is not important for proper disjunction, but rather the chiasmata which form at sites of recombination play an important role in tethering the homologous

chromosomes during MI segregation (Koehler *et al*., 1996). In yeast, the position of crossovers during meiosis is important for chromosome segregations: crossovers either too close (Ross *et al*., 1996) or too far (Sears *et al*., 1995) from the centromere increase susceptibility to nondisjunction. Similarly in the fly *Drosophila* crossovers either too close or too far from the centromere are at risk for nondisjunction (Koehler *et al*., 1996). Mutations that reduce the number of crossovers have been shown to increase nondisjusction in both the worm *C. elegans* (Zetka and Rose, 1995) and mice (Yuan *et al*., 2002). Several methods have been developed to analyze recombination in humans. These include indirect assays such as gene-linkage analysis, and direct assays in which cells at specific stages of meiosis are analyzed to observe crossovers.

1.3.2.1 Gene-linkage analysis

Recombination can be studied indirectly using gene-linkage analysis, in which alleles at two or more marker locations on the same chromosome are examined in a family. When two alleles occur consecutively that were originally from separate grandparents, a crossover can be inferred (Lynn *et al*., 2004). Warren *et al*. (1987) were the first to apply this technique to trisomic conceptions, and found that recombination on chromosome 21 was significantly reduced in children with Down syndrome. A similar relationship between recombination and nondisjunction has been shown in trisomies 13, 15, 16, 18, 21, 22 and sex chromosome aneuploidies of both maternal and paternal origin (Table 1.4; reviewed in Lamb *et al*., 2005). These studies have shown that both the number and position of crossovers is important for ensuring the proper segregation of chromosomes. This technique has also been adapted to examine recombination in aneuploid sperm. Shi *et al*. (2001) used FISH to identify 24,XY sperm, and then performed single sperm PCR to study recombination between the sex chromosomes. As in XXY human conceptions, 24,XY sperm showed reduced recombination between the sex chromosomes (Shi *et al*., 2001). Gene-linkage analysis has provided important insight into the relationship between nondisjunciton and the number and position of crossovers. Nevertheless the major limitations of this technique are that recombination can only be examined over specific chromosomal regions at one time, and the analysis of recombination is limited by hereditary/transmission constraints.

Table 1.4 Recombination in trisomic human conceptuses originating during meiosis I [a]

Reference	Trisomy	No. of cases	Parental origin	Reduced recombination?	Abnormal placement of crossovers?
Hall *et al*., 2007a	13	28	Maternal	Reduced recombination, 25% lacked a crossover vs. 12% in controls	n/a
Robinson *et al*., 1998	15	97	Maternal	Reduced recombination, 21% lacked a crossover vs. 0% in controls	No
Hassold *et al*., 1995	16	62	Maternal	Reduced recombination, increase in 1-exchange events, decrease in 3-exchange events	Increase in telomeric exchanges
Bugge *et al*., 1998	18	50	Maternal	Reduced recombination, 30% lacked a crossover vs. 0% in controls	No
Lamb *et al*, 1996; Lamb *et al* ; 2005	21	207	Maternal	Reduced recombination, 40% lacked a crossover vs. 16% in controls	Increase in telomeric exchanges
Savage *et al*., 1998	21	22	Paternal	Reduced recombination, increase in achiasmate chromosomes	n/a
Hall *et al*., 2007b	22	45	Maternal	Reduced recombination, 25% lacked a crossover vs. 0% in controls	No
Thomas *et al*., 2001	XXX and XXY	68	Maternal	Reduced recombination, 47% lacked a crossover	Increase in pericentromeric exchanges
Thomas *et al*., 2000	XXY	28	Paternal	Reduced recombination, 67% lacked a crossover	n/a

[a]Adapted from Lamb *et al*., 2005

1.3.2.2 Chiasmata analysis

In addition to indirect assays for recombination, there exist methods for directly observing crossovers in meiotic cells of humans. Observing chiasmata during diakinesis of prophase I was the first method for studying recombination in humans. This technique provided the first information on the frequency and distribution of crossovers in humans (Hulten, 1974). Chiasmata analysis on infertile men has also provided some evidence that defective

recombination may be associated with male infertility (Chaganti *et al*., 1979; Chaganti *et al*., 1980; Micic *et al*., 1982). These studies were only able to provide an imprecise analysis of the frequency and location of crossovers because of several limitations of chiasmata analysis. First, diakinesis is a short stage and therefore the number of cells that can be analyzed is very small. Second, diakinesis spreads are extremely difficult to analyze, specific chromosomes are difficult to identify and it is difficult to localize chiasma to specific chromosomal regions. Finally, any method that directly examines crossovers is limited by the acquisition of appropriate tissues: in males recombination occurs in spermatocytes in the testes, whereas in females recombination is initiated in the fetal oocytes and diakinesis occurs just prior to ovulation.

1.3.2.3 Immunofluorescent analysis

The identification of numerous meiotic proteins has lead to the development of antibody-based immunocytogenetic techniques for studying recombination. This method for analyzing meiotic cells can provide a detailed visualization of important meiotic events, including synapsis and recombination. Antibodies against SYCP1, SYCP3 have been used to observe synapsis, whereas antibodies against MLH1, a late-mismatch repair protein, have been used to visualize the sites of recombination (Barlow and Hulten, 1998). The introduction of these new techniques has allowed researchers to address questions that were previously difficult to answer, such as the range of inter-individual variation in recombination rates and whether factors such as paternal age have an effect on recombination rates. Immunofluorescent analyses of recombination have shown that there are approximately 50 crossovers per spermatocyte in normal males (Barlow and Hulten, 1998; Lynn *et al*., 2002; Hassold *et al*., 2004; Sun *et al*., 2005b; Ma *et al*., 2006a), and approximately 70 crossovers per oocyte in the female (Tease *et al*., 2002; Lenzi *et al*., 2005). To date, several studies have shown that there is significant inter-individual variation in crossover frequencies among normal men, with mean recombination rates ranging from 42.5 to 55.0 crossovers per cell (Barlow and Hulten, 1998; Lynn *et al*., 2002; Hassold *et al*., 2004; Sun *et al*., 2005b). This variation in recombination frequencies may account for the variability in the frequency of sperm aneuploidy in humans (Templado *et al*., 2005). The effect of age on recombination rates is a contentious subject, with one study observing a decrease in

recombination as age increases (Sun *et al*., 2006), while others have found no effect (Lynn *et al*., 2002; Hassold *et al*., 2004; Gonsalves *et al*., 2004).

The combination of immunoflourescent techniques and FISH has allowed for chromosome-specific recombination patterns to be studied. The distribution of crossovers along all chromosomes have been examined in twelve men, and have confirmed observations from diakinesis studies showing that, in general, crossovers are preferentially located near the distal parts of the chromosomes, and crossovers appear to be repressed towards the centromeres (Sun *et al*., 2006; Codina-Pascual *et al*., 2006). Furthermore, each chromosome arm generally has at least one crossover, except for the short arms of acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22), which seldom have a crossover. Crossovers also appear to display positive interference, with two crossovers rarely occurring in close proximity to one another (Sun *et al*., 2004; Codina-Pascual *et al*., 2006). While the general rules of crossover distribution appear to be consistent between individuals, there are significant inter-individual variations in both the frequency and distribution of crossovers. Using immunofluorescent and FISH techniques it has also been possible to study the relationship between synaptic errors and recombination. Sun *et al*. (2005a) found that chromosomes displaying asynapsed regions in the heterochromatic regions around the centromeres display more distal crossovers, suggesting that synapsis may have a *cis* effect on the distribution of crossovers. However, more surprisingly, Sun *et al*. (2007a) observed that asynapsed regions on chromosome 9 were associated with a reduction in recombination on chromosome 5, suggesting that synapsis may also have a *trans* effect on the frequency of recombination.

Testicular biopsies are now routinely performed on infertile men to retrieve sperm for ICSI, providing the appropriate tissue to examine whether infertile men display abnormalities in synapsis and recombination. Several studies have begun to address these questions by using immunofluorescent techniques to study meiosis in infertile men (Table 1.5). Gonsalves *et al*. (2004) performed the first analysis of recombination in infertile men, and found that 10% of the men with NOA that they examined displayed reduced recombination when compared to the control men. They also identified 4 men who showed either complete or partial meiotic arrest at the zygotene stage. Similarly, Sun *et al*. (2007) studied synapsis and recombination in infertile

men and also identified men displaying zygotene arrest and defective recombination. However, studies by Codina-Pacual *et al*. (2005) and Topping *et al*. (2006) have not observed decreased recombination in their infertile populations, although the sample sizes were relatively small. Taken together, these studies have shown that approximately 9.1% of men with impaired spermatogenesis display reduced recombination (Table 1.5). Synaptic defects leading to meiotc arrest have also been observed in infertile men. Topping *et al*. (2006) identified three men with different synaptic defects that lead to either complete or partial meiotic arrest: 1) in one man the chromosomes failed to undergo synapsis and no pachytene stage cells were observed; 2) a second man displayed at least partial synapsis in most cells, however no recombination was observed and cells appeared to be arrested at the zygotene/pachytene boundary; and finally 3) a third man showed a few pachytene cells with recombination, however many of these cells showed large asynapsed regions on most of the chromosomes. The subtle difference in the meiotic phenotypes of these infertile men suggest that there may be no single cause of meiotic defects in human males, but rather rare mutations in several different meiotic genes (Topping *et al*., 2006). Furthermore, Ma *et al*. (2006a) were the first to provide evidence of an association between the absense of recombination between the sex chromosomes and an extremely high incidence of sex chromosome aneuploidy in a single infertile man.

Table 1.5: Immunoflourescent analyses of meiosis in infertile men

Number of men with impaired spermatogenesis[a]	Percentage of men with no meiotic cells (Sertoli Cell Only Syndrome)	Percentage with partial or complete arrest at zygotene	Percentage of infertile men showing reduced recombination when compared to fertile men	Reference
40	52.5% (21/40)	10.0% (4/40)	10.0% (4/40)	Gonsalves *et al.* 2004
11	n/a	0%	0%	Codina-Pascual *et al.* 2005
26[b]	34.6% (9/26)	11.5 % (3/26)	0%	Topping *et al.* 2006
4	0	0	25.0% (1/4)	Ma *et al*., 2006a Ma *et al*., 2006b
29[b]	51.7% (15/29)	3.4% (1/29)	17.2% (5/29)	Sun *et al*. 2007b
Total: 110	**40.9% (45/110)**	**7.3% (8/110)**	**9.1% (10/110)**	

[a]spermatogenesis diagnosed as either maturation arrest, hypospermatogenesis of SCOS
[b]pathology diagnoses of spermatogenesis were not reported

1.4 HYPOTHESES AND SPECIFIC OBJECTIVES

Recent advances in immunocytogenetic techniques have provided a method for performing detailed meiotic studies on infertile men. Several studies have now identified meiotic defects among men with non-obstructive azoospermia. We suspect that meiotic defects in the infertile population may explain the link between infertility and sperm aneuploidy. Meiotic defects may lead to spermatogenic arrest and infertility in some cells; however, other cells may be able to progress through meiosis, but have an increased susceptibility to aneuploidy. Thus, our hypotheses are that: (1) abnormalities in synapsis, recombination and the progression through meiosis may be more prevalent in infertile men; (2) abnormalities in recombination may be associated with increased rates of sperm aneuploidy and unsuccessful ICSI outcomes; and (3) asynapsed chromosomal regions may be transcriptionally silenced and play a role in male infertilty. While several studies have identified infertile men with meiotic defects, it is unknown if these men are at an increased risk of producing aneuploid sperm. A case report by Ma *et al*. (2006a) suggests that there may be a relationship between defective recombination and an increased risk of aneuploidy sperm in infertile men, although the relationship has not been studied in a larger sample size. Furthermore, these studies have not examined the crossover frequency and distribution on specific chromosomes in infertile men. The position of crossovers may be important for ensuring the proper segregation of chromosomes during meiosis, and examining crossover positions will be important for determining if the regulation of crossover distribution is altered in infertile men. Finally, there is little information on the role of asynapsed chromosomal regions in infertility. In model organisms it has been shown that asynapsed chromosomal regions are transcriptionally silenced; however, it is unknown if such a process occurs in humans. The work presented in this thesis will begin to address the following specific objectives:

Objective 1a: To determine if abnormalities in the progression through meiosis, synapsis or recombination are more prevalent among infertile men.
Objective 1b: To determine if reduced rates of recombination are associated with the production of aneuploid sperm.

Objective 2: To study the frequency and position of crossovers on chromosomes 13, 18 and 21 in infertile men.

Objective 3a: To determine if carriers of reciprocal translocations display alterations in synapsis or recombination.
Objective 3b: To determine if asynapsed regions undergo transcriptional silencing.

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CHAPTER II: MEIOTIC RECOMBINATION, SYNAPSIS AND SPERM ANEUPLOIDY IN INFERTILE MEN[1]

2.1 INTRODUCTION:

Improper segregation of chromosomes during meiosis can result in the production of genetically unbalanced sperm or oocytes. If these gametes participate in fertilization, the resulting embryo will be aneuploid, with either too many chromosomes (trisomy) or too few (monosomy). Aneuploidy is the most common chromosomal abnormality in humans, occurring in 5% of all pregnancies, and 0.3% of livebirths (Hassold *et al*., 1996). Although the majority of numerical chromosomal abnormalities are of maternal origin, paternal errors account for the majority of sex chromosome aneuploidies. Prenatal testing of ICSI pregnancies has shown an increase in de novo sex chromosome abnormalities (Bonduelle *et al*., 2002), the majority of which are of paternal origin (Van Opstal *et al*., 1997). Studies on the chromosomal constitution of sperm from infertile men have shown that this population may be at an increased risk of producing aneuploid sperm (Moosani *et al*., 1995; Bernardini *et al*., 1997; Pang *et al*., 1999; Kirkpatrick *et al*., 2008). Thus, the increased aneuploidy in the sperm of infertile men is likely one of the major sources of chromosomal abnormalities in ICSI pregnancies, and the transmission of these aneuploidies may be facilitated via ICSI (Tang *et al*., 2004).

During the first meiotic division homologous chromosomes must undergo pairing and synapsis, in which a protein structure known as the synaptonemal complex (SC) forms between the two homologs. It is along this protein structure that meiotic recombination occurs. Studies have identified aberrant meiotic recombination as an important molecular factor causing meiotic nondisjuction (Warren *et al*., 1987; Hassold *et al*., 1991; Savage *et al*., 1998; Shi *et al*., 2001). Meiotic recombination not only serves to generate genetic diversity, but crossovers also tether homologous chromosomes, thus facilitating the proper segregation of chromosomes during meiosis. Abnormalities in the frequency and location of crossovers are associated with non-disjunction of homologous chromosomes and the production of aneuploid gametes (Savage *et al*.,

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1998; Hassold *et al*., 1995; Lamb *et al*., 1996). Thus, the increased frequency of chromosomal abnormalities in the sperm of infertile men may be the result of defects in critical meiotic events during spermatogenesis. Defects in synapsis or recombination may be caught by meiotic checkpoints, leading to a loss of germ cells and subsequent infertility (Baker *et al*., 1995; Edelmann *et al*., 1996; Yuan *et al*., 2000). However, some cells may be able to progress through meiosis, resulting in an increased proportion of sperm with chromosomal abnormalities.

Using antibodies against SYCP3 (axial elements) and SYCP1 (transverse elements) to visualize the SC, antibodies against Mut-L homologue 1 (MLH1) for localization of crossovers, and CREST (calcinosis/Raynaud's phenomenon/esophageal dysmotility/ sclerodactyly/ telangiectasia) antiserum to observe the centromeres of chromosomes, detailed meiotic events can now be studied in human males (Barlow and Hulten 1998; Lynn *et al*., 2002). Recent immunofluorescent studies have shown that defects in synapsis and recombination are a significant cause of male infertility (Gonsalves *et al*., 2004; Codina-Pascual *et al*., 2005; Topping *et al*., 2006; Sun *et al*., 2007). Nevertheless, these studies have not addressed whether infertile men showing defective synapsis and recombination are at an increased risk of producing aneuploid sperm. Furthermore, there is little information on the chromosome-specific patterns of recombination in infertile men. Thus, we combined immunofluorescent techniques and FISH on spermatocytes to study recombination and synapsis in infertile men. Chromosomes 13, 18, 21 and the sex chromosomes were studied specifically, as aneuploidies of these chromosomes are a major cause of spontaneous abortions and abnormalities in liveborns. FISH on testicular sperm from these same men was performed to determine if certain meiotic phenotypes were particularly at risk of producing aneuploid sperm.

2.2 MATERIALS AND METHODS:

2.2.1 Patients and tissue collection

Ethical approval was obtained from the University of British Columbia Ethics Committee before initiating this study (see Appendix I). Testicular tissue was collected and analyzed from twenty-one infertile men seeking fertility treatment and ten proven fertile men who were

undergoing vasectomy reversals (see Appendix II). All infertile men presented at the fertility clinic with idiopathic forms of infertility, and had normal 46,XY karyotypes, no microdeletions on the Y chromosome, and no CFTR mutations. The testicular tissue was used for a pathology diagnosis, sperm extraction for subsequent ICSI cycles, and a small portion was used for the meiotic analyses reported in this study. Patients C1, C3, OA6, NOA4, NOA5 and OAT1 were previously published in Ma *et al*. (2006a) and Ma *et al*. (2006b).

2.2.2 Preparation of testicular tissue

The tissue was processed using a modification of the method used by Barlow and Hulten (1998). In room temperature phosphate-buffered saline (PBS) (pH 7.4) the seminiferous tubules were separated and cut into 3-5 mm segments. The tissue was incubated for 45-60 min at room temperature in a freshly prepared hypo-extraction buffer [30 mM Tris, 50mM sucrose, 17mM citric acid, 5 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulphonyl fluoride (PMSF); pH 8.4]. The tissue was then deposited on a microscope slide with 20 µl of 100 mM sucrose (pH 8.2). Using fine forceps, the tubules were squeezed to release their contents, and 10 µl of the gem cell/sucrose slurry was transferred to a new slide overlain with 1% paraformadehyde with 0.2% Triton X. The slides were then incubated for 24 hours at 37^{o}C in a humid chamber.

2.2.3 Fluorescence immunostaining

The slides were air dried for 30 min and washed twice in 0.4% PhotoFlo (Kodak 200 solution). The slides were then soaked in an antibody diluting buffer (ADB) [1% donkey serum, 0.3% bovine serum albumin, 0.005% Triton X, PBS; pH 7.2] at room temperature for 30 min. The primary antibody cocktail [rabbit antihuman MLH1 (Oncogene, San Diego, CA, USA), 1:37.5; mouse antihuman SYCP3 (produced by P. Moens, York University), 1:300; mouse antihuman SYCP1 (produced by P. Moens), 1:300; human CREST antisera, 1:25; 1 x ADB] was applied to drained by not dry slides. A cover slip was applied and the slide was incubated in a humid chamber at 37^{o}C for 24 hours. The slides were then soaked for 20 min in 1 x ADB, followed by a subsequent wash in 1 x ADB for 48 hours at 4^{o}C. The secondary antibody

cocktail [Flourescein isothiocyanate (FITC) labeled donkey antirabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) 1:50; tetramethyl rhodamine isothiocyanate (TRITC) labeled goat antimouse IgG (Jackson ImmunoResearch) 1:100; aminomethyl coumarin acetic acid (AMCA) labeled donkey antihuman IgG (Jackson ImmunoResearch) 1:50; 1 x ADB] was applied to the slide and incubated for 90 min in a 37^{o}C humid chamber. The slides were then washed three times in PBS (10, 20 and 30 min) with agitation every 5 min. Slides were drained, antifade was added and a coverslip was applied. Slides were scanned with a Zeiss Axioplan epifluorescent microscope equipped with appropriate filters. Images of the SYCP3/SYCP1 fragments, MLH1 and CREST sites were captured using Cytovision V2.81 Image Analysis software (Applied Imaging International, San Jose, CA, USA). Pachytene cells were captured if MLH1 foci were clear and the sex chromosomes were identifiable. Cell co-ordinates were recorded and prints were analyzed for the numbers of MLH1 and abnormalities in the SC.

