Investigation of Meiotic Defects in Male Infertility

by

He Ren

B.Sc., The University of British Columbia, 2013

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTGRADUATE STUDIES

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2016

ABSTRACT

Formation of the synaptonemal complex (synapsis), and crossing over of DNA (recombination) is important for chromosome segregation during meiosis. However, reduced rates of recombination have been observed in infertile men. Our previous study linked decreased recombination on sex chromosomes to increased XY disomy in sperm. This finding elicited our interest in the relationship between autosomal recombination and sperm disomy. We hypothesize that a lack of recombination on smaller chromosomes (21, X and Y) may most likely lead to aneuploid sperm. Using immunofluorescence, and fluorescent *in situ* hybridization, we examined synaptic errors, recombination, and sperm aneuploidy in infertile, and fertile men. When all infertile men were pooled, the frequency of recombination on sex chromosomes and bivalent 21 negatively correlated to rates of corresponding sperm disomy. Our unprecedented finding suggest that meiotic defects may indeed be leading to infertility, and increasing sperm aneuploidy.

Moreover, we previously showed changes in crossover distribution in some infertile men. In this thesis, we examined whether this population display specific crossover distributions that may cause chromosome missegregation. Using FISH, we analyzed chromosome-specific crossover distributions, discovering that some infertile men had increased crossovers in regions where they are normally inhibited, which may disrupt structural proteins involved in segregation. We were also interested in the mechanisms behind meiotic defects, and hence studied telomere homeostasis in infertile men. We found deficiencies in telomere association with telomerase in this population, suggesting that defective telomere function may promote improper synapsis and recombination.

Lastly, we examined the meiotic behaviour and sperm aneuploidy rate in an infertile man with a mosaic 45,X(50%)/46,XY karyotype. We found that only 25% of spermatocytes were 45,X, suggesting that half of these cells were arrested. We also noted unpaired sex chromosomes in 12% of spermatocytes. The X:Y sperm ratio was increased, indicating that some 45,X cells may give rise to X-bearing sperm. Even though the patient had higher rates of sperm aneuploidy, the vast majority were of normal constitution. Thus, stringent checkpoints appear to ensure the production of sperm with correct chromosomal complement, and extraction of normal sperm for ICSI may be possible in cases of sex chromosomal mosaicism.

PREFACE

This dissertation is based on one of the ongoing research projects at Dr. Sai Ma's laboratory, and is the subject of a collaboration with past graduate students at the Ma lab. Ethical approval from University of British Columbia (UBC) was obtained prior to initiation of all studies (H06-03490). This study is funded by the Canadian Institutes of Health Research, Ottawa, Canada, grant numbers MOP-93801 and OBM-101387 to S.M. Author contribution, and publications for each chapter are outlined below:

Chapter 2 and 3:

The experiments in Chapter 2 were conceived by my supervisor, Dr. Sai Ma. The experiments in Chapter 3 were conceived by Dr. Sai Ma and Kyle Ferguson. The majority of the experiments and data analysis was performed by the author, He Ren. Of the twenty infertile men reported in this thesis, Tanya Vinning and Christa Darr performed the immunostaining and fluorescent *in situ* hybridization (FISH) on spermatocytes in eight men, as well as FISH on sperm in five men. Dr. Victor Chow from the Department of Urology, University of British Columbia (UBC), performed the testicular biopsies. Dr. Peter Moens from York University, Toronto, provided the SCP1 primary antibodies. With the permission of Dr. Sai Ma, previously published data (sixteen infertile men and twelve control men; Ren *et al*., 2016) were included in this thesis in order to examine the relationship between meiotic defects, and sperm aneuploidy. A version of this chapter was published. Ren, H., Ferguson, K., Kirkpatrick, G., Vinning, T., Chow, V., & Ma, S. (2016). Altered Crossover Distribution and Frequency in Spermatocytes of Infertile Men with Azoospermia. *PloS one*, 11(6),

e0156817. All figures were prepared by He Ren, and the manuscript was written by He Ren and Dr. Sai Ma.

Chapter 4:

The experiments were conceived by Dr. Sai Ma and He Ren. Data collection and analysis was performed by He Ren. All figures were prepared by He Ren, and the manuscript was written by He Ren and Dr. Sai Ma.

Chapter 5:

The experiments were conceived by Dr. Sai Ma. Christa Darr performed immunostaining and FISH on sperm for the infertile man. The remaining experiments, and all of the data analysis were performed by He Ren. Dr. Victor Chow performed the testicular biopsy on the 45,X/46,XY infertile man. With the permission of Dr. Sai Ma, previously published data (45,X/46,XY infertile men; Ren *et al*., 2015) were included in order to examine the meiotic behaviour, and sperm aneuploidy in the infertile man. A version of this chapter was published: Ren, H., Chow, V., & Ma, S. (2015). Meiotic behaviour and sperm aneuploidy in an infertile man with a mosaic 45, X/46, XY karyotype. *Reproductive biomedicine online*, *31*(6), 783-789. All figure preparation was performed by He Ren. The manuscript was written by He Ren and Dr. Sai Ma.

TABLE OF CONTENTS

ABSTRACT ii
PREFACE iv
TABLE OF CONTENTS vi
LIST OF TABLES x
LIST OF FIGURES xi
LIST OF ABBREVIATIONS xii
ACKNOWLEDGEMENTS xvii
CHAPTER 1: INTRODUCTION 1
1.1 Spermatogenesis 2
1.1.1 Regulation of spermatogenesis 9
1.2 Introduction to meiosis 10
1.2.1 Meiosis I 10
1.2.1.1 Chromosome pairing 13
1.2.1.2 Synapsis and the synaptonemal complex 15
1.2.1.3 Meiotic recombination 18
1.2.1.3.1 Mechanism of meiotic recombination 18
1.2.1.4 Meiotic inactivation of sex chromosomes 23
1.2.1.5 Segregation of chromosomes 26
1.2.2 Meiosis II 29
1.2.3 Meiotic checkpoints 30
1.2.4 Sex differences in meiosis 32
1.2.5 Origin of aneuploidy 33
1.2.5.1 Nondisjunction 38
1.2.5.2 Premature sister chromatid segregation 40
1.3 Male infertility 44
1.3.1 Overview of male infertility 44
1.3.1.1 Semen analysis parameters 44
1.3.1.2 Testicular histology 46
1.3.2 Genetic factors in male infertility 47
1.3.2.1 Y chromosome microdeletions 48

1.3.2.2 Cystic fibrosis 49
1.3.2.3 Chromosomal abnormalities 50
1.3.2.4 Defects in meiotic genes 51
1.3.3 Use of intracytoplasmic sperm injection to treat male infertility 52
1.4 Sperm aneuploidy and meiotic defects in infertile men 53
1.4.1 Sperm aneuploidy in infertile men 53
1.4.2 Meiotic defects in infertile men 56
1.4.2.1 Errors in synapsis 57
1.4.2.2 Errors in crossover frequency 58
1.4.2.2.1 Absence of crossovers 60
1.4.2.3 Alterations in crossover distribution 60
1.5 Rationale, hypotheses, and objectives 61
CHAPTER 2: ASSOCIATION BETWEEN SYNAPSIS, RECOMBINATION AND SPERM ANEUPLOIDY IN INFERTILE MEN 65
2.1 Introduction 65
2.2 Materials and methods 67
2.2.1 Patient information and tissue collection 67
2.2.2 Preparation of testicular tissue 67
2.2.3 Fluorescence immunostaining 68
2.2.4 FISH on immunostained spermatocytes 69
2.2.5 FISH on testicular spermatozoa 70
2.2.6 Statistical analyses 71
2.3 Results 72
2.3.1 Analysis of synapsis 72
2.3.2 Analysis of genome-wide recombination 76
2.3.3 Chromosome-specific crossover frequencies and sperm aneuploidy 77
2.4 Discussion 86
2.4.1 Errors in recombination and synapsis in azoospermic men 86
2.4.2 Linking recombination and sperm aneuploidy in azoospermic men 88
CHAPTER 3: CROSSOVER DISTRIBUTION IN INFERTILE MEN 91
3.1 Introduction 91
3.2 Materials and methods 92
3.2.1 Meiotic analyses 92

3.2.2 Statistical analyses 95
3.3 Results 95
3.3.1 Frequency of crossovers, and sperm aneuploidy in azoospermic men 95
3.3.2 Distribution of crossovers in fertile men 95
3.3.3 Distribution of crossovers in OA men 96
3.3.4 Distribution of crossovers in NOA men 100
3.3.5 Crossover distance to telomere 103
3.4 Discussion 105
3.4.1 Increase in crossovers near centromere and telomeres in azoospermic men 105
3.4.2 Increased crossover distance to telomeres in azoospermic men 106
3.4.3 Altered crossover frequency and distribution in azoospermic men 107
CHAPTER 4: TELOMERE HOMEOSTASIS, AND RECOMBINATION IN INFERTILE MEN 110
4.1 Introduction 110
4.2 Materials and methods 111
4.2.1 Fluorescence immunostaining 111
4.2.2 Statistical analyses 113
4.3 Results 113
4.3.1 Analysis of genome-wide recombination 113
4.3.2 Analysis of telomere homeostasis 113
4.3.3 Genome-wide recombination and telomere homeostasis 115
4.4 Discussion 117
4.4.1 Deficiency in TERT association with telomeres in azoospermic men 117
4.4.2 Linking levels of telomere-associated TERT with recombination 119
CHAPTER 5: MEIOTIC BEHAVIOUR AND SPERM ANEUPLOIDY IN AN INFERTILE MAN WITH 45,X/46,XY MOSAICISM 121
5.1 Introduction 121
5.2 Materials and methods 123
5.2.1 Patient information and tissue collection 123
5.2.2 Fluorescence immunostaining 124
5.2.3 FISH on immunostained spermatocytes 124
5.2.4 FISH on testicular spermatozoa 126
5.2.5 Statistical analyses 126

5.3 Results 126
5.3.1 Meiotic sex chromosome configurations 126
5.3.2 Testicular sperm aneuploidy 127
5.3.3 MSCI of unpaired sex chromosomes 129
5.3.4 Genome-wide recombination and synaptic errors 130
5.4 Discussion 132
5.4.1 Fate of 45,X cell line in the testis 132
5.4.2 Sex chromosome asynapsis in 46,XY cells 133
5.4.3 Recombination and synapsis in mosaic 45,X/46,XY cells 134
CHAPTER 6: SUMMARY AND CONCLUSIONS 137
6.1 Summary and future directions 137
6.1.1 Meiotic recombination and sperm aneuploidy 137
6.1.2 Telomere homeostasis and recombination 139
6.1.3 The fate of sex chromosomal mosaicism in the testes 140
6.2 Conclusion 141
BIBLIOGRAPHY 143
APPENDIX I: PATIENT FLOW CHART 165

LIST OF TABLES

Table 1.1 Aneuploidy in humans at different stages……35

Table 1.2 Origins of human aneuploidies……37

Table 1.3 World Health Organization (WHO) revised diagnoses of semen parameters…….46

Table 1.4 Immunofluorescent analyses of spermatocytes in infertile men……..59

Table 2.1 Analysis of recombination and synaptic errors in azoospermic and control men….74

Table 2.2 Analysis of crossover frequencies on chromosome 13, 18 and 21 in azoospermic and control men…….80

Table 2.3 Testicular sperm aneuploidy in the azoospermic and control men…….82

Table 4.1 Percentage of TRF2-bound TERT in azoospermic and fertile men……..115

Table 5.1 Meiotic sex chromosome configuration in the infertile 45,X/46,XY patient and fertile man……..127

Table 5.2 Testicular sperm aneuploidy in the 45,X/46,XY patient and control men……128

Table 5.3 Genome-wide recombination, synaptic errors and sex body recombination in the 45,X/46,XY patient and control men…….131

LIST OF FIGURES

Figure 1.1 Migration of mammalian primordial germ cells (PGCs) 3
Figure 1.2 Spermatogenesis. 5
Figure 1.3 Development of spermatogonium into spermatozoa in the seminiferous tubules... 7
Figure 1.4 Stages of prophase I during meiosis I. 12
Figure 1.5 Telomere movement during prophase I of meiosis I. 14
Figure 1.6 Structure of the synaptonemal complex (SC). 17
Figure 1.7 Mechanisms of meiotic recombination. 20
Figure 1.8 Meiotic spindle orientation and cohesin during metaphase and anaphase I and II. 28
Figure 1.9 Nondisjunction as a mechanism for the production of aneuploidy gametes. 42
Figure 1.10 Premature sister chromatid segregation as a mechanism for the production of aneuploid gametes. 43
Figure 2.1 Immunofluorescence and FISH analysis of pachytene cells. 73
Figure 2.2 Relationship between the frequency of recombination on the sex body and XY disomy in the sperm of azoospermic men (n = 20). 84
Figure 2.3 Relationship between the frequency of recombination on bivalent 21 and disomy 21 in the sperm of azoospermic men (n = 20). 85
Figure 3.1 Diagram depicting meiotic crossovers in regions along a chromosome. 94
Figure 3.2 Chromosomes 21, and 13 displaying altered crossover distributions in OA men. 98
Figure 3.3 Chromosome 18 displaying altered crossover distributions in OA men. 99
Figure 3.4 Chromosome 21 displaying altered crossover distributions in NOA men. 101
Figure 3.5 Chromosome 18 displaying altered crossover distributions in NOA men. 102
Figure 3.6 Average crossover distance to telomere (+SD) on chromosomes 18 and 21 in OA, NOA and control men. 104
Figure 4.1 Cartoon depiction of telomerase (TERT) association with telomeres in spermatocytes. 114
Figure 4.2 Relationship between percentage of TRF2-bound TERT and mean rate of genome-wide recombination in azoospermic and fertile men (n=6). 116
Figure 5.1 Immunofluorescence analysis of 46,XY and 45,X pachytene spermatocytes to visualize the SC (red), MLH1 foci (green) and centromeres (blue). 125
Figure 5.2 Immunofluoresence and FISH analysis of 46,XY and 45,X pachytene spermatocytes. 125
Figure 5.3 Meiotic sex chromosome inactivation in 46,XY and 45,X pachytene spermatocytes. 129

LIST OF ABBREVIATIONS

The abbreviations used in this thesis follow the rules established by the Human Genome Organization. Human genes are noted in all capital letters, whereas mouse genes only have the first letter capitalized. Genes are indicated by italicized fonts, while proteins are indicated by non-italicized fonts.

γH2AX	Phosphorylated H2AX
2n	Diploid
1n	Haploid
Ad	Dark type A (spermatogonia)
Ap	Pale type A (spermatogonia)
ADB	Antibody diluting buffer
AMCA	Aminomethyl coumarin acetic acid
APC/C	Anaphase-promoting complex or cyclosome
ART	Assisted reproductive technologies
ATR	Ataxia telangiectasia and Rad3 related
AURKC	Aurora kinase C
AZF	Azoospermic factor
BMP	Bone morphogenetic protein
BMP8B	Bone morphogenetic protein 8B precursors
BRCA1	Breast cancer 1, early onset
BPY2	Basic protein Y2
Bub	Benzimidizole protein
C	Centromere
C. elegans	*Caenorhabditis elegans*, nematode
CBAVD	Congenital absence of the vas deferens
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
DBY	Dead box on the Y protein
D-loop	Displacement loop
DAPI	4,6-diamidino-2 phenylindole
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DTT	Dithiothreitol
EDTA	5mM ethylene diamine tetraacetic acid
FISH	Fluorecent in-situ hybridization
FITC	Fluorescein isothiocyanate
Fkbp6	FK506 binding protein
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
H2AX	H2A histone family, member X
H3K4me3	H3 trimethylated at position K4
HTF	Human tubal fluid
ICSI	Intracytoplasmic sperm injection
IgG	Immunoglobulin G
IVF	In vitro fertilization
LH	Luteinizing Hormone
LSI	Locus-specific identifier
Mad	Mitotic arrest-deficient

MGD	Mixed gonadal dysgenesis
MI	Meiosis I
MII	Meiosis II
min	Minute
MLH1	Mut-L homolog 1
MLH3	Mut-L homolog 3
Mps1	Monopolar spindle 1 protein
MRC	Meiotic recombination checkpoint
MRE11	Meiotic recombination 11 homolog A
mRNA	Messenger ribonucleic acid
MRE11-RAD50-NBS1	MRN complex
MSCI	Meiotic sex chromosome inactivation
MSH4	MutS homolog 4
MSH5	MutS homolog 5
MSUC	Meiotic silencing of unsynapsed chromatin
No.	Number
NP-40	Nonyl phenoxylpolyethoxylethanol-40
NOA	Non-obstructuve azoospemria
OA	Obstructive-azoospermia
OAT	Oligoasthenoteratozoospermia
p	Short chromosome arm
P1	Protamine 1
P2	Protamine 2
PAR	Pseudoautosomal region
PBS	Phosphate-buffered saline
RAD9-RAD1-HUS1	PCNA-like 9-1-1 complex
PGCs	Primordial germ cells
PMSF	Phenylmethylsulphonyl fluoride

PSCR	Post-meiotic sex chromosome repression
PSCS	Premature sister chromatid segregation
q	Long chromosome arm
RAD51	RAD51 homolog (RecA homolog E. coli)
Rec8	Rec8 meiotic recombiation protein
RNA	Ribonucleic acid
RPA	Replication protein A
S. cerevisiae	*Saccharomyces cerevisiae*, budding yeast
SAC	Spindle assembly checkpoint
SC	Synaptonemal complex
SCC	Saline sodium citrate
SCOS	Sertoli cell only syndrome
SCP1	Synaptonemal complex protein 1
SCP2	Synaptonemal complex protein 2
SCP3	Synaptonemal complex protein 3
SDSA	Synthesis-dependant strand-annealing
Sgo11	Shugoshin
SPO11	SPO11 meiotic protein covalently bound to DSB homolog
SRY	Sex determining region Y
ssDNA	Single-strand DNA
Tam1/Ndj1	Telomere associated meiotic protein
TERT	Telomerase
TESE	Testicular sperm extraction
TEX11	Testis expressed 11 protein
TEX15	Testis expressed 15 protein
TRITC	Tetramethyl rhodamine isothiocyanate
TRF2	Shelterin protein complex
TTY2	Testis transcript Y2

USP9Y	Ubiquitin specific peptidase 9, Y-linked protein
WHO	World Health Organization
XCI	X chromosome inactivation
XIST	X-inactive specific transcript

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to the faculty, staff, and my fellow colleagues at UBC, who have helped and encouraged me during graduate studies. I owe particular gratitude to my supervisor Dr. Sai Ma for her continuous guidance and support, and for providing me with incredible opportunities during my time in her laboratory. I would like to extend my gratitude to the members of my supervisory committee, Dr. Deborah Money, Dr Tim Rowe, and Dr. Petrice Eydoux, for their constructive feedback and suggestions. I would also like to acknowledge the past and current members of the Ma laboratory. In particular, I offer my sincere gratitude to Kyle Ferguson, Gordon Kirkpatrick, Tanya Vinning, and Christa Darr for their ideas and contributions to this project over the years. I would also like to thank my current lab members, Kate Watt, Kenny Louie, Richard Ng, Luke Gooding, Samuel Schafer, and Ei-Xia Mussai for their endless support and friendship. The completion of this project was also dependent on the collaboration with Dr. Victor Chow, who performed the testicular biopsies necessary to the experiments presented in this thesis. I am also thankful for Dr. Peter Moens and his laboratory for providing the SCP1 primary antibodies used in the studies of this thesis.

Most importantly, I would like to express my deepest gratitude to my family, especially my mother and father. Without their unconditional love and care, I would not be where I am today. Finally, I would like to thank my friends and my boyfriend, Damon, for their unwavering support and love. It was these sources of continuous encouragement and motivation that allowed me to continue my studies in this field.

CHAPTER 1: INTRODUCTION

The genetic material in humans is organized into highly structured units of DNA and protein complexes known as chromosomes. There are two sets of chromosomes in the somatic cells of the human body, one derived from the father and one derived from the mother. During sexual reproduction, the genetic information in the parents is passed to the offspring. In order to preserve the normal adult chromosome content in the offspring, sexual reproduction begins with the creation of haploid gametes with half the number of chromosomes (n) as the parent cell (2n). Subsequent fertilization involves the fusion of gametes to form a diploid (2n) cell with the normal chromosome complement, which then divides to form an embryo. There are two types of cell division involved in sexual reproduction, termed mitosis and meiosis. Mitosis results in daughter cells with the same number of chromosomes as the parent cell; it is responsible for the division of somatic cells in the embryo and adult, and the proliferation of germinal stem cells in the gonads. Meiosis is specific to gametogenesis, and is responsible for dividing the chromosome content in half to produce haploid gametes. Consequently, the proper progression and completion of meiosis is important for gametogenesis and thus fertility, as well as for ensuring the production of gametes with the correct chromosome complement. This thesis will aim to address how errors during meiosis may contribute to impaired sperm production, and/or the production of chromosomally abnormal sperm in infertile men. To begin, I will outline the processes of spermatogenesis, and explain the specific features in meiosis that deem this specialized cell division crucial for normal sperm production.

1.1 Spermatogenesis

Spermatogenesis describes the development of mature spermatozoa from a self-renewing pool of germinal stem cells (Amann, 2008). This process begins in the seminiferous tubules in the testes, and is aided by two differentiated cell types: Sertoli cells and Leydig cells. Sertoli cells reside in the seminiferous tubules, and mainly function to nourish the developing spermatocytes. Leydig cells are found in the interstitial spaces in the testes, adjacent to the seminiferous tubules, and are responsible for androgen production. The early embryonic development of these key cell types and the testes in general is important for establishing the framework for spermatogenesis to occur later in life.

In the early stages of mammalian embryo development, a small group of cells in the wall of the yolk sac are induced by a set of mRNAs and proteins to form primordial germ cells (PGCs), which are cells that eventually form the germ line of the mammal (Ewen-Campen *et al.*, 2010; Richardson and Lehmann, 2010; Fig. 1.1). After a period of proliferation, PGCs begin to actively migrate into the extra-embryonic mesoderm, through the hindgut and dorsal mesentery, and into the two genital ridges that will eventually become the gonads (Ewen-Campen *et al*., 2010; Richardson and Lehmann, 2010; Fig. 1.1).

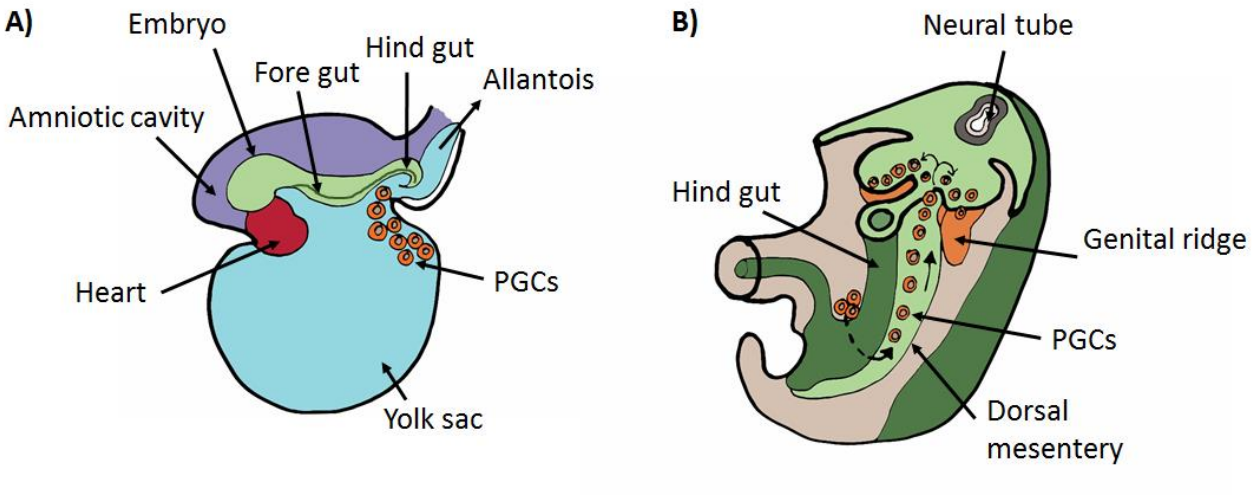

Figure 1.1 Migration of mammalian primordial germ cells (PGCs)

A) During early embryo development, a group of cells in the yolk sac, near the attachment to the allantois, differentiate into PGCs. B) PGCs migrate into the extra-embryonic mesoderm, through the hindgut and dorsal mesentery, and into the two genital ridges.

At this point, PGCs are incorporated into the sex cords and are committed to differentiating into either eggs or sperm, depending on the signals from the developing gonads. Whether the genital ridge develops into female or male gonads, namely the ovaries or testes, depends on the sex chromosome constitution in the somatic cells. The *sex determining region* (*SRY*) gene on the Y chromosome is the determining factor, where its expression signals the differentiation of the testis (Ewen-Campen *et al*., 2010; Richardson and Lehmann, 2010). In response to *SRY* expression, the sex cords morph into seminiferous tubules, while the epithelial cells in the tubules form Sertoli cells (Gilbert, 2014). Interestingly, Sertoli cells have been shown to be important regulators of fetal Leydig cell development (Wen *et al*., 2016). However, the exact origin of fetal Leydig cells remain unclear as these cells are thought to originate from multiple progenitor cells (Wen *et al*., 2016). After the differentiation of the fetal testis, environmental cues begin to signal the differentiation of the PGCs into spermatogonial stem cells, referred to as spermatogonia

(Sutton, 2002). Spermatogonia are the pool of self-renewing cells that maintain spermatogenesis throughout life starting at puberty (Sutton, 2002). In humans, each cycle of spermatogenesis takes approximately 64 days to complete (Amann, 2008), and can be divided into three stages: (1) mitotic division of spermatogonia, (2) meiotic division resulting in haploid spermatids, and (3) differentiation of spermatids into mature spermatozoa in a process known as spermiogenesis (Fig. 1.2).

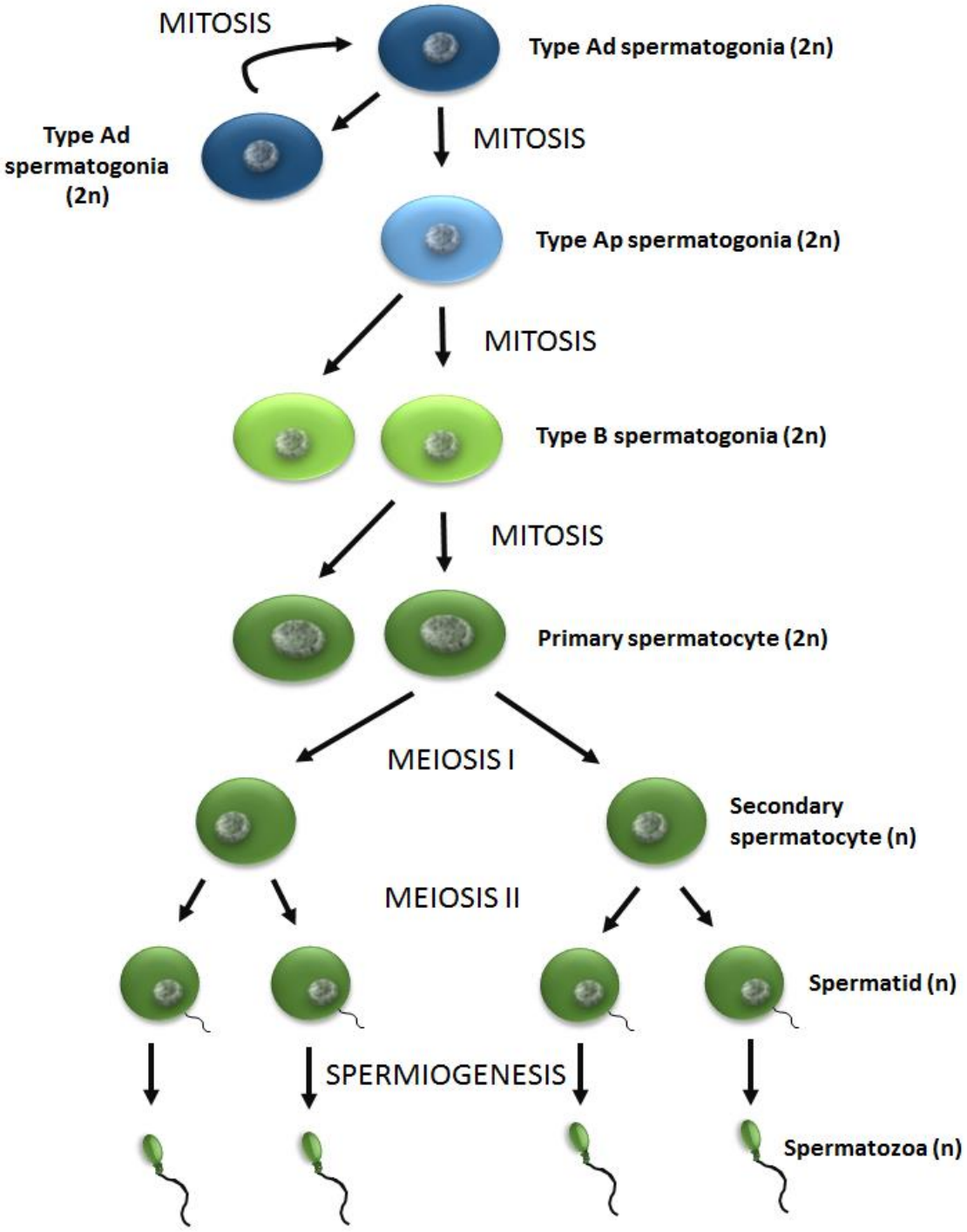

Figure 1.2 Spermatogenesis.

Spermatogenesis is characterized by the differentiation of spermatogonial stem cells through mitosis, meiosis I and meiosis II to reduce the chromosome content by half [from diploid (2n) to haploid (n)], and finally spermiogenesis, where the spermatid differentiates into functional, mature spermatozoa

The development of spermatogonia into more committed cell types occurs in the compartmentalized spaces of the seminiferous tubules, where the least differentiated cells are at the outer edge of the germinal epithelium, while spermatozoa are released into the lumen of the tubules (Fig. 1.3). There are three types of spermatogonia at the outermost layer of the seminiferous tubules: dark type A (Ad), pale type A (Ap), and type B spermatogonia (Amann, 2008; Fig. 1.2). Ad cells are undifferentiated, and function in replenishing the pool of spermatogonia by undergoing mitosis to give rise to more Ad cells. In mammals, the bone morphogenetic protein (BMP) family, consisting of an array of signaling molecules, is thought to play a role in initiating spermatogenesis during puberty (Ghasemzadeh-Hasankolaei *et al*., 2014). In mouse studies, the release of bone morphogenetic protein 8B precursors (BMP8B) by Ad cells have been shown to trigger their differentiation into Ap cells (Ghasemzadeh-Hasankolaei *et al*., 2014). Differentiating Ad cells continue to secrete increased levels of BMP8B, which in turn further induce the differentiation of neighbouring Ad cells into Ap cells (Ohinata, 2009). Ap cells subsequently differentiate into type B cells through mitotic divisions, followed by further differentiation into primary spermatocytes for entry into meiosis. Meiosis consists of two cell divisions, termed meiosis I (MI) and meiosis II (MII). During MI, the primary spermatocyte (2n) undergoes reductional division to segregate the homologous chromosomes, essentially splitting the chromosome content in half, to give rise to two haploid secondary spermatocytes (n). During MII, the secondary spermatocytes (n) undergo equational division to segregate the sister chromatids. This process does not reduce the chromosome content as the four spermatids that result remain haploid (n). This specialized cell division will be discussed in detail in section 1.2.

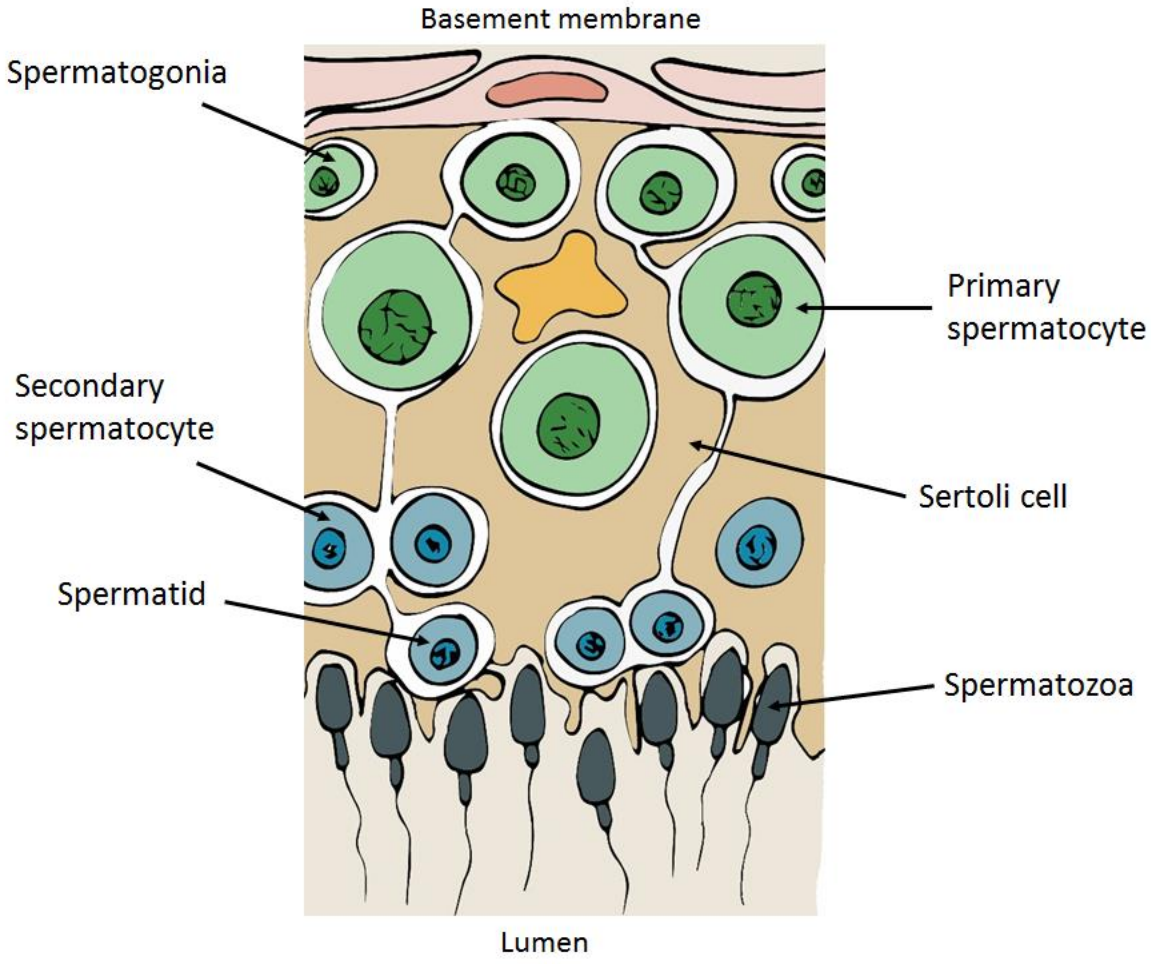

Figure 1.3 Development of spermatogonium into spermatozoa in the seminiferous tubules.

The least differentiated cells, spermatogonium, are at the outer edge of the germinal epithelium, while spermatozoa are released into the lumen of the tubules

The last stage of spermatogenesis is spermiogenesis, which comprises of a series of remarkable events that transform round spermatids into streamlined, functional spermatozoa over a period of 24 days in humans (O'Donnell, 2016). The initial changes in the round spermatid entering spermiogenesis involve the formation of an acrosome cap from the Golgi apparatus and flagellum from the centriole. Both of these structures are necessary for motility and fertilization (O'Donnell, 2016). In the next phase, the nucleus and acrosome cap shift to one side of the cell in preparation for the elongation of the sperm head; a delicate process that is facilitated by nuclear compaction, and chromatin condensation (O'Donnell, 2016). The condensation of chromatin is achieved by replacing DNA-associated histones with small proteins called protamines (Bao and Bedford, 2016; Agarwal *et al*., 2016; O'Donnell, 2016). The human sperm has two types of protamines: protamine 1 (P1) and a family of protamine 2 (P2). Although the function of protamine replacement in sperm is not fully understood, it is hypothesized that this process aims to condense the chromatin so that the sperm head can assume a compact structure, as well as protect the DNA from damage (Gilbert, 2014; Bao and Bedford, 2016; Agarwal *et al*., 2016; O'Donnell, 2016). However, only 85% of DNA in sperm is modified by protamines, while 15% of DNA remains associated with histones (Oliva, 2006). A balanced level of protamine replacement may be necessary to sperm function; studies have shown that decreased levels of protamine replacement are associated with DNA integrity, sperm viability, and male infertility (Oliva, 2006; Ravel *et al*., 2007). When this crucial step in spermiogenesis is complete, the mature spermatozoa are released into the lumen of the seminiferous tubules and transported to the epididymis where they will reside for approximately two weeks (Upadhyay *et al*., 2012; O'Donnell, 2016). It is during this period that the spermatozoa gain motility, and the ability to fertilize an oocyte.

1.1.1 Regulation of spermatogenesis

From puberty and onward, the progression of spermatogenesis is intricately regulated by a network of hormones starting at the hypothalamus, which releases gonadotropin releasing hormone (GnRH) in regular intervals. GnRH induces the anterior pituitary to release follicle stimulating hormone (FSH) and lutenizing hormone (LH), which target the Sertoli cells and Leydig cells in the testes, respectively. FSH induces Sertoli cells to secrete various factors that are critical for spermatogenesis. In particular, glycoprotein secretion is important for the physical integrity of the seminiferous tubules, transport of ions and hormones, and movement of spermatocytes through the compartments of the tubule (Sherwood, 2015; Dimitriadis *et al*., 2015). Sertoli cells also possess androgen receptors (AR), while germ cells do not (Dimitriadis *et al*., 2015). Since Sertoli cells are in direct contact with the developing germ cells, it is theorized that androgens indirectly affect spermatogenesis through Sertoli cells (Dimitriadis *et al*., 2015). In fact, mouse studies showed that perturbed AR on Sertoli cells led to spermatogenic arrest at the spermatocyte or spermatid stage (Gendt *et al*., 2004; Holdcraft and Braun, 2004). Another important factor released by Sertoli cells in response to FSH is inhibin, which mainly acts in a negative feedback fashion to decrease FSH release by the anterior pituitary. Inhibin also possesses two heterodimers that are capable of enhancing or repressing testosterone production by Leydig cells (Martin, 2016). In addition to regulation by inhibin, Leydig cells are also controlled by LH to maintain the high level of testosterone that is needed for testis development, masculinization, and spermatogenesis. As testosterone builds up in the circulation, a negative feedback loop is activated to repress GnRH release by the hypothalamus (Sherwood, 2015; Martin, 2016). Due to the interconnection of hormones involved in spermatogenesis, an

imbalance in any component could negatively impact sperm production and fertility. Consequently, serum FSH and testosterone levels are routinely tested in the case of male infertility. Elevated FSH levels in particular have been shown to be a cause of impaired spermatogenesis (Dimitriadis *et al*., 2015). Abnormal hormone profiles may therefore affect crucial steps in spermatogenesis, such as meiosis, and lead to spermatocyte arrest. To better understand how errors during this step may contribute to male infertility, the next section will focus on the basic events in meiosis.

1.2 Introduction to meiosis

Meiosis marks a key feature of spermatogenesis, where it ensures correct chromosomal content in the sperm. This specialized cell division involves a round of DNA replication, followed by two successive cell divisions, MI and MII.

1.2.1 Meiosis I

During the first meiotic division, the homologous chromosomes in the diploid parent cell (2n) segregate to give rise to two haploid daughter cells (n) with half the chromosomal content. MI can be subdivided into four stages: prophase I, metaphase I, anaphase I, and telophase I based on the events that occur. From a genetic perspective, prophase I is considered the most important step in meiosis. During this period, the homologous chromosomes pair up and then join together in a process known as synapsis. This is mediated by the formation of the synaptonemal complex (SC), a ladder-like structure built from two axial/lateral elements and a transverse element, between the homologous chromosomes. Once synapsis is complete, the exchange of genetic material between the homologous chromosomes begins in a process known as recombination. Pairing, synapsis, and

recombination occur progressively during prophase I, which can be further subdivided into 5 stages: leptotene, zygotene, pachytene, diplotene, and diakinesis according to the synaptic status of the homologous chromosomes (Fraune *et al*., 2012; Fig. 1.4). During leptotene, the chromosomes condense and begin to seek their homologous counterpart. Meanwhile, short fragments of the SC begin to form along the sister chromatids. During zygotene, the lateral elements of the SC have fully formed along the sister chromatids, while the transverse elements of the SC are just starting to form between the homologous partners. The start of pachytene is defined by the completion of synapsis, where the SC has linked the homologous chromosomes from end to end. This sets the stage for meiotic recombination to occur between the homologous chromosomes. During diplotene and diakinesis, the SC disintegrates, leaving the homologous chromosomes tethered solely at chiasmata, which are structures resulting from the recombination sites that formed during pachytene. In the following sections, we will further examine how the homologous chromosomes pair, synapse, and engage in meiotic recombination.

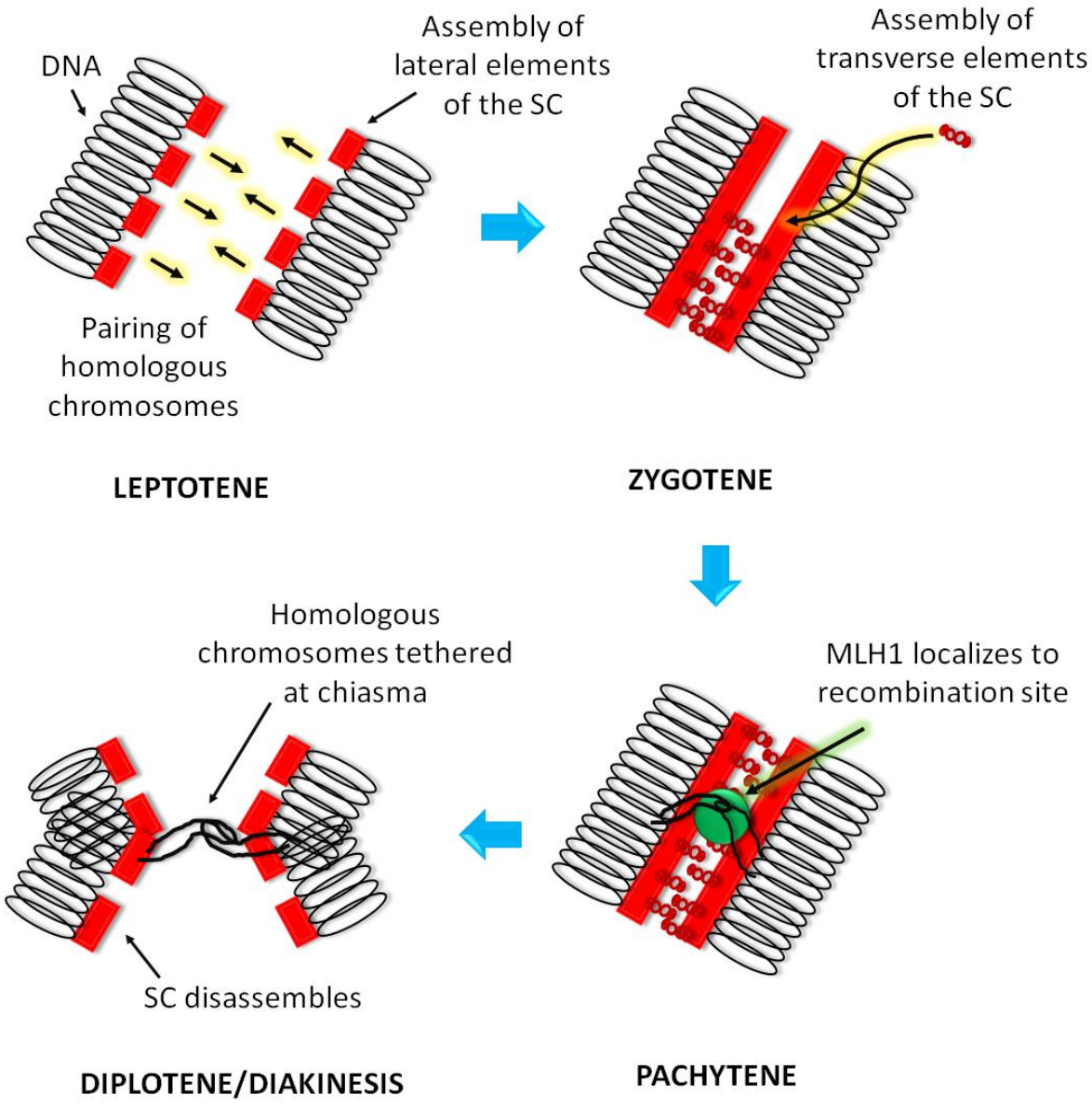

Figure 1.4 Stages of prophase I during meiosis I.

The homologous chromosomes undergo homology search during leptotene; the lateral elements of the synaptonemal complex (SC) also starts to form. During zygotene, the transverse elements of the SC begin to form; synapsis is completed at the pachytene stage, followed by the completion of meiotic recombination which is characterized by the localization of the DNA mismatch repair protein MLH1 in humans. At the diplotene/diakinesis stages, the SC dissembles while the homologous chromosomes start to separate; however, the chromosomes are tethered at the chiasma.

1.2.1.1 Chromosome pairing

Entering meiosis, the chromosomes are spatially separated from their homologous counterparts within the nucleus. The pairing of homologous chromosomes is characterized by overcoming this physical distance so that the chromosomes can pair with their partners based on their DNA sequence homology. Although how the homologous chromosomes find and recognize each other remains one of the mysteries in meiosis, the first step in this process is believed to be reducing the space between the homologous chromosomes (Zickler and Kleckner, 2016). Nearly all organisms rely on a telomere-led mechanism for bringing the homologous chromosomes in close proximity. The orientation of the centromeres and telomeres play an initial role where the telomeres fan out at one side of the nucleus opposite to the centromeres (Zickler and Kleckner, 2016; Fig. 1.5). A cytoskeleton-mediated process then moves the telomeres along the nuclear envelope to group them together, culminating in a tight bundle called a bouquet. The faithful attachment of the telomeres to the nuclear envelope depends on a functional telomere as well as an appropriate association with its structural proteins (Chikashige *et al*., 2007; Conrad *et al*., 2008). Numerous studies have shown that defects in telomere length and telomeric proteins may not only compromise synapsis, but also have a negative downstream effect on meiotic recombination (reviewed in Naranjo, 2012; Reig-Viader *et al*., 2014).

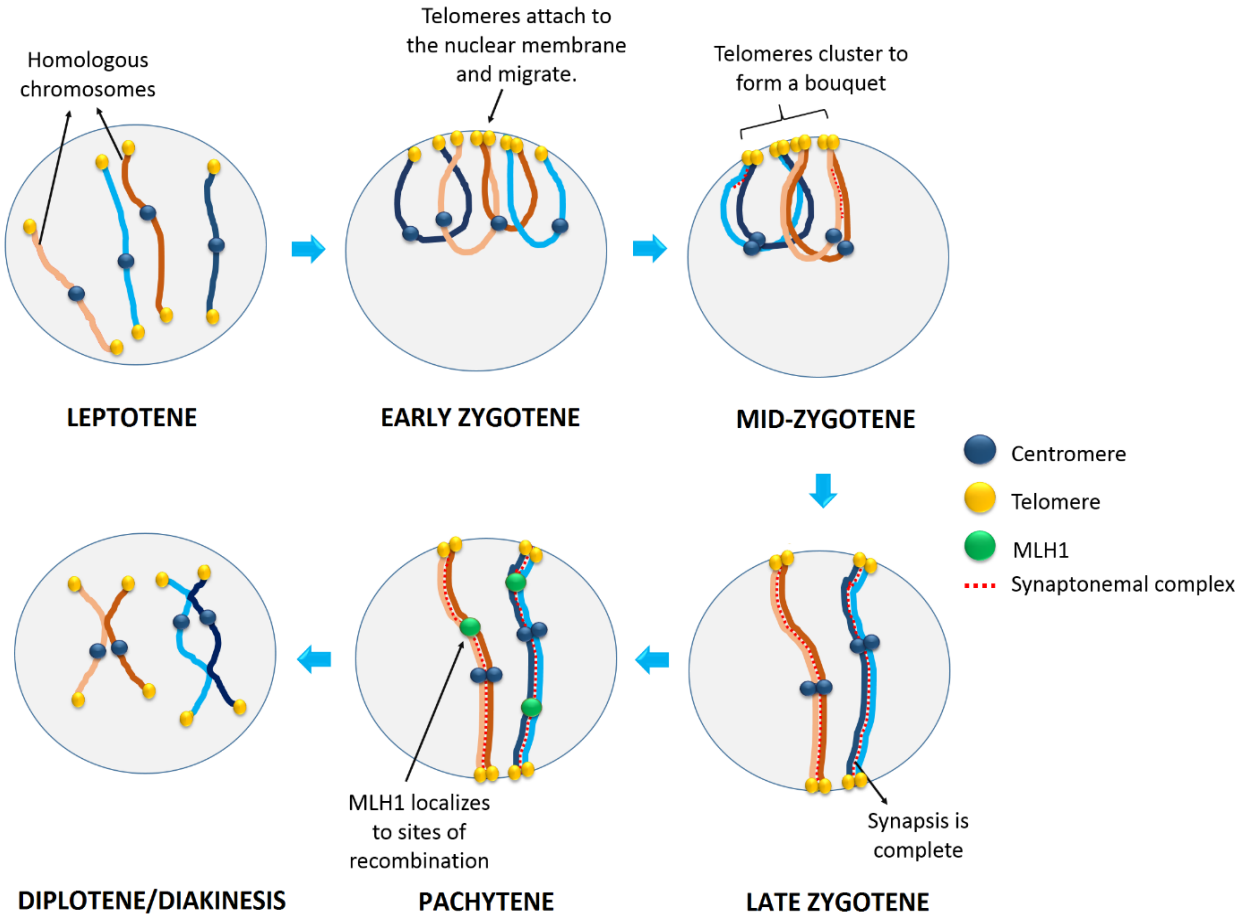

Figure 1.5 Telomere movement during prophase I of meiosis I.

In leptotene, the homologous chromosomes are spatially distant from each other. The telomeres begin to attach to the nuclear membrane and move the chromosomes in to close proximity by early zygotene. By mid-zygotene, the telomeres are bundled closely together to form a bouquet; it is during this time that the transverse elements of the synaptonemal complex forms. During late zygotene, the telomeres disperse, but remain attached at the nuclear membrane; meiotic recombination follows during the pachytene stage. Finally, at the diplotene/diakinesis stage, the telomeres detach from the nuclear membrane.

The movement of the chromosomes into close association is only one step of the pairing process. The homologous chromosomes still need to pair up based on sequence homology, a process that occurs concomitantly with telomere bouquet formation. Studies in different model organisms have yielded a surprising variety of mechanisms for homology search (Naranjo, 2012; Zickler and Kleckner, 2016). The chromosomes of the worm *Caenorhabditis elegans* possess a chromosome-specific pairing center that consists of a DNA-protein complex (Naranjo, 2012; Zickler and Kleckner, 2016). The fission yeast *Schizosaccharomyces pombe* employs a meiosis-specific non-coding RNA to facilitate homology detection (Naranjo, 2012; Zickler and Kleckner, 2016). There are also recombination-dependent strategies identified in the yeast *Saccharomyces cerevisiae,* plants, and mammals (Naranjo, 2012; Zickler and Kleckner, 2016). In these species, the topoisomerase-like protein Spo11 catalyzes the formation of DNA double-strand breaks (DSBs) on the homologous chromosomes during early prophase I. Although DSBs are the foundation for recombination at the later stages of prophase I, they seem to be equally important for homology search and subsequent SC formation as studies in budding yeast, *Sordaria,* and mouse mutants have revealed a relationship between DSBs formation and SC formation (Kauppi *et al.,* 2013; Rockmill *et al.,* 2013). To further suggest the importance of DSB formation on homology recognition, there is evidence that DSBs are more abundant in species that require them for homology recognition compared to those that do not (Lam and Keeney, 2014; Borde and deMassy, 2013).

1.2.1.2 Synapsis and the synaptonemal complex

After the chromosomes pair with their homologous partners, they undergo synapsis where the SC forms to build a bridge between the homologous chromosomes. The SC was

first described in 1956 (Moses), and is a protein structure that is unique to synapsis (Moses, 1958). The two lateral elements of this tripartite run along the length of the homologous chromosomes and are identified as the synaptonemal complex protein 2 (SCP2) and synaptonemal complex protein 3 (SCP3) in mammals (Heyting *et al*., 1985; Zickler and Kleckner, 2016). During synapsis, SCP2 and SCP3 directly interact with the chromosomes through DNA-binding domains (Offenberg *et al*., 1998; Zickler and Kleckner, 2016). The third component of the SC is the transverse filaments, made up of synaptonemal complex protein 1 (SCP1) in mammals, which joins the lateral elements together to form a ladder-like structure (Iersel *et al*., 1992; Zickler and Kleckner, 2016). SCP1 is made up of a middle coiled-coil segment, flanked by a globular N-terminal domain and a globular C-terminal domain (Page and Hawley, 2003; Zickler and Kleckner, 2016; Fig. 1.6). This protein structure facilitates the dimerization of two parallel SCP1s, such that their N-termini interact with each other and reside in the center of the SC, while the C-termini interact with the lateral elements of the SC (Page and Hawley, 2003; Zickler and Kleckner, 2016; Fig. 1.6).

While the structure of the SC is highly conserved in eukaryotes, the order in which synapsis and recombination occur seems to vary drastically between species (Zickler and Kleckner, 2016). Some eukaryotes, such as *Drosophila* and *C. elegans*, require the complete formation of the SC prior to meiotic recombination (McKim *et al.*, 1998; Dernburg *et al.*, 1998; Zickler and Kleckner, 2016). Conversely, many other species such as *S. cerevisiae*, humans, and other mammals initiate recombination through the formation of DSBs, before the SC forms (Mahadevaiah *et al*., 2001; Lenzi *et al*., 2005; Zickler and Kleckner, 2016).

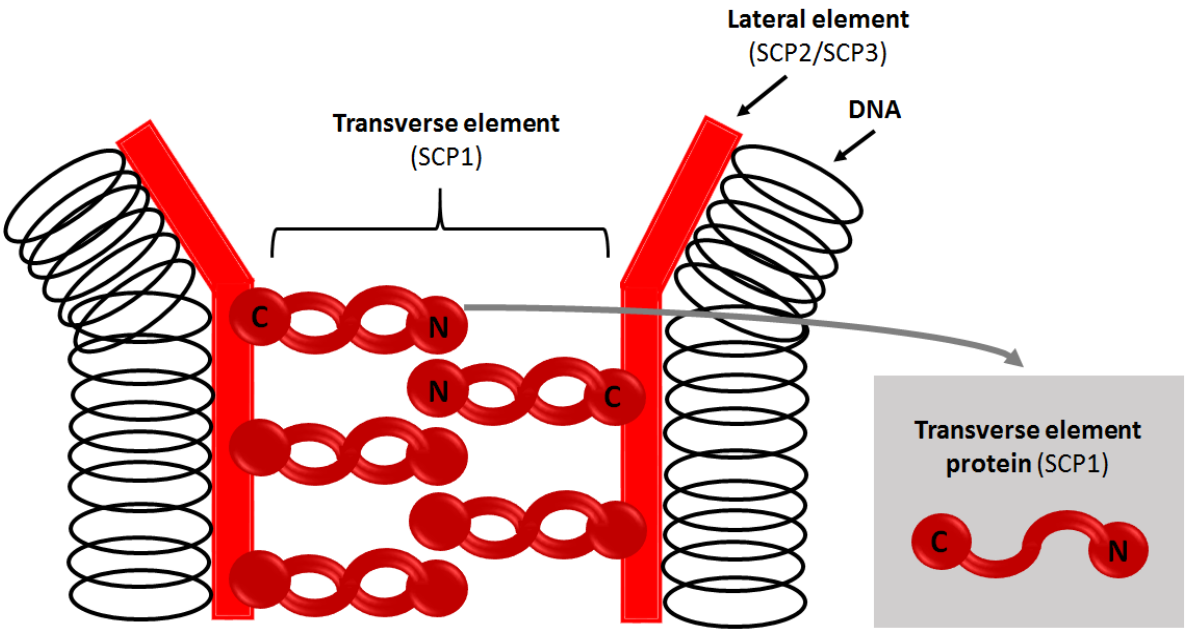

Figure 1.6 Structure of the synaptonemal complex (SC).

The lateral elements (SCP2 and SCP3 in humans) form along the homologous chromosomes. The transverse element (SCP1 in humans) contain a central region that is rich in coiled-coils, flanked by a globular C-terminal domain and a globular N-terminal domain. The C-termini embeds in the lateral elements, while the N-termini form the center of the SC.

1.2.1.3 Meiotic recombination

Meiotic recombination, or crossing over, is characterized by the reciprocal exchange of DNA between homologous chromosomes during prophase I. This process plays two prominent roles: (1) increasing genetic diversity during sexual reproduction, and (2) ensuring the faithful segregation of homologous chromosomes during the first meiotic division. Although many proteins involved in recombination have been described, our understanding of the exact mechanisms of how crossovers form, and how the location of crossovers are regulated remains incomplete.

1.2.1.3.1 Mechanism of meiotic recombination

Meiotic recombination starts with the formation of DNA DSBs, or the physical breakage of DNA along the homologous chromosomes. These DSBs can be rejoined in either a crossover pathway to give a recombinant product, or a non-crossover pathway. Since the number of DSBs is observed to be vastly greater than the number of crossovers that form, it is theorized that most DSBs are resolved through a non-crossover pathway (Baudat *et al*., 2013).

Recombination is initiated at the onset of prophase I with the generation of DSBs along the homologous chromosomes by the nuclease Spo11 (Keeney *et al*., 1997; Fig. 1.7, step 1). There are two isoforms of Spo11: Spo11β and Spo11α. Spo11β is suggested to catalyze the majority of DSBs, namely those on autosomes (Bellani *et al*., 2010; Baudat *et al*., 2013). On the other hand, Spo11α is thought to catalyze DSBs specifically along the sex chromosomes, as suggested from studies in Spo11α null mice, which revealed impaired synapsis and recombination between the sex chromosomes (Kauppi *et al*., 2011; Baudat *et*

al., 2013). After DSBs are formed, Spo11 is released and an unknown exonuclease creates a 3' overhang in the DNA (Hunter and Kleckner, 2001; Fig. 1.7, step 2). Currently, the most probable exonuclease involved in the 3' overhang cleavage is the DSB repair protein MRE11 (Jing *et al*., 2005). These 3' overhangs then undergo homology search and strand invasion into the homologous counterpart (Fig. 1.7, step 3), aided by the recruitment of numerous proteins including replication protein A (RPA), RAD51, DMC1 and BRCA1 (Sinohara and Shinohara, 2004; Baudat *et al*., 2013). This process, known as single end invasion, generates a displacement loop (D-loop) structure that anneals to the single-strand overhang of the homologous chromosome (Baudat *et al*., 2013). Subsequent DNA synthesis at the invading end then connects the homologous chromosomes by two DNA junctions, termed the double Holliday junction (Fig. 1.7, step 4A). The double Holliday junctions are then resolved, depending on the orientation of the cleavage, to give rise to either a crossover or non-crossover (Fig. 1.7, step 5A-B). Several DNA-mismatch repair proteins are involved in the resolution of the double Holliday junction, including the heterodimers formed by mutS homologs MSH4 and MSH5 (de Vries *et al*., 1999; Kneitz *et al*., 2000; Baudat *et al*., 2013), as well as the mutL homologues MLH1 and MLH3, which are associated with crossover resolution (Hunter and Borts, 1997; Hoffmann and Borts, 2004; Baudat *et al*., 2013).

Another pathway for how non-crossovers arise was suggested by several studies in various organisms such as *S. cerevisiae* and *Drosophila* (Do *et al*., 2014), which provided evidence that crossovers and non-crossovers may not necessarily arise from the same double Holliday junction (Borner, 2004; Terasawa *et al*., 2007; Miura *et al*., 2012). In fact, non-crossovers are suggested to predominantly occur through a process called synthesis-dependent strand-annealing (SDSA) (Cromie and Smith, 2007; Terasawa *et al.,* 2007; Miura

et al., 2012). SDSA is characterized by strand displacement after the formation of the D-loop, where the extended single-strand DNA (ssDNA) end that forms following strand displacement anneals to the ssDNA on the homologous partner (Fig. 1.7, step 4B). The gap between the strands are then filled by DNA synthesis and ligation (Fig. 1.7, step 5C).

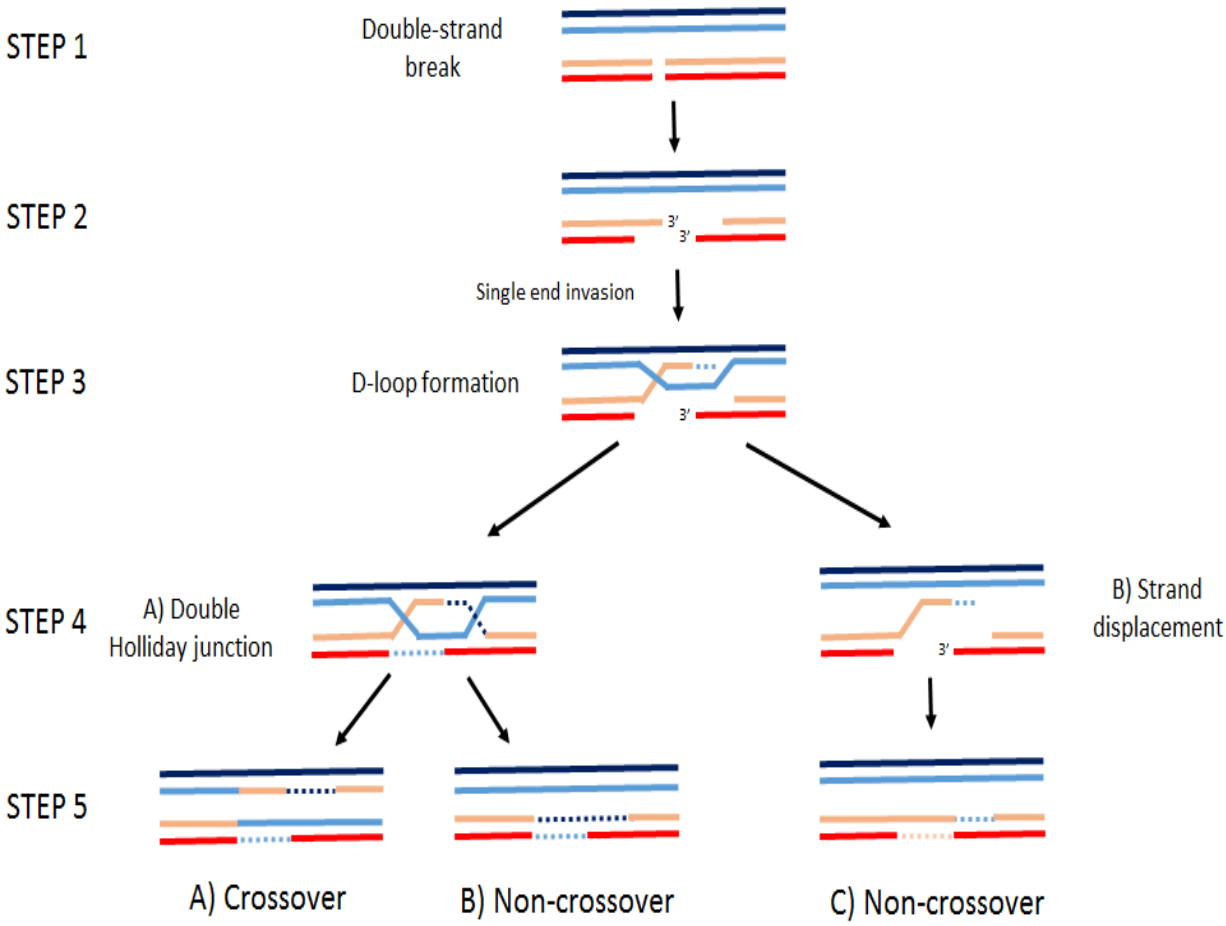

Figure 1.7 Mechanisms of meiotic recombination.

In step 1, double-strand breaks (DSBs) are initiated by exonuclease Spo11. Next, an unknown exonuclease creates 3' overhangs (Step 2). In step 3, the 3' overhang undergoes homology search, single end invasion, and generates a D-loop. In step 4, resolving the D-loop can involve either DNA synthesis to create a double Holliday junction (4A), or single strand displacement (4B). The double Holliday Junction can be resolved through DNA synthesis into a crossover (5A), or non-crossover (5B), whereas the single strand displacement method can only create a non-crossover through DNA synthesis and ligation (5C).

Distribution of crossovers

Crossovers are non-randomly distributed along the pairs of homologous chromosomes (bivalents) during meiosis. Cytological studies that examined the distribution of crossovers in humans have revealed several trends: (1) there are regions of the genome that are most susceptible to crossovers, termed hotspots, (2) at least one crossover is formed on each bivalent, (3) crossover formation is inhibited near the centromeres and telomeres, (4) crossover formation in the subtelomeres (region adjacent to telomeres) is favored, and (5) when two crossovers are found on the same chromosome, they are spaced apart due to crossover interference (Mezard *et al*., 2015).

A comparison between genome-wide maps of DSBs and crossover sites in mice and humans demonstrated that the crossover landscape generally mirrors that of DSBs (Smagulova *et al*., 2011; Pratt *et al*., 2014). However, the correlation between the two maps did not span the entire chromosomes, suggesting that the heterogeneity in DSB formation is only one of the factors governing crossover distribution (Smagulova *et al*., 2011; Pratt *et al*., 2014). It has been long speculated that the majority of crossover events occur in short chromosomal intervals, at so-called hotspots. Recent techniques using genome-wide mapping of the crossover landscape finally shed light on this theory. Over 25,000 crossover hot spots have been revealed in humans with the majority of these located outside of genes (Ségurel *et al*., 2011; Baudat *et al*., 2013; Border and de Massy, 2013).

In humans, the number of DSBs that form in the leptotene stage of prophase I is ten times greater than the number of crossovers that result at the pachytene stage (Baudat *et al*., 2013). The frequency of crossovers is highly regulated such that at least one crossover forms per bivalent. Since the homologous chromosomes are tethered by the chiasmata at the site of

recombination until they segregate during anaphase I, the presence of at least one chiasmata per bivalent is important for ensuring the proper segregation of the homologous chromosomes, and the progression of meiosis (discussed further in section 1.2.5.1).

Aside from the regulation of the frequency of crossovers, the positions of the crossovers are also strategically placed. There are relatively low incidences of crossover events near the centromere in humans, most likely due to the compaction of chromatin. In humans, the distal regions of the chromosomes are largely composed of euchromatin and much more susceptible to recombination than the heterochromatin rich regions near the centromeres (Mezard *et al*., 2015). Analysis of DSBs in *S. cerevisiae* revealed abundant DSBs near the centromeres, but an inhibition of crossover formation during DSB repair (Blitzblau *et al*., 2007). The prevention of crossover formation near the centromeres is thought to protect the faithfulness of meiotic chromosome segregation (discussed further in section 1.2.5.2). Crossovers are also typically suppressed near the vicinity of the telomeres, possibly to prevent damage to the repetitive DNA (Blitzblau *et al*., 2007). In the past decade, studies have shed light on the importance of telomeres in meiotic recombination, where telomere-guided mechanisms have been shown to ensure sufficient DSB formation near the subtelomeres of the chromosomes in *S. cerevisiae* (Peoples-Holst and Burgess, 2005; Blitzblau *et al*., 2007). In human males, increased DSB activity and crossover formation have been observed near the subtelomeres compared to the rest of the chromosomes (Mezard *et al*., 2015). However, subtelomeric crossovers are less frequent in females (Blouin *et al.*, 1995; Badge *et al.*, 2000; Mezard *et al*., 2015). This difference in crossover distribution may be due to the difference in the initiation of synapsis between the sexes; in males, synapsis

begins at the subtelomeric regions of the chromosomes, while in females, synaptic initiation is commonly located at the interstitial regions of the chromosomes (Mezard *et al*., 2015).

Indeed, the timing of synapsis along with DSB formation seem to influence the crossover landscape, where early DSBs are preferentially selected for repair as crossovers (Higgins *et al*., 2012; Pratto *et al*., 2014). Once the first crossover forms, any subsequent crossovers generally do not form in the adjacent areas, such that all crossovers are adequately spaced apart in a phenomenon called crossover interference (Higgins *et al*., 2012; Pratto *et al*., 2014). Finally, there is recent evidence of crossover events corresponding with methylated histones H3K4me3, and local DNA hypomethylation in *S. cerevisiae*, mice and humans (Melamed-Bessudo *et al*., 2012; Macaulay *et al*., 2012; Mirouze *et al*., 2012; Colome-Tatche *et al*., 2012). These suggest that epigenetic mechanisms such as DNA and histone modifications that alter the chromatin's accessibility to proteins may also affect the distribution and timing of DSBs, and thus the distribution of crossovers.

1.2.1.4 Meiotic inactivation of sex chromosomes

In mammals, female somatic cells undergo a process of sex chromosome inactivation where one of the X chromosomes is randomly transcriptionally silenced in order to achieve gene dosage compensation between the sexes. Another form of sex chromosome inactivation occurs in male germ cells during meiosis, where the sex chromosomes are inactivated upon entering prophase I. Human males have two distinct sex chromosomes, X and Y, which only share a small region of homology at the tip of the chromosome arms termed the pseudoautosomal region (PAR). Similar to meiotic recombination on the autosomes, at least one crossover event must occur between the sex chromosomes on the PAR to ensure their proper segregation during the first meiotic division. To achieve this, the X and Y

chromosomes must pair and synapse at the PAR during prophase I. Meanwhile, the unsynapsed regions on the sex chromosomes undergo chromatin compaction and form a condensed structure called the sex body (reviewed in Handel, 2004). The remodeling of the unpaired chromosome regions into heterochromatin during the formation of the sex body leads to the transcriptional silencing of the genes in that region, through a mechanism known as meiotic sex chromosome inactivation (MSCI).