2.2.4 FISH on immunostained spermatocytes

After capturing images of the SC and MLH1 foci, FISH was performed on the same spermatocytes to identify chromosomes 13, 18 and 21. Coverslips were removed and slides were soaked twice for 5 min in 4 x saline sodium citrate (SCC)/0.05% Tween-20 solution, followed by drying in an ethanol series (70, 80, 90, 100%). After air drying, slides were washed for 5 min in PBS, re-fixed in 10% formalin phosphate for 5 min, washed 5 min in PBS and dried in an ethanol series (70, 80, 90, 100%). Directly labeled single-stranded DNA probes for chromosomes 18 [CEP18 (SpectrumAqua)], chromosome 13[LSI 13 (SpectrumGreen)] and chromosome 21 [LSI 21(SpectrumOrange)] (Vysis Inc., Downer Grove, IL, USA) were added to slides, coverslips were added and sealed with rubber cement. Slides were co-denatured on a hotplate for 5 min at 75^{o}C, then placed in a 37^{o}C humid chamber overnight. Coverslips were removed and slides were washed at 75^{o}C in 0.4 x SSC/0.3% NP-40 solution for 2 min and 2 x SSC/0.1% NP-40 at room temperature for 30 seconds. After air drying, antifade and a coverslip were added. The cells captured beforehand were relocated and chromosomes 13, 18 and 21 were identified.

2.2.5 FISH on testicular sperm

Slides of testicular cells containing sperm which were not previously immunostained, were used for FISH analysis of testicular sperm aneuploidy. Sperm were pre-treated with a 5 min wash in 2 x SSC, followed by 15-30 min incubation in 10mM DTT/100mM Tris (pH 8.0) to decondense sperm nuclei to enhance hybridization efficiency. When sufficient decondensation was achieved slides were washed in 2 x SSC followed by a wash in PBS and dehydrated in an ethanol series (70, 80, 90, 100%). Slides were denatured in 70% formamide/2 x SSC at 75^{o}C for 5 min and dried in an ethanol series (70, 80, 90, 100%). A probe mixture containing directly labeled single-stranded DNA probes specific to the alpha-satellite repeat clusters in the centromeric regions of chromosome 18 [CEP 18 (SpectrumAqua), chromosome X [CEP X (SpectrumGreen)] and chromosome Y [CEP Y (SpectrumOrange)] (Vysis Inc.) was added to the slides. A coverslip was added, sealed with rubber cement and incubated overnight at 37^{o}C. Following hybridization the slides were washed in 0.4 x SSC/0.3% NP-40 at 73^{o}C for 2 min and 2 x SSC/0.1% NP-40 at room temperature for 30 seconds. Slides were counterstained with 4,6-diamidino-2 phenylindole (DAPI) (Vysis Inc.) and analyzed with a Zeiss Axioplan epifluorescent microscope. The same slide was then re-hybridized with a probe mixture for chromosomes 13 and 21. Coverslips were removed and slides were soaked for 5 min in 4 x saline sodium citrate (SCC)/0.05% Tween-20 solution, followed by drying in an ethanol series (70, 80, 90, 100%) to remove probes. A new probe mixture containing directly labelled single-stranded DNA probes for chromosomes 13 [13q24 (SpectrumGreen)] and chromosome 21[21q22.13-22.2 (SpectrumOrange)] (Vysis Inc.) was hybridized to slides using the same protocol as used for the chromosome 18/X/Y probe set.

Scoring of sperm nuclei was performed in areas of the slide where hybridization was consistent. Only nuclei with intact morphology and long sperm tails were scored to exclude scoring artifacts and to ensure that overlapping sperm were not counted. Two signals of the same colour were scored as disomy (two copies of the same chromosome), if the signals were of equal size, comparable brightness and separated from each other by a distance longer than the diameter of each signal. We attempted to score at least 1,000 sperm per patient for each probe set.

2.2.6 Statistical Analyses

The Kruskall-Wallace test with a Dunn's post test was used to compare means between groups, and the Mann-Whitney test was used to compare means between individual men. Individual values that were beyond the 95% confidence interval of the control group were considered significantly different from the controls. The Fisher exact test was used to compare the proportion of cells with XY recombination and the Chi-Square test was used to compare the number of crossovers on chromosomes 13, 18 and 21. Statistical analyses of recombination rates in Table 2.2 were performed in the same way as Gonsalves *et al*., 2004, Codina-Pascual *et al*., 2005 and Sun *et al*., 2007 to allow for comparisons between studies. The Pearson's correlation test was used to identify a relationship between recombination rates and sperm disomy rates. Statistical analyses were performed using the GraphPad Prism V5.0 program (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant.

2.3 RESULTS:

2.3.1 Classification of subgroups

Testicular tissue was collected and analyzed from thirty-one men seeking fertility treatment. These men were classified into four groups. Group I was the control group, consisting of ten fertile men who had undergone a vasectomy after fathering a child naturally. The remaining men presented at the fertility clinic with idiopathic forms of infertility, and were classified into three groups based on their pathological diagnosis. Four standard pathological diagnoses for spermatogenesis were used: normal spermatogenesis, hypospermatogenesis (a reduced number of germ cells showing normal maturation), maturation arrest (germ cells present up to a certain stage), and Sertoli cell only (a lack of germ cells). The twenty-one infertile men were divided into three groups: group II (obstructive azoospermia [OA]), men diagnosed with normal spermatogenesis with no sperm in the semen (n=7); group III (non-obstructive azoospermia [NOA]), men with abnormal spermatogenesis without sperm in the semen (n=12), including 8 men diagnosed with either hypospermatogenesis or maturation arrest, and 4 men with Sertoli cell only syndrome; and group IV (oligoasthenoteratozoospermia [OAT]), men with

a very low sperm count in the ejaculate, combined with abnormal sperm morphology and motility (n=2).

2.3.2 Progression through prophase I in infertile men

Prophase cells were classified into four stages: 1) leptotene, if only short fragments of the SC were present; 2) zygotene, if synapsis between homologous chromosomes was evident but not complete; 3) zygotene/pachytene if synapsis was complete, but no MLH1 foci were present; and 4) pachytene, if synapsis was complete and MLH1 foci were present (Figure 2.1). Cells at the diplotene stage were extremely rare in all groups of men. The mean frequencies of cells in the control group at the leptotene, zygotene, zygotene/pachytene and pachytene stages were 9.9%, 1.5%, 8.1% and 80.6%, respectively (Table 2.1). The OA group did not significantly differ from the control group ($p>0.05$, Kriskal-Wallace test). However, almost all of the men in the NOA group and both OAT men showed a significantly increased proportion of cells at the leptotene stage, as well as a significantly decreased proportion of cells at the pachytene stage when compared to the control group (Table 2.1).

2.3.3 Analysis of genome-wide recombination

Immunostaining of spermatocytes with antibodies to mark sites of recombination (MLH1) and the SC (SYCP1 and SYCP3) allowed us to assess rates of recombination and synaptic errors in spermatocytes of fertile and infertile men (Figure 2.2A). A total of 705 pachytene nuclei were analyzed from the control group, and 1,390 pachytene nuclei from the three infertile groups. The control group showed an average of 47.7 crossovers per cell, with individual mean rates of recombination ranging from 44.3 to 51.3 (Table 2.2). The recombination levels that we observed in our control men are within those reported by others (Lynn et al. 2002; Hassold et al. 2004; Sun et al. 2005). The average recombination rate was 47.7 (range: 42.1 to 48.8) in the OA group, 45.1 (range: 34.0 to 50.2) in the NOA group, and 33.9 in the OAT group. We observed significant inter-individual variation among men in the OA and NOA groups. One man in the OA group (OA14) showed significantly reduced recombination when compared to all individual control men ($p<0.01$, Mann-Whitney test). Five men (NOA10, NOA13, NOA 16, OAT1, OAT8)

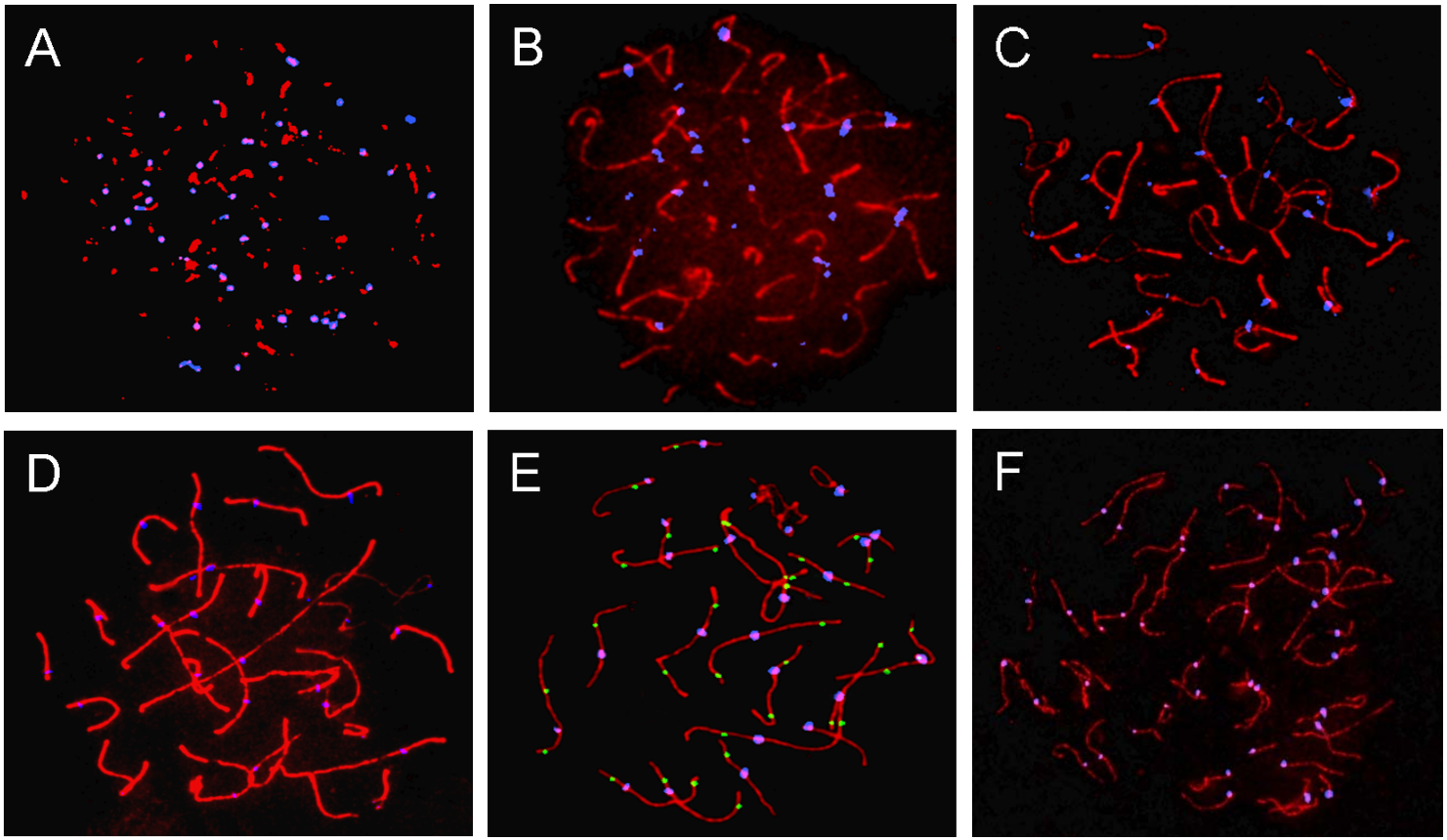

Figure 2.1: Stages of prophase I. (A) leptotene, (B) early zygotene, (C) late zygotene, (D) zygotene-pachytene transition, and (E) pachytene. At the diplotene stage (F) homologous chromosomes desynapse, but remain connected at chiasmata.

Table 2.1: Analysis of the progression through prophase I in fertile and infertile men.[a]

	Testicular Pathology	Number of cells	Leptotene (%)	Zygotene (%)	Zygotene/Pachytene (%)	Pachytene (%)
Group 1: Control (n=10)						
C1	Norm.	358	9.5	0.8	11.7	78.0
C3	Norm.	645	11.0	1.6	4.2	83.3
C4	Norm.	629	9.7	1.8	8.4	80.1
C5	Norm.	620	8.4	0.8	9.6	81.0
C6	Norm.	289	12.1	1.0	9.0	77.9
C7	Norm.	365	11.2	1.9	8.8	78.1
C8	Norm.	620	11.0	2.1	5.0	81.9
C9	Norm.	585	10.6	1.0	7.5	80.9
C10	Norm.	630	6.0	2.4	8.4	83.2
C11	Norm.	495	9.3	1.4	8.1	81.2
Mean			**9.9**	**1.5**	**8.1**	**80.6**
(95% CI)			**(8.6-11.1)**	**(1.1-9.6)**	**(6.5-9.6)**	**(79.1-82.0)**
Infertile men (n=17)						
Group 2: OA (n=7)						
OA6	Norm.	460	8.0	0.7[b]	9.8	81.5
OA7	Norm.	546	9.9	2.0	9.5	78.6[b]
OA9	Norm.	543	8.5	1.1	5.3[b]	85.1
OA11	Norm.	333	6.0	2.1	3.6[b]	88.3
OA14	Norm.	430	8.1	1.6	9.3	80.9
OA19	Norm.	577	10.6	1.6	7.3	80.6
OA20	Norm.	458	11.6[b]	2.6	8.7	77.1[b]
Mean			**9.0**	**1.7**	**7.6**	**81.7**
(95% CI)			**(7.2-10.7)**	**(1.1-2.3)**	**(5.4-9.8)**	**(78.2-85.3)**
Group 3: NOA (n=8)						
NOA4	Hypo	761	20.9[b]	1.2	3.6[b]	74.4[b]
NOA5	PMA	794	14.4[b]	1.8	6.2[b]	77.7[b]
NOA10	MA	610	21.3[b]	6.6	9.8[b]	62.3[b]
NOA13	MA	745	17.4[b]	4.7	22.8[b]	55.0[b]
NOA15	Hypo	566	11.3[b]	1.4	6.7	80.6
NOA16	Hypo	459	15.3[b]	1.7	5.7[b]	77.3[b]
NOA18	Hypo	582	8.9	2.1	8.8	80.2
NOA21	Hypo	177	13.6[b]	6.2	11.3[b]	68.9[b]
Mean			**15.4[b]**	**3.2**	**9.4**	**72.1[b]**
(95% CI)			**(11.8-19.0)**	**(1.3-5.1)**	**(4.4-14.3)**	**(64.3-79.8)**
Group 4: OAT (n=2)						
OAT8	MA	142	28.9[b]	5.6	9.9[b]	55.6[b]
OAT1	PMA	779	32.3[b]	1.4	9.8[b]	56.5[b]

Norm= normal spermatogenesis; Hypo=hypospermatogenesis; MA=maturation arrest; PMA=partial maturation arrest

[a]Cells were classified as leptotene, zygotene, zygotene/pachytene or pachytene (see Figure 2.1) and the frequency of each cell type was calculated.

[b]$p<0.05$ when compared to the control group

(group means were compared using the Kruksall-Wallace with Dunn's posttest; individual values were considered significantly different from the control group if they were beyond the 95% confidence interval of the control group).

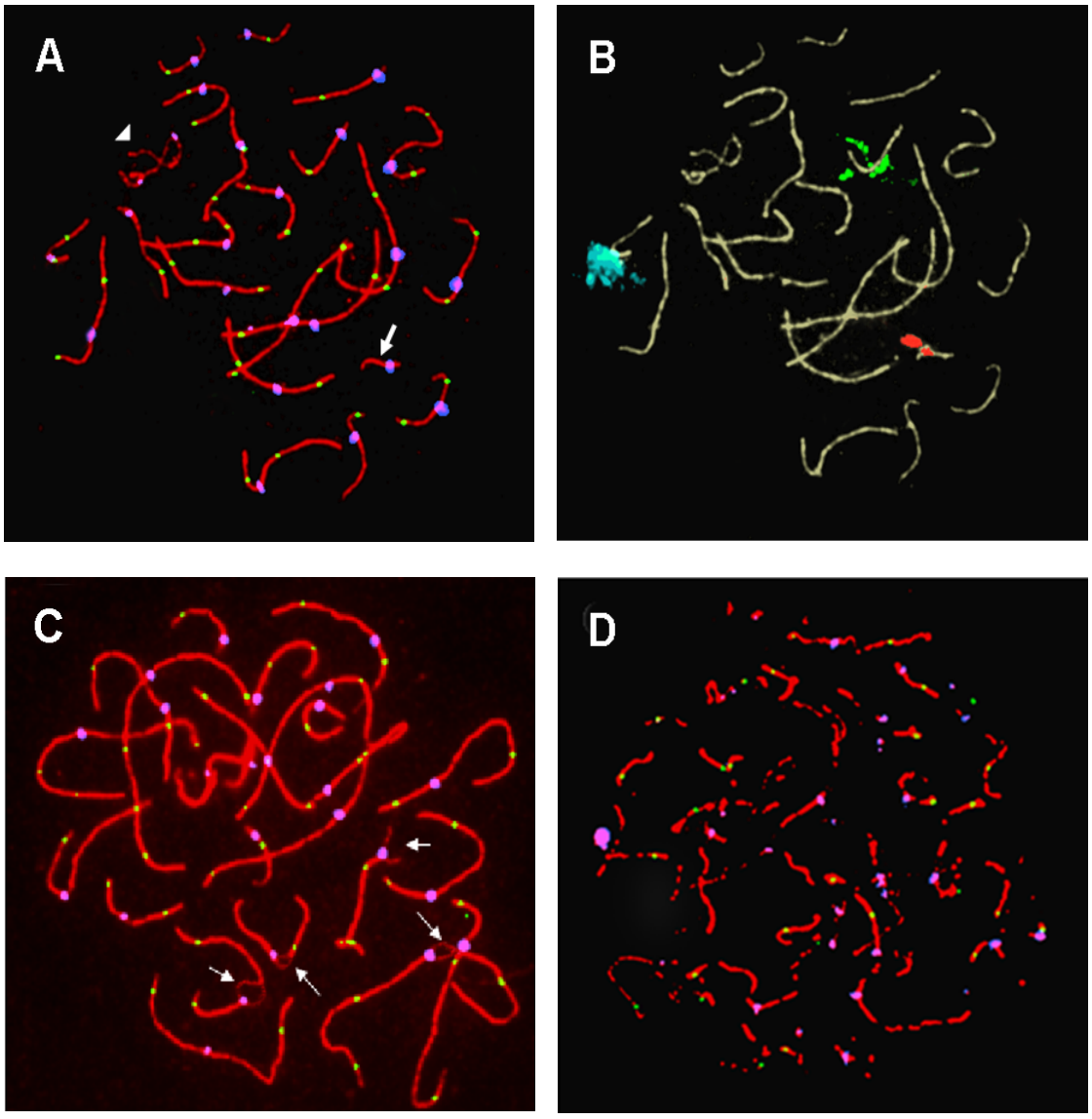

Figure 2.2: Immunofluorescent and FISH analysis of pachytene nuclei. **(A)** Spermatocytes were immunolabelled to visualize the SC (red), MLH1 (green) and centromeres (blue). **(B)** Subsequent FISH on the same spermatocytes allowed us to characterize recombination on chromosome 13 (green), chromosome 18 (blue) and chromosome 21 (red). In **(A)**, the chromosome 21 bivalent (arrow) and XY bivalent (arrowhead) display no recombination. Other meiotic defects were observed, including **(C)** unsynapsed regions of the SC (arrows); and **(D)** fragmentation of the SC, which was observed in two infertile man (OAT8, NOA21).

in the NOA and OAT groups displayed reduced recombination when compared to all control men ($p<0.01$, Mann-Whitney test). However, recombination was only significantly reduced in three of the men (NOA13, OAT1, OAT9) from the NOA and OAT groups when compared to all men in the OA group ($p<0.01$, Mann-Whitney test).