The mechanism of MSCI is distinct compared to that of X chromosome inactivation (XCI) in female somatic cells. In females, the functional RNA, *inactive X specific transcript (XIST)*, transcribed from the X chromosome is responsible for initiating XCI through the localization of *XIST* to the X chromosome (Bontenbal and Gribnau, 2016; Payer, 2016). The binding of *XIST* recruits various proteins involved in chromatin remodeling, which function to compact the chromatin through epigenetic changes such as hypermethylation, and deacetylation of histones (Bontenbal and Gribnau, 2016; Payer, 2016). In males, *XIST* is exclusively expressed in the testis in mice (Dilworth and McCarrey, 1992), and have been shown to localize along the X chromosome (Ayoub *et al.*, 1997). However, the deletion of *XIST* in mice did not affect MSCI, suggesting that other mechanisms are responsible for the male meiosis-specific process (McCarrey *et al*., 2002; Turner, 2007; Checchi and Engebretch, 2011).

At the onset of prophase I, the sex chromosomes are transcriptionally active until the initiation of DSBs induces the temporary phosphorylation of H2AX (histone family, member X) on all chromosomes (Baudat *et al*., 2013). However, once the X and Y chromosomes have synapsed at the PAR at the zygotene to pachytene transition stage, BRCA1 proteins localize to the unsynapsed regions of the sex chromosomes, and subsequently recruit kinase ATR to

specifically phosphorylate H2AX to γH2AX in these regions. The phosphorylation of H2AX is thought to trigger chromatin condensation and transcriptional repression (Turner, 2007; Checchi and Engebretch, 2011). The inactivation of the unsynapsed regions on the sex chromosomes are later completed by additional chromatin modifications such as histone H3 and H4 deacetylation (Khalil *et al*., 2004) and ubiquitylation of H2A (Baarends *et al*., 1999; Checchi and Engebretch, 2011). These modifications remain throughout meiosis, whereas BRCA1, γH2AX, and ATR are displaced after the diplotene stage of prophase I. In fact, there is evidence that parts of the sex chromosomes remain transcriptionally silenced beyond meiosis in a process termed post-meiotic sex chromosome repression (PSCR) (Namekawa *et al.,* 2006; Checchi and Engebretch, 2011). There are also new revelations suggesting that the event of transcriptional silencing during the pachytene stage is not only limited to the sex body, but any unsynapsed region along homologous chromosomes during prophase I (Baarends *et al*., 2005; Turner, 2007; Checchi and Engebretch, 2011). Therefore, it has been hypothesized that MCSI may be the result of the more general mechanism, meiotic silencing of unsynapsed chromatin (MSUC), where the transcriptional status of the chromosomes is dependent on their synaptic status (Ferguson *et al.,* 2008). For example, it appears that sex chromosomes that do undergo full synapsis can escape MSCI. This phenomenon is evident in XYY mice where the two Y chromosomes fully synapse and test negative for γH2AX (Turner, 2007; Checchi and Engebretch, 2011). Furthermore, studies on XO mice have demonstrated that the lone X chromosome without a pairing partner is transcriptionally inactivated during meiosis, leading to meiotic arrest and infertility (Turner, 2007; Checchi and Engebretch, 2011). It is apparent that normal MSCI is imperative for cells to proceed past prophase I onto the next steps in MI: the segregation of the homologous chromosomes.

1.2.1.5 Segregation of chromosomes

At the diplotene stage of prophase I, the SC begins to disassemble, leaving the homologous chromosomes solely tethered at chiasmata, the spots where crossovers had formed. Bivalents that did not experience a crossover event are therefore untethered, and at risk of segregation errors in the first meiotic division. Recent evidence suggests that fragments of SC remain at the centromeres between bivalents at this time, which is speculated to promote the correct segregation of achiasmate bivalents (Kurdzo and Dawson, 2014).

Moving from prophase I to metaphase I, the homologous chromosomes begin to align along the equatorial plate of the cell. Microtubules extend from the centrioles and attach to the kinetochores on the centromeres of the sister chromatids. The sister kinetochores must attach to microtubules originating from the same spindle pole in a monopolar fashion in order for the sister chromatids to be pulled in the same direction (Hauf and Watanabe, 2004; Nambiar and Smith, 2016; Fig. 1.8). At the same time, the kinetochores on the homologous counterparts must attach to microtubules from the opposite spindle poles, so that the homologous chromosomes are pulled to opposite sides of the cell to form separate daughter cells (Hauf and Watanabe, 2004; Nambiar and Smith, 2016). In eukaryotes, ring-shaped protein complexes called cohesins also play a part in the segregation of chromosomes. Cohesins are established between sister chromatids along their entire length prior to meiosis and function to hold the sister chromatids together until they are ready to be separated (Fig. 1.8). Since the homologous chromosomes at anaphase I are tethered at chiasmata, which covalently attaches a sister chromatid from one homologous chromosome to a sister chromatid from the homologous counterpart, cohesins along the arms of the sister chromatids

are released in order to resolve the chiasmata and allow the homologous chromosomes to segregate (Fig. 1.8). However, cohesins around the centromeres remain in order to prevent the premature segregation of the sister chromatids (Riedel *et al*., 2006, discussed in detail in section 1.2.2). The homologous chromosomes are then pulled to opposite sides of the cell through the shortening of the microtubules. At last, the microtubules disassemble and nuclear membranes form around each set of haploid chromosomes during telophase I. Cytokinesis follows by pinching the cell membranes to give rise to two haploid daughter cells, each containing two sister chromatids. These cells that arise from MI are called secondary spermatocytes, and are ready to enter the second meiotic division.

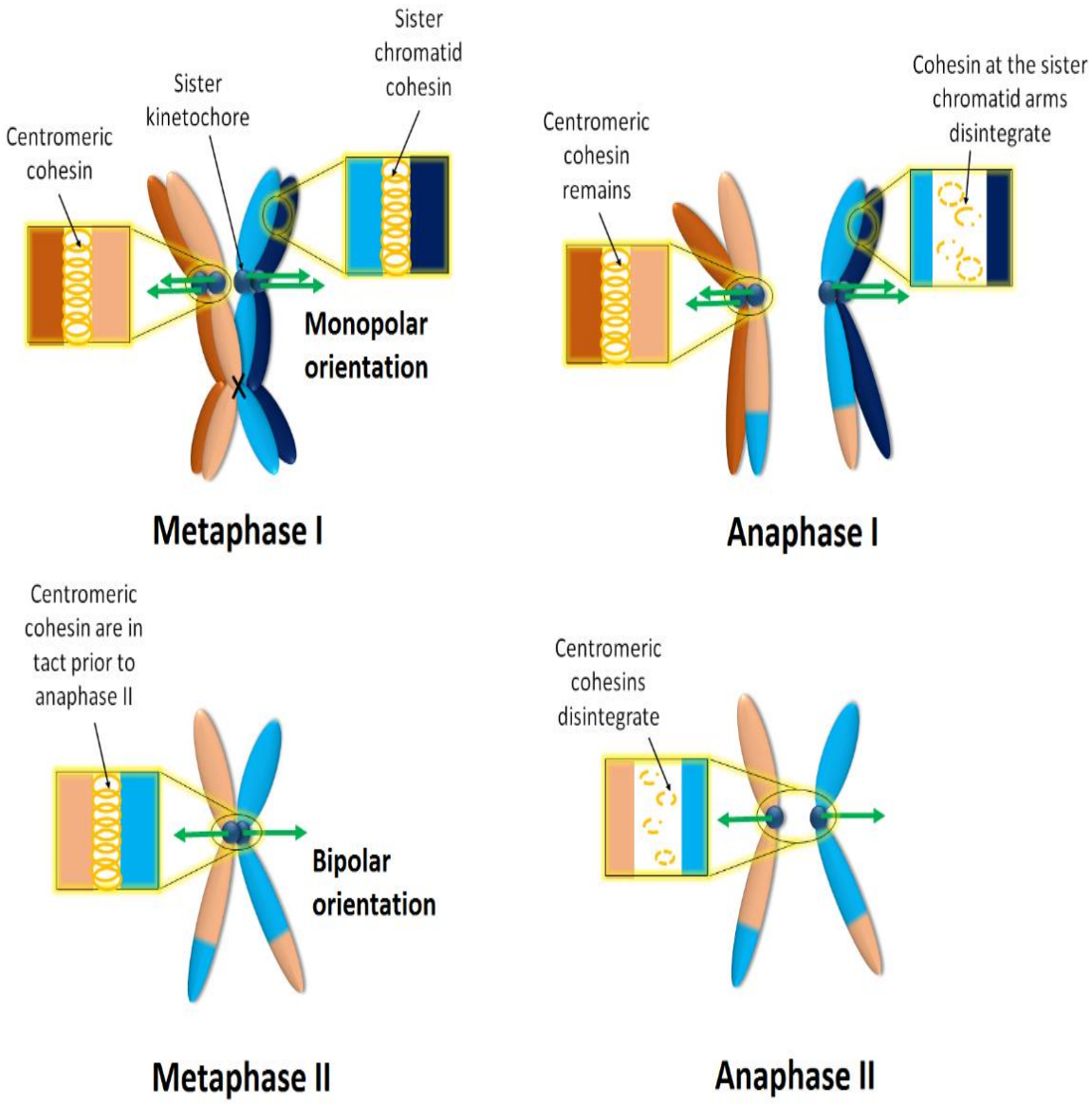

Figure 1.8 Meiotic spindle orientation and cohesin during metaphase and anaphase I and II.

During metaphase I, the kinetochores of the sister chromatids must attach to spindles originating from the same pole (monopolar orientation). In anaphase I, cohesins on the arms of the sister chromatids are cleaved so that the homologous chromosomes can resolve chiasmata and segregate. During metaphase II, the kinetochores on the sister chromatids must attach to spindles arising from opposite poles (bipolar orientation). During anaphase II, cohesins between the centromeres of the sister chromatids are cleaved to allow the segregation of the sister chromatids.

1.2.2 Meiosis II

During the second meiotic division, the sister chromatids in the secondary spermatocytes are segregated such that each daughter cell receives one of the two sister chromatids to form a haploid gamete. Like the first meiotic division, MII can also be subdivided into four stages: prophase II, metaphase II, anaphase II, and telophase II. It is important to note that unlike in MI, DNA replication does not occur prior to prophase II. During metaphase II, the chromosomes align at the equatorial plate, with the kinetochores on the sister chromatids pointing toward opposite poles. This time, the sister kinetochores must attach to microtubules originating from the opposite spindle poles in a bipolar fashion in order for the sister chromatids to be pulled in the opposite directions (Yamamoto and Hiraoka, 2003; Hauf and Watanabe, 2004; Fig. 1.8). At the onset of anaphase II, cohesins that were tethering the centromeres of the sister chromatids together dissociate so the sister chromatids can segregate into separate daughter cells (Fig. 1.8). Similar to the events in MI, telophase II and cytokinesis then occur to give result in two daughter cells, each with one set of chromosomes.

The fidelity of MII is dependent on the integrity of cohesins holding the sister chromatids together, such that the sister chromatids do not separate until anaphase II. As previously described, even though cohesins along the arms of the sister chromatids are dispelled during anaphase I, it is crucial that cohesins at the centromeres of the sister chromatids are retained until anaphase II. During meiosis, many eukaryotes rely on a cohesin complex that includes the protein Rec8 to enforce sister chromatid cohesion. Mounting evidence shows that Rec8 along the arms of sister chromatids are cleaved by the enzyme separase at anaphase I (Allshire, 2004). Interestingly, Rec8 on the centromeres of the sister

chromatids are protected from this cleavage, such that they remain until late metaphase II (Allshire, 2004; Wassmann, 2013; Marston, 2015). In 2004, Katajima *et al.* identified a protein, called shugoshin (Sgo1), in fission yeast that appears to protect centromeric Rec8 from degradation (Wassmann, 2013; Marston, 2015). It turns out that the phosphorylation of Rec8 is essential for its cleavage by separase (Marston, 2015). The presence of Sgo1 at the centromeres counteracts this phosphorylation by recruiting protein phosphatase 2A to prevent the cleavage of Rec8 (Marston, 2015). Proteins with similar sequences to Sgo1 have also been identified in *Drosopholia* and mammals, suggesting that the function of Sgo1 may be widely conserved (Allshire, 2004; Wassmann, 2013; Marston, 2015). Our understanding of how Sgo1 homologues in mammals operate is only beginning to be understood. Future studies on the role of this protector protein and cohesins can further elucidate the mechanisms underlying the second meiotic division, and how errors may arise if these proteins are altered.

1.2.3 Meiotic checkpoints

The progression of meiosis is carefully monitored through checkpoints, in order to avoid the creation of defective gametes with an aberrant number of chromosomes. Checkpoints are regulatory processes that block erroneous cells from completing meiosis by targeting the cell for arrest and apoptosis. There are two well-defined checkpoints during meiosis: the pachytene checkpoint, or meiotic recombination checkpoint (MRC), and the spindle assembly checkpoint (SAC). The first checkpoint during meiosis occurs at the pachytene stage of prophase I (Subramanian and Hochwagen, 2014). The first evidence for this checkpoint came from observations in mouse studies where mice with different mutations for proteins involved in synapsis and recombination showed meiotic arrest at the

pachytene stage (Subramanian and Hochwagen, 2014). The main proteins involved in the MRC also function in the canonical DNA damage response pathways in mitosis. Two critical players are the checkpoint sensor kinases ATM and ATR, which are activated by DNA damage, such as unrepaired DSBs, as well as unsynapsed chromosomes during meiosis (Subramanian and Hochwagen, 2014). Both proteins require cofactors for the detection of DNA damage. ATM detects the end of DSBs with the help of the MRN complex (MRE11-RAD50-NBS1) (Subramanian and Hochwagen, 2014). ATR detects ssDNA following its activation by ATRIP, as well as ssDNA/dsDNA junctions with the aid of the PCNA-like 9-1-1 complex (RAD9-RAD1-HUS1) (Subramanian and Hochwagen, 2014).

When germ cells successfully pass the MRC and head to metaphase I, a second checkpoint awaits them to ensure that the homologous chromosomes are aligned properly at the equatorial plate, in preparation for chromosome segregation later on. The SAC blocks the transition from metaphase I to anaphase I if defective spindles are detected, or if there is insufficient mechanical tension exerted on the kinetochores by the spindle microtubules (Gorbsky *et al.,* 2015). The mechanisms of the SAC in meiosis and mitosis are thought to be analogous (Gorbsky *et al.,* 2015). Mainly, kinetochores lacking microtubule tension or attachment trigger the accumulation of several checkpoint signaling proteins such as the mitotic arrest-deficient (Mad) proteins, budding uninhibited by benzimidizole (Bub) proteins, and monopolar spindle 1 (Mps1) protein (Jia *et al.,* 2013; Sacristan and Kops, 2014; Gorbsky *et al*., 2015). These proteins function as a unit to inhibit the anaphase-promoting complex or cyclosome (APC/C), which is necessary for the transition into anaphase I (Gorbsky *et al*., 2015). As we build understanding toward the mechanics of meiotic checkpoints, it became apparent that males and females may display sex specific variations when it comes to the

efficiency of checkpoints. The sex specific differences in meiosis will be discussed in the following section.

1.2.4 Sex differences in meiosis

Although the sex specific differences in meiotic sex chromosome inactivation was previously addressed, there are three other main differences between male and female meiosis: (1) the temporal progression of meiosis, (2) the frequency of recombination and (3) the efficiency of checkpoints.

The most apparent difference between male and female meiosis is the timing. In males, meiosis is initiated after puberty and proceeds without interruption. In females, meiosis begins during fetal development, but oocytes are arrested part-way through MI. They do not resume meiotic division until ovulation, when they finally complete MI and are arrested once more part-way through MII. The second meiotic division is completed when the oocyte is fertilized by a sperm (Paoloni-Giacobino *et al*., 2000).

A molecular difference in female and male meiosis involves the frequency of crossover formation at the pachytene stage of prophase I. In males, spermatocytes have been shown to contain approximately 50 crossovers per cell, whereas female oocytes contain approximately 70 crossovers per cell (Tease and Hulten, 2004). Surprisingly, studies in XY sex-reversed female mice showed that the recombination rate in their XY germ cells mimicked that of an XX female, suggesting that the rate of recombination is determined by the sex chromosome content of the somatic cells surrounding the developing germ cell, instead of the sex chromosome content of the germ cell itself (Lynn *et al*., 2005). Females also display longer SCs when compared to those in males (Tease *et al*., 2004), which may

partly explain the difference in crossover frequency between the sexes as crossover frequency has been shown to positively correlate to SC length (Lynn *et al.,* 2002; Baudat *et al*., 2013). The shorter SC lengths in males may also signify a more condensed chromosome state, which is less accessible to DSB machinery, and thus exhibit a lower rate of crossover formation (Baudat *et al*., 2013).

There is increasing evidence that meiotic checkpoints operate at different efficiency in males and females (Touati and Wassmann, 2016). In contrast to males, female germs cells are particularly prone to errors in chromosome segregation (Hunt and Hassold, 2000). In fact, knock out studies on mice have demonstrated vastly different results between the sexes when mutations in proteins involved in synapsis and recombination were introduced. For example, mutations in the meiotic gene *Scp3* led to spermatogenic arrest and infertility in male mice, while in females, fertility was retained despite a reduction in litter size and increase in chromosomal abnormalities in the offspring (Yuan *et al*., 2000). Likewise, mutations in the protein Fkpb6, which associates with the SC during prophase, resulted in spermatogenic arrest in male mice, and retained fertility in female mice (Crackower *et al*., 2003). It seems that meiotic checkpoints in females are less stringent than those in males. While the majority of erroneous cells are arrested during male meiosis, female meiosis allows a high portion of erroneous cells to complete meiosis, potentially resulting in aneuploid oocytes.

1.2.5 Origin of aneuploidy

Aneuploidy refers to changes in the chromosome content of a cell, where there is a gain or loss of whole chromosomes. This condition can arise from errors in chromosome or sister chromatid segregation during meiotic or mitotic divisions. When segregation goes awry in either MI or MII cell division, the gamete that is produced may be aneuploid. The

subsequent fertilization of an aneuploid gamete will then give rise to an embryo with an abnormal chromosome constitution. Alternatively, a normal embryo may experience segregation errors during mitotic divisions during its growth, resulting in aneuploidy down the road. Segregation errors during cell division are relatively common, with aneuploidy being detected in 5% of human pregnancies (Hassold and Hunt, 2001; Nagaoka *et al*., 2012). As the human genome is intricately balanced and particularly sensitive to abnormal doses of genetic content, perturbations in the chromosome complement are for the most part incompatible with embryo survival, generally leading to pregnancy loss. In fact, aneuploidy is responsible for approximately 35% of spontaneous abortions and 4% of stillbirths, marking it the leading genetic cause of human pregnancy loss (Nagaoka *et al*., 2012, Table 1.1). When it comes to live births, the incidence of aneuploidy is dramatically decreased to 0.3%. This number is nevertheless significant as aneuploidy is the most common known cause of mental retardation and leading cause of congenital malformations (Nagaoka *et al*., 2012).

Table 1.1 Aneuploidy in humans at different stages[a]

Population	Methodology	Incidence of aneuploidy (%)	Most common aneuploidies	References
[b]Oocytes or polar bodies	Karyotyping	10-35	+16; +17; +18; +21; +22	Pellestor *et al*., 2006; Pacchierotti *et al.*, 2007
	FISH	20->70	Various	Pellestor *et al*., 2006; Pacchierotti *et al*., 2007
Sperm	CGH, SNP array, CGH array	30-70	+15; +16; +21; +22	Fragouli *et al.*, 2011; Gabriel *etla.*, 2011; Garaedts *et al.*, 2011; Obradors *et al*., 2011
	Karyotyping	1-4	XY disomy; +21; +22	Martinand Rademaker, 1990; Martin *et al*., 1991
	FISH	1-3	XY disomy; +13; +21; +22	Templado *et al*., 2011
[c]Preimplantation embryos	Karyotyping	20-40	+16; +17; +18	Zenzes and Casper, 1992
	FISH	25->70	Various	Magli *et al*., 2001; Staessen *et al*., 2004; Munne et *al.*, 2007; Ercelen *et al.*, 2011
	CGH, SNP array, CGH array	30-60	+15; +16; +21; +22	Fragouli *et al.*, 2011; Gutierrez-Mateo *et al.*, 2011; Treff *et al.*, 2011; Treff *et al.*, 2010
Spontaneous abortions	Karyotyping	>35	45,X; +15; +16; +21; +22	Hassold *et al*., 1996
Stillbirths	Karyotyping	4	45,X; +13; +18; +21; XXX; XXY	Hassold *et al*., 1996
Newborns	Karyotyping	0.3	+13; +18; +21; XXX; XXY; XYY	Hassold *et al.*, 1996

[a]Table adapted from Nagaoka et al., 2011

[b]Oocytes in these studies were retrieved after superovulation. The data therefore may not be reflective of oocytes under natural conditions.

[c]Embryos in these studies arose from in vitro fertilization. The data therefore may be reflective of embryos under natural conditions.

Theoretically, segregation errors can occur to any of the autosomes and sex chromosomes in humans. However, only some trisomies (gain of a chromosome) and one type of monosomy (loss of a chromosome) may be viable in the embryo. The most common cases of aneuploidy in live births are trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome), trisomy 21 (Down syndrome), and sex chromosomal aneuploidies such as XXY (Klinefelter syndrome), XYY, and monosomy X (Turner syndrome). With the use of polymorphic DNA markers, researchers have attempted to identify the parental origin of abnormal chromosomes in individuals with aneuploidy (Hall *et al*., 2007b, Table 1.2). The findings showed that the vast majority of aneuploidies originate from errors during the first meiotic division in the mother (Hassold *et al*., 1996; Ioannou and Tempest, 2015). Interestingly, not all chromosomes experience similar likelihoods for error; for example, trisomies 16 and 22 almost always originate from MI errors, while trisomy 18 originates more readily from MII errors (Bugge *et al.*, 1998; Hassold and Hunt, 2001; Hall *et al*., 2007b). On the other hand, aneuploidy for chromosomes 7 and 8 seem to originate most frequently during mitotic divisions in the embryo (Zaragoza *et al*., 1998; James and Jacobs, 1996; Karadima *et al*., 1998).

Table 1.2 Origins of human aneuploidies[a]

Aneuploidy	*n*	Maternal		Paternal		Post-zygotic mitosis	References
		MI (%)	MII (%)	MI (%)	MII (%)		
+2	18	53.4	13.3	27.8	0	5.6	Zarogoza *et al*., 1998
+7	14	17.2	25.7	0	0	57.1	Zarogoza *et al*., 1998
+8	12	50.0		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	Zarogoza *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	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*., 2006b
[b]did not distinguish between MI and MII errors
[c]cannot distinguish between MI and MII errors in XO females

About 90% of trisomies arise from meiotic errors in either the mother or father (Hassold *et al*., 2007). Because the majority of aneuploidies arise through maternal contribution, the importance of paternally derived aneuploidies has been largely overlooked. While the paternal contribution to autosomal trisomies is minor (0-30% depending on the chromosome), sex chromosome aneuploidies do frequently originate from fathers (Hall *et al*., 2007b, Hall *et al*., 2007a; Uroz and Templado, 2012). In fact, paternal origins have been indicated in approximately 50% of XXY males (MacDonald *et al*., 1994; Ioannou and Tempest, 2015), 70 to 80% of 45,X females, and 100% of 47,XYY males (Jacobs *et al*., 1997; Hall *et al*., 2007a; Uroz and Templado, 2012; Ioannou and Tempest, 2015). Moreover, there is accumulating evidence that infertile men display higher levels of aneuploidy in their sperm (Tang *et al.*, 2004; Hansen *et al*., 2005; Ma *et al.*, 2006a; Ma *et al.*, 2006b; Kirkpatrick *et al*., 2008; Ferguson *et al*., 2009; Kirkpatrick *et al.*, 2015; Ren *et al.*, 2015), which is reflected in the higher incidences of aneuploidy in the embryo and offspring conceived through assisted reproduction by this population (Ioannou and Tempest, 2015, discussed in detail in section 1.4.1). Hence, when it comes to the infertile male population, paternally derived aneuploidies may possess greater clinical weight than traditionally perceived. The next sections will examine the mechanisms of how segregation errors may arise during cell division to give rise to aneuploidy.

1.2.5.1 Nondisjunction

The leading cause of aneuploidy is nondisjunction during meiosis, where the chromosomes or sister chromatids fail to separate during anaphase (Fig. 1.9). If the homologous chromosomes fail to segregate during the first meiotic division, all of the resulting gametes will be aneuploid; half of these gametes will contain an extra chromosome,

while the other half of the gametes will be missing a chromosome. Nondisjunction may also occur during the second meiotic division. In this case, only half of the gametes produced will be abnormal; one will have an extra chromosome, while another will be missing a chromosome.

Currently, there are two theories regarding the molecular mechanics of nondisjunction, which involve errors with either meiotic recombination, or the spindle apparatus. Studies in the last two decades have shed light on the importance of proper meiotic recombination when it comes to chromosome segregation during MI. Homologous chromosomes that lack a crossover event, and therefore are not tethered by chiasmata after prophase I, have a significantly greater risk of missegregation during the first meiotic division. These homologous chromosomes essentially enter metaphase I without a partner and align at the equatorial plate as univalents. Univalents, or chromosomes that are not physically linked to their homologous counterpart, have been shown to segregate in unpredictable, random ways to either side of the cell (Hassold and Hunt, 2001; Uroz and Templado, 2012). Females appear to experience nondisjunction due to the lack of recombination on bivalents more frequently than males. In males, almost all spermatocytes show at least one crossover per bivalent (Nagaoka *et al*., 2012). Strikingly, over 10% of human oocytes contain at least one bivalent without a crossover (Nagaoka *et al*., 2012). It is estimated that half of these oocytes will result in aneuploidy (Nagaoka *et al*., 2012). The lack of crossovers may be one of the explanations for the higher incidence of aneuploidy seen in oocytes compared to sperm (Table 1.1).

As previously mentioned, the meiotic checkpoint during anaphase I, SAC, acts to ensure that all of the homologous chromosomes are aligned at the equatorial plate and

correctly attached at the kinetochore to spindle microtubules before APC/C is activated to signal the shortening of the microtubules. If one of the kinetochores on the sister chromatids in the homologous pair fails to attach to a spindle, the homologous chromosomes will fail to separate and be simultaneously pulled to one side of the cell. Normally, SAC should detect errors in kinetochore to spindle attachment and target the cell for meiotic arrest. However, if the faulty cell escapes the checkpoint, nondisjunction will likely occur (Sun and Kim, 2011).

1.2.5.2 Premature sister chromatid segregation

The premature loss of cohesins at the centromeres of sister chromatids can also lead to segregation errors during MI or MII cell divisions (Ma *et al.*, 1989). This cause of aneuploidy is primarily observed in female meiosis. Numerous studies have linked increasing maternal age to an increased incidence of aneuploidy for most chromosomes (Hassold *et al*., 1995; Lamb *et al*., 1996; Eichenlaub-ritter, 2012). This increased risk has been speculated to be caused by the deterioration of cohesins over time. During female fetal development, cohesins are loaded onto newly replicated chromosomes prior to the initiation of meiosis (Eichenlaub-ritter, 2012). As oocytes mature, their ability to reload cohesins onto the sister chromatids decline, leading to a progressive loss of coheins (Eichenlaub-ritter, 2012). Without attachment between the centromeres of sister chromatids, the chromatids behave as univalents and separate independently during anaphase. This can result in the sister chromatids being pulled to the opposite sides of the cell in anaphase I, or the same side of the cell in anaphase II, producing aneuploid gametes (Fig. 1.10). Indeed, studies in the oocytes of women undergoing assisted reproduction have suggested that premature sister chromatid segregation (PSCS) is a major contributor to aneuploidy in women of advanced maternal age (Nagaoka *et al*., 2012).

Recent investigation into the distribution of meiotic crossovers have presented a new perspective on how PSCS may occur. An increase in crossover formation near the centromeres of chromosome 21 have been proposed to cause MI and MII segregation errors in oocytes implicated in cases of trisomy 21 (Oliver *et al*., 2014). Whether this trend is observed in paternally derived trisomy 21 remains unclear (Oliver *et al.*, 2009). Nevertheless, crossover formation near the centromeres is thought to interfere with centromeric cohesins, and thus disturb the attachment of sister chromatids. Future studies on whether crossovers near the centromeres on chromosomes other than 21 are linked to increased risks of PSCS, and aneuploidy is warranted.

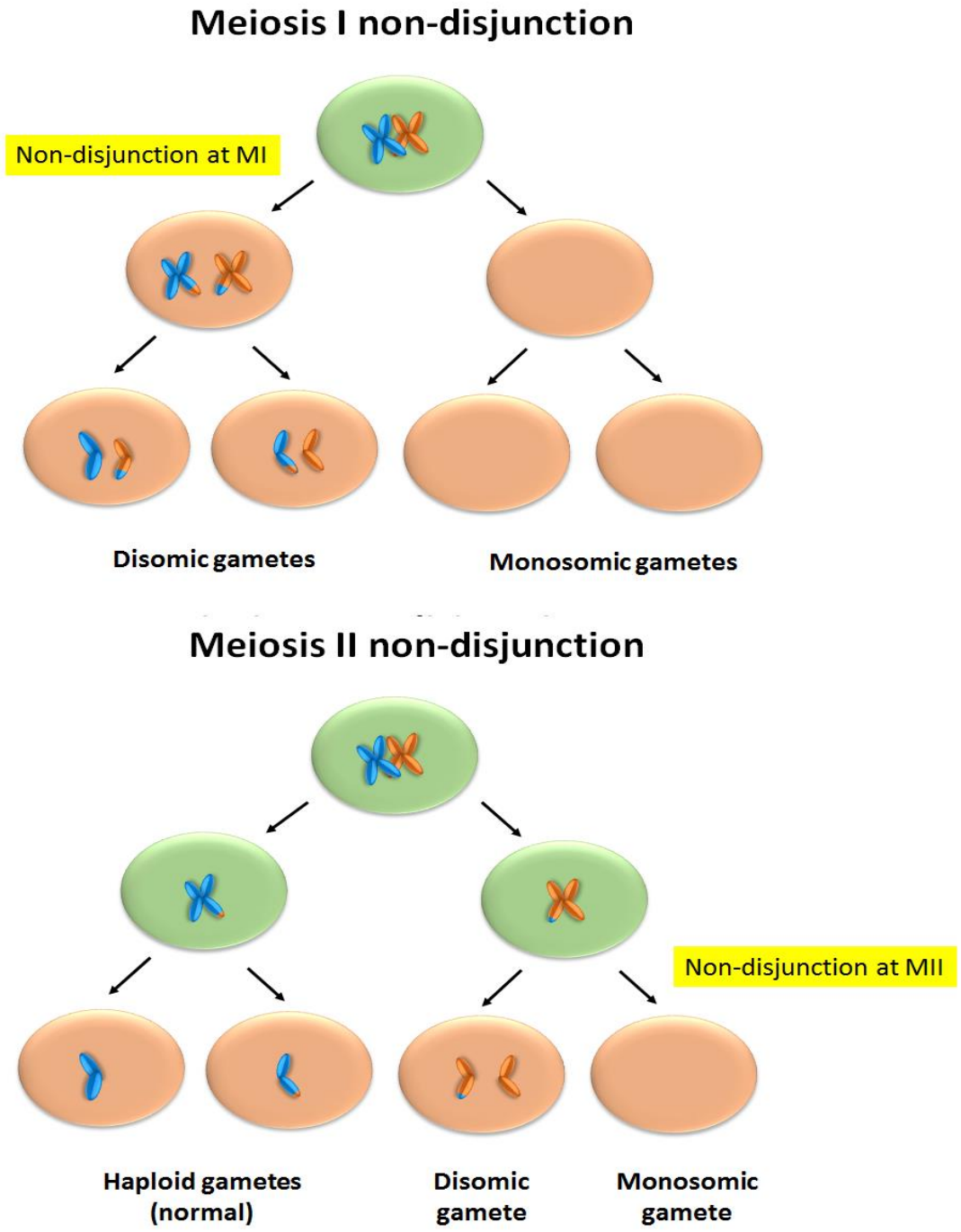

Figure 1.9 Nondisjunction as a mechanism for the production of aneuploidy gametes.

Meiosis I nondisjunction produces disomic (extra chromosome) and nullisomic (missing a chromosome) gametes, where all of the gametes are aneuploidy. On the other hand, nondisjunction at meiosis II creates haploid gametes (normal), disomic, and nullisomic gametes, where only half of the gametes produced are aneuploid.

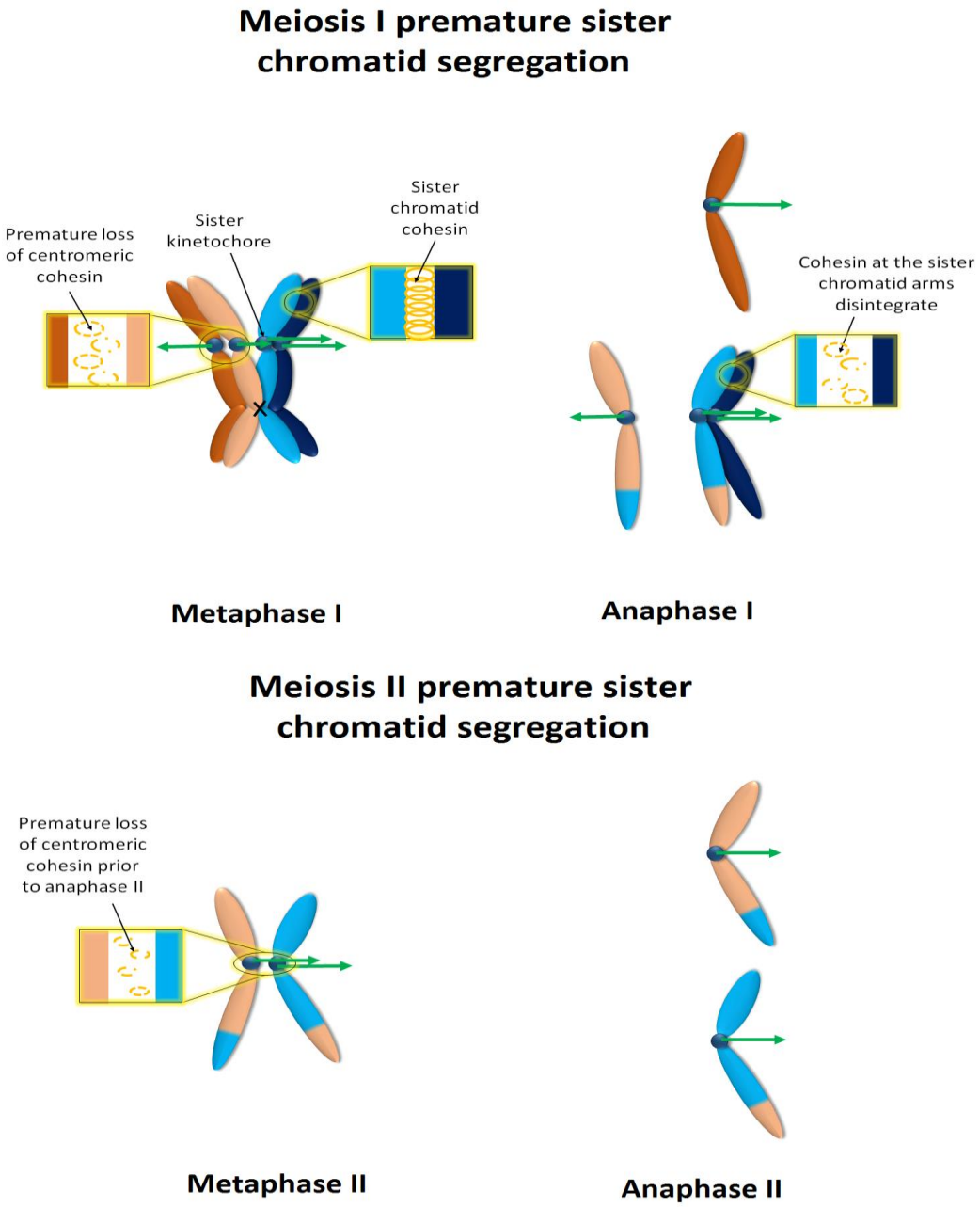

Figure 1.10 Premature sister chromatid segregation as a mechanism for the production of aneuploid gametes.