The proportion of pachytene cells with a crossover in the sex body ranged from 71.0%-92.7% in the control group, and 71.0%-93.2% in the OA group (Table 2.2). However, recombination between the sex chromosomes was particularly disrupted in the NOA and OAT groups, with four of the men (NOA10, NOA13, OAT1, OAT8) displaying significantly reduced recombination when compared to the lowest control ($p<0.001$, Fisher test). Two of these men (NOA10, OAT1) showed no recombination between the sex chromosomes in any of the pachytene nuclei observed. This absence of recombination between the X and Y chromosomes has not been observed in other meiotic studies on infertile men (Gonsalves *et al*., 2004; Codina-Pascual *et al*., 2005; Topping *et al*., 2006; Sun *et al*., 2007). Previous meiotic studies on azoospermic men have observed impaired recombination on autosomal chromosomes, with an increase in pachytene nuclei in which at least one autosomal chromosome is lacking a crossover (Gonsalves *et al*., 2004; Sun *et al*., 2007). We also observed a similar increase in pachytene cells containing at least one achiasmate autosomal chromosome in the NOA and OAT groups (Table 2.2). Five of these men (NOA10, NOA13, NOA15, OAT1, OAT8) displayed a significant increase in pachytene nuclei with at least one autosomal chromosome lacking a crossover, when compared to the men in the control group.

2.3.4 Synaptic anomalies

Two types of synaptic anomalies were observed during pachytene analyses in all groups of men: 1) discontinuities in the SC, in which both the lateral (SYCP3) and transverse (SYCP1) elements were absent in regions of the SC, and 2) unsynapsed regions of the SC in which meiotic pairing of the homologous chromosomes was incomplete, and only the lateral elements (SYCP3) were present (Figure 2.2C). Discontinuities in the SC were the more common of the synaptic

Table 2.2: Analysis of MLH1 foci in fertile and infertile men.

	Age	# of cells analyzed	Mean Recombination foci per cell (95% CI)	Range of recombination foci	Recombination in XY bivalent (%)	Proportion of cells with an autosomal bivalent lacking an MLH1 focus (%)
Group 1: Controls (n=10)						
C1	52	46	49.5 (48.3-50.7)	39-59	82.6	-
C3	35	100	48.0 (47.1-48.9)	39-56	81.0	3.0
C4	41	30	47.3 (45.6-49.0)	37-57	80.0	3.3
C5	34	41	45.2 (43.9-46.5)	37-53	80.5	2.4
C6	46	46	48.6 (47.4-49.8)	39-58	78.3	0
C7	45	41	46.2 (44.6-47.8)	34-59	92.7	0
C8	40	100	51.3 (50.2-52.4)	30-66	87.0	3.0
C9	38	100	44.3 (43.2-45.4)	28-55	71.0	5.0
C10	34	101	48.3 (47.2-49.4)	31-61	76.2	5.9
C11	39	100	47.9 (47.1-48.7)	35-58	82.0	1.0
Mean (95% CI)			**47.7 (46.2-49.1)**		**81.1 (77.0-85.3)**	**2.6 (1.0-4.2)**
Infertile Men (n=21)						
Group 2: OA (n=7)						
OA6	30	52	48.2 (47.3-49.2)	42-59	73.1[e]	-
OA7	31	100	47.6 (46.7-48.5)	36-58	76.0[e]	1.0
OA9	34	100	47.4 (46.5-48.3)	35-57	73.0[e]	2.0
OA11	39	73	46.5 (45.4-47.6)	25-59	93.2	5.5[e]
OA14	37	100	42.1 (41.2-43.0)[a]	25-49	86.0	6.0[e]
OA19	42	100	53.2 (52.4-54.0)	42-62	86.0	0
OA20	36	100	48.8 (48.0-49.6)	42-61	71.0[e]	2.0
Mean (95% CI)			**47.7 (44.7-50.7)**		**79.8 (71.8-87.7)**	**2.8 (0.2-5.3)**
Group 3: NOA (n=12)						
NOA2	30			No meiotic cells		
NOA3	35			No meiotic cells		
NOA12	35			No meiotic cells		
NOA17	29			No meiotic cells		

Table 2.2 (continued)

	Age	# of cells analyzed	Mean Recombination foci per cell (95% CI)	Range of recombination foci	Recombination in XY bivalent (%)	Proportion of cells with an autosomal bivalent lacking an MLH1 focus (%)
NOA4	43	113	48.9 (48.3-49.6)	42-59	79.5	-
NOA5	34	99	50.2 (49.2-51.2)	37-67	70.1[e]	-
NOA10	28	51	42.0 (40.7-43.3)[a]	30-56	0[c, e]	11.8 [d, e]
NOA13	32	100	34.0 (33.4-34.6)[a,b]	25-39	30.0[c, e]	19.0 [d, e]
NOA15	31	61	45.2 (43.6-46.8)	25-59	72.1[e]	14.8 [d, e]
NOA16	48	53	41.7 (40.6-42.8)[a]	30-48	69.8[e]	3.8
NOA18	39	100	49.9 (49.0-50.8)	39-60	75.0[e]	3.0
NOA21	39	33	49.1 (47.8-50.4)	43-56	75.8[e]	0
Mean (95% CI)			**45.1 (40.4-49.9)**		**59.0 (28.5-10.1)**	**8.7 (0.8-16.7)**
Group 4: severe OAT (n=2)						
OAT1	41	133	38.3 (37.4-39.3) [a,b]	19-52	0 [c,e]	35.3 [d, e]
OAT8	38	22	29.5 (25.2-33.8)[a,b]	13-56	13.6 [c,e]	90.9 [d, e]

Abbreviations: C, control; NOA, non-obstructive azoospermia; OAT, oligoasthenoteratozoospermia
[a]p<0.001, recombination significantly reduced when compared to all controls, Mann-Whitney test
[b]p<0.001, recombination significantly reduced when compared to all OA men, Mann-Whitney test
[c]p<0.001, significantly reduced when compared to all control and OA men, Fisher exact test
[d]p<0.001, significantly increased when compared to all control and OA men, Fisher exact test
[e]Individual value beyond the 95% confidence interval of the control group.

Table 2.3: Frequency of synaptic errors in fertile and infertile men.

	# of cells	Proportion of cells with gaps in the SC (%)	Proportion of cells with unsynapsed regions (%)
Group I: Control men (n=9)			
C3	100	31.0	3.0
C4	30	26.7	0
C5	41	22.0	2.4
C6	46	23.9	6.5
C7	41	56.1	2.4
C8	100	37.0	9.0
C9	100	10.0	2.0
C10	101	5.9	1.0
C11	100	13.0	2.0
Mean (95% CI)		**25.1 (13.2-36.9)**	**3.1 (1.0-5.3)**
Infertile Men (n=14)			
Group 2: OA (n=6)			
OA7	100	37.0	3.0
OA9	100	57.0[a]	26.0[a]
OA11	73	16.4	8.2[a]
OA14	100	26.0	3.0
OA19	100	17.0	17.0[a]
OA20	100	20.0	3.0
Group 2: NOA (n=6)			
NOA10	51	21.6	11.8[a]
NOA13	100	22.0	14.0[a]
NOA15	61	11.5	1.6
NOA16	53	17.0	1.9
NOA18	100	20.0	14.0[a]
NOA21	33	Fragmentation of the SC	
Group 4: severe OAT (n=2)			
OAT1	133	26.3	14.3[a]
OAT8	22	Fragmentation of the SC	

[a]Individual values were considered significantly different from the control group if they were beyond the 95% confidence interval of the control group.

anomalies, with a mean frequency of 25.1% (range: 5.9-56.1%) in the control group, although high interindividual variation was observed (Table 2.3). The frequency of SC discontinuities was also highly variable in the OA group (range: 16.4-57.0%), as well as the NOA group (range:11.5-22.0%). Unsynapsed meiotic chromosomes were less frequent than discontinuities, with a mean frequency of 3.1% (range: 0-9.0%) in the control group (Table 2.3). Three men in the OA group (OA9, OA11, OA19) showed a significant increase in cells with unsynapsed chromosomes when compared to the control group, however, only two of these men were beyond the range observed in the control group. We also observed a significant increase in cells with unsynapsed region in four men in the NOA or OAT groups (NOA10, NOA13, NOA18, OAT1) when compared to the control group. We also identified two men (OAT8, NOA21) in which synaptic anomalies could not be accurately assessed, as fragmentation of the SC was observed in almost all pachytene nuclei (Figure 2.2D).

2.3.5 Chromosome-specific recombination frequencies and sperm aneuploidy

After observing that genome-wide recombination rates were reduced in some of the infertile men, we were interested in analyzing chromosome-specific patterns of recombination. The combination of FISH and immunofluorescent techniques enabled us to characterize the frequency of recombination on chromosomes 13, 18 and 21 (Figures 2.2A-B and Table 2.4). From the control group we analyzed 423 pachytene nuclei from 7 men, and from the infertile groups we analyzed 812 pachytene nuclei from 12 men. We observed no significant differences in the chromosome-specific frequencies of recombination between the control group and the OA group. However, chromosome-specific recombination frequencies appeared to be altered in the NOA and OAT groups. In the control group chromosome 13 displayed two MLH1 foci in 84.9% of pachytene nuclei, while chromosome 18 displayed two MLH1 foci in 69.8% of pachytene nuclei (Table 2.4). Recombination frequencies on chromosome 13 bivalents were altered in four infertile men (OAT1, NOA10, NOA13, NOA16), while recombination frequencies on chromosome 18 were altered in three infertile men (OAT1, NOA10, NOA13) ($p<0.05$, Chi-square test). Chromosome 21 bivalents were achiasmate in 0.5% of pachytene nuclei from the control group. We identified four infertile men (OAT1, NOA10, NOA13, NOA15) who showed a significant increase in achiasmate chromosome 21 bivalents when compared to the control

Table 2.4: Analysis of crossovers on chromosomes 13, 18 and 21 in fertile and infertile men.

	# of cells	Chromosomes 13 [*f*% (*n*)]			Chromosome 18 [*f*% (*n*)]			Chromosome 21 [*f*% (*n*)]	
		0 foci	1 foci	≥ 2 foci	0 foci	1 foci	2 foci	0 foci	1 foci
Group 1: Controls (n=7)									
C3	56	0% (0)	19.6%(11)	80.4% (45)	0% (0)	42.9% (24)	57.1% (32)	1.7% (1)	98.3% (55)
C4	23	0% (0)	8.7% (2)	91.3 % (21)	0% (0)	26.1% (6)	73.9% (17)	0% (0)	100% (23)
C6	37	0% (0)	18.9% (7)	81.1% (30)	0% (0)	32.4% (12)	67.6% (25)	0% (0)	100% (37)
C8	70	0% (0)	15.7% (11)	84.3% (59)	0% (0)	8.6% (6)	91.4% (64)	0% (0)	100% (70)
C9	73	0% (0)	15.1% (11)	84.9% (62)	0% (0)	38.4% (28)	61.6% (45)	1.4% (1)	98.6% (72)
C10	79	0% (0)	17.7% (14)	82.3% (65)	0% (0)	32.9% (26)	67.1% (53)	0% (0)	100% (79)
C11	85	0% (0)	9.4% (8)	90.6% (77)	1.2% (1)	27.1% (23)	71.8% (61)	0% (0)	100% (85)
Total	**423**	**0%**	**15.1% (64)**	**84.9% (359)**	**0.2% (1)**	**29.6% (125)**	**70.2% (297)**	**0.5% (2)**	**99.5% (421)**
Infertile men (n=12)									
Group 2: OA (n=6)									
OA7	97	0% (0)	17.5% (17)	82.5% (80)	0% (0)	33.0% (32)	67.0 % (65)	0% (0)	100% (97)
OA9	66	0% (0)	12.1% (10)	84.8% (56)	1.6% (1)	18.2% (12)	77.3% (53)	0% (0)	100% (66)
OA11	66	0% (0)	19.7% (13)	80.3% (53)	1.5% (1)	24.4% (16)	74.2% (49)	1.5% (1)	98.5%(65)
OA14	47	0% (0)	23.4% (11)	76.5% (36)	0% (0)	44.7% (21)	55.3% (26)	2.1% (1)	97.9% (46)
OA19	70	0% (0)	1.4% (1)	98.6% (69)	0% (0)	10.0% (7)	90.0% (63)	0% (0)	100% (70)
OA20	63	0% (0)	3.2% (2)	96.8% (61)	0% (0)	25.4% (16)	74.6% (47)	1.6% (1)	98.4% (62)
Total	**409**	**0%**	**13.4% (54)**	**88.1% (355)**	**0.5% (2)**	**25.8% (104)**	**75.2% (303)**	**0.7% (3)**	**99.3% (400)**
Group 3: NOA/severe OAT (n=6)									
OAT1	113	3.5%[a] (4)	50.4%[a] (57)	46.0%[a] (52)	0.9%[a] (1)	61.1%[a] (69)	38.1%[a] (43)	10.6%[c] (12)	89.3%[c] (101)
NOA10	45	0%[a] (0)	31.0%[a] (14)	69.0%[a] (31)	2.0%[b] (1)	38.0%[b] (17)	60.0%[b] (27)	4.4%[c] (2)	95.6%[c] (43)
NOA 13	92	3.3%[a] (3)	84.8%[a] (78)	12.0%[a] (11)	1.1%[a] (1)	91.2[a] (84)	7.6%[a] (7)	4.3%[c] (4)	95.7%[c] (88)
NOA15	39	0%(0)	20.5% (8)	79.5% (31)	0% (0)	38.5% (15)	61.5% (24)	5.1%[c] (2)	94.9%[c] (37)
NOA16	26	0%[a] (0)	65.4%[a] (17)	34.6%[a] (9)	0% (0)	46.2% (12)	53.8% (14)	0% (0)	100% (26)
NOA18	88	0% (0)	6.8% (6)	93.2% (82)	0% (0)	29.5% (26)	70.5% (62)	0% (0)	100% (88)
Total	**403**	**1.7% (7)**	**44.7% (180)**	**53.6% (216)**	**0.7% (3)**	**55.3% (223)**	**42.4% (171)**	**5.0% (20)**	**95.0% (383)**

[a] $p<0.01$, Chi-square test with 2 degrees of freedom.
[b] $p<0.05$, Chi-square test with 2 degrees of freedom.
[c] $p<0.01$, Chi-square test with 1 degree of freedom.

group ($p<0.001$, Chi-square test). Two infertile men (NOA13, OAT1) showed a significant increase in the frequency of achiasmate chromosome 13 when compared to the control group ($p<0.005$,Fisher test); however, no increase in the frequency of achiasmate chromosome 18 was observed in any of the infertile men.

Recombination is thought to play a critical role in ensuring the proper disjunction of chromosomes during meiosis. Thus, we were interested in determining if the infertile men with altered recombination rates were at an increased risk of producing aneuploid sperm. To assess sperm aneuploidy, we performed FISH for chromosomes 18, X and Y, followed by FISH for chromosomes 13 and 21 on sperm extracted from the testicular tissue. A total of 24,566 sperm were scored from the control men, and 25,372 sperm from the three infertile groups. No sperm could be found in the testes in two of the infertile men with meiotic cells (NOA10, NOA13), and thus aneuploidy could not be assessed in these men. The mean frequency of disomy in sperm from the control, OA, NOA groups, respectively, were 0.14%, 0.23%, 0.15% for XX and YY; 0.26%, 0.33%, and 0.71% for XY; 0.14%, 0.26%, and 0.09% for chromosome 13; 0.11%, 0.20%, and 0.19% for chromosome 18; and 0.28%, 0.29%, and 0.26% for chromosome 21 (Table 2.5). Although there was a trend of increased XY disomy in the NOA group, when the mean disomy rates were compared between the three groups, no significant differences were observed ($p>0.05$, Kruskal-Wallace test). Nevertheless, four of the men in the OA group, and seven of the men in the NOA group showed a significant increase in XY disomy when compared to the control group. Furthermore, the two men with OAT displayed much higher disomy rates for the sex chromosomes and all autosomes studied (Table 2.5). OAT1 showed the most dramatically increased sperm aneuploidy of all the men studied, particularly for XY disomy which was present in 25.24% of the sperm (previously published in Ma *et al*., 2006a).

When all of the men (control and infertile) were combined, we observed an inverse correlation between the frequency of recombination between the sex chromosomes and XY disomy in the sperm, with sperm disomy rates dropping as recombination increased ($p<0.001$, $r=-0.74$, Pearson's test). We thought that two of the infertile men (OAT1, OAT8) with the most disrupted XY recombination may have been skewing the results; however, a relationship was still observed even when these two men were excluded ($p=0.046$, Pearson's test, Figure 2.3).

Table 2.5: Testicular sperm aneuploidy rates in fertile and infertile men.

	# of cells	Frequency of disomy % (*n*) XX or YY	XY	18	# of cells	Frequency of disomy % (*n*) 13	21
Group 1: Controls (n=10)							
C1	1568	0.32 (3)	0.38 (4)	0.07 (1)	-	-	-
C3	3090	0.13 (4)	0.23 (7)	0.10 (3)	3235	0.40 (13)	0.77 (25)
C4	1064	0.28 (3)	0.38 (4)	0.19 (2)	1048	0.38 (4)	0.29 (3)
C5	1021	0.10 (1)	0.10 (1)	0	1007	0	0.20 (2)
C6	1028	0.29 (3)	0.39 (4)	0	1019	0.10 (1)	0.29 (3)
C7	986	0	0.20 (2)	0.20 (2)	1029	0.19 (2)	0.29 (3)
C8	1143	0.09 (1)	0.26 (3)	0.17 (2)	1030	0.10 (1)	0.19 (2)
C9	1007	0.20 (2)	0.40 (4)	0.10 (1)	1012	0	0
C10	1153	0	0.09 (1)	0.09 (1)	1107	0.09 (1)	0.36 (4)
C11	1013	0	0.20 (2)	0.20 (2)	1006	0	0.10 (1)
Mean (95% CI)		**0.14 (0.05-0.23)**	**0.26 (0.18-0.35)**	**0.11 (0.06-0.17)**		**0.14 (0.02-0.26)**	**0.28 (0.11-0.44)**
Infertile men (n=17)							
Group 2: OA (n=7)							
OA6	1177	0.34 (4)[a]	0.68 (8)[a]	0.18 (2)	-	-	-
OA7	1079	0.46 (5)[a]	0.56 (6)[a]	0.37 (4)[a]	1020	0.66 (7)[a]	0.49 (5)[a]
OA9	1023	0.30 (3)[a]	0.49 (5)[a]	0.30 (3)[a]	1019	0.49 (5)[a]	0.39 (4)
OA11	993	0	0.20 (2)	0.20 (2)[a]	1008	0.20 (2)	0.20 (2)
OA14	1134	0.44 (5)[a]	0.62 (7)[a]	0.26 (3)[a]	1112	0.18 (2)	0.36 (4)
OA19	1011	0	0.20 (2)	0	1008	0	0.20 (2)
OA20	1005	0.10 (1)	0.20 (2)	0.10 (1)	1003	0	0.10 (1)
Mean (95% CI)		**0.23 (0.05-0.42)**	**0.33 (0.09-0.56)**	**0.20 (0.09-0.32)**		**0.26 (0-0.54)**	**0.29 (0.14-0.44)**
Group 3: NOA (n=8)							
NOA4	1636	0.12 (2)	1.04 (17)[a]	0	-	-	-
NOA5	568	0	1.23 (7)[a]	0.53 (3)[a]	-	-	-
NOA15	1236	0.40 (5)[a]	0.49 (6)[a]	0.40 (5)[a]	1130	0.27 (3)	0.53 (6)[a]
NOA16	987	0.30 (3)[a]	0.71 (7)[a]	0.20 (2)[a]	966	0.10 (1)	0.21 (2)
NOA18	1009	0.10 (1)	0.20 (2)	0	1000	0	0
NOA21	357	0	0.56 (2)[a]	0	345	0	0.29 (1)
NOA10			No sperm in testes				
NOA13			No sperm in testes				
Mean (95% CI)		**0.15 (0-0.32)**	**0.71 (0.31-1.10)**	**0.19 (0-0.43)**		**0.09 (0-0.30)**	**0.26 (0-0.61)**
Group 4: OAT (n=2)							
OAT1	416	0.72 (3)[a]	25.24 (106)[a]	0.96 (4)[a]	314	4.14 (13)[a]	3.82 (12)[a]
OAT8	1008	0.69 (7)[a]	1.09 (11)[a]	0.60 (6)[a]	808	0.99 (8)[a]	0.99 (8)[a]

Mean disomy rates were compared between the control group, OA group and NOA group using the Kruskall-Wallace test with the Dunn's post-test. Disomy rates for individual men were considered significantly increased if they were beyond the 95% CI of the control group mean.