The premature loss of centromeric cohesin at meiosis I can result in the sister chromatids being segregated to opposite sides of the cell; one daughter cell will have an extra chromosome, whereas the other will be missing a chromosome. The premature loss of centromeric cohesin at meiosis II can result in both sister chromatids being pulled to the same pole of the cell; one daughter cell will have an extra chromosome, whereas the other will be missing a chromosome.

1.3 Male infertility

1.3.1 Overview of male infertility

Although some errors during meiosis may result in the production of sperm with an abnormal chromosome complement as discussed above, most erroneous cells are detected by meiotic checkpoints and arrested. With regards to spermatogenesis, meiotic arrest may cause diminished or even halted sperm production, leading to male infertility. Infertility is defined by the World Health Organization (WHO) as the inability to conceive a clinical pregnancy after 12 months or more of regular, unprotected sexual intercourse. Over the past decade, the incidence of infertility in Canada has doubled to approximately 16%, and continues to be on the rise (Bushnik *et al*., 2012). The cause of infertility can be female factor (~35%), male factor (~30%), combined factor (~20%), or unexplained (~15%) (Crosignani and Rubin, 1996; Kumar and Singh, 2015). Male factor infertility can be further classified into four etiologies: (1) blockage of the reproductive tract, (2) immunological dysfunction, (3) sexual disorders, and (4) aberrancies in sperm production (Witczak *et al.*, 2014). To assess the severity of male infertility and treatment options, semen analyses and testicular biopsies are routinely performed.

1.3.1.1 Semen analysis parameters

The first line of assessment for infertile men is usually semen analysis, which is a simple procedure that provides insight into the severity of male infertility. As defined by WHO, semen parameters are judged based on three characteristics of sperm: concentration, motility, and morphology. Sperm concentration is measured by the number of sperm in millions per milliliter of seminal fluid. Sperm motility is graded as rapid progressive (> 25

μm/s at 37ºC), slow progressive, non-progressive (<5 μm/s at 37ºC), or immotile (WHO, 2010). Sperm morphological defects concern either the head, neck and midpiece, tail, or absence of cytoplasmic droplets in the midpiece (WHO, 2010). Clinically, sperm concentration and motility may be the most important semen parameters in predicting the potential for establishing a natural pregnancy. According to WHO's revised semen parameters published in 2010, a sperm concentration of $<15x10^6$/ml, and progressive motility of <32% is considered the lower limit (Cooper *et al*., 2010). On the other hand, sperm morphology is relatively more challenging to interpret due to the level of subjectivity involved. Although the lower limit of morphology is <4% normal, there have been cases of men with normal sperm concentration and motility, but poor morphology scores (<3%) who nevertheless fathered children naturally (Murray *et al*., 2012).

Semen parameters are useful for classifying the types of male infertility (Table 1.3), such as oligozoospermia (low sperm count, $<15x10^6$/ml), asthenozoospermia (low sperm motility, <32% normal), and teratozoospermia (abnormal sperm morphology, <4% normal). A combination of all three sperm defects is termed as oligoasthenoteratozoospermia (OAT). About 15% of infertile men show an absence of sperm in the ejaculate, a condition known as azoospermia (Boback and Schlegel, 2014). Azoospermia is subdivided into obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). An examination of the testicular histology is required to tell these cases apart.

Table 1.3 World Health Organization (WHO) revised diagnoses of semen parameters

	Sperm parameters		
	Concentration (106/ml)	**Motility (%)**	**Normal morphology (%)**
Normal sperm parameters	≥15	≥32%	≥4%
Types of male infertility			
Oligozoospermia	<15	Normal	Normal
Asthenozoospermia	Normal	<32%	Normal
Teratozoospermia	Normal	Normal	<4%
Oligoastenotearatozoospermia (OAT)	<15	<32%	<4%
Azoospermia	Absence of sperm in the ejaculate		
Aspermia	No ejaculate		

1.3.1.2 Testicular histology

When a semen analysis shows a lack of sperm in the ejaculate, a histological evaluation of the testes following a testicular biopsy can reveal the level of spermatogenesis present in the testes; this information is helpful for identifying the type of azoospermia, as well as predicting the success rate of sperm retrieval for assisted reproduction. OA, which is prevalent in 40% of azoospermic cases, is characterized by normal spermatogenesis and a normal hormone profile (Wosnitzer *et al*., 2014). In this case, the lack of sperm in the ejaculate may be the consequence of numerous causes including the absence of vas deferens, obstructions due to inflammatory diseases, physical blockage in the reproductive tract, or ejaculatory problems (Wosnitzer *et al*., 2014). On the other hand, NOA, which comprises 60% of azoospermic cases, is characterized by impaired spermatogenesis (Wosnitzer *et al*.,

2014). Histological analysis of the testes can reveal three levels of impaired spermatogenesis: hypospermatogenesis, germ cell/maturation arrest, or Sertoli-cell only syndrome (SCOS). Hypospermatogenesis is diagnosed if all stages of spermatogenesis are present, but at a reduced level. Maturation arrest is diagnosed in the presence of total cell arrest at any stage of spermatogenesis. Finally, SCOS is defined by the complete lack of germ cells in the seminiferous tubules. Early maturation arrest or SCOS often imply poor prognosis for successful testicular sperm retrieval (TESE), while hypospermatogenesis and partial maturation arrest may lead to higher chances of success (McLachlan *et al*., 2007; Wosnitzer *et al*., 2014). There are various factors contributing to NOA, classified as either congenital causes (such as abnormal testis development, endocrinological abnormalities, genetic mutations or chromosomal abnormalities), or acquired causes (such as exposure to radiation or environmental toxins, or inflammation of the testes) (Boback and Schlegel, 2014). Genetic contributors of male infertility however, are particularly concerning as they have the potential to be passed to the offspring if the infertile couple chooses to conceive via assisted reproduction.

1.3.2 Genetic factors in male infertility

Genetic abnormalities account for approximately 15-30% of male infertile cases (O'Flynn O'Brien *et al*., 2010; Bukulmez, 2012), and can influence a variety of processes that are vital for fertility such as hormone homeostasis, spermatogenesis, and sperm quality (O'Flynn O'Brien *et al*., 2010). Although many genetic factors remain to be elucidated, it is important to appraise the currently known, common genetic causes of male infertility, which include Y chromosome microdeletions, cystic fibrosis, chromosomal abnormalities, and defects in meiotic genes.

1.3.2.1 Y chromosome microdeletions

Y chromosome microdeletion is the second leading genetic cause of male infertility (Krausz and Degl'Innocenti, 2006; Krausz *et al*., 2014). The relatively high prevalence of deletions on the Y chromosome may be attributed to its unstable structure. The Y chromosome originally evolved from the X chromosome by gaining testis-determining genes and large-scale inversions (Bachtrog, 2013). This change in genetic composition prevented the male-specific Y region on the Y chromosome from pairing with the X chromosome during meiosis, which is thought to have facilitated the formation of tandem duplications and inverted repeats (Bachtrog, 2013). Because of this, the inverted repeats on the Y chromosome now have the potential to pair with each other and form a hair pin shape that is susceptible to intra-chromosomal recombination during meiosis or the detection by DNA replication machinery, subjecting it to deletion (Bachtrog, 2013).

The most commonly deleted region in infertile men is at the Yq11 locus, termed the azoospermia factor (AZF). The genetic significance of the AZF region in spermatogenesis has been described since 1976 (Vogt *et al*., 1996). There are three non-overlapping sub-regions in the AZF termed AZFa, AZFb, and AZFc. A fourth region termed AZFd has also been suggested to exist between AZFb and AZFc (Kent-First *et al*., 1999). Microdeletions in AZFa are rare, in which deletions solely in this region account for 5% of cases (Krausz *et al*., 2014). Candidate genes in AZFa that cause infertility upon their deletion are the *dead box on the Y (DBY)* and *USP9Y* genes (Krausz *et al*., 2014). These deletions are typically associated with SCOS (Krausz *et al*., 2014). Deletions involving AZFb and either AZFa or AZFc comprises 35% of cases (Krausz *et al*., 2014), while deletions in AZFc are the most common, occurring in 80% of infertile men with AZF microdeletions (Kuhnert et al., 2004; Lo Giacco

et al., 2014). Genes in AZFc that are critical for spermatogenesis include the *testis transcript Y2* (*TTY2)* and *basic protein Y2 (BPY2)* genes (Lahn and Page, 1997). The complete deletion of AZFc, with or without the deletion of AZFb, often leads to SCOS or pre-meiotic cell arrest (Krausz *et al*., 2014). It is important to note that infertile men usually show a variety of partial or complete deletions along the AZF, which can manifest as a range of phenotypes from azoospermia to oligospermia.

1.3.2.2 Cystic fibrosis

Another genetic defect that is associated with male infertility is the autosomal recessive disease called cystic fibrosis (CF), which is caused by mutations in a gene that encodes for a chloride-conducting transmembrane channel called the *cystic fibrosis transmembrane regulator* (*CFTR*) gene (Elborn, 2016). The disease manifests as mucus retention, chronic local infection in the airways, and pancreatic insufficiency (Elborn, 2016). With recent advancements in treatment, the quality of life in CF patients has dramatically improved, with median life expectancy reaching over 40 years old (Elborn, 2016).

Since CF follows a recessive inheritance pattern, the symptoms only manifest in individuals with two copies of the mutated *CFTR* allele. Individuals who are heterozygous for the *CFTR* mutation have one mutated *CFTR* allele, and one normal *CFTR* allele; although they do not have CF, they may experience other symptoms such as infertility. Over 95% of male CF patients have obstructive azoospermia, due to a congenital bilateral absence of vas deferens (CBAVD) (Elborn, 2016). Heterozygous men with only one mutated *CFTR* allele may also display CBAVD and be infertile, comprising 60% of CBAVD cases (Elborn, 2016). For this reason, infertile couples with OA are strongly advised to screen for *CFTR* mutations when seeking fertility treatment. If both parents are heterozygous for the *CFTR* mutation,

there is a 25% chance of conceiving a baby with CF. Genetic screening for infertile couples can therefore prevent the transmission of *CFTR* mutations onto offspring (Gazvani and Lewis-Jones, 2006).

1.3.2.3 Chromosomal abnormalities

Studies have shown an eight to ten fold higher incidence of chromosomal abnormalities in infertile men compared to fertile men (Johnson, 1998; Egozcue *et al*,. 2000; Bulkumez, 2012; Stouffs *et al*., 2014). This frequency increases to 15% in azoospermic men. Studies on 9766 azoospermic and severely oligozoospermic men found sex and autosomal chromosomal abnormalities in 4.2% and 1.5% of the cases compared to 0.14% and 0.25% of the control population, respectively (Johnson, 1998; Bulkumez, 2012).

Chromosomal aberrancies consist of two types: structural or numerical. Structural chromosomal abnormalities are characterized by large-scale changes in chromosome content, including extra or missing genetic material, or rearranged sections of the chromosome. Chromosomes may also be altered numerically with the most prevalent condition being aneuploidy, the gain or loss of whole chromosomes (O'Flynn O'Brien, 2010). Some carriers of aneuploidy may be mosaic, where not all cells contain the additional or missing chromosome. Although aneuploidy can involve either sex or autosomal chromosomes, the most common cases involve extra or missing sex chromosomes such as Klinefelter Syndrome (47,XXY), XYY syndrome, and mixed gonadal dysgenesis (Bulkumez, 2012). Klinefelter Syndrome occurs in about 1 in 500 male live births and is the most prevalent genetic cause of azoospermia, occurring in about 14% of cases (Steinman *et al*., 2009). The classic phenotypes associated with the syndrome include testicular atrophy, a diminution of germ cells, and infertility. It is thought that the extra X chromosome poses lethal gene dosage,

resulting in germ cell death (Steinman *et al*., 2009). About 10% of Klinefelter men have a mosaic karyotype of 47,XXY/46,XY, in which case sperm production may be present due to the 46,XY germ cells that are able to complete meiosis (Bulkumez, 2012; Stouffs *et al*., 2014). 47,XYY men are observed in 1 in 1000 live male births. Although the incidence of infertility is slightly increased in this population, most 47,XYY men are fertile (Stouffs *et al*., 2014). Finally, mixed gonadal dysgenesis is a rare condition that is characterized by a unilateral testis and contralateral streak gonad (Bulkumez, 2012; Flannigan *et al*., 2014). Men with mixed gonadal dysgenesis can have a karyotype of 45,X/46,XY or 46,XY, and are generally infertile depending on the level of abnormal germ cells in the testes (Bulkumez, 2012).

1.3.2.4 Defects in meiotic genes

Although the infertile population shows an increased incidence of chromosomal abnormalities, the majority of infertile men have a normal karyotype. Nonetheless, increased rates of numeric chromosomal abnormalities in sperm have been observed in the infertile population (Bernardini *et al*., 2000; Egozcue *et al*., 2003; Ferguson *et al*., 2007; Kirkpatrick *et al*., 2008). Since meiosis is responsible for producing sperm with the correct chromosome content, perturbations in this cell division event have been suggested to be the culprit behind some cases of male infertility. In fact, an estimated 5-10% of NOA cases may be due to meiotic arrest (Topping *et al*., 2006). Although mutations in meiosis-specific genes have been implicated in a small percentage of male infertility (Yang *et al*, 2015; Hann *et al*., 2011), our understanding of how meiotic errors cause infertility remains elusive. Studies that screened large cohorts of infertile men found modest evidence for the association of mutations in *SCP3* (facilitates chromosome synapsis) and *Spo11* (involved in DSB

formation) with NOA (Miyamoto *et al*., 2003; Arabi *et al*., 2006; Sanderson *et al*., 2008). In 2009, Dieterich *et al*. showed that a deletion in the *aurora kinase C* (AURKC) gene, which functions in spindle assembly during meiosis, gave rise to sperm with irregular morphology and infertility. However, a knock-out of the gene in mice resulted in poor sperm morphology and retained fertility (Kimmins *et al*., 2007). Recently, studies on azoospermic men have identified mutations in *TEX11* and *TEX15*, which are genes shown to regulate meiotic recombination in mice (Yang *et al*., 2008; Ruan *et al*., 2012; Yang *et al*., 2015). Evidently, mutations in meiotic genes may be an emerging explanation for some cases of male infertility. However, further investigation is warranted to identify the mutations that are most clinically relevant for assessing the genetic risk of infertile couples undergoing assisted reproduction.

1.3.3 Use of intracytoplasmic sperm injection to treat male infertility

The introduction of intracytoplasmic sperm injection (ICSI) in the early 1990s as a type of assisted reproductive technology (ART) has revolutionized the treatment of male infertility. Prior to the inception of ICSI, men with severe infertility could not successfully establish a pregnancy through the conventional type of ART, *in vitro* fertilization (IVF), due to an inadequate amount of sperm for use in the procedure. ICSI overcame this obstacle by employing the injection of a single sperm into an oocyte (Ma and Ho Yuen, 2000). A review by Devroey and Steirteghem (2004) looking at ICSI cycles in twelve European countries summarized that more than 67% of injected oocytes become successfully fertilized when using ejaculated sperm. The fertilization rate using testicular sperm in the case of NOA was lower at 50% (Devroey and Steirteghem, 2004). When compared to conventional IVF methods, there were fewer unexpected fertilization complications in the ICSI cohort

(Devroey and Steirteghem, 2004). According to publications from fertility clinics across Europe, ICSI comprised 40% of all ART procedures in 2001 to 2002 (reviewed in Devroey and Steirteghem, 2004). The popularity of ICSI is also observed worldwide, where this procedure has replaced conventional IVF in many clinics even when sperm parameters are normal (Devroey and Steirteghem, 2004). As a result, there is a growing population of children born via ICSI, which has emphasized the importance of evaluating the possible genetic repercussions. As a portion of male infertility is caused by genetic factors, there is concern regarding the transmission of these genetic defects to the next generation. Over the past decades, our understanding of the sperm chromosome content and germ cells in infertile men has also rapidly improved. This has helped us to evaluate some of the potential complications associated with the use of sperm from infertile men in ICSI.

1.4 Sperm aneuploidy and meiotic defects in infertile men

1.4.1 Sperm aneuploidy in infertile men

Although an early method for studying the chromosomal constitution of human sperm was developed in 1978 by Rudak *et al*., it could not be applied to men with severe infertility since the sperm used in the technique must be present in a sufficient concentration and be able to fertilize an egg. Therefore, it was not until the advent of a molecular cytogenetic technique called fluorescent *in situ* hybridization (FISH) that the study of chromosome content in the sperm of infertile men took off. FISH involves the hybridization of fluorescently labeled DNA probes to target DNA on specific chromosomes. This technique is efficient at detecting numerical chromosomal abnormalities, and allows for the study of sperm aneuploidy in large cohorts of fertile and infertile men. A review conducted by Templado *et al*. (2011) looked at the FISH analyses in the sperm of 388 healthy men, and

reported disomy frequencies for 18 of the 24 chromosomes, with the estimated rate of total aneuploidy to be 4.5%. With respect to individual chromosomes, the average frequency of aneuploidy was 0.1%, ranging from 0.03% for chromosome 8 to 0.47% for chromosome 22. The review also confirmed that chromosome 21 (0.17%), 22 (0.47%), and the sex chromosomes (0.27%) were most susceptible to segregation errors during meiosis, showing a two to three-fold higher rate of aneuploidy compared to the other autosomes.

When the chromosome content of infertile men was examined, it became evident that infertile men with abnormal karyotypes had elevated rates of aneuploidy in the sperm (Wong *et al*., 2008; Kirkpatrick *et al*., 2008; Kirkpatrick *et al*., 2012; Kirkpatrick *et al*., 2015). The high rates of sperm aneuploidy in this population is thought to be due to the improper or unbalanced segregation of the abnormal chromosome during meiosis (Templado *et al*., 2013). Beyond this revelation, the study of sperm in infertile men yielded a more surprising result, showing that infertile men with normal karyotypes may nevertheless carry a higher rate of sperm aneuploidy compared to fertile men. The first study to reveal this phenomenon found an increased rate of numerical chromosomal abnormalities in the sperm of men with severe oligozoospermia (Moosani *et al*., 1995). This trend was further confirmed in large cohorts of severely oligozoospermic men by other laboratories (Ferguson *et al*., 2008; Kirkpatrick *et al.*, 2008; reviewed in Templado *et al*., 2013). In general, these studies observed a two to ten-fold increase in numerical chromosomal anomalies in the sperm of infertile men when compared to fertile men (Templado *et al*., 2013). Furthermore, the severity of infertility may be proportional to the production of aneuploid sperm as demonstrated by several studies including Nagvenkar *et al.* (2016), who found higher incidences of aneuploid sperm in severely oligozoospermic men when compared to

oligozoospermic men (Vegetti *et al.,* 2000; Hansen *et al.,* 2005; Templado *et al.,* 2013; Chatziparasidou *et al.,* 2014; Nagvenka *et al.,* 2016). There are also various FISH studies performed on sperm retrieved from the testes of azoospermic men, where most studies have found higher levels of aneuploidy in this population when compared to fertile men (Bernardini *et al.*, 2000; Burrello *et al.,* 2002; Rodrigo *et al.,* 2004; Kickpatrick *et al.,* 2008; Vozdova *et al*., 2012). When it comes to the different types of azoospermia, NOA men have been shown to display higher incidences of aneuploid sperm than OA men, possibly due to the underlying causes of spermatogenic failure in the NOA population (reviewed in Burrello *et al*., 2005; Sun *et al*., 2008; Rodrigo *et al*., 2011; Vozdova *et al*., 2012).

All in all, it appears that some infertile men are highly predisposed to producing sperm with numerical chromosomal abnormalities. Further investigation into which subset of infertile men may carry the highest risk of aneuploid sperm can benefit genetic counselling for couples undergoing ICSI. The level of aneuploid sperm in the infertile man may act as one of the predictors for the success rate of establishing a healthy pregnancy after ICSI; if an aneuploid sperm is used to fertilize an oocyte, the resulting aneuploid embryo has an extremely high risk of perishing *in utero*. In the rare chance that the embryo survives to birth, the newborn may exhibit a wide range of phenotypes, including congenital birth defects, depending on which chromosome was affected, and the severity of aneuploidy. Several case reports have in fact suggested the transmission of numerical chromosomal abnormalities from the sperm to the fetus after ICSI, including a 47,XXY pregnancy of paternal origin that was conceived using sperm with an increased frequency of XY disomy (Moosani *et al*., 1999). Carrell *et al.* (2001) reported a trisomy 15 pregnancy of paternal origin that arose from a father with elevated rates of disomy 15 in the sperm (4.03% vs. 0.4% in control men).

Furthermore, Tang *et al*. (2004) reported a 45,X abortus that resulted from a man with exceptionally high rates of sex chromosome aneuploidy in the sperm (40% vs 0.4% in control men). Although there is mounting evidence of elevated incidences of chromosomal abnormalities in newborns conceived via ICSI (ranging from 1.5% to 12.7%) compared to natural conceptions (0.9%) (reviewed in Hansen *et al*., 2005 and Lie *et al*., 2005), there remains a shortage of studies examining whether paternal factors are significant contributors. Notwithstanding, the increased prevalence of aneuploid sperm in infertile men is concerning with regards to the safety of ICSI, and may potentially be contributing to paternal origins of chromosomal abnormalities in ICSI births. Therefore, ongoing research on the mechanisms underlying the production of aneuploid sperm in infertile men is direly warranted. In this respect, errors in meiotic recombination, and other defects in meiosis, have become an emerging explanation for the generation of aneuploid sperm in male reproduction.

1.4.2 Meiotic defects in infertile men

Until relatively recently, the vast majority of our understanding of meiotic events, including synapsis and recombination, was derived from studies in lower eukaryotes such as *S. cerevisiae* and *Drosophila* (Martin, 2010). However, recent advancements in immunocytogenetic approaches have allowed the visualization of meiotic proteins in humans, using antibodies against the proteins involved in the SC (SCP1 and SCP3), recombination (MLH1), and centromeres (Ma *et al*., 2006a; Ma *et al*., 2006b; Ferguson *et al*., 2007). Notably, by combining immunofluorescent techniques and FISH, researchers were able to construct recombination maps on individual chromosomes in human meiotic cells (Sun et al., 2004; Sun et al., 2006b). The findings unraveled the interconnection between chromosome synapsis and recombination, where the frequency of crossovers was shown to

be proportional to the SC length (Sun et al., 2004; Sun et al., 2006b). Longer chromosomes, such as chromosome 1, display an average of 3.9 crossovers, whereas shorter chromosomes such as 21, 22, and the sex chromosomes only display a single crossover (Sun et al., 2004; 2006c). Sun *et al*. (2005a) also demonstrated that disruptions in chromosome synapsis can influence recombination. The study noted that unsynapsed regions near the centromere are shown to have a *cis* effect on crossover distribution with crossovers forming farther from the centromeres on the affected chromosome. Another study unveiled a more surprising result, showing that unsynapsed regions on chromosome 9 were associated with a reduction in crossover formation on chromosome 5, and thus hinting at the possibility of a *trans* effect of asynapsis on recombination (Sun *et al*., 2007a).

While data on chromosome synapsis and meiotic recombination in fertile men was being generated, studies have also begun to examine these meiotic events in infertile men. Since spermatocytes from the testes are required to study meiosis, most studies thus far have been performed on azoospermic men. Testicular tissue from these cases can be obtained with patient consent when the infertile men undergo testicular biopsies for sperm retrieval for the use in ICSI. These studies have demonstrated that infertile men may display several meiotic defects including errors in: 1) chromosome synapsis, 2) crossover frequency, and 3) crossover distribution.

1.4.2.1 Errors in synapsis

By visualizing the SC using antibodies against the lateral and transverse element proteins, problems with SCP1 assembly can be detected as splits within the SC, whereas simultaneous errors in SCP1 and SCP3 can be observed as gaps. With this approach, Ferguson *et al.* (2007) observed a significantly higher frequency of SC gaps in NOA men

(32.7%) and OAT men (38.1%) when compared to fertile men (26.6%). Moreover, Sun *et al*. (2007b) found an elevated incidence of SC splits in NOA men (24%) when compared to fertile men (10%). Numerous studies have suggested that synaptic errors may lead to partial (Gonsalves *et al*., 2004; Judis *et al*., 2004) or complete (Sun et al., 2004) spermatogenic arrest in infertile men. Additionally, errors in synapsis may negatively impact the formation of crossovers as shown by Ferguson *et al*. (2009), where 60% of NOA men who displayed elevated rates of synaptic errors also showed reduced crossover frequencies compared to fertile men.

1.4.2.2 Errors in crossover frequency

The investigation of the frequency of crossovers in infertile men unveiled several interesting trends. Pachytene is observed to be the longest stage in prophase I, where 80-90% of cells observed in a testicular sample are found at this stage in fertile men (Ferguson *et al*., 2007; Martin, 2010). Strikingly, a large percent of NOA men (45-53%) show a complete absence of meiotic cells in the testes (Sun *et al*., 2005b; 2007b; Topping *et al.*, 2006; Ferguson *et al.*, 2007), or reduced frequency of pachytene cells (10% vs. 80-90%) due to partial or complete meiotic arrest at the zygotene stage (Gonsalves *et al*. 2004; Sun *et al.* 2004b; 2007b; Ferguson *et al*. 2007). In addition to the disruption in the progression of meiosis, most studies showed that NOA men also display a reduced frequency in crossover formation when compared to fertile men (reviewed in Martin, 2010; Table 1.4). The first such study found that 10% of its NOA cohort showed a significantly reduced rate of recombination when compared to controls (Gonsalves *et al*., 2004). Similarly, reports by Ma *et al*. (2006a; 200b) and Ferguson *et al*. (2007) identified reduced recombination in 25% and 63% of infertile men with impaired spermatogenesis, respectively. Conversely, there are two

studies that did not find a decrease in the frequency of recombination in their infertile cohort, although this may be due to the small sample sizes (Codina-Pascual *et al.,* 2005; Topping *et al*., 2006). Combining all the meiotic analyses in infertile men with impaired spermatogenesis, it is estimated that approximately 20.8% of these men display a reduced rate of recombination (Table 1.4). Interestingly, OA men have also been shown to display slight reductions in the frequency of crossovers when compared to controls, although the rate of recombination is higher than that of NOA men (Sun et al., 2004; Sun *et al*., 2007b; Ferguson *et al*., 2007; Ren *et al.,* 2016). Contrary to NOA men, OA men do not show impaired spermatogenesis. Thus, the errors in recombination in OA men is thought to possibly arise from epigenetic changes in the cells due to the prolonged obstruction of the reproductive tract (Martin, 2010; Minor *et al.,* 2011).

Table 1.4 immunofluorescent analyses of spermatocytes in infertile men

Number of men showing impaired spermatogenesis	Number of men with reduced recombination compared to controls	Percentage of infertile men with reduced recombination compared to controls (%)	References
40	4	10	Gonsalves *et al*., 2004
11	0	0	Codina-Pascual *et al*., 2005
26	0	0	Topping *et al*., 2006
4	1	25	Ma et al., 2006a; Ma *et al*., 2006b
29	5	17.2	Sun *et a*l., 2007b
8	5	62.5	Ferguson *et al*., 2007
6	4	66.7	Ferguson *et al*., 2009
4	3	75	Reig-Viader et al., 2014
16	8	50	Ren *et al*., 2016
Total: 144	**30**	**20.8**	

1.4.2.2.1 Absence of crossovers

An extreme form of recombination error has also been observed in the infertile male population, which is the complete lack of recombination on a bivalent (achiasmate bivalent) during pachytene (Ma *et al*., 2006a). This occurrence carries particular clinical significance since achiasmate bivalents may readily undergo nondisjunction at the first meiotic division. Although achiasmate bivalents are present in only 0.1-5% of cells in fertile men (Sun et al., 2006a; 2005b; 2007b; Ferguson *et al.*, 2007), the frequency is significantly higher in NOA men, occurring in up to 29% of cells (Martin 2006; Sun *et al*. 2007b; Ferguson *et al*., 2007). These studies note that chromosomes 21, 22, X, and Y are most likely to lack a crossover (Tempest, 2010). Interestingly, Ma *et al.* (2006a) also reported an infertile man who did not display any recombination on the sex chromosomes. This study was also the first to show a negative correlation between the frequency of recombination on the sex chromosomes and XY disomy in the sperm. Similarly, Sun *et al.* (2008) found a relationship between reduced recombination on the sex chromosomes and an increase in sex chromosome aneuploidy in NOA men. It is not yet clear whether the absence of recombination on autosomes show a similar relationship with regards to sperm disomy for the corresponding chromosome.

1.4.2.3 Alterations in crossover distribution

Thus far, studies of recombination errors in infertile men have primarily focused on the differences in the frequency of crossovers between infertile and fertile groups. However, crossover formation is meticulously regulated in not only number, but also position. Meiotic chromosomes are uniquely structured to facilitate their proper migration during chromosome pairing, synapsis, and segregation. Thus, a disruption in the chromosome structure and its associated proteins may lead to missegregation. As aforementioned in section 1.2.5.2, the

formation of crossovers is generally inhibited near the centromeres to avoid the disruption of centromeric cohesins, which aid in proper chromosome segregation. Intriguingly, Ferguson *et al*. (2009) reported that 30% of infertile men from the study (n=10) showed altered crossover distributions on chromosome 13, 18, or 21 compared to controls, where there was a shift in crossover formation toward the centromere, and decrease in crossover formation in the subtelomeric regions. This finding aligned with early chiasma studies that examined crossover distribution in a small group of infertile men (Hulten *et al*., 1970; Micic *et al*., 1982; Codina-Pascual *et al*., 2006). Furthermore, Ferguson *et al*. (2009) identified two infertile men who had normal recombination rates, and nevertheless displayed altered crossover distributions. This observation was initially reported in a single infertile man in an early chiasma study (Laurie and Hulten, 1985). These limited studies posed the curious question of whether subsets of infertile men may show alterations in the localization of crossovers, regardless of changes in the number of crossovers, and whether this phenomenon has a negative effect on chromosome segregation.

1.5 Rationale, hypotheses, and objectives

So far, we have seen that meiosis is a key process in the production of sperm with the correct number of chromosomes, effectively dividing a diploid spermatocyte (2n) into four haploid spermatids (n). The reductional division of the spermatocyte ultimately boils down to the segregation of the homologous chromosomes during MI. Two events in the prophase I stage are particularly crucial for this step: 1) the synapsis of the chromosomes with their homologous counterpart, and 2) the crossing over of genetic material between the homologous chromosomes. Over the past decade, meiotic studies have shown that infertile men may exhibit errors in these two important processes, where increased rates of synaptic

errors, reduced rates of recombination, and alterations in crossover distribution have been noted. Nonetheless, it remains unanswered whether these defects may be contributing to the concerning increase in numerical chromosomal abnormalities in the sperm of infertile men when compared to fertile men. To date, only one study has reported a link between reduced rate of genome-wide recombination and increased rate of sperm aneuploidy in an infertile man (Ma *et al*., 2006a). While Ferguson *et al*. (2007) and Sun *et al.* (2008) have linked achiasmate sex chromosomes to an increase in sex chromosomal aneuploidies in the sperm of infertile men, it remains unclear whether such a relationship exists between achiasmate autosomes and disomy in the sperm. Aside from the frequency of crossovers, changes in the position of crossovers have also been implicated in some infertile men (Laurie and Hulten, 1985; Ferguson *et al*., 2009; Ren *et al*., 2006). However, it remains uninvestigated whether this population show specific aberrant trends in crossover distribution, which may give rise to chromosome segregation errors.

Thus, our work in this thesis aims to investigate whether meiotic defects, in the form of changes to crossover frequency or distribution, may explain the link between male infertility and the higher rate of sperm aneuploidy present in this population. We speculate that errors during meiosis may lead to the spermatogenic arrest of most cells, resulting in an infertile phenotype in the individual. However, some cells may escape meiotic arrest, and are at higher risk of chromosome missegregation and producing aneuploid sperm. We hypothesize that: (1) infertile men may display reduced rates of recombination, which may be associated with increased rates of sperm aneuploidy within the same individual; and (2) infertile men may also display changes in crossover distribution, where increased or

decreased crossover formation in certain regions of the chromosomes may facilitate chromosome missegregation.

We are next interested in uncovering the underlying mechanisms behind the recombination defects observed in the infertile cohort. Crossover formation has been shown to be most frequent near the subtelomeres of the chromosomes in normal males, where telomere-guided mechanisms have been proposed to establish crossovers in this region (Blitzblau *et al*, 2007). However, previous studies on the crossover distribution of infertile men have found a decrease in crossover formation near the subtelomeres of some infertile men (Ferguson *et al*., 2009; Ren *et al*., 2016). Since assessment of telomeres in infertile men have shown defects in length and stability (Reig-Viader *et al.*, 2014a), we sought to determine whether defects in telomeres may be leading to synapsis and recombination errors in infertile men. We hypothesized that telomeric stability, in terms of the association of telomeres with its structural protein, may be linked to the level of recombination observed in infertile and fertile men.

Finally, our understanding of meiosis in infertile men has been relatively restricted to men with normal karyotypes, as it is rare to obtain testicular sample from infertile men with abnormal karyotypes due to the low prevalence of chromosomal abnormalities. However, infertile men with chromosomal abnormalities may face increased risks when undergoing ICSI as the chromosome complement in their sperm may be unpredictable. Therefore, the last objective of our study aims to examine the meiotic behaviour and sperm aneuploidy patterns in carriers of sex chromosomal abnormalities. Our findings hope to offer clinical significance for genetic counsellors and patients with similar sex chromosomal mosaicism. The work in this thesis will begin to address the following specific objectives:

Objective 1a. To assess the rate of synaptic errors, and frequency of crossovers in spermatocytes of infertile men compared to fertile men. **1b.** To determine if reduced crossover formation is associated with the increased production of aneuploid sperm in infertile men.

Objective 2. To determine whether the distribution of crossovers in infertile men is altered compared to fertile men.

Objective 3. To correlate telomeric protein homeostasis with crossover frequency in infertile and fertile men.

Objective 4a. To determine the meiotic behaviour and sperm aneuploidy rates in a mosaic 45,X/46,XY infertile man.

CHAPTER 2: ASSOCIATION BETWEEN SYNAPSIS, RECOMBINATION AND SPERM ANEUPLOIDY IN INFERTILE MEN

2.1 Introduction

During the first meiotic division, homologous chromosomes undergo pairing and synapsis, in which a ladder-like protein structure called the synaptonemal complex (SC) forms between them. This interaction facilitates the crossover or exchange of DNA between the homologous chromosomes in a process known as recombination. Meiotic recombination is important for not only genetic diversity, but also for the proper segregation of the homologous chromosomes during meiosis. Specifically, the crossovers form structures called chiasmata, which tether the homologous chromosomes at the sites of recombination, and ensure proper tension for chromosome segregation in anaphase I. Thus, crossover formation is regulated such that at least one crossover forms on each chromosome pair (bivalent) (Ma *et al*., 2006a; Martin, 2010). Anomalies in the frequency of crossovers, particularly achiasmate bivalents that are lacking a crossover, have been proposed to lead to an increased risk of chromosome missegregation, and the subsequent production of aneuploid gametes (Ma *et al.*, 2006a; Ma *et al.*, 2006b; Ferguson *et al*., 2007; Ferguson *et al.*, 2009; Ren *et al.*, 2016).

The development of immunofluorescent techniques for the analysis of germ cells has allowed for the study of meiosis in humans. A series of studies have demonstrated that infertile men may experience defects in meiosis (Gonsalves *et al*., 2004; Codina-Pasual *et al.*, 2005; Topping *et al.*, 2006; Ma *et al.*, 2006a; Sun *et al.*, 2007). Numerous studies have also confirmed the decrease in recombination levels and increase in synaptic errors that are often reported in infertile men (Ma *et al.*, 2006a; Ma *et al.*, 2006b, Ferguson *et al.*, 2007; Ferguson *et al*., 2009; Ren *et al.*, 2016). It is proposed that the vast majority of defects in synapsis or

recombination are caught by meiotic checkpoints, which leads to the meiotic arrest of germ cells, and consequently infertility (Tempest *et al*., 2011). However, we speculate that some faulty cells may escape arrest, and are predisposed to chromosome segregation errors, thus potentially leading to an increase in aneuploidy in the sperm. Our hypothesis is supported by the abundant evidence of increased incidences of aneuploidy in the sperm of infertile men (Ferguson *et al*., 2008; Kirkpatrick *et al.,* 2008; reviewed in Tempest *et al.,* 2001 and Templado *et al*., 2013). However, there remains a lack of investigation into whether there is an association between the increased rates of meiotic defects and sperm aneuploidy observed in infertile men. In this respect, Ferguson *et al. (*2007) was the first to examine the chromosome-specific patterns of recombination in infertile men, in order to correlate the crossover frequencies on particular chromosomes to their disomy rates in the sperm within the same individual. The results showed that the frequency of recombination between the sex chromosomes is negatively correlated to the rate of XY disomy in the sperm of fertile and infertile men (Ferguson *et al.,* 2007). However, due to the limitation in sample size (n=18), the analysis could not be accurately performed on the infertile cohort alone. Moreover, the study did not find a significant relationship between recombination on the autosomes analyzed and their disomy rates in the sperm. Building on these previous findings, we are largely interested in further investigating the chromosome-specific rates of recombination in a larger sample size (n=34), with a focus on elucidating the relationship between recombination and aneuploidy in infertile men. Through the use of immunofluorescent and FISH techniques, we studied synapsis, genome-wide recombination, and chromosome-specific recombination in the spermatocytes of infertile men. We focused on chromosomes 13, 18, 21, and the sex chromosomes as aneuploidies in these chromosomes are a major

cause of spontaneous abortions, as well as abnormalities in livebirths. Finally, we also assessed the rates of aneuploidy in sperm for these chromosomes in order to determine if infertile men with certain meiotic defects are at an increased risk of producing aneuploid sperm.

2.2 Materials and methods

2.2.1 Patient information and tissue collection

Testicular tissue was collected from twenty-four azoospermic, infertile men seeking fertility treatment and fourteen proven fertile men who were undergoing vasectomy reversals (see Appendix I). Fertile men whose vasectomy lasted for more than ten years were excluded from the study. All infertile men were diagnosed with idiopathic forms of infertility, had normal 46,XY karyotypes, no microdeletions on the Y chromosome, and no CFTR mutations. The testicular tissue was used for pathology diagnosis, sperm extraction for ICSI, and a small portion was used for the meiotic analyses presented in this study. The pathology report diagnosed two infertile men with SCOS, where there was a lack of germ cells. Ten infertile men were classified as NOA; two of these men showed maturation arrest in the testes and did not have enough germ cells for meiotic analysis. The remaining eight NOA men were diagnosed with either hypospermatogenesis or partial maturation arrest, and had enough germ cells for analysis. Twelve infertile men were diagnosed as OA, and showed normal spermatogenesis despite having no sperm in the semen.

2.2.2 Preparation of testicular tissue

Testicular tissue was processed according to a modified version of the protocol described by Barlow and Hulten (1998). On a 37°C thermal plate, seminiferous tubules were

separated in human tubal fluid (HTF) and cut into 3-5mm segments. Segmented tubules were incubated at 37°C for 45-60 min in freshly prepared hypo-extraction buffer [30mM Tris, 50mM sucrose, 17mM citric acid, 5mM ethylene diamine tetraacetic acid (EDTA), 0.5mM dithiothreitol (DTT), and 0.1mM phenylmethylsulphonyl fluoride (PMSF); pH 8.4]. Tissue was subsequently deposited onto a microscope slide with 20µl of 100mM sucrose (pH 8.2). Using fine forceps, tubules were minced in order to release their contents, in which 20µl of the germ cell/sucrose slurry was transferred to a new slide coated with 200µl of 1% paraformaldehyde with 0.2% Triton X. Slides were incubated for 24 hours at 37°C in a humid chamber.

2.2.3 Fluorescence immunostaining

The germ cell-containing slides were immunostained according to a previously published protocol by Ma *et al.*, (2006b). Slides were air dried prior to two washes in 0.4% PhotoFlo (Kodak 200 solution). Slides were next incubated in antibody diluting buffer (ADB) [1% donkey serum, 0.3% bovine serum albumin, 0.005% Triton-X, PBS; pH 7.2] for 30 min at room temperature. Primary antibody cocktail [mouse anti-human MLH1 (BD Pharmagen, San Diego, CA, USA), 1:12; rabbit anti-human SCP3 (Abcam, Cambridge, MA, USA), 1:20; rabbit anti-human SCP1 (produced by P. Moens), 1:60; human CREST antisera, 1:25; 1 x ADB] was then applied to the drained slides. After applying a cover slip and sealing it using rubber cement, slides were incubated in a humid chamber at 37°C for 24 hours. Slides were washed in 1 x ADB for 20 min, followed by a second wash in 1 x ABD for 48 hours at 4°C. Secondary antibody cocktail [Fluorescein isothiocynate (FITC) labeled donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) 1:12; tetramethyl rhodamine isothiocynate (TRITC) labeled donkey anti-rabbit IgG (Jackson

ImmunoResearch) 1:15; aminomethyl coumarin acetic acid (AMCA) labeled donkey anti-human IgG (Jackson ImmunoResearch) 1:25; 1 x ADB] was applied to the slides, which were then incubated for 90 min at 37°C in a humid chamber. Slides were sequentially washed three times in 1 x PBS (10, 20, and 30 min) with agitation every 5 min. Finally, slides were drained before antifade was added. Slides were examined using a Zeiss Axioplan epifluorescent microscope equipped with the appropriate filters in order to identify pachytene cells. Cytovision V2.81 Image Analysis software (Applied Imaging International, San Jose, CA, USA) was used to capture the SCP3/SCP1, MLH1, and CREST signals on the pachytene cells. Pachytene cells were captured if MLH1 foci were clear and the sex chromosomes were identifiable. The X and Y chromosomes are distinguishable due to its unique structure during pachytene, where the two chromosomes condense to form a sex body (Fig. 2.1A). The number of MLH1 foci per cell, frequency of synaptic errors, and cell coordinates were recorded.

2.2.4 FISH on immunostained spermatocytes

After capturing images of the SC and MLH1 foci, FISH was performed on the previously immunostained slides according to the methods reported by Ma *et al.* (200b). Coverslips were removed before soaking slides twice in 4 x saline sodium citrate (SSC)/0.05% Tween-20 solution for 5 min each time. Slides were dried in a series of ethanol solutions (70%, 80%, 90%, 100%) for 2 min each. After air drying, slides were soaked for 5 min in 1 x PBS, re-fixed in 10% formalin phosphate for 5 min, washed for 5 min in 1 x PBS and air dried again in an ethanol series of solutions (70%, 80%, 90%, 100%) for 2 min each. Directly labeled single-stranded DNA probes: LSI 13 (SpectrumGreen), CEP 18 (SpectrumAqua) and LSI 21 (SpectrumOrange) (Vysis Inc., Downers Grove, IL, USA) were

added to slides. Coverslips were added and sealed with rubber cement. Slides were then co-denatured on a hotplate for 5 min at 76°C, and subsequently placed in a humid chamber overnight at 37°C. Coverslips were then removed, and slides were washed at 75°C in 0.4 x SSC/0.3% NP-40 solution for 2 min, followed by 2 x SSC/0.1% NP-40 at room temperature for 30 seconds. Finally, slides were air dried before antifade and coverslips were added. The pachytene cells that were captured beforehand were relocated in order to identify the chromosomes 13, 18, and 21.

2.2.5 FISH on testicular spermatozoa

Slides that were not previously immunostained were used for FISH on testicular sperm to examine the rate of aneuploidy. Slides were washed for 5 min in 2 x SSC, and incubated for 15-20 min in 10mM DTT/100Mm Tris (pH 8.0) to decondense the sperm nuclei. Slides were washed for 5 min in 2 x SSC, soaked in 1 x PBS, and dehydrated in an ethanol series (70%, 80%, 90%, 100%) for 2 min. A probe mixture of directly labeled single-stranded DNA probes: CEP 18 (SpectrumAqua), CEP X (SpectrumGreen), and CEP Y (SpectrumOrange) (Vysis Inc.) was applied to the slides. Coverslips were added and sealed with rubber cement. Slides were co-denatured on a hotplate for 5 min at 76°C, and subsequently placed in a humid chamber overnight at 37°C. Coverslips were removed, and slides were washed at 75°C in 0.4 x SSC/0.3% NP-40 solution for 2 min, followed by 2 x SSC/0.1% NP-40 at room temperature for 30 seconds. Slides were air dried and counterstained with 4,6-diamidino-2 phenylindole (DAPI) (Vysis Inc) before antifade and coverslips were added. Slides were analyzed with a Zeiss Axioplan epifluorescent microscope. Only non-overlapping sperm nuclei with intact morphology, and visible sperm tails were scored. Two signals of the same colour were scored as disomy if the signals were

of comparable intensity and size, and were separated from each other by a distance equal or greater than the diameter of each signal.

After scoring the signals for chromosomes 18, X, and Y, the same slide was re-hybridized with probes for chromosomes 13 and 21. Coverslips were removed and slides were washed for 5 min in 4 x SSC/0.05% Tween-20 solution, and dried in an ethanol series (70%, 80%, 90%, 100%) for 2 min each. A probe mixture of directly labelled single-stranded DNA probes: LSI 13 (SpectrumGreen), and LSI 21 (SpectrumOrange (Vysis Inc.) was applied to the slides using the same steps that were used for the CEP 18/X/Y probe mixture. Scoring of the chromosome 13 and 21 signals were performed using the same criteria as those used for chromosomes 18, X, and Y.

2.2.6 Statistical analyses

The Mann-Whitney test was used to compare the mean rate of genome-wide recombination between individual infertile men and the control group. The rate of synaptic errors in the infertile men were considered significantly different if they were beyond the 95% confidence interval of the control group. The Fisher exact test was used to compare the proportion of cells with XY recombination. Statistical analyses for genome-wide recombination rates were performed according to the methods used by Gonsalves *et al*., 2004, Codina-Pascual *et al*., 2005, Sun *et al*., 2007b and Ferguson *et al.*, 2009 in order to allow for the comparison of results. The Chi-square test with two degrees of freedom was used to compare the frequencies of crossovers on chromosomes 13 and 18, while a Chi-square test with one degree of freedom was used to compare the crossover frequencies on chromosome 21 between individual infertile men and the control group. The Fisher exact test was used to compare the rates of disomy in the sperm. The Pearson's correlation test was

used to correlate recombination frequencies on chromosomes 13, 18, 21, and the sex chromosomes to the corresponding sperm disomy rates in the infertile men. $P < 0.05$ was considered significant.

2.3 Results

2.3.1 Analysis of synapsis

With fluorescent immunostaining, we were able to assess the rate of synaptic errors and genome-wide recombination in the spermatocytes of infertile and fertile men by visualizing the SC (SCP3/SCP1), crossover sites (MLH1), and centromeres (CREST) using antibodies against the associated proteins (Fig. 2.1A). A total of 707 pachytene nuclei from the control group, 587 pachytene nuclei from the OA group, and 539 pachytene nuclei from the NOA group were analyzed. In our analyses, we observed two types of synaptic anomalies in the pachytene cells: 1) discontinuities in the SC cause by the absence of both SCP1 and SCP3 in the same region, and 2) unsynapsed regions in the SC in which only the lateral elements (SCP3) were present (Fig. 2.2). A cell containing either type of anomalies in the SC structure was noted to contain synaptic errors. The frequency of synaptic errors ranged from 0% to 9.8% in the control group, with a mean of 3.0% (Table 2.1). The OA group and NOA group showed an average rate of synaptic errors of 4.4% and 5.1% respectively, which were not significantly different than the rate observed in the controls. However, three OA men (OA28, OA31, OA34) and three NOA men (NOA24, NOA29, NOA30) individually showed an increased frequency of synaptic errors when compared to controls.

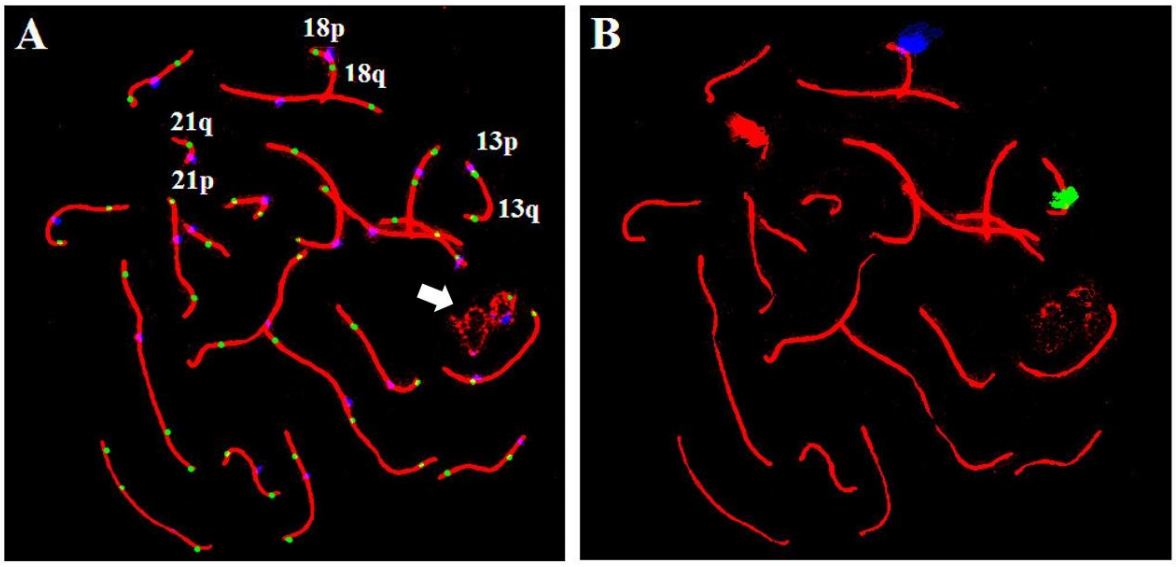

Figure 2.1 Immunofluorescence and FISH analysis of pachytene cells.

(A) Cells were immunostained using antibodies against SCP3/SCP1, MLH1, and CREST to visualize the SC (red), crossover sites (green) and centromeres (blue). A spermatocyte with 45 crossovers is shown. The sex body is indicated by the arrow, and shows one crossover. (B) Subsequent FISH was performed to identify chromosomes 13 (green, LSI 13), 18 (blue, CEP 18), and 21 (red, LSI 21) in the same cell.

Table 2.1 Analysis of recombination and synaptic errors in azoospermic and control men

	No. of cells	Mean rate (± SD) of genome-wide recombination	Proportion of cells with achiasmate sex body (%)	Proportion of cells with unsynapsed regions (%)
Control men (*n* = 14)				
C20	50	46.1 ± 4.0	6.0	6.0
C21	51	51.4 ± 4.7	7.8	2.0
C22	50	47.5 ± 4.7	12.0	4.0
C23	50	49.7 ± 4.42	6.0	6.0
C24	51	50.2 ± 3.6	7.8	9.8
C25	50	48.1 ± 4.7	14.0	0
C26	50	45.4 ± 4.8	6.0	2.0
C27	52	47.5 ± 4.6	9.6	3.8
C28	50	48.1 ± 4.3	12.0	2.0
C29	50	53.2 ± 3.8	14.0	0
C30	49	50.2 ± 5.8	14.3	0
C31	50	52.2 ± 3.2	18.0	4.0
C32	51	51.2 ± 4.2	10.0	2.0
C33	53	55.1 ± 4.1	17.0	0
Mean (95% CI)		49.7 (48.3-50.9)	11.0 (8.9-13.2)	3.0 (1.5-4.5)
Obstructive azoospermic men (*n* = 12)				
OA26	28	46.0 ± 4.7	10.7	0
OA27	32	46.8 ± 7.1	6.3	3.1
OA28	58	50.8 ± 3.5	**15.5[b]**	**10.3[c]**
OA29	40	**42.5 ± 3.1[a]**	11.8	2.5
OA30	41	47.0 ± 4.5	**22.0[b]**	4.9
OA31	39	**43.0 ± 3.2[a]**	12.8	**10.3[c]**
OA32	42	48.5 ± 4.9	11.9	2.4
OA33	45	50.5 ± 6.1	**17.8[b]**	2.2
OA34	62	**45.2 ± 3.1[a]**	9.7	**6.5[c]**
OA35	99	50.7 ± 6.2	8.6	3.0

	No. of cells	Mean rate (± SD) of genome-wide recombination	Proportion of cells with achiasmate sex body (%)	Proportion of cells with unsynapsed regions (%)
OA36	50	49.7 ± 3.2	8.0	4.0
OA37	51	50.1 ± 4.5	7.8	3.9
Mean (95% CI)		47.6 (46.0-49.1)	11.9 (9.5-14.3)	4.4 (2.8-6.1)
Non-obstructive azoospermic men (*n* = 8)				
NOA24	87	**44.4 ± 3.1[a]**	**25.3[b]**	**10.3[c]**
NOA25	35	**41.4 ± 3.7[a]**	**31.4[b]**	2.9
NOA26	100	53.8 ± 5.7	10	2.0
NOA27	86	52.1 ± 3.9	9.3	1.2
NOA28	101	46.6 ± 6.6	**16.8[b]**	4.0
NOA29	45	**40.9 ± 3.5[a]**	**17.8[b]**	**8.9[c]**
NOA 30	47	**40.5 ± 3.2[a]**	**25.5[b]**	**8.5[c]**
NOA 31	38	**42.5 ± 4.2[a]**	**18.4[b]**	2.6
Mean (95% CI)		**45.3 (42.6-48.0)[a]**	**19.3 (15.3-23.4)[b]**	5.1 (3.2-6.9)

[a]P < 0.05, recombination significantly reduced when compared with controls, Mann-Whitney Test.
[b]P < 0.01, proportion of cells with aschiamate sex body is significantly higher when compared with controls, Fisher exact test
[c]Proportion of cells with unsynapsed regions was considered significantly different from controls if it was beyond the 95% CI of the control group

2.3.2 Analysis of genome-wide recombination

The control group displayed an average of 49.7 crossovers per cell, with individual mean rates ranging from 45.4 to 55.1 (Table 2.1). The OA group showed an average of 47.6 crossovers per cell (ranging from 42.5 to 50.8), which was not significantly different from the controls. On the other hand, the NOA group showed a significantly reduced average of 45.3 crossovers per cell when compared to the controls ($P < 0.05$, Mann-Whitney test), with a range of 40.5 to 53.8. We also noted large inter-individual variations among the azoospermic, infertile men, where three OA men (OA29, OA31, OA34) and five NOA men (NOA24, NOA25, NOA29, NOA30, NOA31) independently showed reduced recombination when compared to the control group ($P <0.01$, Mann-Whitney test).

The proportion of pachytene cells displaying an achiasmate sex body (showing absence of crossovers) ranged from 6.0% to 18.0% in the control group, and 6.3% to 22.0% in the OA group (Table 2.1). The average frequency of achiasmate sex bodies was not significantly different between the OA and control groups (11.9% vs. 11.0%). Three OA men (OA28, OA30, OA33), however, individually showed an increased proportion of cells with achiasmate sex body when compared to the controls ($P <0.01$, Fisher exact test). It appears that recombination on the sex body was particularly disrupted in the NOA group, where the frequency of achiasmate sex bodies ranged from 9.3% to 31.4%. The average frequency of achiasmate sex bodies was significantly higher in the NOA group than the controls (19.3% vs. 11.0%, $P < 0.01$, Fisher exact test). In this group, six NOA men (NOA24, NOA25, NOA28, NOA29, NOA30, NOA31) independently displayed a significantly higher proportion of cells with achiasmate sex body when compared to the controls ($P <0.01$, Fisher exact test).

2.3.3 Chromosome-specific crossover frequencies and sperm aneuploidy

After noting reduced levels of genome-wide recombination in some infertile men, we attempted to analyze the crossover frequencies on specific chromosomes. By combining immunofluorescence and FISH, we were able to assess the frequency of crossovers on chromosomes 13, 18, and 21 (Figure. 2.1B and Table 2.2). A total of 667 pachytene nuclei from the control group, 471 pachytene nuclei from the OA group, and 416 pachytene nuclei from the NOA group were analyzed. In the control group, two or more crossovers were observed on chromosome 13 in 86.1% of pachytene cells, and on chromosome 18 in 78.1% of cells. Furthermore, 99.1% of pachytene cells in the control group showed one crossover on chromosome 21. We did not observe significant differences in chromosome-specific crossover frequencies between the control group and either of the infertile groups. Nevertheless, recombination frequencies were altered in four infertile men (OA34, NOA25, NOA29, NOA30) on chromosome 13, and in six infertile men (OA26, OA31, OA34, NOA25, NOA29, NOA30) on chromosome 18 ($P < 0.05$, Chi-square test). These infertile men showed reduced frequencies of two or more crossovers on chromosome 13 or 18 ($P < 0.05$, Chi-square test). Furthermore, crossover frequencies on chromosome 21 were altered in eight infertile men (OA26, OA27, OA33, OA34, NOA24, NOA25, NOA29, NOA30), in which the frequency of achiasmate bivalent 21 was increased ($P < 0.05$, Chi-square test).

Since recombination is thought to be important for the proper segregation of chromosomes during the first meiotic division, we were interested in whether reduced recombination rates in infertile men may correlate to an increased risk of sperm aneuploidy. Using FISH, we examined the rate of aneuploidy for chromosomes 13, 18, 21, and the sex chromosomes in the testicular sperm of infertile and fertile men (Table 2.3). A total of 14574

sperm nuclei from the control group, 10854 sperm nuclei from the OA group, and 5682 sperm nuclei from the NOA group were scored. The mean frequency of disomy in sperm in the control group was 0.13% for XX or YY, 0.23% for XY, 0.12% for chromosome 18, 0.17% for chromosome 13, and 0.26% for chromosome 21. The OA group did not show a significant difference in sperm disomy from the controls. However, the NOA group showed an increased mean rate of disomy XY (0.62%) and disomy 18 (0.32%) when compared to controls ($P < 0.05$, Fisher exact test). Overall, seven OA men, and five NOA men showed increases in sex chromosomal aneuploidy when compared to the controls ($P < 0.05$, Fisher exact test). Three OA men (OA28, OA29, OA31), and two NOA men (NOA24, NOA30) showed increased rates of disomy 13 ($P < 0.05$, Fisher exact test). Three OA men (OA26, OA31, OA34), and three NOA men (NOA24, NOA25, NOA30) showed increased rates of disomy 18 ($P < 0.05$, Fisher exact test). Furthermore, four OA men (OA26, OA31, OA33, OA34), and four NOA men (NOA24, NOA25, NOA29, NOA30) showed increased rates of disomy 21 ($P < 0.05$, Fisher exact test). Two NOA men (NOA 24 and NOA30) showed elevated levels of disomy for all chromosomes in the study, with particularly high rates of XY disomy (around 1.0% in both men).

When we combined all of the infertile men, we observed an inverse correlation between the frequency of recombination on the sex body and XY disomy in the sperm (Fig. 2.3; $P < 0.001$, $r=-0.79$, Pearson's test). Next, we wanted to assess the relationship between chromosome-specific recombination rates on the autosomes, and disomy in the sperm. We observed a negative correlation between the frequency of recombination on bivalent 21 and disomy 21 in the sperm (Fig. 2.4; $P < 0.001$, $r=-0.68$, Pearson's test). However, there did not

appear to be a relationship between crossover frequencies and sperm disomy rates for chromosomes 13 and 18.

Table 2.2 Analysis of crossover frequencies on chromosome 13, 18 and 21 in azoospermic and control men

		Chromosome 13			Chromosome 18			Chromosome 21		
	No. of Cells	**0 foci**	**1 focus**	**≥ 2 foci**	**0 foci**	**1 focus**	**≥ 2 foci**	**0 foci**	**1 focus**	**≥ 2 foci**
Control men (*n* = 14)										
C20	43	0% (0)	18.6% (8)	81.4% (35)	0% (0)	25.6% (11)	74.4% (32)	2.3% (1)	98.2% (42)	0% (0)
C21	49	0% (0)	12.2% (6)	87.8% (43)	0% (0)	18.4% (9)	81.6% (40)	0% (0)	100% (49)	0% (0)
C22	48	0% (0)	12.5% (7)	87.5% (49)	1.8% (1)	28.6% (16)	69.6% (39)	0% (0)	100% (56)	0% (0)
C23	50	0% (0)	14.0% (7)	86.0% (43)	2.0% (1)	14.0% (7)	84.0% (42)	2.0% (1)	98.0% (49)	0% (0)
C24	51	0% (0)	17.6% (9)	82.4% (42)	0% (0)	13.7% (7)	86.2% (44)	3.9% (2)	96.1% (49)	0% (0)
C25	45	0% (0)	11.1% (5)	88.9% (40)	0% (0)	17.8% (8)	82.2% (37)	0% (0)	100% (45)	0% (0)
C26	42	0% (0)	11.9% (5)	88.1% (37)	0% (0)	16.7% (7)	83.3% (35)	0% (0)	100% (42)	0% (0)
C27	45	0% (0)	11.1% (5)	88.9% (40)	0% (0)	24.4% (11)	75.6% (34)	0% (0)	100% (45)	0% (0)
C28	50	0% (0)	14.0% (7)	86.0% (43)	2.0% (1)	20.0% (10)	78.0% (39)	0% (0)	100% (50)	0% (0)
C29	45	0% (0)	17.8% (8)	82.2% (37)	2.2% (1)	31.1% (14)	66.7% (30)	2.2% (1)	97.8% (44)	0% (0)
C30	48	0% (0)	8.3% (4)	91.7% (44)	0% (0)	20.8% (10)	79.2% (38)	2.1% (1)	97.9% (47)	0% (0)
C31	50	0% (0)	10% (5)	90.0% (45)	0% (0)	24.0% (12)	76.0% (38)	0% (0)	100% (50)	0% (0)
C32	48	0% (0)	18.8% (9)	81.3% (39)	0% (0)	16.7% (8)	83.3% (40)	0% (0)	100% (48)	0% (0)
C33	53	0% (0)	15.1% (8)	84.9% (45)	0% (0)	22.6% (12)	77.4% (41)	0% (0)	100% (53)	0% (0)
Total	667	0% (0)	13.9% (93)	86.1% (574)	0.6% (4)	21.3% (142)	78.1% (521)	0.9% (6)	99.1% (661)	0% (0)
Obstructive azoospermic men (*n* = 12)										
OA26	20	0% (0)	10.0% (2)	90.0% (18)	**0% (0)****	**45.0% (9)****	**55.0% (11)****	**15.0% (3)****	**85.0% (17)****	**0% (0)****
OA27	29	3.4% (1)	13.8% (4)	82.8% (24)	0% (0)	24.1% (7)	75.9% (22)	**10.3% (3)****	**86.2% (25)****	**3.4% (1)****
OA28	49	0% (0)	8.2% (4)	91.8% (45)	0% (0)	18.4% (9)	81.6% (40)	0% (0)	100% (49)	0% (0)
OA29	35	0% (0)	8.6% (3)	91.4% (32)	0% (0)	28.6% (10)	71.4% (25)	0% (0)	100% (35)	0% (0)
OA30	40	0% (0)	5.0% (2)	95.0% (38)	0% (0)	15.0% (6)	85.0% (34)	2.5% (1)	97.5% (39)	0% (0)
OA31	35	0% (0)	14.3% (5)	85.7% (30)	**0% (0)****	**51.4% (18)****	**48.6% (17)****	2.9% (1)	97.1% (34)	0% (0)
OA32	40	0% (0)	25.0% (10)	75.0% (30)	0% (0)	20.0% (8)	80.0% (32)	2.5% (1)	97.5% (39)	0% (0)

	No. of Cells	Chromosome 13			Chromosome 18			Chromosome 21		
		0 foci	**1 focus**	**≥ 2 foci**	**0 foci**	**1 focus**	**≥ 2 foci**	**0 foci**	**1 focus**	**≥ 2 foci**
OA33	43	0% (0)	14.0% (6)	86.0% (37)	0% (0)	25.6% (11)	74.4% (32)	**14.0% (6)****	**86.0% (37)****	**0% (0)****
OA34	41	**0% (0)****	**31.7% (13)****	**68.3% (28)****	**0% (0)*****	**58.5% (24)*****	**40.5% (17)*****	**4.9% (2)****	**95.1% (39)****	**0% (0)****
OA35	67	0% (0)	10.4% (7)	89.6% (60)	0% (0)	11.9% (8)	88.1% (59)	1.5% (1)	98.5% (66)	0% (0)
OA36	41	0% (0)	12.2% (5)	87.8% (36)	0% (0)	26.8% (11)	73.2% (30)	0% (0)	100% (41)	0% (0)
OA37	32	0% (0)	9.4% (3)	90.6% (29)	0% (0)	21.9% (7)	79.1% (25)	0% (0)	100% (32)	0% (0)
Total	472	0.2% (1)	13.6% (64)	86.2% (407)	0% (0)	27.1% (128)	72.9% (344)	3.8% (18)	96.0% (453)	0.2% (1)
Non-obstructive azoospermic men (*n* = 8)										
NOA 24	50	0% (0)	20.0% (10)	80.0% (40)	0% (0)	18.0% (9)	82.0% (41)	**14.0% (7)****	**86.0% (43)****	**0% (0)****
NOA 25	30	**0% (0)*****	**83.3% (25)*****	**16.7% (5)*****	**0% (0)*****	**60.0% (18)*****	**40.0% (12)*****	**6.7% (2)****	**93.3% (28)****	**0% (0)****
NOA 26	88	0% (0)	8.0% (7)	92.0% (81)	0% (0)	10.2% (9)	89.8% (79)	0% (0)	100% (88)	0% (0)
NOA 27	57	0% (0)	5.3% (3)	94.7% (54)	0% (0)	19.3% (11)	80.7% (46)	0% (0)	100% (57)	0% (0)
NOA 28	95	0% (0)	10.5% (10)	89.5% (85)	0% (0)	16.8% (16)	83.2% (79)	0% (0)	100% (95)	0% (0)
NOA 29	40	**2.5% (1)*****	**82.5% (33)*****	**15.0% (6)*****	**0% (0)*****	**57.5% (23)*****	**42.5% (17)*****	**5.0% (2)****	**95.0% (38)****	**0% (0)****
NOA 30	35	**0% (0)****	**34.3% (12)****	**65.7% (23)****	**0% (0)*****	**74.3% (26)*****	**25.7% (9)*****	**8.6% (3)****	**91.4% (32)****	**0% (0)****
NOA 31	21	0% (0)	9.5% (2)	90.5% (19)	0% (0)	23.8% (5)	76.2% (16)	0% (0)	100% (21)	0% (0)
Total	416	0.2% (1)	24.5% (102)	75.2% (313)	0% (0)	28.1% (117)	71.9% (299)	3.4% (14)	96.6% (402)	0% (0)

***P < 0.001, Chi-square test with two degrees of freedom

**P < 0.01, Chi-square test with two degrees of freedom

*P < 0.05, Chi-square test with two degrees of freedom

Table 2.3 Testicular sperm aneuploidy in the azoospermic and control men

	No. of Cells	Frequency of disomy % (*n*)			No. of cells	Frequency of disomy % (*n*)	
		XX or YY	XY	18		13	21
Control men (*n* = 14)							
C20	1028	0.29% (3)	0.39% (4)	0.10% (1)	1022	0.39% (4)	0.49% (5)
C21	1010	0.30% (3)	0.20% (2)	0.10% (1)	1005	0.20% (2)	0.30% (3)
C22	1090	0.18% (2)	0.28% (3)	0.10% (1)	1235	0.16% (2)	0.43% (5)
C23	1034	0.29% (3)	0.19% (2)	0.19% (2)	1041	0.38% (4)	0.29% (3)
C24	1021	0.10% (1)	0.10% (1)	0% (0)	1001	0% (0)	0.20% (2)
C25	1023	0.29% (3)	0.20% (2)	0% (0)	1000	0.10% (1)	0.30% (3)
C26	1008	0% (0)	0.30% (3)	0.20% (2)	1009	0.20% (2)	0.20% (2)
C27	1203	0.08% (1)	0.25% (3)	0.17% (2)	1013	0.30% (3)	0.20% (2)
C28	1000	0.10% (1)	0.30% (3)	0.10% (1)	1002	0.10% (1)	0% (0)
C29	1015	0% (0)	0.10% (1)	0.30% (3)	1006	0.10% (1)	0.40% (4)
C30	1000	0% (0)	0.30% (3)	0.10% (1)	1002	0.10% (1)	0.20% (2)
C31	1113	0.10% (1)	0.18% (2)	0.18% (2)	1171	0.26% (3)	0.10% (1)
C32	1004	0.10% (1)	0.30% (3)	0% (0)	1030	0.10% (1)	0.29% (3)
C33	1025	0% (0)	0.20% (2)	0.20% (2)	1002	0% (0)	0.30% (3)
Total	14574	0.13% (19)	0.23% (34)	0.12% (18)	14539	0.17% (25)	0.26% (38)
Obstructive azoospermic men (*n* = 12)							
OA26	980	0% (0)	**0.41% (4)***	**0.41% (4)***	956	0.21% (2)	**0.52% (5)***
OA27	978	0% (0)	0.20% (2)	0% (0)	947	0% (0)	0.32% (3)
OA28	870	**0.34% (3)***	**0.57% (5)****	0.23% (2)	852	0.23% (2)	0.12% (1)
OA29	988	**0.30% (3)***	**0.40% (4)***	0.20% (2)	956	0.21% (2)	0.31% (3)
OA30	1000	0.20% (2)	**0.40% (4)***	0.20% (2)	957	0.21% (2)	0.10% (1)
OA31	800	**0.38% (3)***	0.38% (3)	**0.63% (5)****	745	**0.40% (3)***	**0.67% (5)****
OA32	789	0% (0)	0.25% (2)	0.25% (2)	821	0.12% (1)	0.12% (1)
OA33	803	0.12% (1)	**0.50% (4)***	0.12% (1)	834	0% (0)	**0.48% (4)***
OA34	856	0.23% (2)	**0.58% (5)****	**0.70% (6)****	914	**0.33% (3)***	**0.55% (5)***
OA35	900	0% (0)	0.11% (1)	0.22% (2)	899	0% (0)	0.22% (2)
OA36	950	0.11% (1)	0.21% (2)	0% (0)	923	0% (0)	0% (0)
OA37	940	0% (0)	0.21% (2)	0.11% (1)	967	0.21% (2)	0.10% (1)
Total	10854	0.14% (15)	0.35% (38)	0.25% (27)	10771	0.16% (17)	0.29% (31)

	No. of Cells	Frequency of disomy % (*n*)			No. of cells	Frequency of disomy % (*n*)	
		XX or YY	**XY**	**18**		**13**	**21**
Non-obstructive azoospermic men (*n* = 8)							
NOA24	450	**0.44% (2)****	**1.11% (5)****	**0.67% (3)****	530	**0.57% (3)****	**0.75% (4)****
NOA25	678	0% (0)	**1.33% (9)****	**0.59% (4)****	589	0.17% (1)	**0.51% (3)***
NOA26	693	0.29% (2)	0.29% (2)	0.29% (2)	789	0% (0)	0.13% (1)
NOA27	762	0% (0)	0.13% (1)	0.13% (1)	758	0% (0)	0.13% (1)
NOA28	760	0% (0)	**0.53% (4)***	0.13% (1)	745	0% (0)	0.13% (1)
NOA29	780	0.26% (2)	**0.64% (5)****	0.26% (2)	827	0.24% (2)	**0.60% (5)****
NOA 30	860	**0.34% (3)***	**1.01% (9)****	**0.47% (4)***	765	**0.52% (4)****	**0.65% (5)****
NOA 31	699	0.14% (1)	0.29% (2)	0.14% (1)	639	0.16% (1)	0.16% (1)
Total	5682	0.18% (10)	**0.62% (35)****	**0.32% (18)***	5642	0.19% (11)	0.37% (21)

**P <0.001, Fisher exact test

*P <0.05, Fisher exact test

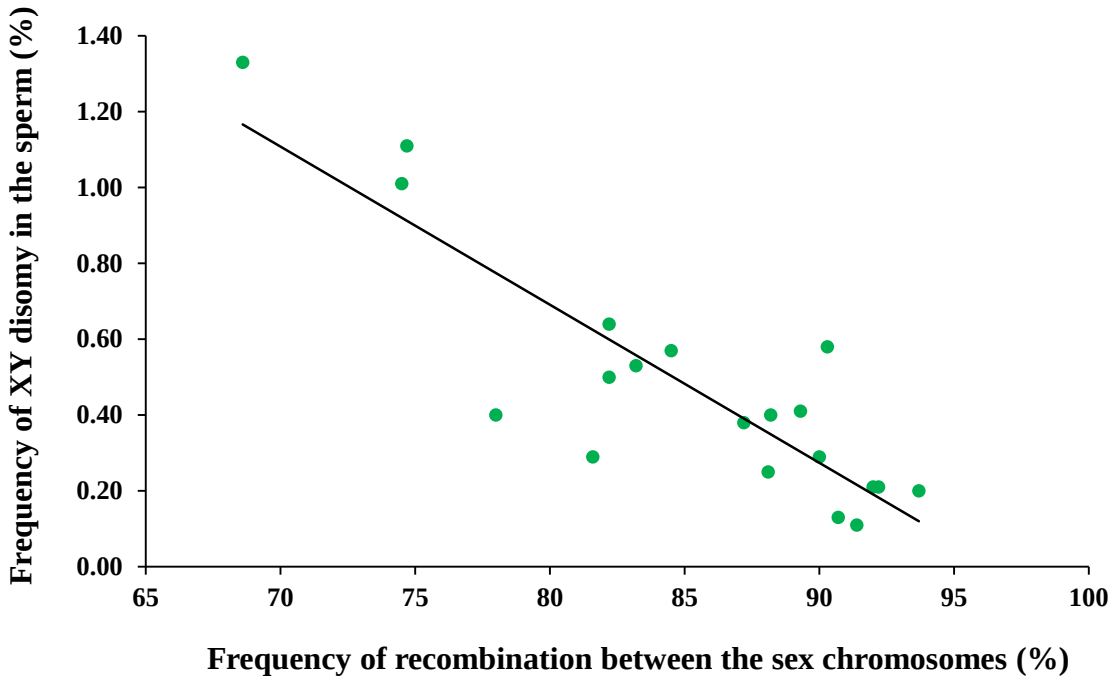

Figure 2.2 Relationship between the frequency of recombination on the sex body and XY disomy in the sperm of azoospermic men (n = 20).