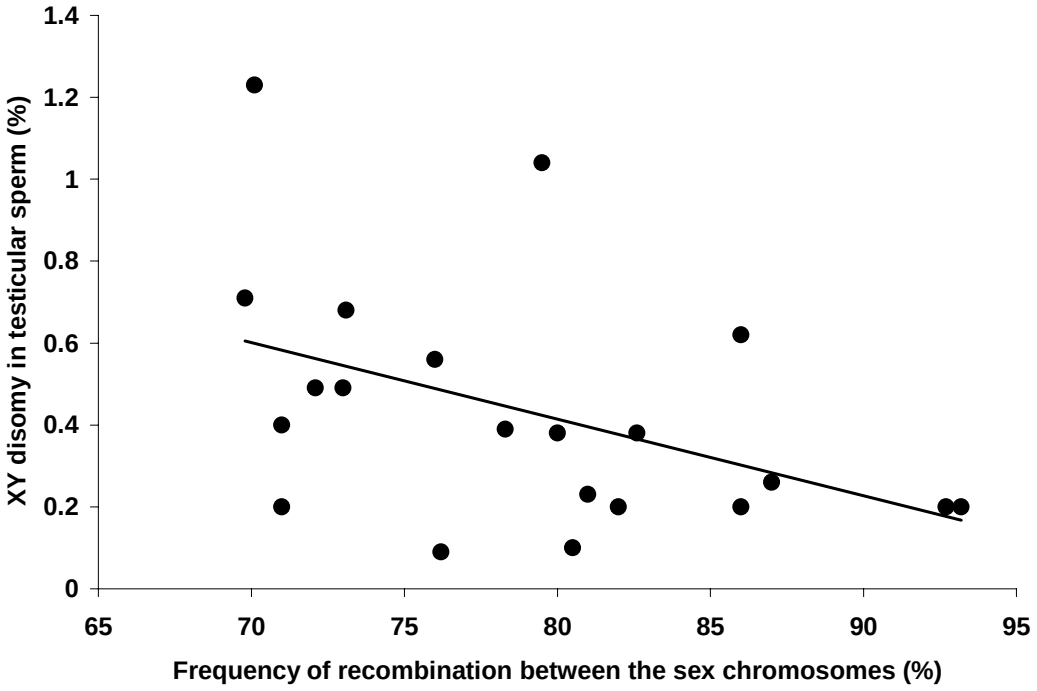

Figure 2.3: Relationship between the frequency of XY recombination and XY disomy in sperm from eighteen fertile and infertile men. Rates of XY disomy were highly variable among men, and appear to be inversely correlated with the frequency of XY recombination in spermatocytes ($p=0.046$, $r=-0.42$). Two men (OAT1 and OAT8) were not included in the correlation due to their extremely low levels of XY recombination.

Next, we wanted to see if there was a relationship between chromosome-specific recombination on autosomal chromosomes and sperm disomy rates. When we combined all of the men in which chromosome-specific rates of recombination were studied (Table 2.4) we observed a significant relationship between the frequency of achiasmate chromosome 21 and disomy 21 in the sperm ($p<0.001$, $r=0.89$, Pearson's test). However, the OAT1 patient appeared to be an outlier in this group, and when he was eliminated no significant relationship was observed ($p=0.16$, $r=0.37$, Pearson's test). Similarly, no relationships were observed between rates of recombination and disomy rates in the sperm for chromosomes 13 and 18.

2.4 DISCUSSION:

A series of recent immunofluorescent studies on infertile men have shown that meiotic defects are routinely observed among infertile men (Gonsalves *et al*., 2004; Codina-Pascual *et al*., 2005; Topping *et al*., 2006; Ma *et al*., 2006a; Sun *et al*., 2007). In this study we set out to further characterize the meiotic defects that have been observed in the infertile population, as well as investigate chromosome-specific deficiencies in recombination. Furthermore, we were interested in determining if abnormalities in recombination were associated with the production of chromosomally abnormal sperm.

Two previous studies have identified infertile men showing complete arrest at the zygotene stage, in which no pachytene cells could be observed, due to severe synaptic defects (Gonsalves *et al*., 2004; Topping *et al*., 2006). We did not observe this phenotype in any of our infertile men; however, when we classified meiotic cells into different stages of prophase, we observed an increase in the proportion of cells at the leptotene stage and a decrease in the proportion of cells at the pachytene stage among men with NOA or severe OAT. This impaired progression through meiosis has also been observed in another study on infertile men (Sun *et al*., 2007). Defective formation or synapsis of the SC leads to male infertility in mice (Yuan *et al*., 2000), possibly through the apoptotic cell death of pachytene cells by stringent meiotic checkpoints (Roeder and Bailis, 2000).

2.4.1 Defects in recombination and synapsis in infertile men

The development of immunofluorescent techniques for the analysis of pachytene cells has provided an efficient method for assessing meiotic recombination frequencies in males. The initial studies performed on normal males have provided a baseline of recombination frequencies in males (Lynn *et al*., 2002; Hassold *et al*., 2004; Sun *et al*., 2005), and more recent studies have identified several infertile men displaying reduced genome-wide levels of recombination (Gonsalves *et al*., 2004; Ma *et al*., 2006a; Sun *et al*., 2007). These studies have confirmed early cytological observations that defects in both meiotic recombination (Hulten *et al*., 1970; Micic *et al*., 1982) and synapsis (Chaganti *et al*., 1980) are associated with male infertility. In this study we have identified six infertile men (OA14, NOA10, NOA13, NOA15, OAT1, OAT8) who displayed reduced levels of genome-wide recombination when compared to all of the control men, thus confirming the observations of Gonsalves and colleagues (2004) that recombination deficiencies are a significant factor in male infertility. Interestingly, we identified two men (NOA10, OAT1) who showed a novel recombination deficiency: a complete absence of recombination between the sex chromosomes in all pachytene cells analyzed. While other studies have identified infertile men displaying reduced recombination between the sex chromosomes (Gonsalves *et al*., 2004; Topping *et al*., 2006; Sun *et al*., 2007), all of the men previously examined showed some degree of XY recombination. The lack of recombination between the sex chromosomes had differing effects on the outcome of spermatogenesis, with one man (NOA13) showing an absence of sperm in the testes, while the other man (OAT1) produced a low number of sperm with extremely elevated rates of sperm aneuploidy. Thus, XY-recombination may be necessary for the completion of spermatogenesis in some men (Hale, 1994), however other men may display less stringent checkpoints and be capable of completing spermatogenesis. While several gene knock-out mice have been found to display altered recombination patterns (Baudat *et al*., 2000; Liu *et al*., 2004; de Vries *et al*., 2005), none display the lack of XY-recombination that we observed.

The frequency of usynapsed regions of the SC were increased in four of the men with NOA or severe OAT. These observations were similar to those of Sun *et al*. (2007) who observed an increase in synaptic errors in their NOA population. Surprisingly, we also observed

increased rates of unpaired regions of the SC in three men whose histological diagnosis reported normal spermatogenesis, and were therefore considered OA. Unpaired regions of meiotic chromosomes are transcriptionally silenced through the recruitment of BRCA1 and γH2AX (Baarends *et al*., 2005; Turner *et al*., 2005; Ferguson *et al*., 2007), and may also trigger meiotic checkpoints (Odorisio *et al*., 1998), and have therefore been suspected to play a role in some cases of infertility. We also observed fragmentation of the SC in most pachytene nuclei from two men (OAT8, NOA21), which may be indicative of spermatocytes undergoing apoptotic cell death. The SC fragmentation that we observed in these men was similar to that observed in fetal oocytes thought to be at risk of atresia (Tease *et al*., 2006) and spermatocytes from men with AZFc deletions on the Y chromosome (Geoffroy-Siraudin *et al*., 2007).

2.4.2 Recombination and sperm aneuploidy

Recently, several studies have combined immunofluorescent and FISH analysis of pachytene nuclei to characterize the recombination patterns on specific chromosomes in normal males (Tease and Hulten, 2004; Codina-Pascual *et al*., 2006; Sun *et al*., 2006). Nevertheless, there is no information on the chromosome-specific frequencies of recombination in infertile men. Recombination in the NOA and OAT groups appeared to be reduced on all three of the autosomal chromosomes studied. Chromosome 13 and 18 bivalents with only one crossover were more prevalent in the NOA and OAT groups when compared to the control group, who displayed double crossovers on the majority of bivalents. Furthermore, a greater proportion of chromosome 21 bivalents lacked a crossover in the NOA and OAT groups. The achiasmate chromosome 21 bivalents may be caught by a checkpoint and induce meiotic arrest (Roeder and Bailis, 2000); however, they may also increase the risk of meiotic nondisjunciton and trisomy 21 in the offspring (Savage *et al*., 1998).

In both model organisms and humans, recombination is thought to be essential for the proper segregation of chromosomes during meiosis (Koehler *et al*., 1996). Thus, the reduced levels of both genome-wide and chromosome-specific recombination that have been observed in some infertile men may increase their risk of producing aneuploid sperm and chromosomally abnormal offspring. Furthermore, the variation in recombination parameters among both fertile

and infertile men may explain the inter-individual variation in sperm aneuploidy rates (Rubes *et al*., 2005). By performing FISH on testicular sperm, we were able to assess disomy rates for chromosomes 13, 18, 21 and the sex chromosomes. The modest increase in XY disomy in testicular sperm from men with NOA compared to normal controls is consistent with other reports (Bernardini *et al*., 2000; Martin *et al*., 2003; Rodrigo *et al*., 2004); however, we were surprised to find that the OA population also displayed an increased rate of XY disomy in the sperm. It appears that men diagnosed with OA may nevertheless display meiotic abnormalities, such as an increased frequency of unsynapsed meiotic chromosomes or a decreased frequency of recombination (OA14), which may increase the risk of aneuploid sperm. It should be noted that, unlike other studies (Gonsalves *et al*., 2004), our group of OA men had no obvious blockages in the seminal tract, such as a congenital absence of the vas deferens. Thus, although a histological analysis of spermatogenesis may appear normal, subtle meiotic defects may be present in both the spermatocytes and sperm of men who are considered OA.

While we found no correlation between rates of recombination and sperm disomy for chromosomes 13, 18 and 21, we found an inverse correlation between rates of sex chromosome recombination and XY disomy in the sperm. Thus, men with higher rates of recombination between the sex chromosomes may face a smaller risk of producing XY disomic sperm. This supports previous observations that most paternally derived 47,XYY males were conceived by sperm in which the sex chromosomes did not undergo meiotic recombination (Hassold *et al*., 1991). This suggests that men with extremely defective XY recombination may be at a greater risk of fathering offspring with a sex chromosome abnormality. Indeed, an attempt at ICSI using sperm from an infertile man who displayed 0% recombination between the sex chromosomes (OAT1) resulted in a 45,X abortus of paternal origin (Ma *et al*., 2006a).

In this study we have contributed to the growing evidence that meiotic defects, including defective recombination, synaptic defects and an impaired progression through prophase I, are associated with male-factor infertility. By examining both the early meiotic events in spermatocytes and the chromosomal constitution of the sperm in the same men, we found that recombination between the sex chromosomes was inversely correlated with XY disomy rates in the sperm. XY recombination rates are variable among men, and this may explain both the inter-

individual variation in sperm aneuploidy rates, as well as the increased sperm aneuploidy rates that have been frequently reported among the infertile population. We also identified a novel meiotic defect in two unrelated men, in which no recombination was observed between the sex chromosomes. In one case, spermatogenic arrest and an absence of sperm in the testes was observed. However, the other case produced sperm with extremely elevated rates of sex chromosome aneuploidy, which resulted in a 45,X abortus after ICSI (Tang *et al*., 2004). This is the first evidence that defective recombination in infertile men may be associated with elevated levels of sperm aneuploidy, and chromosomally abnormal offspring (Ma *et al*., 2006a). Further meiotic studies on infertile men will provide important insights into the genetic basis of idiopathic male infertility, and could also provide useful information for predicting rates of sperm aneuploidy, which could aid in risk assessment for couples undergoing assisted reproduction.

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CHAPTER III: DISTRIBUTION OF MLH1 FOCI AND INTER-FOCAL DISTANCES IN INFERTILE MEN[1]

3.1 INTRODUCTION:

Meiotic crossovers play an essential role in both the generation of genetic diversity, as well as in ensuring the proper segregation of homologous chromosomes during the first meiotic division. In humans, gene-linkage analysis has been used to examine the crossover position of non-disjoined chromosomes in trisomic conceptions, providing insight into the configurations of meiotic recombination that increase the susceptibility to nondisjunciton. These studies have found that both the number and position of meiotic crossovers are important in ensuring proper segregation of the chromosomes (reviewed in Lamb *et al*., 2005). Thus, abnormalities in either the number or position of crossovers during meiosis may increase the risk of non-disjunction and chromosomal abnormalities in offspring.

Recent developments in immunocytological techniques have allowed for the analysis of meiotic recombination in spermatocytes of severely infertile men. Several studies have found that a significant proportion of men with impaired spermatogenesis display abnormalities in the number of crossovers (Gonsalves *et al*., 2004; Sun *et al*., 2007a; Ferguson *et al*., 2007), while others have found no abnormalities in the number of crossovers in their infertile populations (Codina-Pascual *et al*., 2005; Topping *et al*., 2006). In Chapter II, we observed an inverse correlation between the frequency of sex chromosome recombination and XY disomy in the sperm from both fertile and infertile men. Thus, we suggested that decreased rates of meiotic recombination may explain the frequently reported increase in the incidence of aneuploid sperm in severely infertile men (Ma *et al*., 2006; Kirkpatrick *et al*., 2008).

The combination of immunostaining and fluorescent in-situ hybridization (FISH) has provided a method for studying the distribution of crossovers on specific chromosomes in both male (Lynn *et al*., 2002; Tease and Hulten, 2004; Codina-Pascual *et al*., 2006; Sun *et al*., 2006)

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and female (Tease *et al*., 2002) meiotic cells. These studies have found that, among normal men there are significant inter-individual variations in both the number of crossovers and distribution of crossovers along specific chromosomes. Laurie and Hulten (1985) used cytological studies of chiasmata to examine the distribution of crossovers in a single infertile male. Altered crossover distribution was observed in this man when compared to a control population, including a higher proportion of crossovers that were farther from the telomeres, as well as an alteration in the inter-chiasma distances (Laurie and Hulten 1985). Nevertheless, there is little information on the distribution of crossovers in infertile men, particularly those displaying a reduced number of crossovers. Abnormalities in both the number and position of crossovers in azoospermic men may contribute to spermatogenic arrest and infertility, as well as an increased risk of aneuploidy in the sperm. In Chapter II we characterized the frequency of recombination on chromosomes 13, 18 and 21. In this Chapter we examine the distribution of meiotic crossovers on chromosomes 13, 18 and 21 in spermatocytes from 5 normal fertile men, as well as 10 infertile men. To our knowledge, this is the first analysis of meiotic crossover distributions in an infertile population.

3.2 MATERIALS AND METHODS:

3.2.1 Meiotic analyses:

Testicular tissue was obtained from 15 men: 10 infertile men seeking fertility treatment, and 5 men who had previously fathered a child and were undergoing a vasectomy reversal at the time of tissue retrieval. Testicular tissue was processed, and spermatocytes were immunostained with antibodies against SYCP3, SYCP1, MLH1 and CREST antisera, as reported in Chapter II. FISH was performed to identify chromosomes 13, 18 and 21, which was also reported in Chapter II. The distribution of MLH1 foci and SC lengths were measured using the image analysis software Micromeasure V3.3, available at: http://www.biology.colostate.edu/MicroMeasure/ (Reeves *et al*., 2001). The SC arms of chromosomes 13, 18 and 21 were divided into 10% intervals, with the centromere at 0% and the telomeres at 100%. The frequency of MLH1 foci in each interval was calculated. For chromosomes 13 and 18, MLH1 distributions were analyzed separately for bivalents with single and double crossovers. Inter-focal distances were calculated by measuring the absolute distance between adjacent MLH1 foci in chromosome 13 and 18

bivalents with double crossovers. Absolute inter-focal distances were then divided by the total length of the SC for that particular chromosome and expressed as a percentage.

3.2.2 Statistical Analyses:

To analyze the distribution of MLH1 foci, each SC arm was divided into 10% intervals and the frequency of MLH1 foci in each interval was calculated. Chromosome 13 and 18 bivalents with single and double crossover configurations were analyzed separately. A Chi-square test with 36 degrees of freedom was used to compare the distribution of MLH1 foci in the five control men for each chromosome arm studied (13q, 18p, 18q and 21q). No differences in MLH1 distribution were observed between the control men for any of the chromosome arms studied. The control men were pooled and a Chi-square test with 9 degrees of freedom was used to compare the distribution of MLH1 foci on specific chromosome arms between individual infertile men and the control group. The Fisher test was used to compare MLH1 frequencies in each 10% SC interval between each infertile man and the control group. All statistical analyses were performed using the GraphPad Prism V5.0 program (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant.

3.3 RESULTS:

Crossover distributions on chromosomes 13, 18 and 21were studied in five normal control men and ten infertile men. Of the ten infertile men, four were diagnosed with normal spermatogenesis and were classified as OA. The other six azoospermic men were diagnosed with either hypospermatogenesis (a reduced number of germ cells showing normal maturation) or maturation arrest (germ cells present up to a certain stage) and were classified as NOA, except for one man who had a few sperm present in the ejaculate and was considered OAT. In total, 327 spermatocytes were analyzed from the control men, and 679 spermatocytes from the two azoospermic groups.

3.3.1 MLH1 frequencies:

Recombination frequencies, synaptic defects and rates of testicular sperm aneuploidy of the infertile population reported on in this study were reported in Chapter II. Of the 10 azoospermic men in this study, five men had displayed reduced genome-wide recombination (Table 2.2). Of these five infertile men, four had impaired spermatogenesis (OAT1, NOA10, NOA13, NOA16), while one was diagnosed with normal spermatogenesis (OA14). Four of the NOA men displayed altered recombination frequencies on chromosome 13 (OAT1, NOA10, NOA13, NOA16), three men displayed altered recombination frequencies on chromosome 18 (OAT1, NOA10, NOA13), and four men displayed altered recombination frequencies on chromosome 21 (OAT1, NOA10, NOA13, NOA15) (Table 2.4).