Rates of XY disomy in sperm were highly variable among azoospermic men, and appears to be negatively correlated with the frequency of XY recombination in pachytene cells ($p < 0.0001$, $r=-0.79$, Pearson's test).

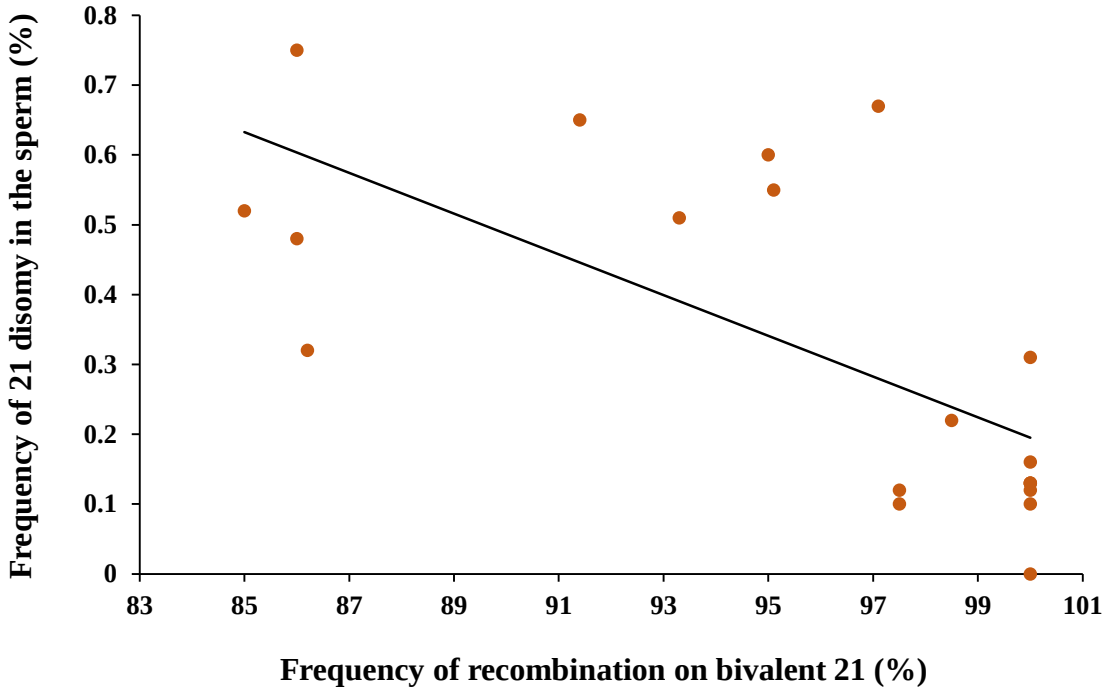

Figure 2.3 Relationship between the frequency of recombination on bivalent 21 and disomy 21 in the sperm of azoospermic men (n = 20).

Rates of disomy 21 in sperm were highly variable among azoospermic men, and appear to negatively correlate with the frequency of recombination on bivalent 21 in pachytene cells ($p < 0.0001$, $r=-0.79$, Pearson's test).

2.4 Discussion

2.4.1 Errors in recombination and synapsis in azoospermic men

Immunocytological studies performed in fertile men have established the frequency of recombination to be around 50 crossovers per cell (Lynn *et al*., 2002; Sun *et al.*, 2005). In this study, the recombination levels we observed in the fertile men were similar to those presented by others (Lynn *et al*., 2002; Sun *et al.,* 2005; Ferguson *et al.,* 2009). There have been several reports of reduced genome-wide levels of recombination in infertile men (Gonsalves *et al.,* 2004; Ma *et al.,* 2006a; Ma *et al.,* 2006b Sun *et al.,* 2007b; Ferguson *et al.,* 2007; Ferguson *et al.,* 2009). We identified eight azoospermic men who displayed reduced rates of genome-wide recombination when compared to the control group. Specifically, 62.5% of the NOA men (5/8), and 25% of the OA men (3/12) showed reduced frequencies of crossovers. Our results add further evidence that errors in recombination are a major factor in male infertility. It was surprising to find higher rates of meiotic errors in the OA cohort. It is important to note that, unlike other studies (Gonsalves *et al.,* 2004), the OA men in this study were classified based on the presence of normal spermatogenesis in the testes, despite having no sperm in the semen. However, it is unclear whether there are physical blockages in the seminal tract. Therefore, although spermatogenesis appears normal in this cohort, there may nevertheless be meiotic defects that may potentially lead to an increased rate of sperm aneuploidy.

We noted nine azoospermic men who showed an increased frequency of achiasmate sex bodies when compared to the controls. The NOA group also displayed such a significance, confirming the observations made by other studies that this group is predisposed to disruptions in recombination on the sex body (Gonsalves *et al*., 2004;

Topping *et al.*, 2006; Sun *et al.*, 2007b; Ferguson *et al.*, 2008). The higher risk of recombination errors faced by sex chromosomes compared to autosomes may be due to their differing experiences during prophase I. Kauppi *et al*., (2011) revealed that two different isoforms of Spo11 catalyze DSBs on the autosomes (Spo11β) versus the X and Y chromosomes (Spo11α). Knockout mice for Spo11α displayed unpaired X and Y chromosomes in up to 70% of pachytene cells, lack of recombination on the sex body, and infertility due to pachytene arrest of spermatocytes (Kauppi et al., 2011). Future investigation into the *Spo11* gene, and its splice variants in humans may elucidate the high level of recombination errors on the X and Y chromosomes in infertile men.

Our observations of synapsis in this study aligned with those reported by others (Sun *et al.*, 2007b; Ferguson *et al.*, 2007; Ferguson *et al.*, 2009), where the frequency of synaptic errors was increased in nine infertile men. Notably, 66.7% of the OA men (2/3) and 60% of the NOA men (3/5) with reduced recombination displayed increased rates of synaptic errors as well, suggesting that some meiotic defects affect both synapsis and recombination. Synapsis and recombination are highly dependent on each other; DSB formation is required for chromosome pairing and thus synapsis, while synapsis in turn provides a platform for recombination to occur. It is therefore interesting that three infertile men showed defects in only synapsis or recombination. Since it has been shown that crossovers occur at short stretches of regions along the chromosome called hotspots (Ségurel et al., 2011; Smagulova *et al.*, 2011), it is possible that synaptic errors that occurred outside of hotspots may noticeably affect the frequency of recombination. Furthermore, errors in recombination, but not synapsis, may be indicative of problems with late recombinant proteins that repair DSBs into crossovers. It has been shown that unpaired regions of chromosomes during pachytene

are transcriptionally silenced through the recruitment of BRCA1 and γH2AX, and may trigger meiotic checkpoints (Baarends *et al.,* 2005; Turner, 2007; Ferguson *et al.,* 2007; Checchi and Engebretch, 2011). Therefore, spermatogenic arrest due to synaptic errors may play a role in some cases of infertility.

2.4.2 Linking recombination and sperm aneuploidy in azoospermic men

Several studies have assessed the chromosome-specific patterns of recombination in normal males (Tease and Hulten, 2004; Codina-Pascual *et al.,* 2006; Sun *et al;* 2006b). However, limited studies have examined the chromosome-specific frequencies of recombination in infertile men (Ferguson *et al.,* 2007; Sun *et al.,* 2008; Ferguson *et al.,* 2009). The 2009 study by Ferguson *et al.* found altered frequencies of crossovers on chromosomes 13, 18, and, 21 in the NOA group when compared to the control group. In this study, we did not find any significance in the crossover frequencies on chromosomes 13, 18, and 21 between the infertile groups and control group. However, 41.7% of the OA men (5/12), and 50% of the NOA men (4/8) showed significantly altered crossover frequencies on at least one of the chromosomes studied when compared to the control group. We found that chromosome 13 and 18 bivalents with only one crossover was more prevalent in infertile men when compared with the control men, who mostly display two or more crossovers on these chromosomes. Notably, 88.9% of the infertile men (8/9) with altered crossover frequency on the chromosomes studied showed an increase in achiasmate bivalent 21 when compared to controls. It is likely that achiasmate bivalents are caught by meiotic checkpoints, and induce meiotic arrest in the cell (Roeder and Bailis, 2000). However, some cells may evade arrest and lead to an increased risk of meiotic nondisjunction and disomy 21 in the sperm (Savage *et al.,* 1998).

There have been two studies that showed a correlation between increased frequency of achiasmate sex bodies and increased sex chromosomal aneuploidy in the sperm (Ferguson *et al.,* 2007; Sun *et al*., 2008), however the sample size for each study was limited to seven infertile men. Our data is in agreement with the previous findings, where we found a negative correlation between the frequency of recombination between the sex chromosomes and rate of XY disomy in the sperm of twenty infertile men. With a significantly larger sample size, our findings provide valuable insight for the risk of sperm aneuploidy in infertile men. It appears that infertile men with a high frequency of achiasmate sex bodies in pachytene cells are particularly susceptible to producing sperm with sex chromosomal aneuploidies. The sex chromosomes have been shown to be the most at risk of both recombination defects (Gonsalves *et al*., 2004; Ma *et al*., 2006a; Ma *et al*., 2006b; Ferguson *et al.,* 2007) and sperm aneuploidy (Templado *et al.,* 2013). The paternal contribution to sex chromosomal aneuploidies in pregnancies is also much higher than that of autosomal aneuploidies, ranging from 50% in XXY males to 100% in XYY males (Ioannou and Tempest, 2015). Thus, there is concern that infertile men with severely defective recombination on the sex chromosomes are more susceptible to conceiving an embryo with sex chromosomal anomalies. Indeed, there have been observations that most paternally derived XYY males were conceived by sperm produced from germ cells that did not undergo recombination on the sex chromosomes (Ioannou and Tempest, 2015). An infertile man who did not display recombination on the sex chromosomes also resulted in a paternally derived 45,X abortus (Ma *et al*., 2006a).

We found no correlation between the rates of recombination and sperm disomy for chromosomes 13 and 18. Notwithstanding, to the best of our knowledge, we found the first evidence of a negative correlation between the frequency of recombination on bivalent 21

and disomy 21 in the sperm of infertile men. A decrease in recombination on bivalent 21 has been observed in oocytes that were implicated in trisomy 21 cases (Oliver *et al.,* 2014). Analysis of such a trend in paternally derived trisomy 21 has shown conflicting results due to small sample sizes (Oliver *et al.,* 2009). Further investigation into the rate of disomy 21 in the sperm of fathers who gave rise to trisomy 21 conceptions may shed light on the risks infertile men with extremely reduced rates of recombination on bivalent 21 may face during ICSI. Further studies that examine meiotic defects in infertile men may elucidate the mechanisms behind the production of aneuploid sperm, as well as aid in the risk assessment for couples undergoing fertility treatments.

CHAPTER 3: CROSSOVER DISTRIBUTION IN INFERTILE MEN

3.1 Introduction

Meiotic recombination is essential for genetic diversity, as well as the correct segregation of chromosomes during the first meiotic division. This process is strictly regulated in part by the non-uniform structure of the chromosomes entering prophase I of meiosis, where accessibility to recombination machinery is restricted to specific regions of the chromosome. The layout of the chromatin, whether it is loosely or highly condensed, therefore allows for close surveillance of where DSBs and resulting crossovers can occur. Studies have examined the crossover positions in trisomic conceptions in humans, and observed that certain distributions of crossovers may increase the susceptibility of chromosome missegregation and give rise to aneuploid gametes (reviewed in Lamb *et al*., 2005).

In Chapter 2, we demonstrated correlations between reduced recombination on bivalent 21 and the sex chromosomes, and an increase in sperm disomy in infertile men for the respective chromosomes. However, aside from changes in the frequency of crossovers, early chiasma work has also observed changes in the position of crossovers in infertile men (Hulten *et al.,* 1970; Micic *et al*., 1982; Laurie and Hulten 1985). In normal males, an inhibition of crossovers near centromeres and the vicinity of telomeres has been observed (Lynn *et al*., 2002; Tease and Hulten, 2004). Studies have also indicated a high frequency of crossovers near the subtelomeres (Sun et al., 2006a; Codin-Pascual *et al*., 2006; Ferguson *et al*., 2009). In a preliminary study, Ferguson *et al*., (2009) identified two infertile men with more frequent crossovers near the centromeres, and fewer crossovers near the subtelomeres on the chromosomes studied. Although an increased frequency of crossovers near the

centromeres has been linked to chromosome missegregation in model organisms (Rockmill *et al*., 2006; Blitzblau *et al*., 2007), the association has not been studied in humans. In this study, we aimed to further investigate whether infertile men display perturbations in crossover localization. To achieve our aims, we analyzed the distribution of crossovers on chromosomes 13, 18, and 21 in the same cohort of infertile and fertile men as those reported in Chapter 2. Furthermore, we assessed the average distance between crossovers and telomeres to determine if there is an overall shift in crossovers. By including the results on chromosome-specific crossover frequencies presented in Chapter 2, our findings may shed light on the consequences of altered crossover frequency and positions on spermatogenic arrest, as well as the production of aneuploid sperm in infertile men.

3.2 Materials and methods

3.2.1 Meiotic analyses

The same infertile and fertile men participated in this study as those reported in section 2.2.1 in Chapter 2. Testicular tissue from the infertile and fertile men were processed, and spermatocytes were immunostained using antibodies against SCP3/SCP1, MLH1, and CREST antisera according to the methods described in section 2.2.3. FISH was performed on previously immunostained slides according to the methods described in section 2.2.4 in order to study the crossover locations on chromosomes 13, 18, and 21 in the spermatocytes. The crossover distribution, represented by the distribution of MLH1 foci, and SC lengths were measured using the image analysis software Micromeasure V3.3, available at: sites.biology.colostate.edu/MicroMeasure/ (Reeves *et al.*, 2001). The SC arms of the chromosomes 13, 18, and 21 were divided into 10% intervals, with the centromeres at 0% and telomeres at 100% (Fig 3.1). The frequency of MLH1 foci in each interval was

calculated. As the number of crossovers along a chromosome significantly influences their distribution (Ferguson *et al.*, 2009), we separately analyzed the crossover distributions on chromosome 13 and 18 bivalents with single and double crossovers (Fig 3.1). The absolute distance between crossovers and telomeres was measured for each arm of chromosomes 13, 18, and 21, and then divided by the length of the SC arm in order to express the distance as a percentage.

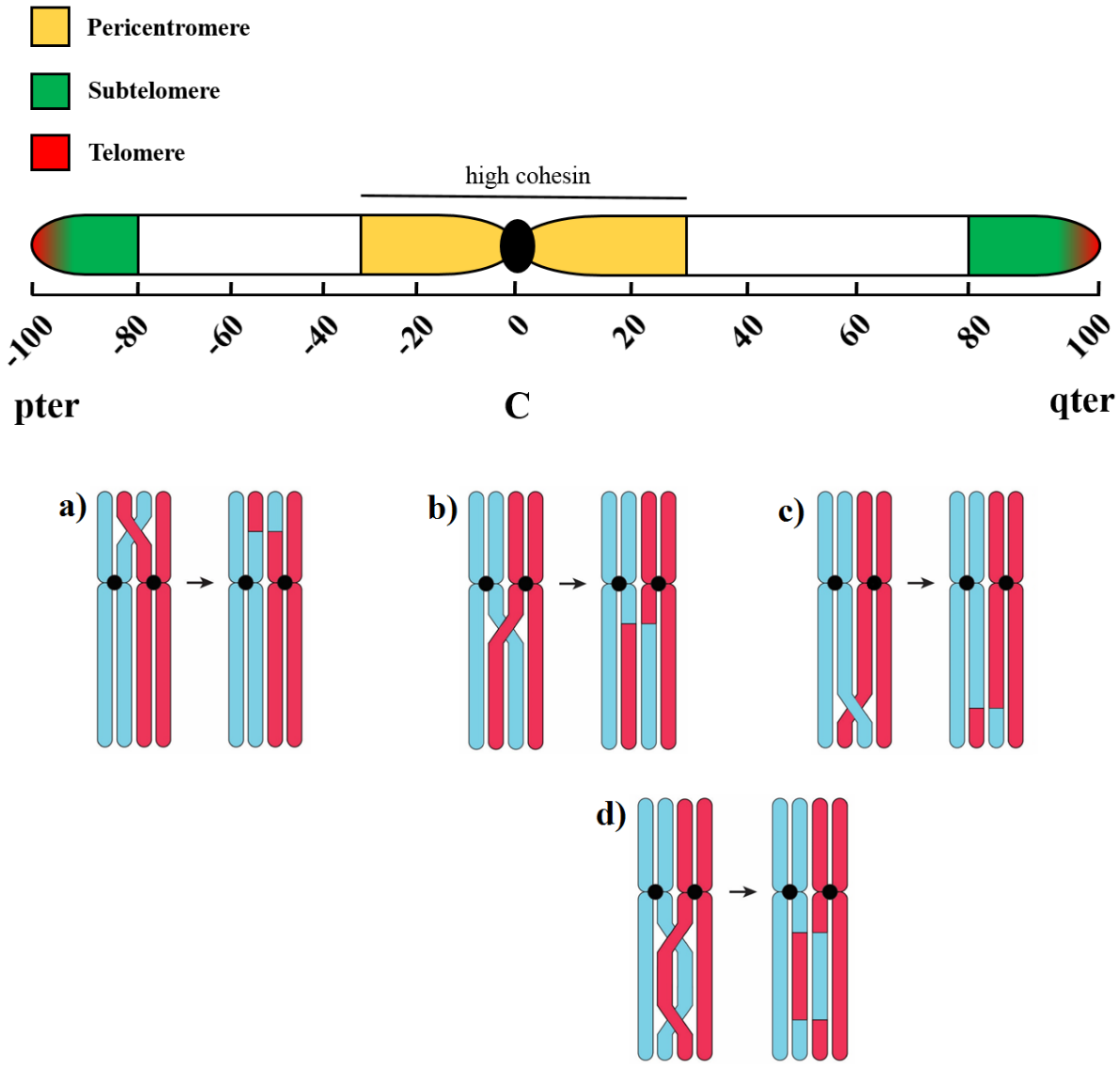

Figure 3.1 Diagram depicting meiotic crossovers in regions along a chromosome.

The p and q arms of the chromosome are divided into 10% intervals, with the centromere (C) at 0%, and telomeres at 100%. The subtelomere is shaded in the 80-100% intervals. The pericentromere, the region surrounding the centromere is shaded in the 10-30% intervals. This region attracts high levels of cohesin which are protein complexes that hold sister chromatids together until they separate during meiosis. Crossover formation in each region of the chromosome, and the resulting recombinant chromosomes are illustrated: a) a single crossover near the telomere on the p-arm; b) a single crossover near the centromere on the q-arm; c) a single crossover near the telomere on the q-arm; d) double crossovers on the q-arm.

3.2.2 Statistical analyses

We used a Chi-square test to compare the crossover distribution in the fourteen control men, and found no significant differences for any of the chromosome arms studied. Thus, the control men were pooled, and a Chi-square test with nine degrees of freedom was used to compare the crossover distribution on specific chromosome arms between individual infertile men and the control group. The Fisher test was used to compare the crossover frequencies in each 10% interval between individual infertile men and the control group. $P < 0.05$ was considered significant. The Mann-Whitney test was used to compare the average crossover distance to telomeres between the NOA/OA group and the control group. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Frequency of crossovers, and sperm aneuploidy in azoospermic men

Frequency of chromosome-specific crossovers in the infertile (n=20) and fertile men (n=14) in this study were reported in section 2.3.3 of Chapter 2. Crossover frequencies were altered in four infertile men (OA34, NOA25, NOA29, NOA30) on chromosome 13, and in six infertile men (OA26, OA31, OA34, NOA25, NOA29, NOA30) on chromosome 18 (Table 2.2). Moreover, eight infertile men (OA26, OA27, OA33, OA34, NOA24, NOA25, NOA29, NOA30) showed elevated incidences of achiasmate bivalent 21 (Table 2.2).

3.3.2 Distribution of crossovers in fertile men

We analyzed the crossover distribution on chromosomes 13, 18, and 21 in a total of 667 pachytene nuclei from the control group. We observed that single crossovers on the long arm of chromosome 21 (21q) were most frequently located near subtelomeres, at relative

distances to the centromere of 70-90% (denoted as intervals) as shown in Figure 3.2A. Similarly, double crossovers on chromosome 18 were most commonly found near subtelomeres, where crossover frequency was highest at the 60-80% intervals on 18p, and 80-90% intervals on the long arm of chromosome 18 (18q) (Fig 3.2C). Furthermore, crossover frequencies were low near the centromere on both arms of chromosome 18, as well as near the vicinity of the telomere on 18p. In contrast, the pattern of crossover distribution was different on 13q, where crossovers were frequently located near the centromere and telomeres (Fig 3.2B). In fact, crossover frequency was lowest around the middle of 13q, at the 50-70% intervals (Fig 3.2B).

3.3.3 Distribution of crossovers in OA men

We examined the crossover distributions in a total of 472 pachytene nuclei from the OA group. Five OA men displayed altered crossover distributions on at least one of the chromosome arms studied when compared to the control group ($P < 0.05$, Chi-square test). OA31 displayed altered crossover distributions on 21q, where there were fewer crossovers near the subtelomere (Fig 3.2A). Out of all the infertile men, only OA35 displayed an altered crossover distribution on 13q containing double crossovers ($P < 0.05$, χ^2 test), showing more frequent crossovers near the telomere at the 90% interval (Fig 3.2B).

We identified three OA men who exhibited changes in crossover distributions on either arms of chromosome 18 containing double crossovers when compared to the controls ($P < 0.05$, Chi-square test). OA26 displayed more frequent crossovers near the centromere, and fewer crossovers near the subtelomere (Fig 3.3A). OA33 showed fewer crossovers near the subtelomere of 18q at the 70% interval (Fig 3.3B). Finally, OA34 showed a much different change in crossover distribution compared to the rest of the infertile men, where

there were fewer crossovers at the subtelomere, but more frequent crossovers at the vicinity of the telomeres at the 100% interval (Fig. 3.3C).

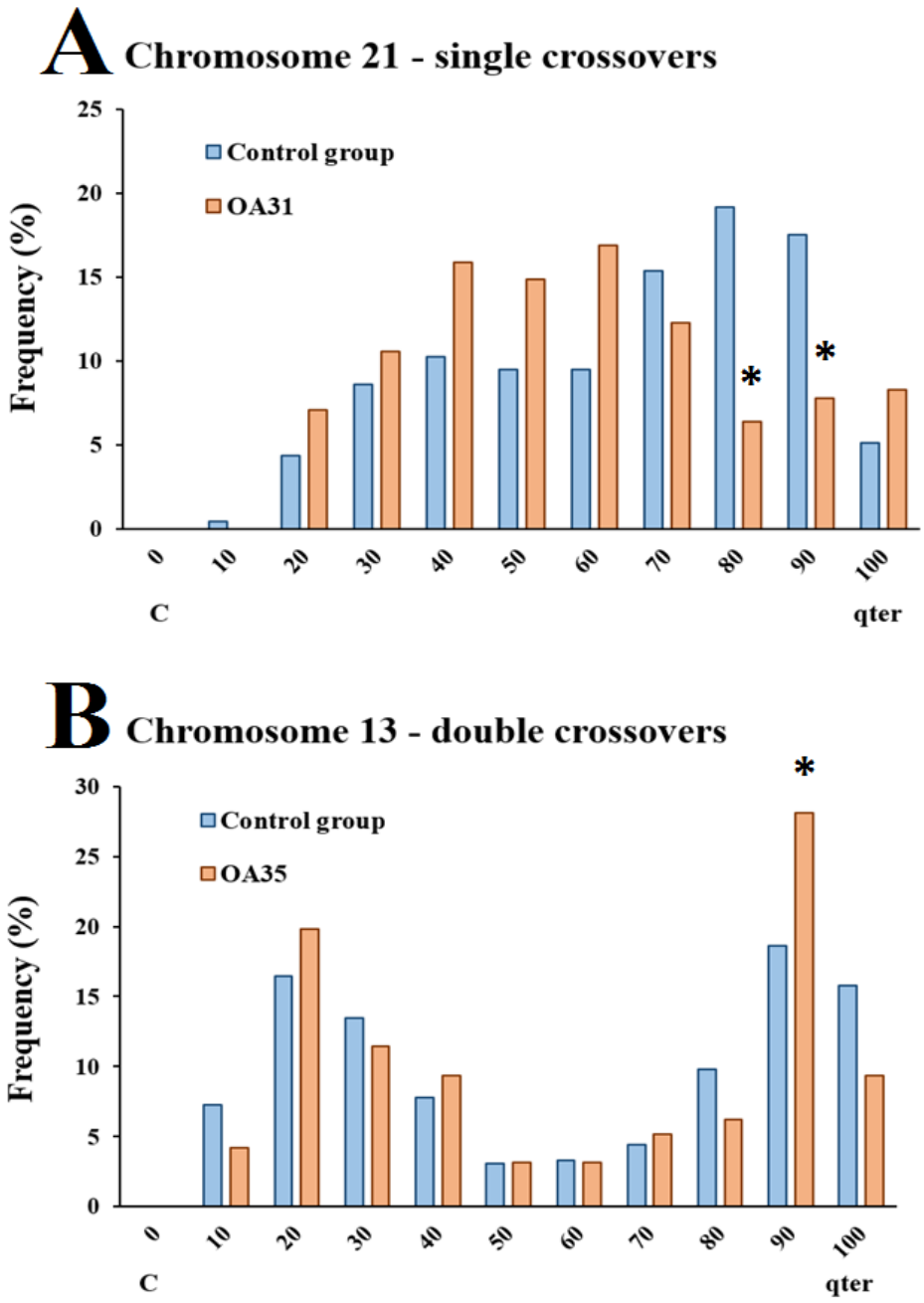

Figure 3.2 Chromosomes 21, and 13 displaying altered crossover distributions in OA men.

The Y-axis represents the frequency of crossovers in each interval. The X-axis represents the relative crossover position from the centromere with the values representing the upper limit of each interval. As crossovers in the p-arm of chromosomes 13 and 21 are extremely rare, the p-arms are not shown. The crossover frequencies in each interval were compared to the control group, and significant differences are indicated by asterisks ($P < 0.05$, Fisher test).

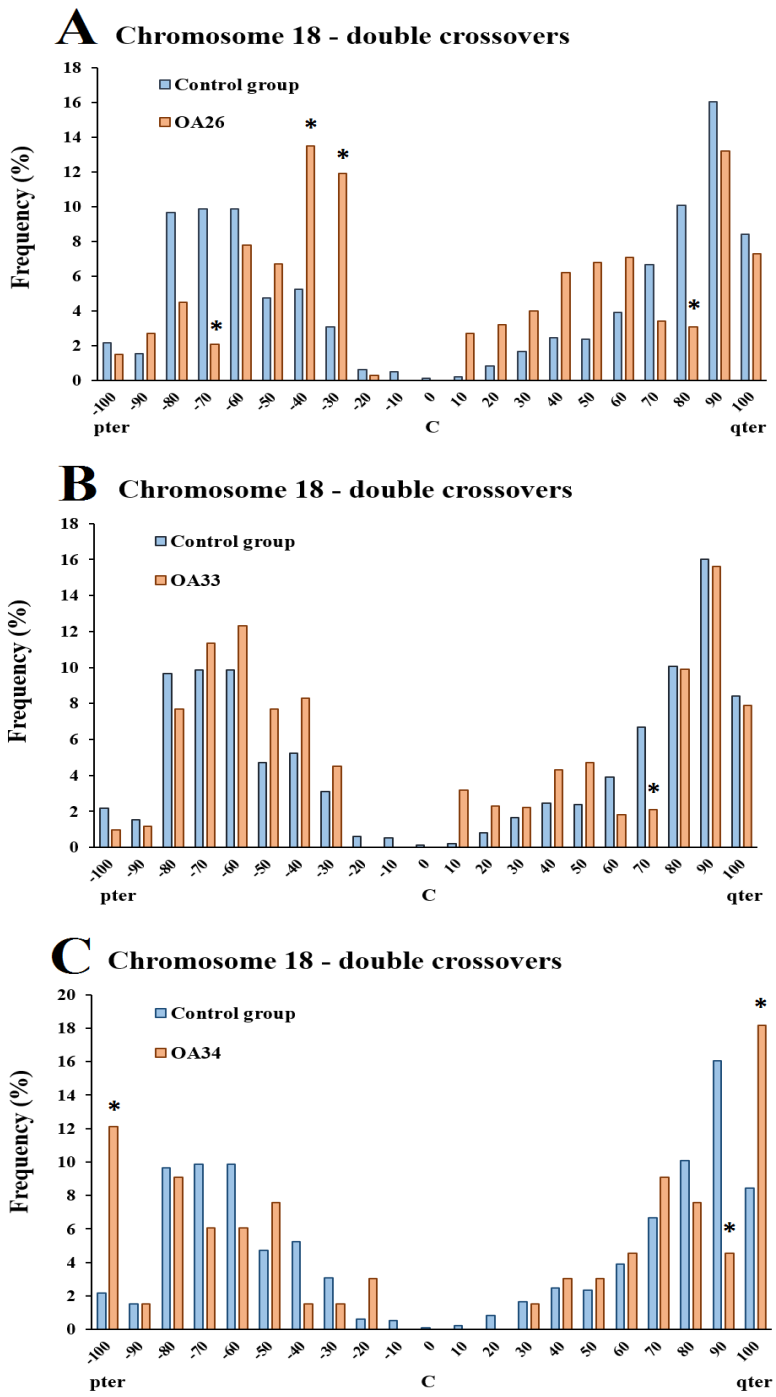

Figure 3.3 Chromosome 18 displaying altered crossover distributions in OA men.

The Y-axis represents the frequency of crossovers in each interval. The X-axis represents the relative crossover position from the centromere with the values representing the upper limit of each interval. The crossover frequencies in each interval were compared to the control group, and significant differences are indicated by asterisks ($P < 0.05$, Fisher test).

3.3.4 Distribution of crossovers in NOA men

A total of 416 pachytene nuclei from the NOA group were analyzed for the distribution of crossovers. We identified four NOA men who displayed altered crossover distributions on at least one of the chromosome arms studied when compared to the control group ($P < 0.05$, Chi-square test). Two of these cases had altered crossover distributions on 21q. NOA24 showed an increase in crossover frequency in the middle of 21q, and a decrease in crossover frequency at the subtelomere (Fig 3.4A). Similarly, NOA30 showed increased crossover formation near the centromere, and decreased crossover formation at the subtelomere (Fig 3.4B). None of the NOA men showed an altered crossover distribution on chromosome 13 when compared to controls.

We observed altered crossover distributions on chromosome 18 with double crossovers in two NOA men when compared to the controls ($P < 0.05$, Chi-square test). NOA25 showed an increase in crossover formation near the centromere and subtelomere (Fig 3.5A), while NOA29 exhibited a decrease in crossover formation at the subtelomere (Fig 3.5B).

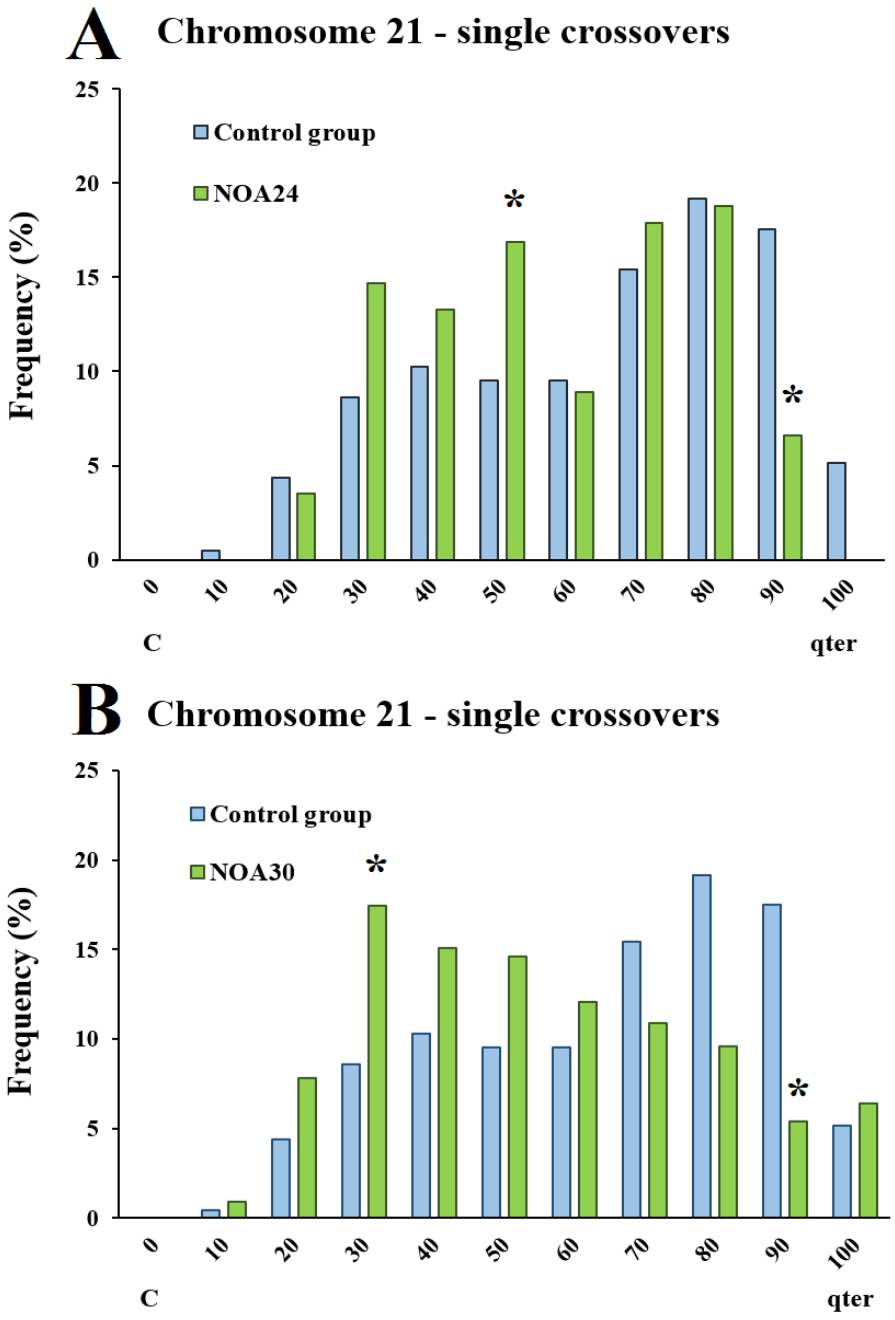

Figure 3.4 Chromosome 21 displaying altered crossover distributions in NOA men.

The Y-axis represents the frequency of crossovers in each interval. The X-axis represents the relative crossover position from the centromere with the values representing the upper limit of each interval. As crossovers in the p-arm of chromosomes 21 are extremely rare, the p-arms are not shown. The crossover frequencies in each interval were compared to the control group, and significant differences are indicated by asterisks ($P < 0.05$, Fisher test).

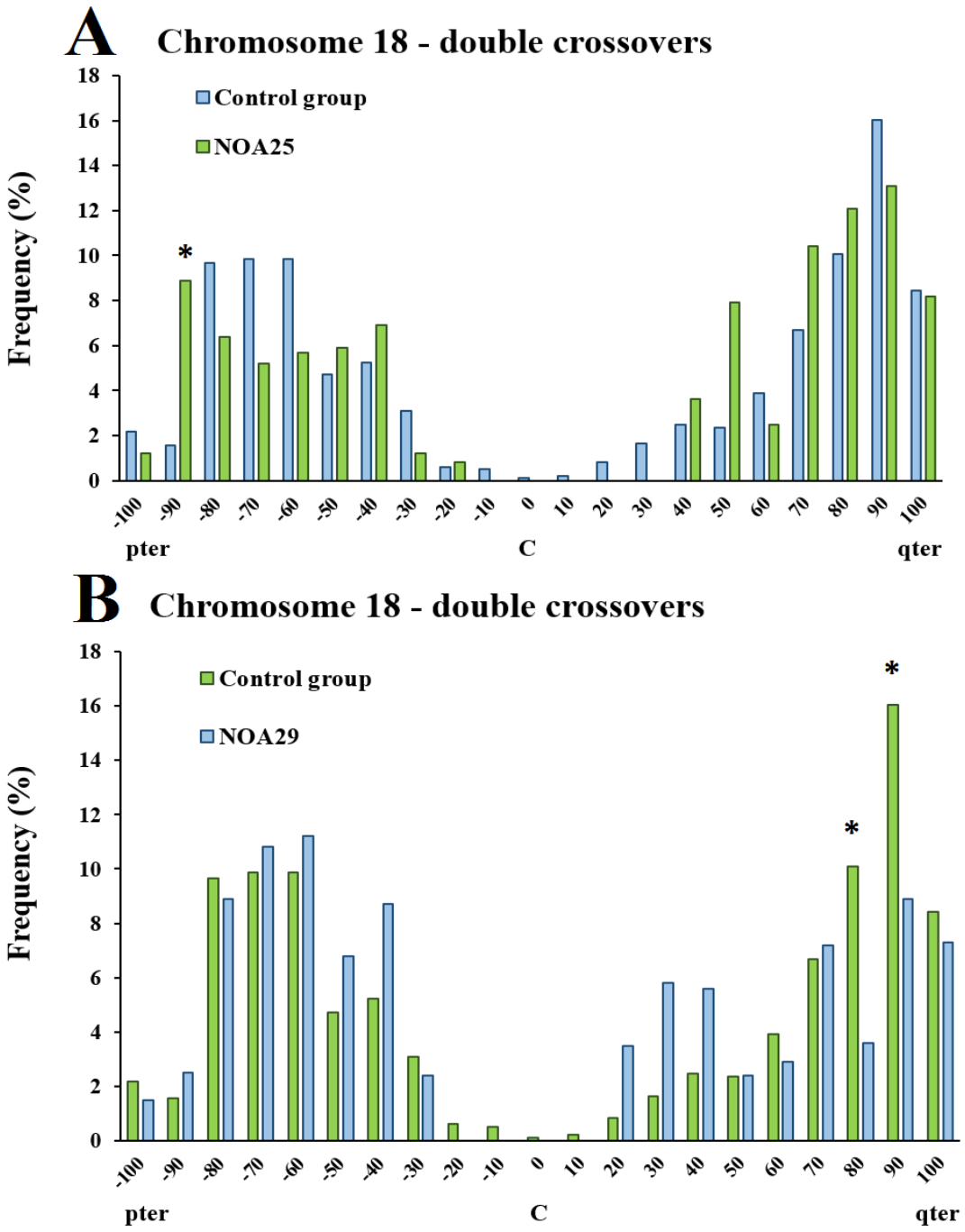

Figure 3.5 Chromosome 18 displaying altered crossover distributions in NOA men.

The Y-axis represents the frequency of crossovers in each interval. The X-axis represents the relative crossover position from the centromere with the values representing the upper limit of each interval. The crossover frequencies in each interval were compared to the control group, and significant differences are indicated by asterisks ($P < 0.05$, Fisher test).

3.3.5 Crossover distance to telomere

We pooled the NOA, OA, and control men into the respective groups, and measured the average crossover distance to the telomere as a percentage of the total SC length. On 21q, the NOA group displayed an increased average distance between crossover and telomere compared to controls (50.8%$\pm$4.8 vs. 37.8%$\pm$4.2, $P < 0.05$, Mann-Whitney test, Fig 3.6A). The OA group did not show any significance in this aspect compared to the control group (Fig 3.6A). Regarding chromosome 18, neither the NOA nor OA group showed a significant difference in mean crossover distance to the telomere on 18p when compared to controls (Fig 3.6B). Nevertheless, the OA group showed an increased average crossover distance to the telomere compared to controls on 18q (58.6%$\pm$7.5 vs. 39.5%$\pm$5.7, $P < 0.05$, Mann-Whitney test, Fig 3.6B). However, the NOA group did not show such significance compared to controls (Fig 5B). Neither the NOA nor OA group showed significantly different average distances between crossover and telomere on 13q compared to controls ($P > 0.05$, Mann-Whitney test).

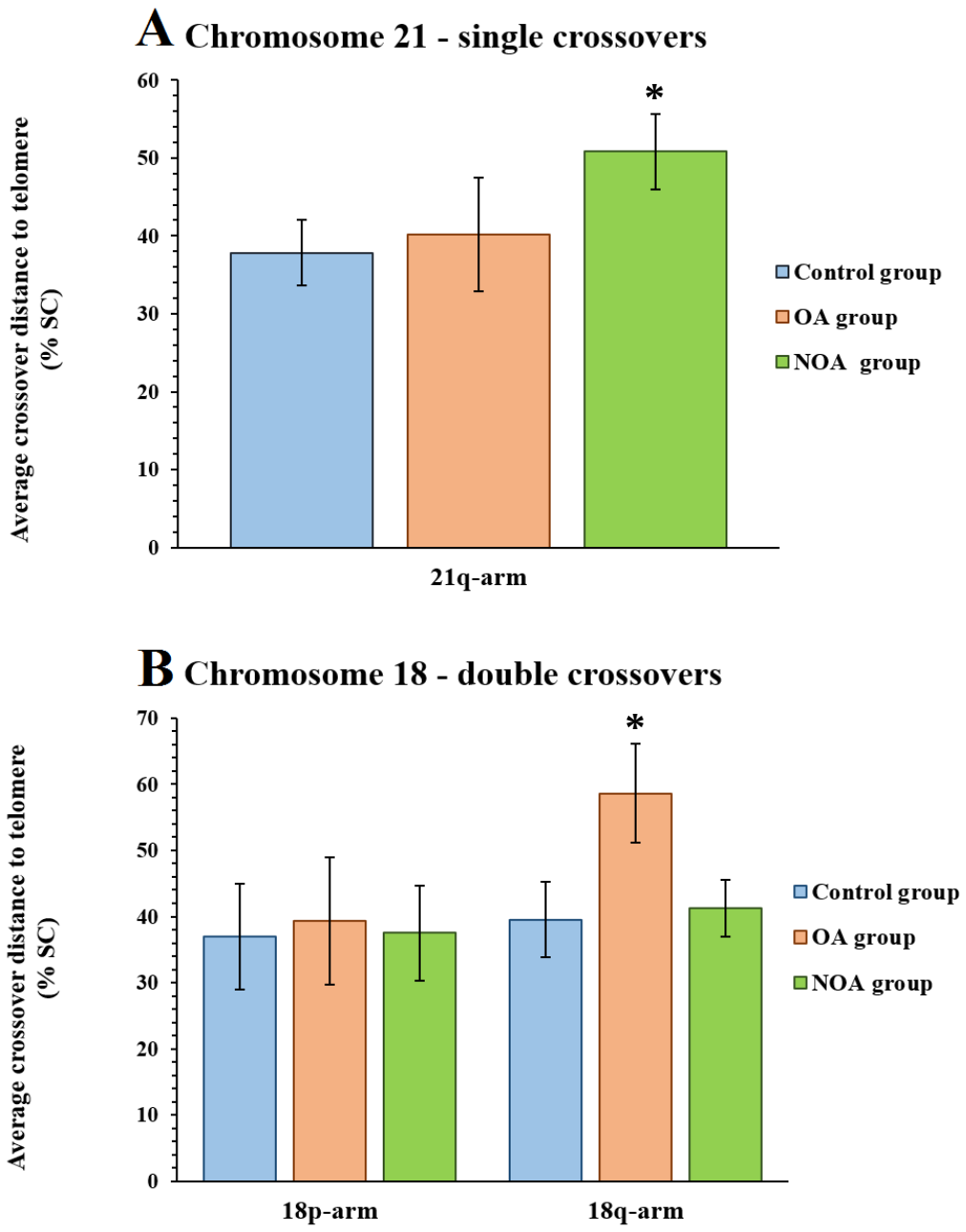

Figure 3.6 Average crossover distance to telomere (±SD) on chromosomes 18 and 21 in OA, NOA and control men.

The absolute distance between crossover and telomere was divided by the total SC arm length in order to express the distance as a percentage. The Y-axis represents the mean distance between crossovers to the telomeres on the chromosome arms 18q-arm, 18p-arm and 21q-arm. The average distances between crossovers to telomeres were compared to the control group and significant differences are indicated by asterisks ($P < 0.05$, Mann-Whitney test).

3.4 Discussion

3.4.1 Increase in crossovers near centromere and telomeres in azoospermic men

It has been shown that the distal regions of the chromosomes in normal men are largely composed of loosely condensed euchromatin, and much more susceptible to recombination events than the highly condensed regions of heterochromatin near the centromeres (Mézard *et al*., 2015). Analysis of meiotic chromosomes in *S. cerevisiae* revealed that crossover formation near the centromeres are generally inhibited, which is thought to be important for chromosome segregation. Namely, an abnormally high prevalence of crossovers near the centromeres may disturb the attachment of cohesins in this region, as shown in *S. cerevisiae,* where crossover formation near the centromeres have been linked to the premature segregation of the sister chromatids (Blitzblau *et al*., 2007; Rockmill *et al.,* 2006). In our study, the distribution of crossovers was altered in five OA and four NOA men when compared to the control group. Out of these nine infertile men, four men (44.4%) displayed an increase in crossover formation near centromeres. Although this shift of crossovers toward the centromere was previously observed in three infertile men (Laurie and Hulten, 1985; Ferguson *et al.,* 2009), this is the first report in a group of NOA and OA men. Furthermore, our observation of a shift in crossovers toward the centromere on chromosome 21 in two NOA men (NOA24, NOA30) may be of particular clinical significance. Human oocytes with increased frequencies of crossovers near the centromere on 21q have been implicated in maternally-derived trisomy 21 (Down syndrome) (Oliver *et al*., 2014). Our observation in spermatocytes may follow a similar pattern, where sperm from some infertile men may introduce an elevated risk of paternally-derived trisomy 21. Although altered crossover distribution as a risk factor for paternally derived trisomy 21 has

not been extensively studied, Oliver *et al*. found weak evidence that an increase in crossovers near the centromere may play a role in paternal chromosome 21 nondisjunction.

Crossovers are also suppressed near the vicinity of the telomeres, possibly to prevent damage to the repetitive DNA (Blitzblau *et al.,* 2007). In our study, one of the nine infertile men (11.1%) with altered crossover distributions demonstrated more frequent crossovers at the telomeres, with extremely high rates of crossover formation near the telomere on both 18q and 18p. In *S. cerevisiae*, crossovers in this region may migrate to the ends of the chromosomes and disrupt the microtubule tension, leading to premature sister chromatid separation (Ross *et al*., 1996; Su *et al*., 2000). Potentially, an increase in crossovers near the centromeric or telomeric regions may disrupt the segregation of chromosomes and play a role in the production of aneuploid sperm in infertile men. Yet, this mechanism may be chromosome-specific as we only observed one infertile man (OA35) with an altered crossover distribution on chromosome 13. It is important to note that the control group displayed a different pattern of crossover distribution on chromosome 13 compared to 18 and 21, where crossovers were most frequent at the centromere and telomere. It will be worth investigating whether chromosomes with a strong inhibition of crossovers at the centromere and telomeres are more prone to disruptions in crossover distribution.

3.4.2 Increased crossover distance to telomeres in azoospermic men

It has been shown in *S. cerevisiae*, mice, and humans that most crossovers occur near the subtelomeres (Peoples-Holst and Burgess, 2005; Blitzblau *et al.,* 2007). The sufficient formation of crossovers in this region is thought to be facilitated by telomeric activity (Peoples-Holst and Burgess, 2005; Blitzblau *et al.,* 2007). Our study demonstrated a decrease in crossover formation near the subtelomeres in three of the nine infertile men (33.3%) with

altered crossover distributions. Moreover, the NOA and OA groups displayed increased crossover distances to the telomeres on 21q and 18q respectively. These results may suggest a link between altered crossover distribution and compromised telomeres in infertile men, where this population shows reduced telomere length and impaired telomere integrity (Thilagavathi *et al*., 2013; Reig-Viader *et al.*, 2014a). The copy number of telomeric or subtelomeric repeats could lead to changes in crossover positions (Barton *et al*., 2003). Moreover, defects in telomere-associated proteins that function in synapsis and DSB repair could reduce recombination rates (Watanabe *et al*., 1998; Joseph and Lustig, 2007; Wu *et al.*, 2006). This trend has been shown in *S. cerevisiae,* where the deletion of a telomere-associated protein, Tam1/Ndj1, altered the distribution, but not frequency of crossovers (Chua *et al*., 1997). Tam1/Ndj1 mutants also displayed an increase in MI and MII nondisjunction, demonstrating the possible effect of impaired telomeres on aneuploidy (Chua *et al*., 1997).

3.4.3 Altered crossover frequency and distribution in azoospermic men

Our results confirmed the limited reports that both NOA and OA men may have meiotic defects that affect the position of crossover, frequency of crossovers, or both (Laurie and Hulten, 1985; Ferguson *et al*, 2009). However, the degree of meiotic defects in these two types of azoospermic men may be different due to distinct etiologies. NOA cases display impaired spermatogenesis in the testes, where a high degree of maturation arrest leads to the absence of sperm in the ejaculate. Although OA cases also lack sperm in the ejaculate, they exhibit normal spermatogenesis. From our data, two of the five OA men with altered crossover distributions, whereas all four of the NOA men with altered crossover distributions showed disrupted crossover frequencies on the same chromosome. It seems that OA men

may possess meiotic defects despite normal spermatogenesis. Possibly, prolonged obstruction of the reproductive tract may alter the microenvironment of the testes, and consequently affect the epigenetic regulation of DSB formation. Our current knowledge regarding the epigenetic dynamics in OA men is scarce, where only a few studies have shown abnormal epigenetic status, in the form of altered DNA methylation, in the testicular tissue of OA men (Minor *et al.,* 2011; Ferfouri *et al.,* 2013).

It appears that NOA men may be at a higher risk of having both alterations in the frequency and position of crossovers. Our findings coincide with previous data that suggested higher incidences of aneuploid sperm in NOA men compared to OA men (Kirkpatrick *et al*., 2008; Ferguson *et al*., 2009). We speculate that altered crossover frequency and distribution may have negative synergistic effects on chromosome segregation, giving rise to sperm with abnormal chromosome count. Meiotic defects in NOA cases may be due to genetic factors associated with impaired spermatogenesis. Mouse studies have shown that mutations in meiosis-specific genes such as *SCP3* and *MRE11,* which function in DNA repair, may alter crossover distributions (Cherry *et al*., 2007). Variations in centromeric heterochromatin can also inhibit or promote crossovers, possibly resulting in the shift in crossovers toward the centromere (Yamamoto *et al*., 1979). Studies on azoospermic men have also identified mutations in *TEX11* and *TEX15*, which are genes shown to regulate recombination in mice (Yang *et al.,* 2008; Ruan *et al.,* 2012; Yang *et al.,* 2015).

In summary, our data is in agreement with our previous findings, indicating that infertile men may possess meiotic defects that affect the frequency and distribution of crossovers. We confirmed several aberrant trends of crossover distribution in infertile men that may facilitate errors in chromosome segregation. Moving forward, we plan to investigate

the relationship between crossover distribution and sperm aneuploidy in hopes of elucidating whether spermatocytes with abnormal crossover localization are arrested at meiotic checkpoints, or progress through meiosis, potentially giving rise to aneuploid sperm. Our study also provides valuable insight for future directions on the role of telomeres in crossover distribution, particularly the establishment of crossovers in subtelomeres. The work from this chapter has built the foundation for our next study presented in the following chapter, which looks at the role of telomeric proteins in relation to meiotic recombination in infertile men.

CHAPTER 4: TELOMERE HOMEOSTASIS, AND RECOMBINATION IN INFERTILE MEN

4.1 Introduction

In recent years, telomeres have become an emerging topic of research with regards to its importance for meiosis and fertility. Telomeres are DNA repeat structures that cap the ends of mammalian chromosomes. Along with its association with numerous structural proteins, telomeres are primarily known to protect the stability of chromosomes. However, it appears that telomeres may also play crucial roles during early meiosis, where they are shown to aid in the processes of chromosome synapsis and meiotic recombination. In detail, telomeres attach to the inner nuclear membrane and migrate towards the cell pole at the onset of the zygotene stage of prophase I (Scherthan *et al*., 2007). This event brings the chromosomes into close proximity, thus facilitating homology search, pairing, and synapsis between homologous chromosomes (Scherthan *et al*., 2007). Recently, studies have focused on how defects in telomere length and integrity may be contributing to infertility. In mice and humans, shortened or damaged telomeres have been linked to meiotic arrest, risks of DNA fragmentation in sperm, as well as increased levels of aneuploidy in oocytes (Keefe *et al*., 2006; Thilagavathi *et al*., 2013).

There are many proteins that are responsible for maintaining the integrity of the telomeres, one of which being the protein complex shelterin (TRF2), which forms part of the telomeric structure (O'Sullivan *et al*., 2010). Another telomere-associated protein, telomerase (TERT), is better known for its role in maintaining telomere length. Aside from elongating the telomere, telomerase can also act as a structural component when bound to the telomeric complex, possibly acting to stabilize its structure (Reig-Viader *et al*., 2013). It is thought that

a careful balance of telomere-bound and free-form TERT is necessary for maintaining the structure, and function of telomeres. A recent report by Reig-Viader *et al*., (2014a) described a disruption in telomere homeostasis, in part due to decreased localization of TERT to telomeres, in the spermatocytes of infertile men. Although the study also showed reduced rates of genome-wide recombination in the infertile men, a relationship between telomere homeostasis and recombination was not analyzed. Furthermore, the study was limited by a small sample size with four men with idiopathic infertility, and one fertile man undergoing vasectomy reversal. As large variability in recombination and other meiotic defects between infertile men have been described in literature (Ma *et al*., 2006a; Ferguson *et al*., 2007; Ferguson *et al*., 2009; Ren *et al.*, 2016), further investigation into telomere homeostasis and its potential effect on recombination is warranted in a larger sample size. Studies in yeast and mice have revealed a relationship between reduced rates of recombination, and defective telomeres (Joseph and Lustig, 2007; Liu *et al*., 2004). From these findings, we postulate that compromised telomeric structure may be contributing to the impaired recombination often seen in infertile men. In this preliminary study, we aim to analyze the linkage between telomere homeostasis, based on the association of TERT to telomeres, and meiotic recombination in infertile men.

4.2 Materials and methods

4.2.1 Fluorescence immunostaining

Spermatocytes from four fertile men (C20, C21, C22, C23) and two OA men (OA29, OA31) reported in section 2.2.1 in Chapter 2 were analyzed for this study. Due to limitations of technique, we currently could not successfully analyze spermatocytes in NOA men for telomere homeostasis. Further optimization of the fluorescence immunostaining protocol is

required in order to analyze the limited number of spermatocytes that is present in NOA testicular samples. Testicular tissue from the infertile and fertile men were processed according to the methods described in section 2.2.2. For the analysis of genome-wide recombination, a slide containing spermatocytes from each man was immunostained using antibodies against SCP3/SCP1, MLH1, and CREST antisera according to the protocol described in section 2.2.3. For the assessment of telomere homeostasis, another slide from each man was incubated with a primary antibody cocktail [mouse anti-TRF2 (Millipore, Ontario, Canada), 1:24; rabbit anti-SCP3/1 (Abcam), 1:20; rabbit anti-TERT (Rockland, Limerick, PA, USA), 1:12] overnight in a 37°C humid chamber. The telomeric regions of the chromosome, and localization of telomerase were visualized by using antibodies against TRF2 and TERT, respectively. The slide was then incubated in a secondary antibody cocktail [FITC labeled donkey anti-mouse IgG (Jackson ImmunoResearch) 1:12; TRITC labeled donkey anti-rabbit IgG (Jackson ImmunoResearch) 1:15] for 90 mins in a 37°C humid chamber, and subsequently washed according to steps detailed in section 2.2.3. Using a Zeiss Axioplan epifluorescent microscope equipped with the appropriate filters, the localization of TRF2 and TERT were detected in the pachytene nuclei. Pachytene nuclei were identified by the state of the SC, where 22 strands of SC (representing pairs of autosomes) were present. Only cells with intact SC were assessed, as non-fragmented SC reveals the completion of synapsis at the pachytene stage. The percentage of telomere-bound TERT was noted by the number of TERT signals that were associated with TRF2 signals divided by the total number of TERT signals in a given spermatocyte. Approximately 50 spermatocytes were evaluated per individual to yield an average percentage of telomere-bound TERT in the individual.

4.2.2 Statistical analyses

We compared the average percentage of telomere-bound TERT in the individual OA men with the pooled controls using the Mann-Whitney test. The frequency of genome-wide recombination in the OA and control men were correlated with the average percentage of telomere-bound TERT using Pearson's correlation test. $P < 0.05$ was considered significant.

4.3 Results

4.3.1 Analysis of genome-wide recombination

The rates of genome-wide recombination in the OA men and fertile men were reported in Table 2.1. The four fertile men in this study displayed genome-wide recombination levels ranging from 46.1 to 51.4 (Table 2.1). Both OA men showed reduced rates of recombination when compared to controls, with OA29 displaying an average of 42.5 crossovers per cell, and OA31 showing an average of 43.0 crossovers per cell ($P < 0.05$, Mann-Whitney test).

4.3.2 Analysis of telomere homeostasis

Using antibodies against the telomere-associated protein complex shelterin (TRF2), telomerase (TERT), and SC (SCP3/1), we were able to visualize the telomeres, denoted by TRF2 signals, as well as the distribution of TERT in the pachytene nuclei of infertile and fertile men. We calculated the percentage of TRF2-bound TERT (TRF2-TERT) in a total of 213 pachytene nuclei in the control group, and 101 pachytene nuclei in the OA group (Fig. 4.1, Table 4.1). The control group displayed an average percentage of 48.2% for TRF2-TERT association, ranging from 42.7% to 56.2%. The OA group displayed a significantly lower average TRF2-TERT level of 29.4% when compared to the controls ($P < 0.05$, Mann-

Whitney Test). With regards to individual infertile men, both OA men showed reduced percentage of TRF2-TERT when compared to controls ($P < 0.05$, Mann-Whitney Test).

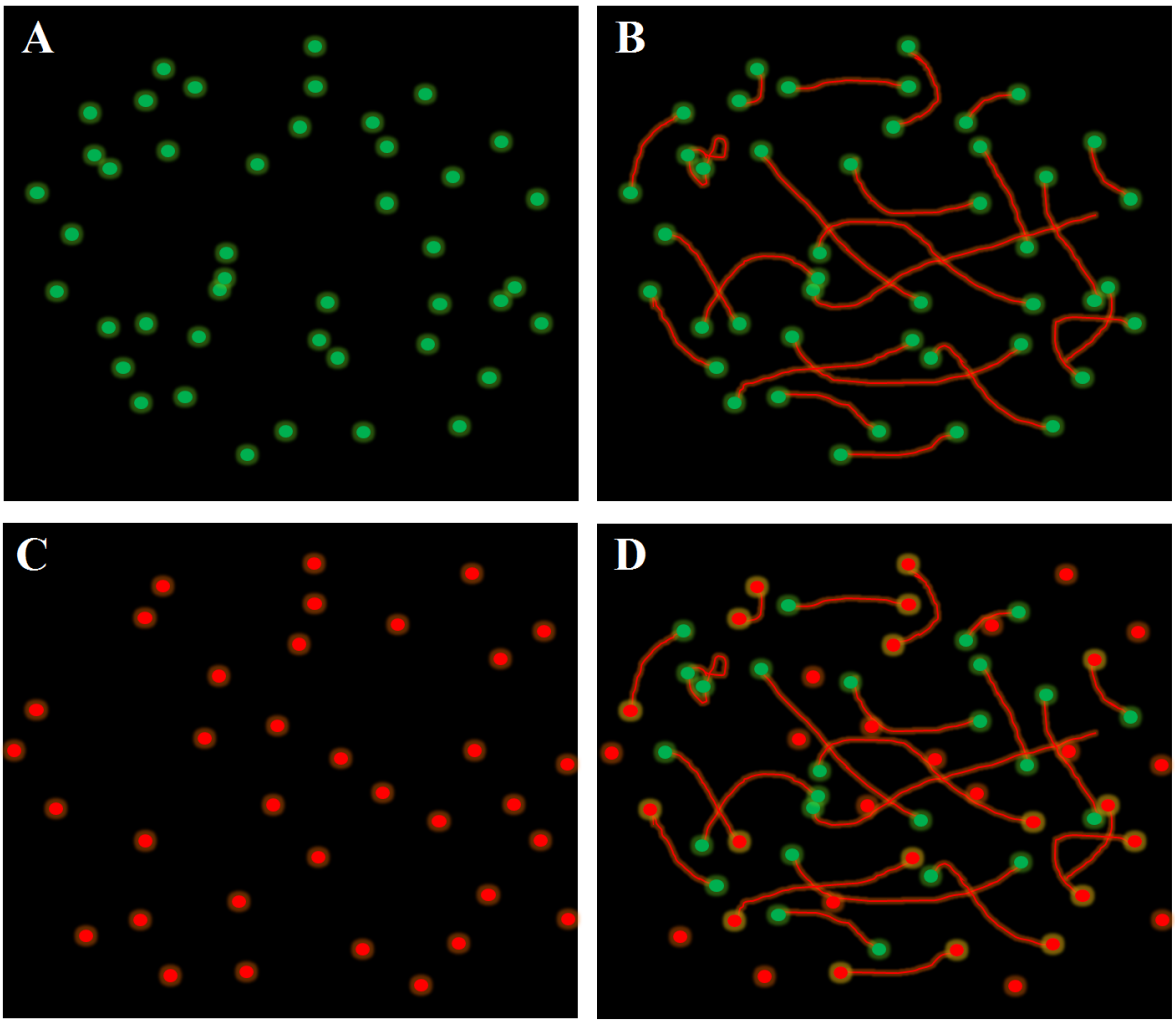

Figure 4.1 Cartoon depiction of telomerase (TERT) association with telomeres in spermatocytes.

Antibodies against the telomeric region of chromosomes (TRF2), telomerase (TERT) and synaptonemal complex (SCP3/1) were used to immunostain spermatocytes. **(A)** TRF2 signals in a given spermatocyte. **(B)** Overlay of TRF2 and SCP1/3 signals to detect the telomeres on the 22 pairs of chromosomes during pachytene (noted by non-fragmented SC). **(C)** The same spermatocyte showing 35 TERT signals. **(D)** Overlay of TERT, TRF2 and SCP allows the assessment of the percentage of TERT that is bound to telomeres. In this spermatocyte, 18 out of the 35 TERT signals are superimposed on TRF2 signals, signifying a 51.4% of telomere-bound TERT.

Table 4.1 Percentage of TRF2-bound TERT in azoospermic and fertile men

	No. of cells	Mean percentage (% ± SD) of TRF2-associated TERT signals
Control men (*n* = 4)		
C20	52	56.2±4.5
C21	51	42.7±5.3
C22	53	45.7±3.2
C23	57	53.1±5.8
Mean (95% CI)		48.2 (40.1-56.3)
Obstructive azoospermic men (*n* = 2)		
OA29	50	33.3±6.2*
OA31	51	25.4±5.1*
Mean (95% CI)		29.4 (21.6-37.1)*

*P <0.05, Man-Whitney test

4.3.3 Genome-wide recombination and telomere homeostasis

When we examined the relationship between the level of genome-wide recombination and TRF2-TERT in the pachytene nuclei of all infertile and fertile men (n=6), we did not observe a significant correlation between the two variables (Fig. 4.2, r =0.618, P =0.191, Pearson's correlation test). Nevertheless, there was a trend of increasing genome-wide recombination as TRF2-TERT levels increased.

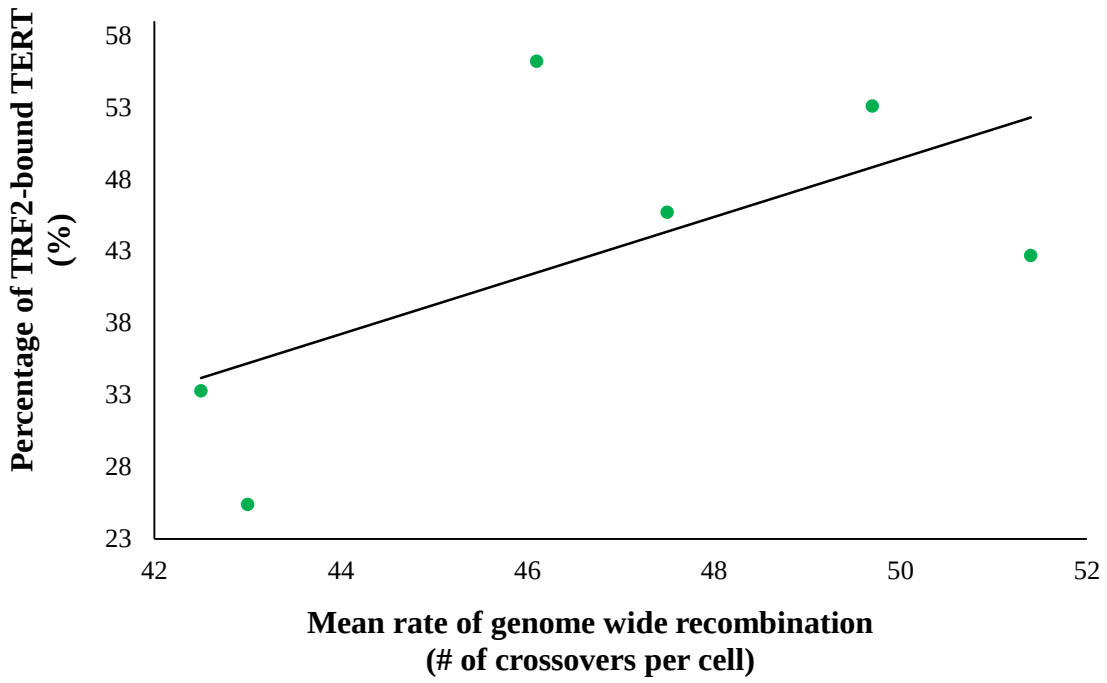

Figure 4.2 Relationship between percentage of TRF2-bound TERT and mean rate of genome-wide recombination in azoospermic and fertile men (n=6).