3.3.2 Distribution of MLH1 foci:

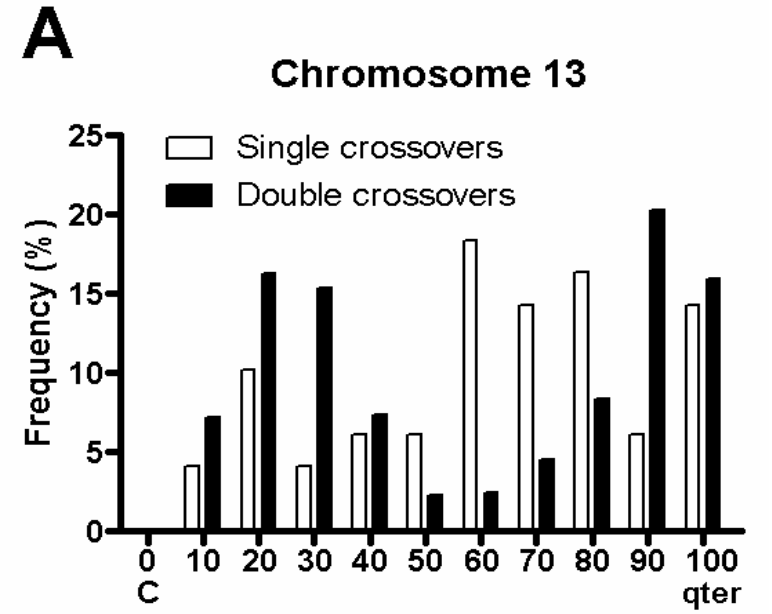
MLH1 distributions were separated into single and double crossover configurations, as the number of crossovers along a chromosome was found to considerably influence the distribution of the crossovers. The distribution of MLH1 foci were examined on chromosome 13 bivalents with single and double crossovers, chromosome 18 bivalents with single and double crossovers, and chromosome 21 bivalents with single crossovers and the results of the control population are presented graphically in Figure 3.1. In the control population chromosome 13 bivalents with a single MLH1 focus showed the highest frequency of crossovers at relative distances from the centromere of 50-80% of the SC q arm; whereas chromosome 13 bivalents with double crossovers showed a first peak at a relative distance of 10-30% and a second peak at a relative distance from the centromere of 80-100% of the SC q arm (Figure 3.1A). Chromosome 18 bivalents with a single MLH1 focus in the control population did not show a distinct peak in crossover distribution, but MLH1 foci were most frequently located at relative distances from the centromere of 30-80% of the SC q arm (Figure 3.1B). Chromosome 18 bivalents with double crossovers in the control population displayed a peak at relative distances from the centromere of 50-80% of the SC p arm, and a second peak at a relative distance from the centromere of 70-90% of the SC q arm (Figure 3.1B). Chromosome 21 bivalents in the control group showed a peak in MLH1 foci at a relative distance from the centromere of 70-90%

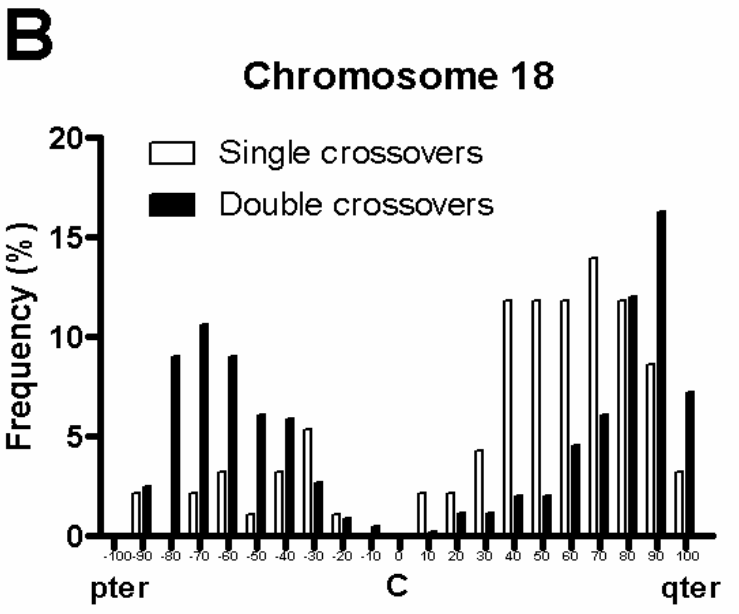
of the SC q arm (Figure 3.1C). No significant differences in MLH1 distribution were observed between any of the control men for all of the chromosome arms studied ($p>0.05$, Chi-square test).

Four of the infertile men displayed altered MLH1 distributions on at least one of the chromosome arms studied when compared to the pooled control group. OAT1 displayed altered recombination on the 13q arm of chromosome 13 bivalents with double crossovers (Figure 3.2A; $p=0.0293$, Chi-square test), showing a decrease in extremely distal crossovers, and an increase in more proximal crossovers at relative distances from the centromere of 30-40% and 60-70% of the q arm SC. This patient also displayed altered MLH1 distributions on chromosome 21 bivalents (Figure 3.2B; $p=0.0002$, Chi-square test), showing a decrease in extremely distal crossovers and a significant increase in more proximal crossovers at relative distances from the centromere of 10-40% of the q arm SC. NOA10 displayed altered MLH1 distribution on the 18p arm in chromosome 18 bivalents with double crossovers (Figure 3.2C; $p<0.0001$, Chi-square test) with an increase in extremely distal exchanges on the p arm. NOA18 displayed altered MLH1 distribution on the 18q arm in chromosome 18 bivalents with double crossovers (Figure 3.2D; $p=0.0361$, Chi-square test) showing an increase in crossovers at the relative distances from the centromere of 10-20% and 60-70% of the q arm SC. OA9 displayed altered recombination on chromosome 21 bivalents (Figure 3.2E; $p=0.0392$, Chi-square test), with a decrease in exchanges at the relative distances from the centromere of 20-40% of the q arm SC.

3.3.3 Inter-focal distances:

Inter-focal distances were calculated by measuring the distance between adjacent MLH1 foci and were reported as a percentage of the entire SC (Table 3.1). In the control men, the mean inter-focal distance between MLH1 foci on chromosome 13 bivalents was 56.5%, while the mean inter-focal distance on chromosome 18 bivalents was 70.5%. Only one infertile man, patient OAT1, displayed a significantly reduced inter-focal distance on chromosome 13 bivalents.





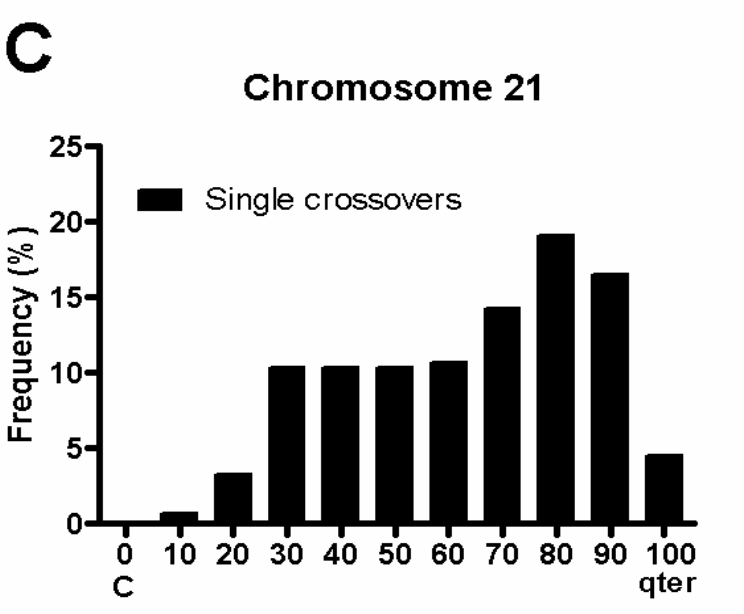
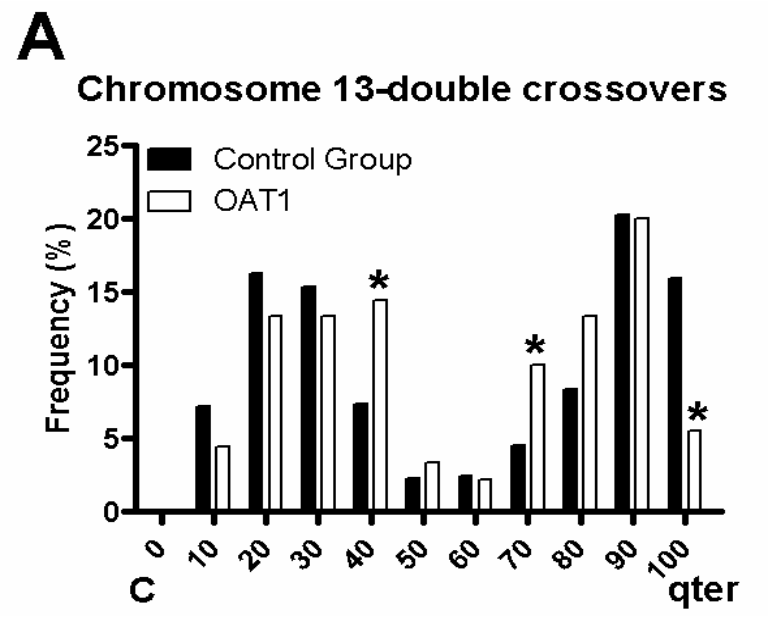
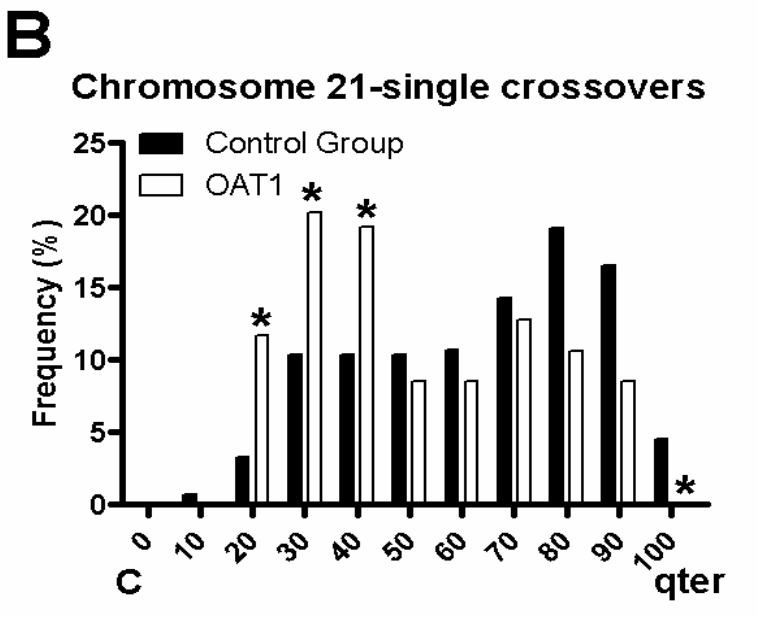


Figure 3.1: MLH1 distribution along chromosome 13, 18 and 21 bivalents with single and double crossovers in normal men.
Chromosome arms were divided into 10% intervals and the frequency of MLH1 foci in each interval was calculated. The Y-axis represents the frequency of MLH1 foci in each interval, and the X-axis represents the relative position from the centromere of the MLH1 foci. Values on the X-axis represent the upper limit of each 10% interval. The centromere is labeled "c", with the p arms to the left of the centromere, and the q arms to the right of the centromere. P arms of chromosomes 13 and 21 are not shown, as foci in these intervals were extremely rare. No difference was observed between the distributions of MLH1 foci in the five control men, and therefore they were pooled into a control group.





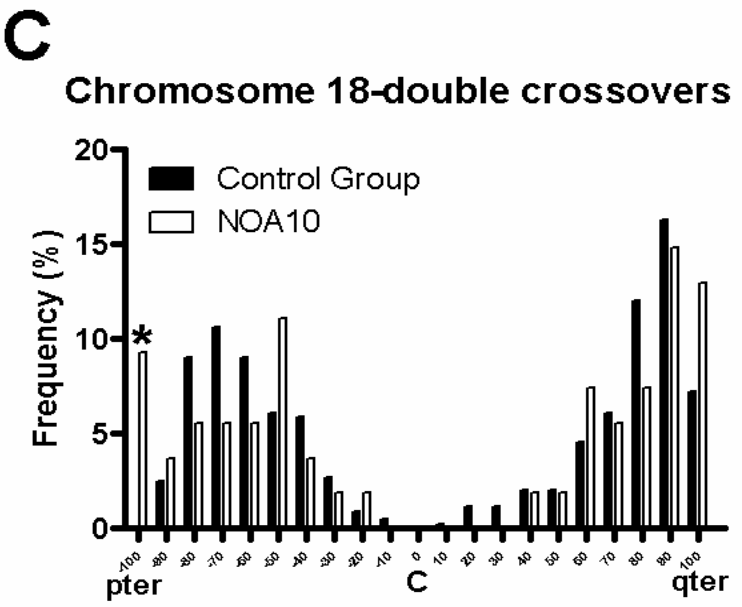


Figure 3.2: Chromosomes displaying altered MLH1 distributions in infertile men. Four infertile men displayed significantly different MLH1 distributions on at least one chromosome studied when compared to the control group ($p<0.05$, Chi-Square test). Black bars indicate the control group and the white bars indicate the individual infertile man. MLH1 frequencies in each interval were compared to the control group and significant differences are indicated by black stars ($p<0.05$, Fisher test).

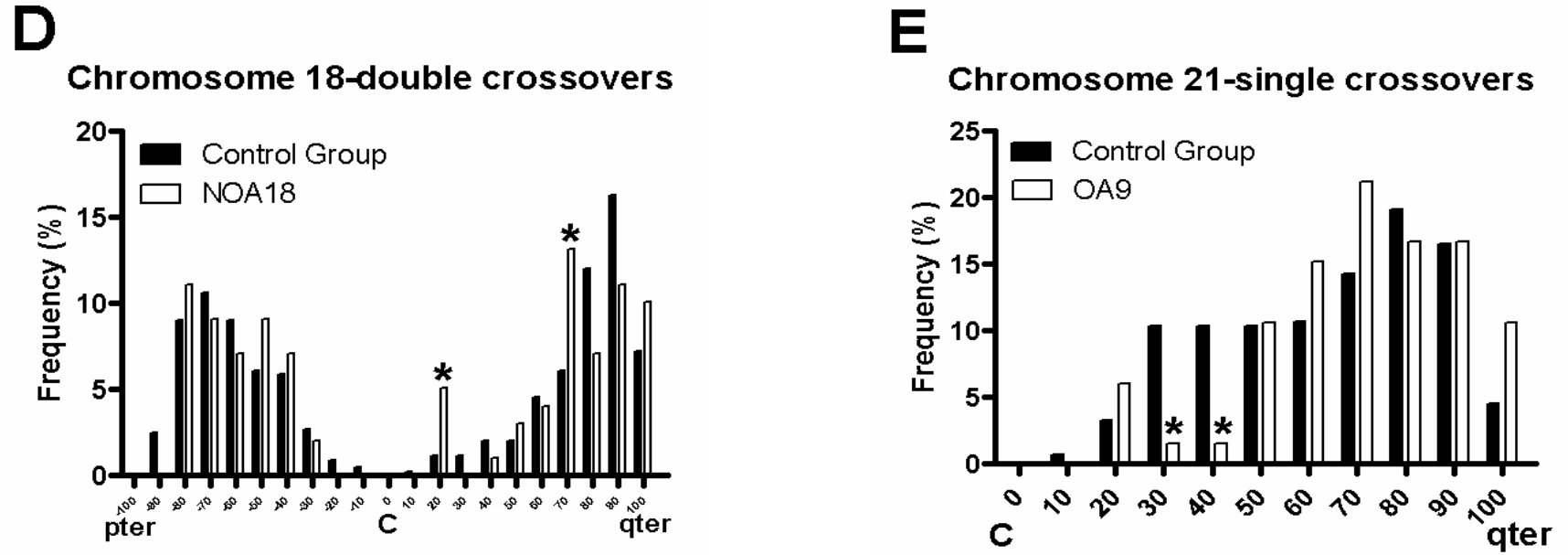


Figure 3.2 (continued): Figure 3-2: Chromosomes displaying altered MLH1 distributions in infertile men.

Table 3.1: Inter-focal distances on chromosome 13 and 18 bivalents with two MLH1 foci.

	Chromosome 13				Chromosome 18			
	No. of intervals	Inter-focal distance (% of SC)	SD	Range	No. of intervals	Inter-focal distance (% of SC)	SD	Range
Control men (n=5)								
C3	44	60.0	7.7	41.7-77.0	31	70.1	11.9	41.1-90.5
C6	29	57.2	7.5	46.6-76.0	26	66.1	13.2	23.2-79.3
C8	78	54.3	13.8	24.1-75.7	60	71.5	17.7	31.4-99.8
C10	60	56.3	14.5	15.0-99.6	48	71.8	14.1	18.0-93.3
C11	71	54.7	13.6	25.3-78.7	55	72.9	12.4	39.7-90.6
Mean		**56.5**				**70.5**		
Obstructive azoospermic (n=4)								
OA7	74	55.2	11.5	20.5-78.0	61	70.8	13.4	33.0-93.2
OA9	55	56.0	12.2	33.2-80.8	51	69.1	15.8	27.6-96.1
OA11	47	58.4	14.9	15.6-81.0	46	76.0	15.2	32.1-98.0
OA14	33	59.4	16.3	17.1-93.2	25	76.0	14.3	40.2-91.3
Non-obstructive azoospermic/severe oligoasthenteratozoospermia (n=6)								
OAT1	46	48.5[a]	12.8	19.2-80.6	37	68.5	15.9	12.2-87.1
NOA10	27	56.1	14.0	29.0-78.1	27	74.4	13.0	47.4-97.5
NOA13	9	57.2	12.6	39.1-73.6	4	64.3	15.8	41.2-76.4
NOA15	34	57.7	14.1	34.7-92.7	24	68.6	13.7	37.9-85.6
NOA16	18	61.4	10.3	41.2-81.4	12	74.7	14.1	43.2-95.8
NOA18	70	60.4	14.8	10.1-88.7	49	63.1	17.7	18.6-99.9

[a]p=0.0295, significantly decreased when compared to all control men, Mann-Whitney test.

3.4 DISCUSSION:

In Chapter II we provided the first analysis of chromosome-specific rates of recombination in infertile men by studying recombination frequencies on chromosomes 13, 18, 21 and the sex chromosomes. In this Chapter, we set out to further characterize meiotic recombination patterns in azoospermic men by examining the position of MLH1 foci and the inter-focal distances on chromosomes 13, 18 and 21 in ten infertile men. Of the ten infertile men examined in this study, five men (OAT1, NOA10, NOA13, NOA16, OA14) had previously displayed abnormal genome-wide or chromosome-specific rates of recombination (Tables 2.2 and 2.4).

3.4.1 Distribution of MLH1 foci in normal men:

Two recent studies have combined immunofluorecent techniques with centromere-specific mulitcolour FISH (cenM-FISH) to examine the distribution of MLH1 foci along all autosomal chromosomes in a small population of normal men (Sun *et al*., 2006; Codina-Pascual *et al*., 2006). In this study we examined the distribution of MLH1 foci along chromosomes 13, 18 and 21 in five normal men. The general trends that we observed in our control population were similar to those observed by Sun *et al*. (2006) and Codina-Pascual *et al*. (2006), including: a peak in crossovers in the proximal and distal regions of the q arm on chromosome 13 bivalents with double crossovers; a peak in crossovers in the distal regions of the p and q arm on chromosome 18 bivalents with double crossovers; and a peak in crossovers in the distal region of the q arm on chromosome 21 bivalents with a single crossover (Figure 3.1). We observed no significant inter-individual variation in MLH1 distribution for any of the chromosomes studied among our five control men. In a previous study Sun *et al*. (2006) examined 10 normal men and found no inter-individual variation in the distribution of MLH1 foci on chromosomes 13 and 18; however, they did observe significant inter-individual variation in foci distribution on chromosome 21.

3.4.2 Altered distribution of MLH1 foci in infertile men:

The distribution of MLH1 was altered in four infertile men when compared to the control group. Patient OAT1 showed a decrease in exchanges in the sub-telomeric regions of chromosomes 13 and 21, and patient NOA18 showed an increase in crossovers in the centromeric region of the chromosome 18 q arm. This shift in crossovers towards the centromere is similar to that observed by Laurie and Hulten (1985) who observed chismata farther from the telomere in an infertile man. However, we also observed infertile men with very different alterations in crossover distribution: patient NOA10 displayed an increase in exchanges near the telomere of the chromosome 18 p arm, and patient OA9 displayed a decrease in proximal exchanges on chromosome 21. Of the four men with altered crossover distribution, two (OAT1 and NOA10) had displayed reduced genome-wide recombination (Table 2.2), as well as reduced recombination on the chromosomes displaying altered MLH1 distributions (Table 2.4), while the other two men (NOA18 and OA9) displayed normal rates of meiotic recombination. Laurie and Hulten (1985) also observed altered crossover distributions in a single infertile man who displayed a normal number of crossovers, further suggesting that some infertile men with normal rates of meiotic recombination may nevertheless display an altered distribution of crossovers. Furthermore, all four of the men that displayed altered MLH1 distribution also displayed a significant increase in unsynapsed autosomal chromosomes during meiosis (Table 2.3). While unsynapsed regions have been shown to have a *cis* effect on the distribution of crossovers on a chromosome (Sun *et al*., 2005), this is unlikely to have contributed to the altered recombination patterns as chromosomes 13, 18 and 21 displayed extremely low levels of asynapsis. Sun *et al*. (2007b) recently observed in fertile men that the unsynapsed regions on chromosome 9 were associated with a reduction in MLH1 foci on other chromosomes, providing evidence that synaptic anomalies may also have a *trans* effect on the number of crossovers on other chromosomes. Nevertheless, Sun *et al*. (2007b) found that these synaptic anomalies had no significant effect on the position of MLH1 foci on other chromosomes, suggesting that the alterations in MLH1 foci distributions that we observed may not be related to the increases in synaptic anomalies on other chromosomes.

The distribution of meiotic recombination is regulated on multiple levels, making it difficult to determine the origin of the abnormal crossover distribution in the infertile men. Variations in DNA sequences, such as the centromeric heterochromatin (Yamamoto, 1979), or the copy number of telomeric or subtelomeric repeat sequences (Barton *et al*., 2003) could account for the changes in the crossover positions. Mutations in meiotic genes could also have contributed to an altered crossover distribution. For example, mutations in SC components such as *Scp3* (Yuan *et al*., 2000; Wang and Höög, 2006) and DNA repair proteins such as meiotic recombination 11 homolog A (Mre11a) (Cherry *et al*., 2007) have been shown to alter the distribution of crossovers and lead to infertility in male mice. Furthermore, alterations in the distribution of crossovers can originate at different stages of early prophase: either early on when the DNA double-strand breaks are induced based on the DNA sequence and chromatin structure (Wu and Lichten, 1994), or later when a small subset of the DNA breaks are converted into crossovers (Blitzblau *et al.*, 2007).

While it is now well established that a sufficient number of crossovers is important for the proper segregation of chromosomes, unfavorable positions of the crossovers may also increase the risk of chromosome non-disjunction. While there is little information on the crossover patterns that increase susceptibility to non-disjunction for chromosomes 13 and 18, chromosome 21 non-disjunction has been more thoroughly studied. In females, crossovers near the telomere of chromosome 21 increase the risk of MI non-disjunction, wherease crossovers shifted towards the centromere of chromosome 21 increase the risk of MII non-disjunction (Lamb *et al*., 1997). Nevertheless, crossover distributions differ significantly between the sexes, and there is little information on the crossover patterns that increase susceptibility to non-disjunction during paternal meioses. Studies on trisomy 21 conceptions of paternal origin suggest that in chromosome 21 non-disjunction occurring during MII there is a slight increase in exchanges in the proximal-medial range, compared to the distal 21q exchanges observed in normal male meioses, while no clear position effect has been observed for chromosome 21 non-disjunction during MI (Savage *et al*, 1998). The patient OAT1 showed a significant increase in proximal-medial exchanges, suggesting that he may be at an increased risk of chromosome 21 disomy in the sperm. In Chapter II we observed that this patient displayed a ten-fold increase in chromosome 21 disomy in the sperm (Table 2.5), however this increase may also be attributed to

the significant increase in achiasmate chromosome 21 bivalents in this patient (Table 2.4 and Ma *et al*., 2006).

3.4.3 Inter-focal distances in infertile men:

Adjacent crossovers display positive interference, which prevents two crossovers from being located too closely together and is an important factor in the regulation of crossover distribution. To investigate whether infertile men display alterations in crossover interference, we measured the relative distance between adjacent MLH1 foci on chromosome 13 and 18 bivalents with double crossovers. Chromosome 13 bivalents almost always display both crossovers on the q arm, allowing us to assess interference within a chromosome arm, while chromosome 18 usually displays one crossover on the p arm and one on the q arm, allowing us to assess interferences across a centromere. Unlike previous studies, we observed no significant inter-individual variation ($p>0.01$, Kruskal-Wallace test) in inter-focal distances in our control population (Tease *et al*., 2004; Sun *et al*., 2006). Inter-focal distances were greater on chromosome 18 than on chromosome 13, supporting previous observations that trans-centromere interference is stronger than intra-arm interference (Laurie and Hulten 1985; Sun *et al*., 2006; Codina-Pascual *et al*., 2006). Furthermore, the inter-focal distances in our control group were almost identical to that observed by Codina-Pascual *et al*. (2006), who found that trans-centromere interference on chromosome arms with two crossovers is approximately 70% of the SC, while intra-arm interference on acrocentric chromosomes is approximately 57% of the SC. Only one man (OAT1) displayed altered crossover interference on chromosome 13, with crossover being spaced more closely together. This patient also displayed an altered recombination distribution on chromosome 13, which may have been related to the decreased interference. This decrease in interference is similar to that observed by Laurie and Hulten (1985) who observed that proximal crossovers were farther from the centromere, and the distal crossovers were farther from the telomeres in an infertile man.

In Chapter II we observed that a subset of infertile men display reduced rates of meiotic recombination. In this study we have found evidence that some infertile men also display alterations in the position of crossovers on chromosomes 13, 18 and 21, and that these

abnormalities may be observed in men displaying normal rates of recombination. These results suggest that recombination defects in infertile men may include alterations in the number of crossovers, the position of crossovers, or both. Alterations in the inter-focal distances were rare, with only one man displaying reduced interference on one of the chromosomes studied. Both the frequency and position of crossovers are important factors in ensuring the proper segregation of chromosomes during meiosis. Further studies on the distribution of crossovers in infertile men will allow us to determine if alterations in the position of crossovers is a risk factor for the production of aneuploid sperm. Detailed studies on the distribution of crossovers in azoospermic men may also provide insight into the nature of the meiotic defects, and provide direction in the search for mutations that contribute to meiotic defects and infertility.

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CHAPTER IV: MEIOTIC STUDIES IN A CARRIER OF A t(8;13) RECIPROCAL TRANSLOCATION[1]

4.1 INTRODUCTION:

Reciprocal translocations are present in 0.1% of phenotypically normal newborns; however, the incidence of reciprocal translocations among infertile men is approximately 10 times greater (De Braekeleer and Dao, 1991). During spermatogenesis in carriers of balanced translocations, sperm with unbalanced chromosomal complements can be produced. The proportion of chromosomally unbalanced sperm produced by translocation carriers can range from 19% to more than 80%, and appears to be dependent on the translocation (reviewed in Benet *et al*., 2005). The increase in chromosomally abnormal sperm can lead to an increased risk of pre- and post-implantation pregnancy loss. Furthermore, if a fetus with an unbalanced chromosomal complement survives to term, it is likely to suffer from mental retardation or congenital malformations. While this greater risk of pregnancy loss contributes to the decreased fertility among translocation carriers, spermatogenesis may also be directly impaired. Sperm parameters are highly variable among translocation carriers, ranging from normozoospermic to a complete absence of sperm in the ejaculate, and are likely dependent on the chromosomes involved and the sites of the breakpoints.

During meiosis, homologous chromosomes undergo synapsis, in which a protein structure known as the synaptonemal complex forms between the paired chromosomes, and meiotic recombination, in which genetic material is exchanged between the chromosomes. These two events are critical to the fidelity of meiosis, and in Chapter II we observed that failures in both of these processes are associated with infertility and may also contribute to the production of aneuploid sperm (Ma *et al*., 2006; Ferguson *et al*., 2007). In order to align their homologous regions during synapsis, reciprocal translocations adopt a quadrivalent structure. The meiotic behaviour of the quadrivalent is thought to have a significant impact on the fertility status of the carrier, and may explain the variation in sperm parameters among male carriers.

[1] A version of this chapter has been published by Oxford University Press. Ferguson, K.A., Chow, V., Ma, S. (2008) Silencing of unpaired meiotic chromosomes and altered recombination patterns in a human male carrier of a t(8;13) reciprocal translocation. *Hum. Reprod.* 23(4): 988-995.

Meiotic studies on infertile male carriers of reciprocal translocations have shown that the regions surrounding the breakpoints often fail to completely synapse (Gabriel-Robez *et al*., 1986, Chandley *et al*., 1986, Johannisson *et al*., 1987, Oliver-Bonet *et al*., 2005a). Furthermore, these asynapsed regions have been found to associate with the sex chromosomes. During the male meiosis the X and Y chromosomes are transcriptionally silenced, forming a condensed sex body. Associations between quadrivalents and the sex body in infertile men have lead to the hypothesis that sex chromosome inactivation may spread to the associated regions of autosomal chromosomes, leading to cell death (reviewed in Oliver-Bonet *et al*., 2005b).

Meiotic sex chromosome inactivation (MSCI) is characterized by the localization of phosphorylated H2AX on the sex chromosomes, which is thought to be critical for chromatin condensation and transcriptional inactivation (Celeste *et al*., 2002). BRCA1 is also critical for MSCI, recruiting ATR to phosphorylate H2AX (Xu *et al*., 2003, Turner *et al*., 2004). Recent studies, however, have suggested that the phenomenon of meiotic inactivation may not be limited to the sex chromosomes: unsynapsed autosomal chromosomes have been shown to undergo a similar transcriptional silencing in the germ cells of mice (Baarends *et al*., 2005; Turner *et al*., 2005). Nevertheless, there is little information on the inactivation of unpaired meiotic chromosomes in humans. Carriers of structural chromosomal abnormalities generally display disturbed homologous synapsis, and thus provide an exceptional model for studying the behaviour of unpaired autosomal chromosomes during human meioses. In this Chapter we present an analysis of synapsis, recombination and transcriptional silencing in spermatocytes from an azoospermic carrier of a t(8;13) reciprocal translocation.

4.2 MATERIALS AND METHODS:

A 45 year-old male had a five year history of infertility. Semen analysis showed an absence of sperm in the semen sample, hormonal profiles were normal, and a physical examination did not reveal any obstruction in the reproductive tract. Routine karyotyping showed that the man was a heterozygous carrier of a t(8;13)(q21;p11) reciprocal translocation. Two testicular biopsies were performed one year apart and on separate testes in order to extract sperm for ICSI. A few sperm were found from the first biopsy, and none from the second. A

histological analysis found germ cell maturation arrest. A small portion of testicular tissues from both biopsies were used for the meiotic analyses reported on in this study. As a control, testicular tissue samples were retrieved during vasectomy reversals on eight proven fertile 46,XY men. Ethical approval was obtained from the University of British Columbia Ethics Committee before initiating this study (see Appendix I).

4.2.1 Meiotic Analyses:

Testicular tissue was processed according to the methods reported in Chapter I. A primary antibody cocktail [rabbit anti-MLH1 (Oncogene, San Diego, CA, USA), 1:37.5; mouse anti-SYCP3 (provided by P.Moens, York University), 1:300; mouse anti-SYCP1 (provided by P.Moens), 1:300; and human CREST antisera, 1:25, was applied to slides and incubated at 37^{o}C overnight. A secondary antibody cocktail [FITC Donkey antirabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) 1:50, TRITC Goat antimouse IgG (Jackson ImmunoResearch) 1:100, AMCA Donkey antihuman IgG (Jackson ImmunoResearch) 1:50, 1 x ADB] was applied and slides were incubated in a 37^{o}C humid chamber for 90 min. Slides were scanned with a Zeiss Axioplan epifluorescent microscope equipped with appropriate filters. Images of the SYCP3/SYCP1 fragments, MLH1 and CREST sites were captured using Cytovision V2.81 Image Analysis software (Applied Imaging International, San Jose, CA, USA). One hundred pachytene cells were captured for each biopsy, cell co-ordinates were recorded and printouts were analyzed for the number of recombination foci and synaptic errors.

FISH was performed on the slides that had been previously immunostained, to identify the arms of the translocated chromosomes. Coverslips were removed and the slides were washed in PBS for 5 min, followed by dehydration in an ethanol series (70, 80, 90, 100%). After drying, a probe mixture of LSI 13 (13q14) SpectrumGreen, CEP 8 SpectrumOrange, and 8q24 LSI MYC Dual Color (SpectrumOrange/SpectrumGreen) (Vysis Inc., Downer Grove, IL, USA) was added to the slides. Slides were co-denatured on a hotplate at 75^{o}C for 5 min, and then incubated overnight in a humid chamber. Coverslips were removed, and slides were washed in 0.4 x SSC/0.3%NP-40 at 72^{o}C for 2 min, followed by 2 x SSC/0.1% NP-40 for 30 sec at room

temperature. After air drying in the dark, antifade and coverslips were added. Cells were then relocated and FISH signals were captured.

Following FISH analysis, a second round of immunostaining was performed on the same slides. FISH probes were washed off by rinsing the slides in phosphate-buffered detergent (PBD) for 5 min, followed by an ethanol series (70, 80, 90, 100%). A primary antibody cocktail of mouse anti-γH2AX (Upstate Biotech, Lake Placid, NY, USA) at 1:1000 dilution and rabbit anti-BRCA1 (Santa Cruz Biotech, CA, USA) at 1:50 dilution was applied to slides. This was followed by a secondary antibody cocktail of FITC Donkey antirabbit IgG (Jackson ImmunoResearch) at a dilution of 1:100 and TRITC Goat antimouse IgG (Jackson ImmunoResearch) at a dilution of 1:100. Cells were relocated and the γH2AX and BRCA1 localization was observed.

To observe the transcriptional activity in the pachytene nuclei, an unused slide from the second biopsy on the t(8;13) patient was immunostained with a primary antibody cocktail of mouse anti-RNA polymerase II (8wg16, Abcam, Cambridge, MA, USA) at 1:50 dilution, rabbit anti-SYCP3 (P. Moens) at 1:50 dilution, rabbit anti-SYCP1 (P. Moens) at 1:50 dilution and CREST antisera at 1:25 dilution. The secondary antibody cocktail contained FITC Donkey antirabbit IgG (Jackson ImmunoResearch) 1:50, TRITC Goat antimouse IgG (Jackson ImmunoResearch) 1:100, and AMCA Donkey antihuman IgG (Jackson ImmunoResearch) 1:100.

4.2.2 Statistical Analyses:

The Mann-Whitney test was used to compare mean rates of genome-wide recombination between the biopsies and the control men. The Fisher test was used to compare the frequency of MLH1 foci on arms of the translocated chromosomes. All statistical analyses were performed using the GraphPad Prism V5.0 program (GraphPad Software, San Diego, CA).

4.3 RESULTS:

4.3.1 Genome-wide and chromosome-specific recombination frequencies

One hundred pachytene nuclei were analyzed from each biopsy after immunostaining to observe the SC and sites of crossover (Figure 4.1A). The mean rates of recombination were 47.3 and 49.6 crossovers per cell in the first and second biopsies, respectively (Table 4.1). Recombination was also assessed in pachytene nuclei from eight fertile control men (Table 4.1). Mean rates of recombination ranged from 44.3 to 51.3 crossovers per cell in the control men. Genome-wide recombination was significantly different between the two biopsies, with the rate of recombination significantly greater in the second biopsy ($p=0.0134$; Mann-Whitney test). Although genome-wide recombination in the second biopsy was beyond the 95% confidence interval of the control group, the mean recombination rates in both biopsies were within the range of recombination rates observed in the control population. Recombination between the sex chromosomes appeared to be unaffected in the t(8;13) carrier, with recombination occurring in 82.0% and 78.0% of spermatocytes in the first and second biopsy, respectively, which were both within the range observed in the control men (Table 4.1). Furthermore, 4.0% and 2.0% of nuclei in the first and second biopsies, respectively, showed at least one achiasmate autosomal bivalent, which were not beyond the range observed in the control men (Table 4.1).

While genome-wide recombination and recombination between the sex chromosomes appeared to be unaffected, we were also interested in quantifying recombination specifically on the translocated chromosomes. Combining immunofluorescent analyses with FISH allowed us to identify the chromosome arms of the quadrivalent (Figure 4.1B). Recombination on chromosome 13 was examined in 50 and 91 pachytene nuclei from the first and second biopsies on the t(8;13) patient, as well as 338 pachytene nuclei from 6 control men (Table 4.2). In the control population, 13q displayed two MLH1 foci in 83.4% of pachytene spermatocytes, which was significantly greater than the 48.0% and 48.4% of pachytene nuclei from the first and second biopsies, respectively ($p<0.01$, Fisher test). Recombination on chromosome 8 was examining in 158 spermatocytes from 2 control men, as well as 50 and 91 spermatocytes from the first and

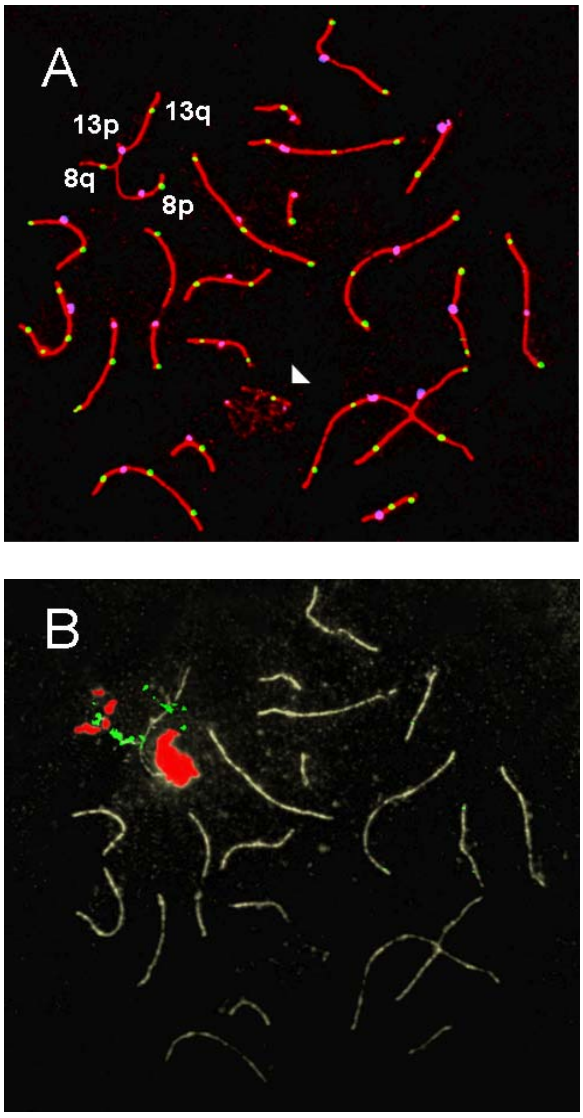

Figure 4.1: Immunofluorescent and FISH analysis of spermatocytes from a t(8;13) carrier. **(A)** Spermatocytes were immunostained to visualize the synaptonemal complex (red), MLH1 foci (green) and centromeres (blue). **(B)** FISH was performed to identify the centromere of chromosome 8 (red), 8q24 (red/green) and 13q14 (green). In **(A)**, a quadrivalent displays complete synapsis and no association with the sex chromosomes (arrow head).

Table 4.1: Analysis of MLH1 foci in an azoospermic carrier of a t(8;13) translocation.

	# of cells analyzed (*n*)	Mean no. of recombination foci per cell (95% CI)	Range of recombination foci	Recombination in XY bivalent (%)	Proportion of cells with an autosomal bivalent lacking an MLH1 focus (%)
Control men					
C1	46	49.5 (48.3-50.7)	39-59	82.6	-
C3	100	48.0 (47.0-48.9)	39-56	81.0	3.0
C4	30	47.3 (45.5-49.1)	37-57	80.0	3.3
C5	41	45.2 (43.9-46.6)	37-53	80.5	2.4
C6	46	48.6 (47.4-49.9)	39-58	78.3	0
C7	41	46.2 (44.5-47.8)	34-59	92.7	0
C8	100	51.3 (50.2-52.4)	30-66	87.0	3.0
C9	101	44.4 (43.2-45.5)	28-55	71.0	5.0
C10	101	48.3 (47.2-49.5)	31-61	76.2	5.9
C11	100	47.9 (47.1-48.7)	35-58	82.0	1.0
Mean (95% CI)		**47.7 (46.2-49.1)**		**81.1 (77.0-85.3)**	**2.6 (1.0-4.2)**
t(8;13) biopsy 1	100	47.3 (46.0-48.6)*	24-56	82.0	4.0
t(8;13) biopsy 2	100	49.6 (48.5-50.7)*	26-64	78.0	2.0

*recombination was significantly different between the two biopsies on the t(8;13) carrier (p=0.013, Mann-Whitney test)

Table 4.2: Analysis of crossovers on chromosome arms involved in the t(8;13) translocation.

		13q [*f*% (*n*)]				8p [*f*% (*n*)]			8q [*f*% (*n*)]		
	# of cells	0 foci	1 focus	≥ 2 foci	# of cells	0 foci	1 focus	≥ 2 foci	0 foci	1 focus	≥ 2 foci
Control men	338	0%	16.6% (56)	83.4% (282)	158	2.5% (4)	94.9% (150)	2.5% (4)	0.6% (1)	62.7% (99)	36.7% (58)
t(8;13) biopsy 1	50	4.0%[b] (2)	48.0%[a] (24)	48.0%[a] (24)	50	4.0% (2)	96.0% (48)	0%	6.0%[c] (3)	74.0% (37)	20.0%[c] (10)
t(8;13) biopsy 2	91	1.1% (1)	50.5%[a] (46)	48.4%[a] (44)	91	2.2% (2)	93.4% (85)	4.4% (4)	1.1% (1)	79.1%[c] (72)	19.8%[a] (18)

[a]$p<0.01$ when compared to the pooled control men, Fisher test
[b]$p<0.02$ when compared to the pooled control men, Fisher test
[c]$p<0.05$, when compared to the pooled control men, Fisher test

second biopsies (Table 4.2). There was no difference in the frequency of crossovers on the 8p arm of the t(8;13) quadrivalent when compared to the control men. However, the 8q arm displayed double crossovers in 36.7% of pachytene nuclei from the control men, which was significantly greater than the 20.0% (p=0.037, Fisher test) and 19.8% (p=0.006, Fisher test) in the first and second biopsies, respectively (Table 4.2). Spermatocytes from the first biopsy displayed achiasmate 8q arms in 6.0% of nuclei, which was significantly greater than the 0.6% of nuclei from the control men (p=0.044, Fisher test).

4.3.2 Synapsis, XY-association and inactivation of quadrivalents

In order to pair homologous regions, the chromosomes involved in a reciprocal translocation adopt a quadrivalent configuration during meiosis (Figures 4.2A and 4.2B). The t(8;13) carrier displayed a high degree of asynapsis in the chromosomal regions adjacent to the breakpoints, with 90% and 87% of quadrivalents from the first and second biopsies, respectively, displaying asynapsed quadrivalents (Table 4.3). These asynapsed quadrivalents were found to associate with the sex chromosomes (Figure 4.2C) in 74% of pachytene nuclei from both biopsies (Table 4.3). Only 10% and 13% of quadrivalents in the first and second biopsy, respectively, displayed heterosynapsis which enabled the quadrivalents to fully synapse, and these configurations displayed no association with the sex chromosomes (Figure 4.2D).

Spermatocytes that were previously immunostained to observe synapsis and recombination were subsequently immunostained to investigate the localization of BRCA1 and γH2AX, two proteins implicated in MSCI (Figure 4.3). Pachytene nuclei from normal 46,XY men displayed BRCA1 and γH2AX localization exclusively on the sex chromosomes, indicating their transcriptional inactivation during meiosis (Figures 4.3A-A'). BRCA1 was observed only on the asynapsed axial elements of the sex chromosomes, but not on the paired pseudoautosomal region (arrow, Figure 4.3A'). Asynapsed regions of the quadrivalent that were associated with the sex chromosomes were positive for both BRCA1 and γH2AX, suggesting that these regions may be inactivated during meiosis (Figures 4.3B-B'). However, association of unpaired autosomal elements with the sex chromosomes does not appear to be necessary for

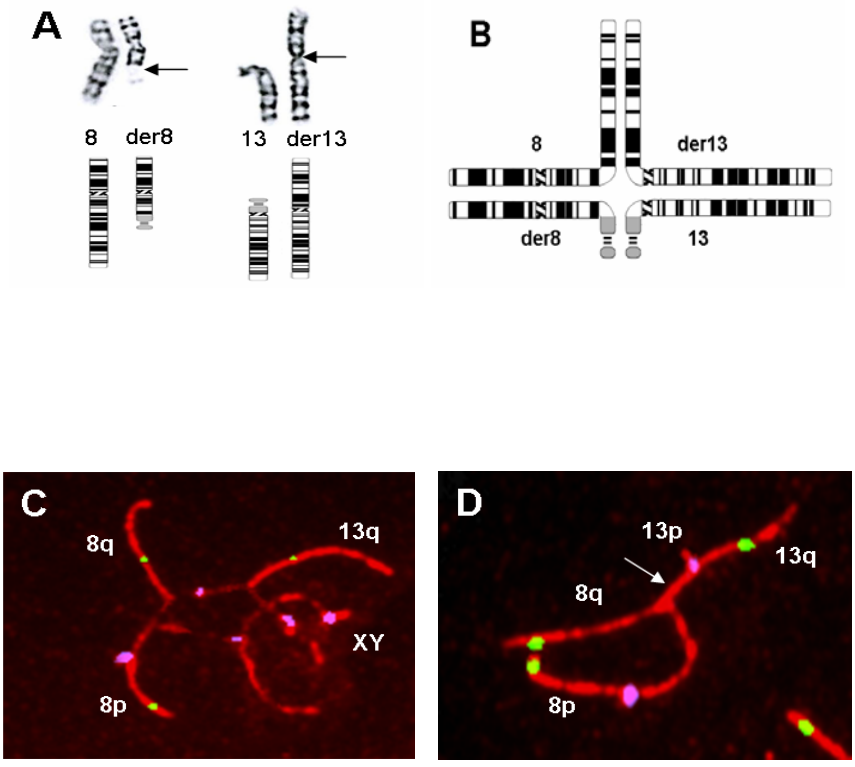

Figure 4.2: Fidelity of synapsis in a t(8;13) reciprocal translocation. (**A**) G-banding on peripheral blood metaphases was performed to identify breakpoints (arrows) of the t(8;13)(q21;p11) reciprocal translocation. **(B)** During the first meiotic division reciprocal translocations adopt a quadrivalent conformation in order to pair all homologous regions. **(C)** Immunostaining of synaptonemal complexes (red), centromeres (blue) and sites of recombination (yellow) showed that the majority of spermatocytes contained asynapsed quadrivalents which were frequently associated with the sex chromosomes. **(D)** Spermatocytes in which the quadrivalents displayed heterosynapsis and complete pairing (arrow) were rare, and these quadrivalents showed no association with the sex chromosomes.

Table 4.3: Comparison of synapsis and recombination in carriers of different reciprocal translocations

Translocation	Semen parameters	Mean recombination foci per cell	% of cells with asynaped quadrivalents	% of cells with XY-quadrivalent associations	Reference
t(8;13)(q21;p11) biopsy 1	azoospermic	47.3 ± 6.5	90	74	Present study
t(8;13)(q21;p11) biopsy 2	azoospermic	49.6 ± 5.6	87	74	Present study
t(11;14)(q13;q32)	azoospermic	48.0 ± 3.5	98	20	Pigozzi *et al*. (2005)
t(13;20)(p11;p11)	azoospermic	45.4 ± 7.6	71	46	Oliver-Bonet *et al*. (2005)
t(10;14)(q24;q32)	normozoospermic	50.0 ± 4.6	30	0	Oliver-Bonet *et al*. (2005)
T(Y;1)(q?;q?)	azoospermic	34.9 ± 18.4[a]	n/a	n/a	Sun *et al*. (2005a)

[a]Recombination significantly reduced when compared to control men

transcriptional inactivation. Asynapsed quadrivalents displaying no association with the sex chromosomes were also positive for BRCA1 and γH2AX (Figures 4.3C-C'). Quadrivalents displaying heterosynapsis and complete pairing of the chromosomes showed no γH2AX staining, and BRCA1 did not appear to be localized along chromosome arms (Figures 4.3D-D').

To further investigate the transcriptional silencing of unsynapsed regions, we immunostained new slides with antibodies against RNA polymerase II and SYCP3 (Figure 4.4). Pachytene nuclei from control men display an absence of RNA polymerase II in the region surrounding the transcriptionally silenced sex chromosomes (Figure 4.4A). Pachytene nuclei in which the t(8;13) quadrivalent was associated with the sex chromosomes also displayed an absence of RNA polymerase II from the sex chromosomes, as well as the attached asynapsed autosomal chromosomes (Figure 4.4B). Similarly, asynapsed quadrivalents that were not directly associated with the sex chromosomes were found to be negative for RNA polymerase II, suggesting that they are also transcriptionally silenced (Figure 4.4C).

4.4 DISCUSSION:

4.4.1 Impaired recombination on translocated chromosomes

To date, recombination frequencies have been studied in four carriers of structural chromosomal abnormalities using immunofluorescent techniques to visualize MLH1 foci (Table 4.3). Only one of the translocations studied so far, an azoospermic t(Y;1) carrier, displayed a rate of genome-wide recombination that was outside the range observed in control men (Sun *et al*., 2005a). Three autosomal-autosomal reciprocal translocations (Oliver-Bonet *et al*., 2005a; Pigozzi *et al*., 2005) displayed rates of recombination that were similar to those observed in normal 46,XY men (Hassold *et al*., 2004; Sun *et al*., 2005b). Similarly, global-recombination rates did not appear to be altered in both biopsies from the t(8;13) carrier that we examined. Interestingly, global-recombination in the second biopsy was significantly greater than the first biopsy. Although recombination has been shown to be highly variable between individuals (Hassold *et al*., 2004; Sun *et al*., 2005b; Sun *et al*., 2006), little is known regarding the variability within an individual. This analysis of recombination in two sites from the same male is the first

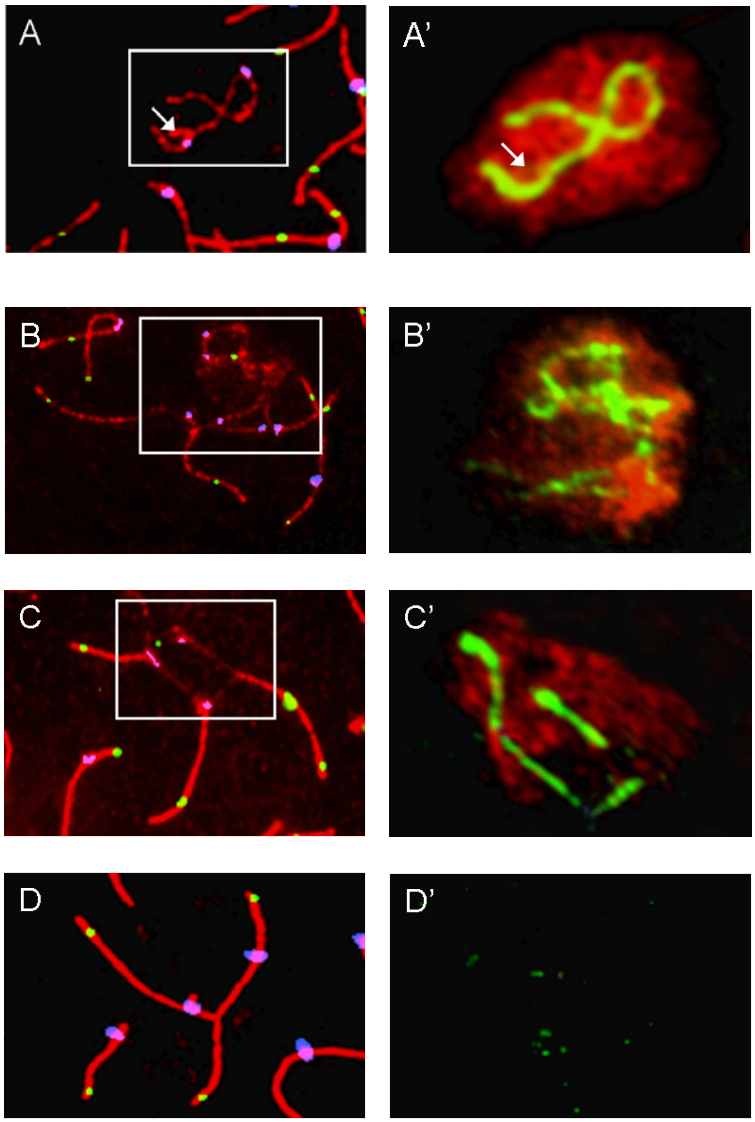

Figure 4.3: BRCA1 and γH2AX localization on meiotic chromosomes. Spermatocytes were immunostained with antibodies against SYCP3 and SYCP1 (red), MLH1 (green) and the centromeres (blue) (**A-D**). The same spermatocytes were then immunostained with antibodies against γH2AX (red) and BRCA1 (green) (**A'-D'**). Sex chromosomes from spermatocytes of control men (**A**, **A'**) display γH2AX labeling, as well as BRCA1 localization on unsynapsed axes, but not at the paired pseudoautosomal region (arrows). Unsynapsed quadrivalents that were associated with the sex chromosomes (**B**, **B'**), as well as unsynapsed quadrivalents not associated with the sex chromosomes (**C**, **C'**) show γH2AX and BRCA1 labelling along unsynapsed regions. Fully synapsed quadrivalents displaying complete heterosynapsis displayed minimal BRCA1 and no γH2AX labeling (**D**, **D'**).

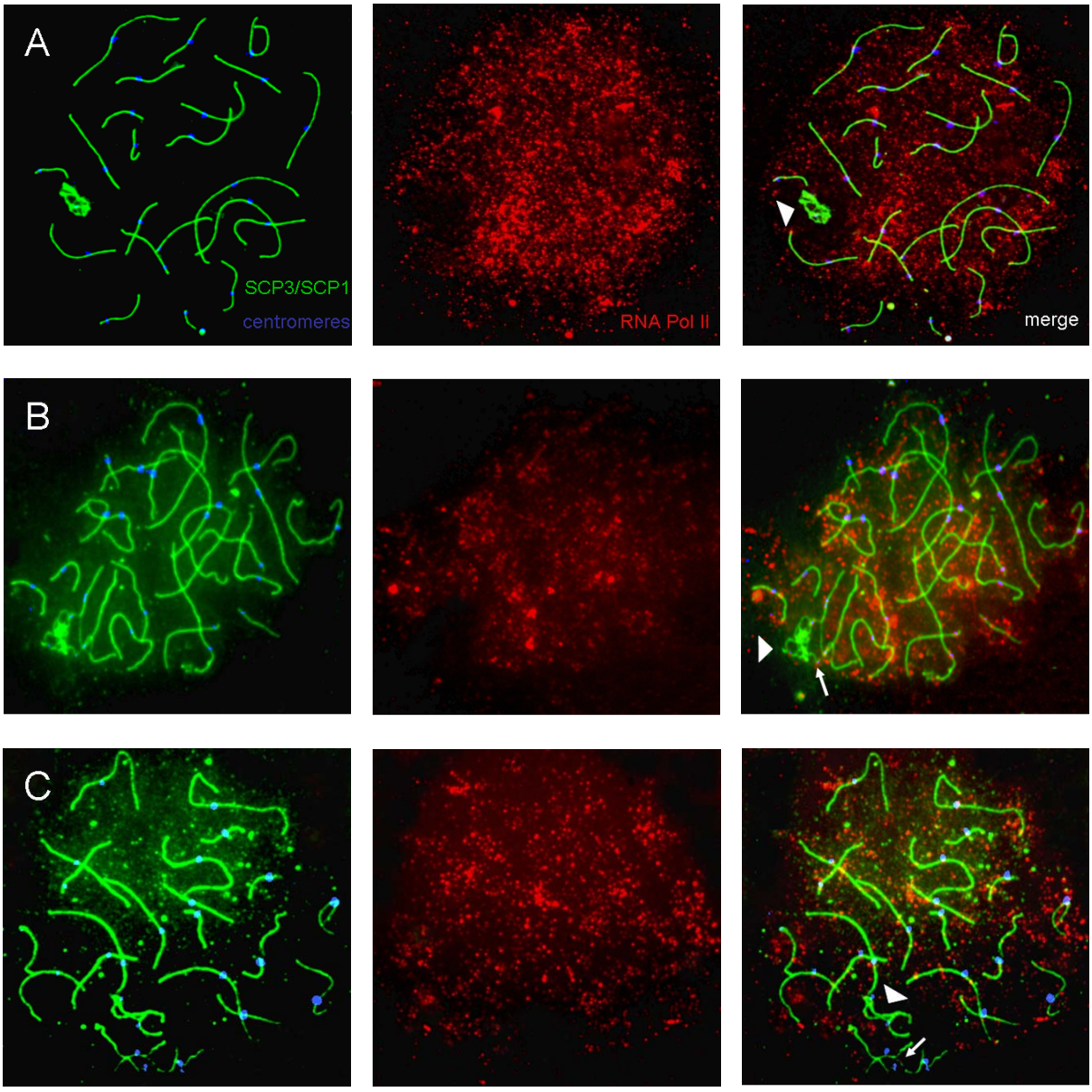

Figure 4.4: Localization of SYCP3/SYCP1 (green) and RNA polymerase II (red) in pachytene nuclei. (**A**) Spermatocytes from control men displayed an absence of RNA polymerase II on the transcriptionally silenced sex chromosomes (arrow head). In (**B**), a spermatocyte from the t(8;13) carrier displays no RNA polymerase II on the sex chromosomes (arrow head), as well as the unpaired quadrivalent associated with the sex chromosomes (arrow). In (**C**), a spermatocyte from the t(8;13) carrier displays an absence of RNA polymerase II on the unpaired quadrivalent (arrow), despite showing no contact with the sex chromosomes.

evidence, to our knowledge, that global-recombination rates are variable within the same individual. The first biopsy was taken from the left testes and, a year later, a second biopsy was performed on the right testes. Thus, due to differences in both the site of tissue retrieval and the time of the biopsies, we cannot attribute the variability to differences between the testes, or environmental factors that may have changed over time. Recombination plays an important role in ensuring the proper segregation of chromosomes during meiosis, and the temporal variation in recombination rates may explain the variation in sperm aneuploidy rates observed in the same men over time (Rubes *et al*., 2005).

FISH on spermatocytes allowed us to quantify recombination on the chromosome arms of the translocated chromosomes. Recombination frequencies on the 8p arm were not altered in either biopsy from the t(8;13) carrier when compared to the control men. However, recombination on the 8q and 13q arms was reduced in both biopsies from the t(8;13) carrier, with a greater proportion of quadrivalents having only a single crossover on these arms, as opposed to the double crossovers that were more common in the control men. It is likely that the high degree of asynapsis on these arms prevented the second crossover from occurring. Similarly, Pigozzi *et al*. (2005) reported a decrease in recombination and a change in the distribution of crossovers on the 14q arm in a t(11;14)(q13;q32) carrier, most likely due to asynapsis. Thus, although genome-wide recombination rates may be normal in translocation carriers, recombination may be disturbed specifically on the rearranged chromosomes.

4.4.2 Behaviour of unpaired meiotic chromosomes

The fidelity of synapsis in male carriers of chromosomal rearrangements is thought to be critical for the completion of spermatogenesis. Unpaired chromosomal regions may be detected by a pachytene checkpoint, leading to apoptotic death of the cell and spermatogenic arrest (Odorisio *et al*., 1998). Indeed, studies on azoospermic carriers of reciprocal translocations have reported a high degree of asynapsis around the breakpoints (Table 4.3). The azoospermic t(8;13) carrier reported on in this study further supports the relationship between asynapsis and infertility, as the majority of quadrivalents from both biopsies failed to pair properly. These asynapsed regions were also found to migrate towards, and pair with the sex chromosomes

during pachytene. It has been suggested that the asynapsed regions of the quadrivalents attempt to pair with the sex chromosomes in order to avoid detection by the pachytene checkpoint which monitors synapsis (Oliver-Bonet *et al*., 2005b). While there is little information on the meiotic behaviour of quadrivalents in normozoospermic translocation carriers, a study by Oliver-Bonet *et al*. (2005a) on a normozoospermic t(10;14) carrier showed a low frequency of asynapsis around the breakpoints. A few sperm were found in the first biopsy of the t(8;13) carrier examined in this study, suggesting that, as long as some spermatocytes contain completely synapsed quadrivalents, a few cells may be able to complete meiosis and produce sperm.

The association between the rearranged chromosomes and the XY body has lead to the hypothesis that MSCI may spread to the quadrivalent, leading to the inactivation of autosomal genes (Jaafar *et al*., 1993). However, recent studies have shown that meiotic silencing may not be limited to the sex chromosomes. Rather, MSCI appears to a be an example of a more generalized mechanism known as meiotic silencing of unsynapsed chromatin (MSUC) that silences any asynapsed regions during meiosis, regardless of whether they are on autosomal or sex chromosomes (Turner *et al*., 2006). In mice, these asynapsed regions are characterized by the localization of BRCA1 along unsynapsed axes, H2AX phosphorylation, and transcriptional silencing (Turner *et al*., 2005). These observations are consistent with our findings in the t(8;13) carrier. Asynapsed regions of the quadrivalents display BRCA1 and γH2AX localization, regardless of whether or not they pair with the sex chromosomes. Conversely, quadrivalents displaying heterosynapsis and complete pairing display no clear BRCA1 or γH2AX labeling. These observations were consistent with those of Sciurano *et al*. (2007), who recently found that the asynapsed regions of quadrivalents show both BRCA1 and γH2AX localization. Immunostaining for RNA polymerase II provided further evidence that pairing with the sex chromosomes is not required for unsynapsed regions to be transcriptionally silenced. Thus, the hypothesis that MSCI spreads to the quadrivalent is not entirely correct. While asynapsed quadrivalents associated with the sex chromosomes are silenced, pairing with the sex chromosomes does not appear to be required for this inactivation. These observations support suggestions that asynapsis is the driving force behind meiotic inactivation in humans, and is not specific to the sex chromosomes (Turner *et al*., 2006). Homolka *et al*. (2007) recently found that pachytene spermatocytes of mice who carried a t(16;17) translocation showed a partial

suppression of genes near the breakpoint on chromosome 17, providing evidence of MSUC at the mRNA level. Surprisingly, they also found an overexpression of genes on the X chromosome, suggesting that the XY-association of the quadrivalent may also contribute to male sterility by disrupting MSCI (Homolka *et al*., 2007).

Carriers of structural chromosomal abnormalities provide a useful model for studying the behaviour of meiotic chromosomes when synapsis is compromised. While global recombination rates do not appear to be dramatically altered in carriers of reciprocal translocations, recombination may be altered on the rearranged chromosomes, possibly due to the presence of large asynapsed regions. By performing biopsies on both testes of the same patient, we have provided evidence that recombination rates may differ either between the testes or may vary over time in the same individual. The azoospermic t(8;13) patient displayed a high frequency of asynapsis in the quadrivalent, providing further evidence that the fidelity of synapsis is a critical factors in determining spermatogenic outcome. In this report we have provided evidence that unpaired regions, regardless of whether or not they pair with the sex chromosomes, are transcriptionally silenced during human meiosis. If genes critical for meiosis are present in the asynapsed regions, meiotic arrest is likely to occur. Both the chromosomes involved in the translocation, and the location of the breakpoints, are likely the determining factors for the fidelity of synapsis, and thus the fertility status of the carrier.

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY AND CONCLUSIONS:

Infertility affects 10-15% of the population, and approximately half of these cases can be attributed to the male partner (World Health Organization, 1997). Nevertheless, little is known regarding the causes of male infertility, as 40-50% of cases are diagnosed as idiopathic (de Kretser and Baker, 1999). With the introduction of novel immunocytogenetic techniques it has become possible to examine detailed meiotic events in spermatocytes from infertile men. The results presented in this thesis (see Appendix III for summary), as well recent studies in the published literature, suggest that defects in early meiotic events may be a major contributor to defective sperm production in humans. In Chapter II we examined recombination and synapsis in ten control men, seven men with OA, and fourteen men with impaired spermatogenesis. Of the men with impaired spermatogenesis, 36% (5/14) displayed reduced rates of genome-wide recombination when compared to the control men. Reduced genome-wide recombination was observed in one man with OA, suggesting that meiotic defects may be observed in some men who otherwise show normal pathological diagnoses of spermatogenesis. Furthermore, other meiotic defects were observed in our populations of men with NOA and severe OAT. Almost all men with NOA and OAT showed a decrease in pachytene stage cells and an increase in leptotene stage cells. An increase in cells with unsynapsed regions was observed in men with NOA, severe OAT and OA. The frequency of XY disomy was the most frequently increased aneuploidy in the sperm, with increases observed in men with NOA, severe OAT and OA. When all men were pooled, we observed an inverse correlation between the frequency of sex chromosome recombination and XY disomy in the sperm. Thus, a lack of recombination between the sex chromosomes may be a useful predictor of rates of sex chromosome aneuploidy in the sperm. Furthermore, we identified two men with a novel meiotic defect: an absence of recombination between the sex chromosomes. One of these men displayed meiotic arrest and subsequently an absence of sperm in the testes, while the other man had severe OAT and showed a low number of sperm in both the testes and ejaculate. Thus, men displaying an absence of recombination between the sex chromosomes may be at an increased risk of sperm aneuploidy and possibly a chromosomally abnormal fetus after ICSI.

In our study of chromosome-specific recombination rates we observed that some men with NOA and severe OAT showed decreased recombination rates on chromosomes 13, 18 and 21. However, we were also interested in determining if the distribution of crossovers were altered in infertile men. Thus, in Chapter III we examined the distribution of crossovers on chromosomes 13, 18 and 21 in five control men, four men with OA, and six men with either NOA or severe OAT. Four of the infertile men (2 with NOA, 1 with OAT, and 1 with OA) displayed an altered crossover distribution on at least one of the chromosome arms studied. Of these four men, two had displayed reduced rates of meiotic recombination on the specific chromosome displaying an altered crossover distribution. One of the OAT men displayed an abnormality in crossover interference, with inter-focal distances reduced on chromosome 13 bivalents. This is the first evidence of altered crossover distributions in an infertile population. Thus, we suggest that recombination defects in infertile men may include alterations in the number of crossovers, the position of crossovers, or both.

In Chapter IV we examined the meiotic behaviour of chromosomes in two biopsies from a carrier of a t(8;13) reciprocal translocation. Genome-wide recombination rates differed between the two biopsies which were taken one year apart; however, recombination rates in both biopsies did not appear to differ from the control group. While global recombination rates were not altered when compared to control men, recombination frequencies were reduced specifically on the rearranged chromosomes, most likely due to the presense of large asynapsed regions. These asynapsed regions were associated with the sex chromosomes in ~90% of spermatocytes from both biopsies. The localization of both BRCA1 and γH2AX, and the absence of RNA PolII suggests that asynapsed regions is undergo transcriptional inactivation during meiosis, even if they are not in contact with the sex chromosomes. The presense of asynapsed region may account for the variability in sperm parameters among male carriers of reciprocal translocations. Chromosomal rearrangements that fail to pair properly may be caught by a meiotic checkpoint; however, there is now evidence that these regions will be inactivated, which could lead to meiotic arrest and infertility.

In summary, meiotic defects such as an impaired progression through prophase I, a reduced number of meiotic crossovers, changes in the distribution of crossovers, and an increase in synaptic defects were observed in men with idiopathic infertility. These meiotic defects were observed in a substantial portion of the infertile men, suggesting that they may constitute a novel cause of impaired spermatogenesis. Reduced recombination between the sex chromosomes appears to be particularly disrupted in infertile men and may be associated with an increased risk of sex chromosome aneuploidy in the sperm, and potentially an increased risk of a chromosomally abnormal offspring after ICSI. Meiotic defects are likely the cause of infertility in male carriers of reciprocal translocations. These men appear to show normal genome-wide recombination rates, but display severe defects in the synapsis of the rearranged chromosomes. Clearly, further studies will be necessary to confirm our present findings in a larger sample size, as well as to address new questions that have arisen based on this work.

5.2 FUTURE DIRECTIONS:

The work presented in this thesis has only begun to address the relationship between meiotic defects, male infertility and the production of aneuploid sperm. Furthermore, the cause of meiotic defects in humans is currently unknown. Early studies in screening meiotic genes for mutations have been met with some success (Miyamoto *et al*., 2003; Zhang *et al*, 2006), while others have been less promising (Westerveld *et al*., 2005; Christensen *et al*., 2005; Zhang *et al*., 2008). While several studies have examined meiosis in infertile populations, and several others have examined gene mutations in meiotic genes, no studies have examined both gene mutations and the meiotic phenotypes. Such studies will be extremely useful for identifying the consequences of meiotic mutations on meiosis. Studies in knock-out mice have provided insights into the resulting phenotypes of mutations in meiotic genes, and will provide an important tool for identifying candidate genes for mutation screening in humans. Furthermore, it may also be useful to examine early recombination proteins in infertile men displaying reduced meiotic recombination. By performing such studies, it may be possible to identify whether reduced recombination originates at the level of the DNA DSBs or later in the processing of the DSBs when they are converted into either crossovers or non-crossovers.

The cause of meiotic defects may not be entirely genetic, as it is also possible that environmental factors play a role. Very little is known regarding the impact of chemical exposures on meiotic events such as synapsis or recombination. A recent study has found that exposure to BPA, a component of polycarbonate plastics, has dramatic effects on synapsis and recombination in mice oocytes (Susiarjo *et al*., 2007). Nevertheless, almost nothing is known regarding the effects of BPA or other chemicals on meiosis in males. A meta-analysis of the incidence of Klinefelter syndrome over the past 30 years has suggested that there has been an increase in the incidence of the chromosomal abnormality (Morris *et al*., 2008). Because Klinefelter syndrome has a strong paternal contribution, this increase may be attributable to the fathers, and may be related to the controversial reports of declining sperm counts in humans (Swan *et al*., 2000; Jensen *et al*., 2002). Both of these phenomenons, if indeed true, likely have strong environmental components which may be acting at the level of meiosis. Investigating the effects of chemical exposure on meiosis would be most feasible in mice, as quantifying chemical exposures in men whose testicular tissue is available would be extraordinarily difficult.

It will also be important to identify the clinical impact of meiotic defects, particularly defective meiotic recombination. A study by Kong *et al*. (2004) examined genome-wide microsatellites data from 23,066 individuals and found a modest, yet positive correlation between recombination rates and the number of children born to a mother. This relationship between recombination rates and reproductive success may also apply to infertile men undergoing ICSI. Recombination parameters may prove to be a useful indicator for prediction ICSI success. Studies that follow the ICSI outcomes in men with known recombination rates will be necessary to determine if such a relationship exists.

There is now evidence that MSUC is observed during meiosis in many organisms, including humans. However, relatively little is known regarding the mechanisms and consequences of such a process. It may be informative to examine the proteins that localize to the sex chromosomes during meiosis and proteins that localize to unsynapsed chromosomes to determine if there are differences between MSCI and MSUC. These studies will allow us to determine if MSCI is a result of the more generalized process of MSUC, or if it is a similar, yet distinct process. Furthermore, it will be important to determine the size of asynapsis required for

silencing. So far, studies examining MSUC in humans and mice have relied on carriers of reciprocal translocations who display large regions of asynapsis. If MSUC also acts on small regions of asynapsis, it may have consequences for men with idiopathic infertility, as the results in Chapter II suggest that these men may display increased rates of small asynapsed regions.

5.3 REFERENCES:

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APPENDIX I: ETHICS APPROVAL CERTIFICATES

The original certificate of full board approval and the certificate of amendment approvals are 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	H06-03490

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Vancouver Coastal Health (VCHRI/VCHA)	Vancouver General Hospital
Vancouver Coastal Health (VCHRI/VCHA)	Lions Gate 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:
Investigation of meiotic defects in infertile men undergoing assisted reproductive technologies

THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES: January 9, 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: January 9, 2007			
DOCUMENTS INCLUDED IN THIS APPROVAL:			**DATE DOCUMENTS APPROVED:**
Document Name	**Version**	**Date**	
Protocol:			
Investigation of meiotic defects in infertile men undergoing assisted reproductive technologies	1.2	December 18, 2006	
Consent Forms:			**January 9, 2007**
subject consent form	1.2	December 18, 2006	
control consent form	1.2	December 18, 2006	

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:

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	H06-03490

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) - "Investigation of Meiotic Defects as an Underlying Cause of Male Factor Infertility"

PROJECT TITLE:
Investigation of meiotic defects as an underlying cause of male factor infertility

REMINDER: The current UBC CREB approval for this study expires: January 14, 2009

AMENDMENT(S):

Document Name	Version	Date
Protocol:		
Protocol	1.3	June 5, 2008
Consent Forms:		
Subject consent form	1.4	June 5, 2008
Control consent form	1.4	June 5, 2008

AMENDMENT APPROVAL DATE:
June 13, 2008

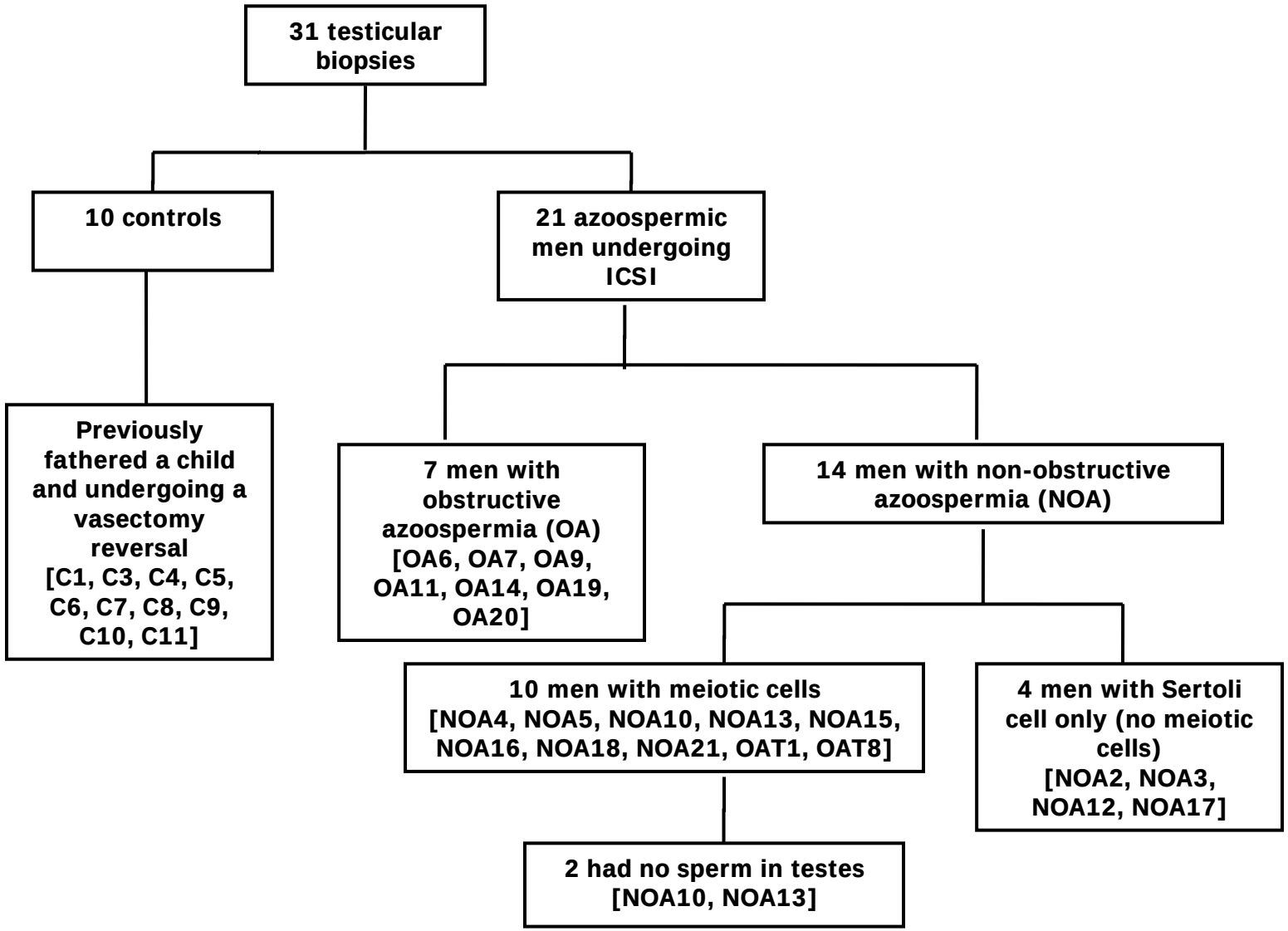
CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with 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:

Dr. James McCormack,
Associate Chair

APPENDIX II: PATIENT FLOW-CHART



APPENDIX III: SUMMARY OF MEIOTIC DEFECTS

Patient	Altered progression through prophase I?	Reduced global recombination?	Increase in unsynapsed regions?	Reduced sex chromosome recombination?	Reduced recombination on chromosomes 13, 18 or 21?	Altered crossover distribution on chromosomes 13, 18 or 21?	Altered inter-focal distances on chromosomes 13 or 18?
OA6	YES	NO	-	YES	-	-	-
OA7	YES	NO	NO	YES	NO	NO	NO
OA9	YES	NO	YES	YES	NO	YES (21)	NO
OA11	YES	NO	YES	NO	NO	NO	NO
OA14	NO	YES	NO	NO	NO	NO	NO
OA19	YES	NO	YES	NO	NO	-	-
OA20	YES	NO	NO	YES	NO	-	-
NOA4	YES	NO	-	NO	-	-	-
NOA5	YES	NO	-	YES	-	-	-
NOA10	YES	YES	YES	YES	YES (13, 18, 21)	YES (18)	NO
NOA13	YES	YES	YES	YES	YES (13, 18, 21)	NO	NO
NOA15	YES	NO	NO	YES	YES (21)	NO	NO
NOA16	YES	YES	NO	YES	YES (13)	NO	NO
NOA18	NO	NO	YES	YES	NO	YES (18)	NO
NOA21	YES	NO	fragmentation	YES	-	-	-
OAT1	YES	YES	YES	YES	YES (13, 18, 21)	YES (13, 21)	YES (13)
OAT8	YES	YES	fragmentation	YES	-	-	-