There appears to be a moderate positive correlation where the recombination rate increases as the percentage of TRF2-bound TERT increases. However, due to the small sample size, the correlation was not statistically significant (p=0.191, Pearson's correlation test).

4.4 Discussion

Although meiotic defects, mainly regarding recombination, have been frequently reported in the infertile male population, the mechanisms behind the errors remain elusive. In this study, we aimed to examine the association between telomere homeostasis and meiotic recombination, in hopes of elucidating potential mechanisms for how recombination may be affected in infertile men.

4.4.1 Deficiency in TERT association with telomeres in azoospermic men

Mouse studies have suggested that a decrease in telomere length due to aberrant TERT activity may lead to gametogenic arrest (Reig-Viader *et al*., 2014a). However, there has been little information on TERT activity in male germ cells, due to strict meiotic checkpoints, where most cases of severe TERT deficiency showed an absence of germ cells at the pachytene stage of prophase I (Ravidranath *et al*., 1997, Riou *et al*., 2005). Furthermore, studies on the human male germ line were limited to the evaluation of TERT activity in whole testicular samples (Fujisawa *et al*., 1998; Schrader *et al*., 2000), which are made up of multiple cell types including somatic cells such as epithelial cells, Leydig cells, and Sertoli cells, as well as germ cells. Because telomere length and TERT activity has been shown to vary dramatically in somatic cells when compared to germ cells (Reig-Viader *et al*., 2014a), the results from these studies offer a level of uncertainty. Notwithstanding, there has been two studies that examined the level of TERT in different stages of spermatogenesis (Yashima *et al*., 1998; Reig-Viader *et al*., 2014b). However, these studies on the general activity of TERT in a given cell may not be showing the full picture when it comes to maintaining telomere stability. A better measure of TERT efficiency is to examine the TERT proteins that are physically associated with the telomeres, and are therefore actively

maintaining telomere structure and function. In this respect, Reig-Viader *et al*. (2014a) provided the first evaluation of the percentage of TERT that is associated with the telomeres in infertile and fertile men. In this study, the percentage of TRF2-TERT in the spermatocytes of fertile men ranged from 42.7% to 56.2%, which is similar to the level reported in the one fertile man assessed by Reig-Viader *et al*. (2014a) at around 48%. Our data thus provides a baseline for the level of telomere-associated TERT in the germ cells of fertile men. Like the results reported in Reig-Viader *et al*. (2014a), we also found significantly decreased percentages of TERT levels associated with the telomeres in the spermatocytes of infertile men. However, the infertile men in our study were diagnosed with OA, whereas the infertile men reported in Reig-Viader *et al*. (2014a) were diagnosed with impaired spermatogenesis. It is also important to note that the levels of telomere-bound TERT in the OA men from our study (ranging from 25.4% to 33.3%) were higher than the levels in the infertile men (<20%) presented by Reig-Viader *et al*. (2014a). Thus, our results may suggest that infertile men with normal spermatogenesis may display subtler disruptions in telomere homeostasis than infertile men with compromised spermatogenesis. Furthermore, the regulation of the nuclear distribution of TERT and its association to the telomeres may be exclusive to meiosis. A study that looked at the level of telomere-bound TERT in testicular cells that were not in prophase I, classified by the absence of the SC, did not show significant differences between infertile and fertile men (Reig-Viader *et al.*, 2014a). From these results, it seems that it is the meiosis-specific mechanisms that are responsible for ensuring proper telomere homeostasis during prophase I that may be compromised in infertile men. For future studies, it would be interesting to evaluate if variable perturbations in telomere homeostasis during prophase I may link to the differing levels of recombination seen in infertile men with NOA and OA.

4.4.2 Linking levels of telomere-associated TERT with recombination

Telomere-guided movement of chromosomes have been verified in lower eukaryote and plant meiosis (Scherthan *et al*., 2000; Bass *et al*., 2000), and it is theorized that a similar mechanism occurs during mammalian meiosis (de Lange, 1998). Thus, telomere function has been speculated to be important for the subsequent steps in meiosis such as chromosome synapsis and recombination. There has indeed been evidence in fission yeast and *C. elegans* to support this theory (Nimmo *et al*., 1998; Ahmed and Hogdkin, 2000). In these studies, the loss of telomeric function led to reduced levels of meiotic recombination, and increased incidences of chromosome missegregation. In this study, we examined the association of telomeres to TERT, which is an important protein for maintaining telomere structure and function when bound. We first found that telomere-associated TERT levels were significantly decreased in infertile men, and next investigated whether the level of telomere-bound TERT was linked to recombination. Our data showed a moderate positive correlation between the percentage of telomere-bound TERT and level of genome-wide recombination in the pachytene nuclei of four fertile and two infertile men. However, due to the limited sample size in this preliminary study, the correlation was not statistically significant. Nevertheless, our preliminary data offers valuable insight into the potential effect of telomere impairment on meiotic recombination in men. To date, our understanding of the role of telomeres in mammalian meiosis, with regards to chromosome synapsis and recombination, is relatively scarce. Male mice with severe TERT deficiency unfortunately show a complete depletion of meiotic cells in the testes due to apoptosis (Zalenskaya *et al.*, 2002). In order to overcome this obstacle, studies have examined the patterns of synapsis and recombination in mice with intermediate deficiencies in TERT, where germ cells exhibit telomere shortening,

but less apoptosis compared to mice with severe TERT deficiencies (Liu *et al.*, 2004). This work found that shortened telomere length was associated with reduced synapsis and recombination, as well as meiotic arrest in female germ cells. On the other hand, male germ cells showed extensive apoptosis prior to prophase I, but the few germ cells that did survive to pachytene showed compromised synapsis and recombination. Therefore, a sex-specific meiotic checkpoint is suggested to exist prior to prophase I to allow only the germ cells with minimum telomere length to proceed. Moreover, the study noted that telomere length was not a sole indicator of synaptic and recombination status; many telomere-associated proteins, including TERT, come into play to stabilize the structure and function of the telomeres during meiosis (Trelles-Sticken *et al*., 2000). Taking these studies into account, we speculate that infertile men who show meiotic cells in the testes may have adequate telomere length, which allowed the germ cells to progress into meiosis. However, the function of the telomeres may be impaired in some infertile men due to disrupted telomere homeostasis, leading to increased synaptic and recombination errors. Consequently, telomere homeostasis may be a novel indicator of infertility, in which changes could affect synapsis and recombination, resulting in the depletion of germ cells in males. Further investigation into the relationship between telomere homeostasis and recombination in a larger sample size may be useful for elucidating novel mechanisms behind the increased rates of recombination errors observed in infertile men.

CHAPTER 5: MEIOTIC BEHAVIOUR AND SPERM ANEUPLOIDY IN AN INFERTILE MAN WITH 45,X/46,XY MOSAICISM

5.1 Introduction

45,X/46,XY mosaicism, where 45,X somatic cells are present at a high level, is very rare in the general population with an incidence rate of around 1.5/10,000 (Moussaif *et al.*, 2011; Chang *et al.*, 1990). Depending on the level of somatic 45,X cells, individuals exhibit a wide range of phenotypic abnormalities from males with apparently normal phenotypes or men with short stature and gonadal failure, to females with Turner syndrome (Newberg *et al.*, 1998; Layman *et al.*, 2009; Gantt *et al.*, 1980; Telvi *et al.*, 1999). Anaphase lag during the first mitotic division of the zygote is the simplest explanation for how 45,X/46,XY mosaicism may arise, where one of the Y sister chromatids fails to be included in the daughter cells (Telvi *et al.*, 1999; Lukasa *et al.*, 1986). This mitotic error may result in a 50:50 split of 45,X cells and 46,XY cells being present in the somatic cells. This type of mosaic karyotype is often accompanied by a rare sexual development disorder termed mixed gonadal dysgenesis (MGD). Adults with MGD may vary in degrees of infertility depending on the level of mosaicism (Brosman, 1979). In rare MGD cases, testicular spermatozoa are present and intracytoplasmic sperm injection (ICSI) is sought for fertility treatment. However, there have been very few MGD cases that resulted in successful conceptions, and the genetic transmission of chromosomal abnormalities in the sperm remains a concern (Arnedo *et al.*, 2005; Bofinger *et al.*, 1999). To better understand the production of aneuploid sperm in these mosaic individuals, it is crucial to study the behaviours of the abnormal cell lines during meiosis.

During the first meiotic division, two events are critical for the fidelity of meiosis 1) Homologous chromosome pairing, or synapsis, characterized by the establishment of the synaptonemal complex (SC) and, 2) The exchange of genetic materials, or DNA recombination, between homologous chromosomes. Our and others' studies have also shown that compromised synapsis or recombination in infertile men may lead to meiotic cell arrest and subsequent infertility, as well as contribute to an increased production of aneuploid sperm (Gonsalves *et al.*, 2004; Ma *et al.*, 2006; Ferguson *et al.*, 2007). In male meiosis, the X and Y chromosomes pair up at a small region of homology called the pseudoautosomal region (PAR). The rest of the X and Y chromosomes form a condensed chromatin structure termed the sex body. While the PAR remains transcriptionally active during meiosis, the sex body is transcriptionally silenced through a mechanism known as meiotic sex chromosome inactivation (MSCI) (Ferguson *et al.*, 2007; Turner, 2007). MSCI is characterized by the localization of phosphorylated histone H2AX and BRCA1, which is thought to trigger chromatin condensation and transcriptional repression (Turner, 2007). Recent studies suggest that transcriptional silencing is not limited to the sex body, but to any unsynapsed regions on autosomal or sex chromosomes through meiotic silencing of unsynapsed chromatin (MSUC) (Turner, 2007; Baarends *et al.*, 2005). In the case of 45,X/46,XY cell lines, transcriptional activity has not been analyzed on the unpaired (univalent) X chromosome in humans.

Until now, meiotic behaviour and sperm aneuploidy in 45,X/46,XY men with a high level of 45,X cells have not been studied. By combining immunofluorescence techniques with fluorescence *in situ* hybridization (FISH), our study sought to analyze the meiotic recombination and synapsis, sex chromosome configurations, MSCI, and sperm aneuploidy in an azoospermic man with a 50:50 split 45,X/46,XY karyotype. By comparing the unique

case to fertile 46,XY males, we aimed to better understand the fate of the 45,X/46,XY cell lines, whether chromosomally abnormal cell lines progress through meiosis, and if they give rise to aneuploid sperm.

5.2 Materials and methods

5.2.1 Patient information and tissue collection

A 27-year-old male and his 25-year-old female partner presented a two year history of primary infertility, where a successful pregnancy had not been achieved following 1.5 years of unprotected intercourse. The female had undergone infertility assessment and displayed no evidence of tubal, ovulatory, or pelvic infertility factors. The male patient was previously diagnosed with MGD. Karyotyping of the man's peripheral blood revealed a 50:50 45,X/46,XY mosaicism. Hormonal profiles revealed elevated follicle-stimulating hormone at 14 IU/L, normal luteinizing hormone, and normal total testosterone. The patient tested negative for Y chromosome microdeletions in the regions AZFa, AZFb, and AZFc. Upon physical examination and semen analysis, he was diagnosed with azoospermia. A testicular biopsy was performed to acquire sperm for ICSI treatment, where a piece of testicular tissue was obtained for this study. Subsequent histological analysis revealed common characteristics of MGD such as the presence of spermatogenesis, thickening of the basement membrane and significant Leydig cell hyperplasia (Flannigan *et al.*, 2014). The patient and his partner were unable to conceive after two rounds of ICSI. Because the level of sperm aneuploidy was low in the patient (reported in section 5.3.2), the failure to conceive was likely not due to sperm quality (Flannigan *et al.*, 2014). Testicular tissues from ten proven fertile, 46,XY men previously reported in Chapter 2 (C20-C29) were used as controls for this study.

5.2.2 Fluorescence immunostaining

Testicular tissue from the patient and control men were processed, fixed onto microscope slides, and immunostained using antibodies against SCP3/SCP1, MLH1, and CREST antisera in order to visualize the SC, recombination sites and centromeres respectively according to the methods described in section 2.2.3.

A previously immunostained slide from the patient was re-stained for γH2AX in order to observe the MSCI according to a previously described protocol (Ferguson *et al.*, 2008). Mouse anti- γH2AX antibodies (Upstate Biotech, Lake Placid, NY, USA) at a dilution of 1:1000 was used as the primary antibody cocktail.

5.2.3 FISH on immunostained spermatocytes

FISH was performed on a previously immunostained slide for the patient as well as a control man (C29) according to the methods described in section 2.2.4 in order to study the chromosomes 18, X, and Y in the spermatocytes. We observed three types of meiotic sex chromosome configurations in the patient: (1) 46,XY cell with XY bivalent (XY) where the sex body was present and the signals for X and Y were in close proximity (Fig. 5.2A), (2) 46,XY cell with univalent X and Y (X+Y) when the X and Y chromosomes were unpaired and their signals were distant from each other (Fig. 5.2B), and (3) 45,X cell with univalent X (X) when the signal for the Y chromosome was absent (Fig. 5.2C).

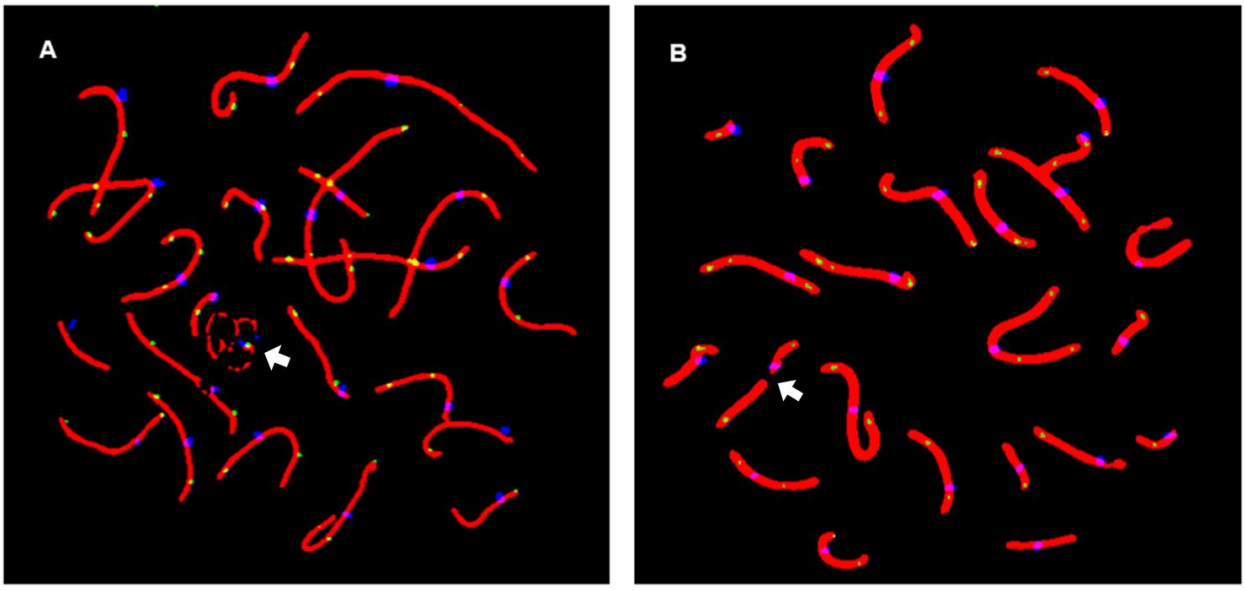

Figure 5.1 Immunofluorescence analysis of 46,XY and 45,X pachytene spermatocytes to visualize the SC (red), MLH1 foci (green) and centromeres (blue).

(A) 46,XY cell with sex body (arrow). (B) 45,X cell with 23 centromeres where a gap in the SC is present (arrow).

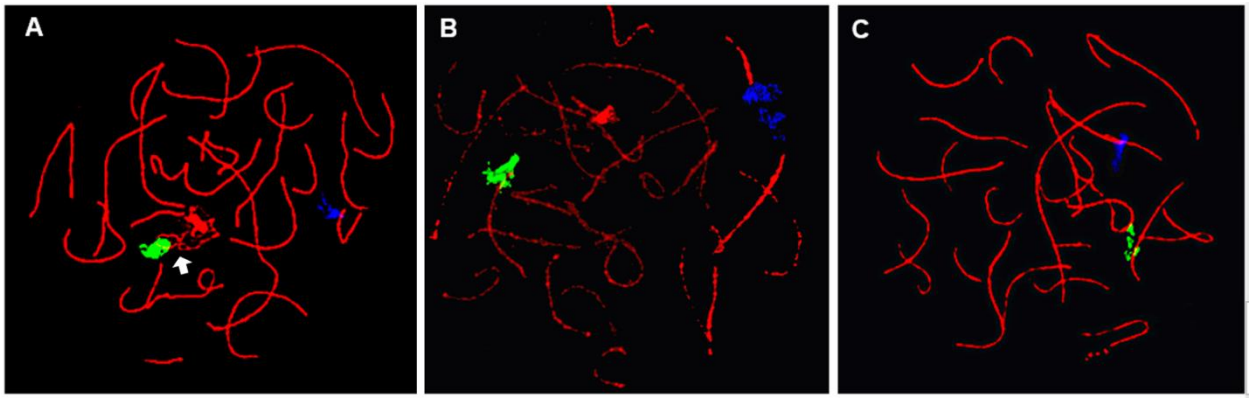

Figure 5.2 Immunofluoresence and FISH analysis of 46,XY and 45,X pachytene spermatocytes.

The SC is immunolabeled in red while chromosomes 18 (blue), X (green), and Y (red) are identified by FISH. (A) 46,XY cell with sex body formation (XY). (B) 46,XY cell with unpaired sex chromosomes (X+Y). (C) 45,X cell with univalent X (X).

5.2.4 FISH on testicular spermatozoa

Slides that were not previously immunostained were used for FISH to examine rates of chromosome 13, 18, 21, and sex chromosome aneuploidy in the testicular sperm of the patient and ten control men according to the protocol outlined in section 2.2.5.

5.2.5 Statistical analyses

The Mann-Whitney test was used to compare the mean rate of genome wide recombination between the patient and control group. Fisher exact test was used to compare the proportion of cells where the sex body lacks a recombination event (achiasmate). The rate of synaptic errors in the patient was considered significantly different if they were beyond the 95% confidence interval of the control group. The Chi-square test with two degrees of freedom was used to compare the frequencies of sperm aneuploidy and X:Y ratio in the patient versus the control group.

5.3 Results

5.3.1 Meiotic sex chromosome configurations

Pachytene spermatocytes from the 45,X/46,XY patient (n=89) and a control man (n=100) were immunostained for the SC, and analyzed by FISH to identify chromosomes 18, X, and Y (Table 5.1). In the patient, 75.3% of cells were 46,XY constitution, and 24.7% were 45,X. The most common meiotic configuration observed was XY (62.9%), while 12.4% were X+Y. 100% of cells analyzed in the control were XY configuration.

Table 5.1 Meiotic sex chromosome configuration in the infertile 45,X/46,XY patient and fertile man

	No. of cells	Frequeny of configuration in pachytene cells (%)		
		XY	X+Y	X
Control (*n* =1)				
C29	100	100	0	0
Patient	89	62.9	12.4	24.7

5.3.2 Testicular sperm aneuploidy

FISH was performed on the testicular sperm of the 45,X/46,XY patient and ten control men to identify chromosomes 18, X, and Y (Table 5.2). 2679 sperm from the patient and 10432 from the control group were scored. The patient showed increased levels of sex nullisomy (1.7% vs.0.24%, P<0.001), XY disomy (2.0% vs. 0.23%, P<0.001), and disomy 18 (0.19% vs. 0.12%, P<0.05) when compared to controls. The ratio of X:Y sperm in the patient was also significantly different compared to controls (2:1 vs. 1:1, P<0.001). A second round of FISH was performed on the same slides to identify chromosomes 13 and 21 (Table 5.2). 2125 sperm from the patient and 10334 from the control group were scored. Increased frequencies of disomy 21 was observed in the patient compared to controls (0.52% vs 0.28%, P<0.05), while the level of disomy 13 was not significantly different.

Table 5.2 Testicular sperm aneuploidy in the 45,X/46,XY patient and control men

	No. of Cells	Frequency of disomy % (*n*)			Frequency of sex nullisomy % (*n*)	X:Y ratio	No. of cells	Frequency of disomy % (*n*)	
		XX or YY	XY	18				13	21
Control men (*n* = 10)									
C20	1028	0.29% (3)	0.39% (4)	0.10% (1)	0.19% (2)	1.05	1022	0.39% (4)	0.49% (5)
C21	1010	0.30% (3)	0.20% (2)	0.10% (1)	0.20% (2)	1	1005	0.20% (2)	0.30% (3)
C22	1090	0.18% (2)	0.28% (3)	0.10% (1)	0.37% (4)	1.01	1235	0.16% (2)	0.43% (5)
C23	1034	0.29% (3)	0.19% (2)	0.19% (2)	0.39% (4)	0.99	1041	0.38% (4)	0.29% (3)
C24	1021	0.10% (1)	0.10% (1)	0% (0)	0.10% (1)	0.98	1001	0% (0)	0.20% (2)
C25	1023	0.29% (3)	0.20% (2)	0% (0)	0.39% (4)	1.02	1000	0.10% (1)	0.30% (3)
C26	1008	0% (0)	0.30% (3)	0.20% (2)	0.10% (1)	1.14	1009	0.20% (2)	0.20% (2)
C27	1203	0.08% (1)	0.25% (3)	0.17% (2)	0.25% (3)	1.05	1013	0.30% (3)	0.20% (2)
C28	1000	0.10% (1)	0.30% (3)	0.10% (1)	0.30% (3)	1.12	1002	0.10% (1)	0% (0)
C29	1015	0% (0)	0.10% (1)	0.30% (3)	0.10% (1)	0.98	1006	0.10% (1)	0.40% (4)
Total	10432	0.16% (17)	0.23% (24)	0.12% (13)	0.24% (25)	1.03	10334	0.19% (20)	0.28% (29)
Patient	2679	0.19% (5)	**1.97% (53) ****	0.19% (5)	**1.72% (46)****	**1.98****	2125	0.28% (6)	**0.52% (11)***

**P <0.001, Chi-square test with two degrees of freedom.

*P <0.05, Chi-square test with two degrees of freedom.

5.3.3 MSCI of unpaired sex chromosomes

Immunostaining for γH2AX was performed to identify the patterns of MSCI in the pachytene spermatocytes of the 45,X/46,XY patient. Pachytene cells with XY configuration displayed γH2AX localization along the sex body, but not the PAR (Fig. 5.3A). However, γH2AX localization was observed along the entire univalent chromosomes in the X and X+Y configurations (Fig. 5.3B).

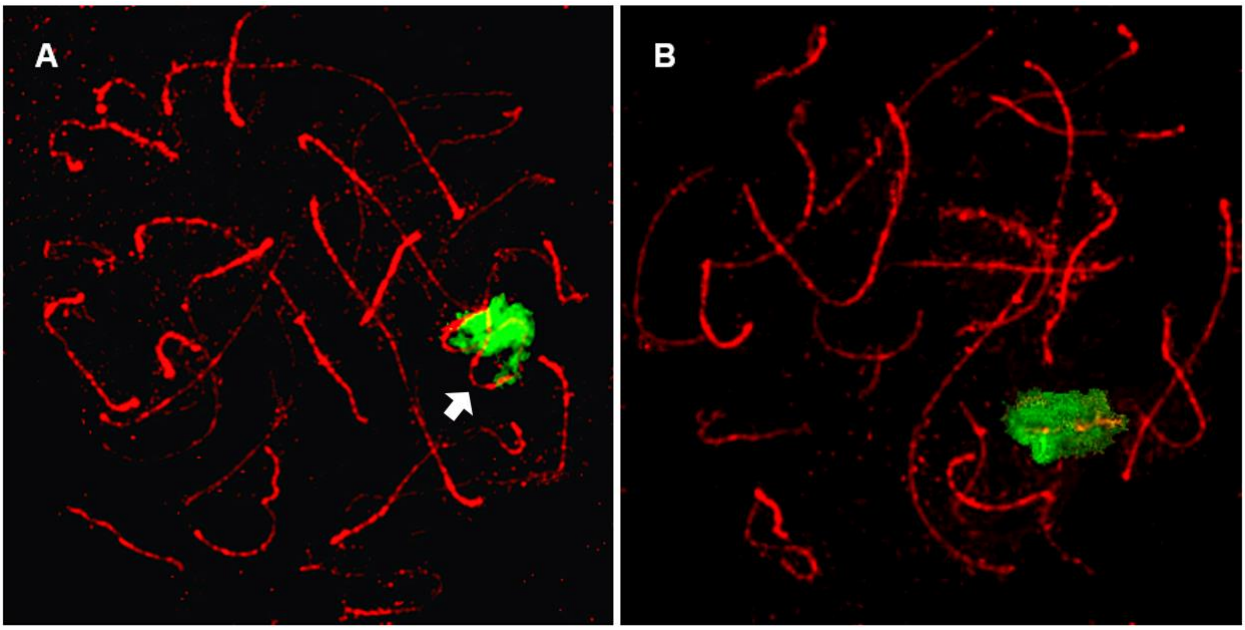

Figure 5.3 Meiotic sex chromosome inactivation in 46,XY and 45,X pachytene spermatocytes.

Spermatocytes are immunolabeled to visualize the SC (Red) and γH2AX (Green) (A) 46,XY cell displaying γH2AX staining along the sex body except for the PAR (arrow). (B) 45,X cell displaying γH2AX staining along the univalent X chromosome.

5.3.4 Genome-wide recombination and synaptic errors

Using immunofluorescence techniques to visualize the recombination sites and SC, we assessed the frequency of genome-wide recombination and synaptic errors in the pachytene spermatocytes of the 45,X/46,XY patient and ten control men (Table 5.3). A total of 101 cells from the patient and 735 from the control group were analyzed. The mean rate of recombination per cell in the control group was 48.7, with the individual mean rates ranging from 45.4 to 53.2. For the patient, the average number of crossovers per cell was 46.6±5.6. There was no significant difference in the mean recombination rate between the patient and controls. There was also no significant difference in the proportion of cells with achiasmate sex body in the patient compared to controls (9.9% vs. 9.2%).

There were two types of synaptic errors observed in the patient and control group: 1) A discontinuous SC where both the SCP1 (transverse) and SCP3 (lateral) proteins are absent resulting in a gap (Fig. 5.1B), and 2) Unsynapsed regions between the homologous chromosomes where the lateral element of the SC (SCP3) is absent. The frequency of cells with synaptic errors in the patient was not significantly different than the controls (5.2% vs. 3.6%).

Table 5.3 Genome-wide recombination, synaptic errors and sex body recombination in the 45,X/46,XY patient and control men

	No. of cells	Mean rate (± SD) of genome-wide recombination	Proportion of cells with achiasmate sex body (%)	Proportion of cells with unsynapsed regions (%)
Control men (*n* =10)				
C20	50	46.1 ± 4.0	6.0	6.0
C21	51	51.4 ± 4.7	7.8	2.0
C22	50	47.5 ± 4.7	12.0	4.0
C23	50	49.7 ± 4.42	6.0	6.0
C24	51	50.2 ± 3.6	7.8	9.8
C25	50	48.1 ± 4.7	14.0	0
C26	50	45.4 ± 4.8	6.0	2.0
C27	52	47.5 ± 4.6	9.6	3.8
C28	50	48.1 ± 4.3	12.0	2.0
C29	50	53.2 ± 3.8	14.0	0
Mean (95% CI)	504	48.7 (47.2 - 50.2)	9.5 (7.5 - 11.5)	3.6 (1.7 - 5.5)
Patient	101	46.6±5.6	9.9	5.2

5.4 Discussion

5.4.1 Fate of 45,X cell line in the testis

Assuming the patient's germ cell chromosomal constitution is consistent with his somatic karyotype, we should expect a 50:50 split of 45,X and 46,XY cells in the spermatogonia. If both cell lines entered meiosis, 50% of pachytene cells in the Prophase I stage should be of 45,X constitution. We observed a smaller than theoretically expected proportion of 45,X pachytene cells at 24.7%, which may be explained by one of two hypotheses: (1) the level of mosaicism in the germ cells is different than that in the somatic cells, or (2) half of the 45,X germ cells were arrested prior to meiosis. Since we did not observe 45,X pachytene cells in the control, it is likely that a proportion of the 45,X germ cells in the patient indeed proceeded to early meiosis I (MI). Mouse studies have shown that XO pachytene oocytes with unpaired X chromosomes are progressively eliminated during meiosis starting at the pachytene checkpoint (Turner, 2007). Nevertheless, it is hypothesized that a proportion of XO pachytene oocytes in mice and rare human cases may complete meiosis by undergoing heterologous X-chromosome pairing with an autosome or itself (Turner, 2007; Speed, 1986). In our study, the increase in X:Y sperm ratio (2:1) may suggest that a proportion of the 45,X pachytene cells progressed through meiosis and gave rise to X-bearing sperm. Presumably, if the 45,X cells (24.7%) completed meiosis, the cell lines would give rise to sex nullisomic and X-bearing sperm at 12.5% each. As we observed a sex nullisomy level (1.7%) much lower than theoretically expected, the majority of the 45,X cells resulting in nullisomic sperm may have been eliminated during meiosis. Due to the scarcity of testicular spermatocytes in the patient, we could not analyze cells in the metaphase I stage,

which may have elucidated whether the 45,X cells could evade the pachytene checkpoint and progress through MI.

5.4.2 Sex chromosome asynapsis in 46,XY cells

The meiotic sex chromosome configuration analysis showed a high frequency of sex chromosome asynapsis in the 46,XY cells of the patient compared to control (12.4% vs 0.0%). Our previous meiotic analysis of an infertile 47,XYY male, and others' studies on infertile men with 47,XYY, 47,XXY, and mosaic 46,XY/47XXY karyotypes have also implicated the presence of unpaired sex chromosomes in the 46,XY pachytene cells (Wong *et al.*, 2008; Blanco *et al.*, 2001; Milazzo *et al.*, 2006). In these cases, a mosaic germ cell composition was observed regardless of a mosaic or non-mosaic somatic karyotype. The etiology of sex chromosome asynapsis seen in the 45,X/46,XY patient and other cases remains unclear and may be due to several factors. Studies have demonstrated that an aberrant testicular environment affects chromosomal behaviour especially in chromosomes with fewer crossover events such as X and Y (Newberg *et al.*, 1998; Mroz *et al.*, 1999; Blanco *et al.*, 2003). The disrupted hormonal and physical testicular environment common in MGD men and men with abnormal sex chromosome karyotypes may cause a general increase in aberrant meiotic behaviours affecting the sex chromosomes and nondisjunction (Newberg *et al.*, 1998; Mroz *et al.*, 1999; Egozcue *et al.*, 2000). Furthermore, we speculate that a mosaic germ cell composition and intercellular effect may play a role in the disrupted X and Y pairing seen in the 45,X/46,XY patient and other men with abnormal sex chromosome karyotypes.

Mouse and other model organism studies such as *Caenorhabditis elegans* have demonstrated the importance of X and Y pairing in male infertility (Burgoyne *et al.*, 1992;

Jaramillo-Lambert and Engebrecht, 2010). Recent studies showed that pachytene spermatocytes of mice lacking *Spo11α*, an evolutionarily conserved protein involved in PAR recombination, displayed a high percentage of asynapsed X and Y chromosomes at 70% (Kauppi *et al.*, 2013; Kauppi *et al.*, 2011). Although the aberrant cells accumulated at Metaphase I, the univalent X and Y chromosomes failed to properly align at the spindles and appeared to undergo spindle checkpoint-induced apoptosis (Kauppi *et al.*, 2013). While some male mice lacking *Spo11α* produced offspring, most were infertile (Kauppi *et al.*, 2013; Kauppi *et al.*, 2011). In our study, it is possible that the X+Y cells observed in the 45,X/46,XY patient followed a similar fate and are meiotically incompetent. Aside from the presence of univalent chromosomes at Metaphase I, we propose that an abnormal transcriptional silencing of the PAR may also trigger meiotic arrest in the X+Y cells. Our analysis of MSCI demonstrated the localization of γH2AX along the entire univalent X chromosomes in the X and X+Y cells, suggesting the transcriptional silencing of the PAR through MSUC. The lack of PAR gene expression during meiosis may cause cell arrest as observed in mice with PAR deletions (McElreavey and Cortes, 2001). Thus, it is likely that the X+Y pachytene cells in the patient do not give rise to viable sperm, or contribute to the sperm aneuploidy rate and X:Y sperm ratio observed.

5.4.3 Recombination and synapsis in mosaic 45,X/46,XY cells

With regards to meiotic DNA recombination, it is thought that the reduced recombination rate and increased synaptic errors observed in infertile men may contribute to an increase in sperm aneuploidy due to MI nondisjunction events (Gonsalves *et al.*, 2004; Ma *et al.*, 2006b; Ferguson *et al.*, 2007; Sun *et al.*, 2007b; Codina-Pascual *et al.*, 2005; Topping *et al.*, 2006; Moosani *et al.*, 1995; Bernardini *et al.*, 2000; Ushijima1 *et al.*, 2000). Our

previous study demonstrated the inverse correlation between the frequency of recombination on the sex body and XY disomy in the sperm (Ferguson *et al.*, 2007). For the 45,X/46,XY patient, the pachytene cells did not show significantly different rates of global recombination, sex body recombination, or synaptic error. The lack of association between sex chromosome aneuploidy and sex body recombination rate suggests that 46,XY cells have the capacity of normal meiotic division. The increase in XY disomy and sex nullisomy seen in the sperm is unlikely to have originated from MI nondisjunction of the 46,XY cell line with paired sex chromosomes. Although the patient displayed increased rates of sex chromosome aneuploidy and disomy 18 and 21, the rates were within the range found in infertile 46,XY men with or without recombination and synaptic errors (Gonsalves *et al.*, 2004; Ferguson *et al.*, 2007; Sun *et al.*, 2007b; Codina-Pascual *et al.*, 2005; Topping *et al.*, 2006; Moosani *et al.*, 1995; Bernardini *et al.*, 2000; Ushijima1 *et al.*, 2000).

To our knowledge, we are the first to apply fluorescence immunostaining to examine the meiotic sex chromosome configuration, recombination, synapsis, and MSCI in a mosaic 45,X/46,XY man. Furthermore, we present the unprecedented report of asynapsed sex chromosomes in such an individual. Despite a mosaic karyotype, the 45,X/46,XY man displayed similar rates of sperm aneuploidy compared to infertile men with normal karyotypes (Gonsalves *et al.*, 2004; Ferguson *et al.*, 2007; Sun *et al.*, 2007b; Codina-Pascual *et al.*, 2005; Topping *et al.*, 2006; Moosani *et al.*, 1995; Bernardini *et al.*, 2000; Ushijima1 *et al.*, 2000). Consistent with previous reports on a 45,X/46,XY mosaic male, as well as 47,XYY and Klinefelter's syndrome men, our study suggests that infertile men with abnormal sex chromosome karyotypes and high levels of mosaicism may nevertheless produce sperm that are primarily normal in chromosomal constitution (Wong *et al.*, 2008;

Blanco *et al.*, 2001; Milazzo *et al.*, 2006). The extraction of normal sperm for ICSI may be possible in men with similar karyotypes.

CHAPTER 6: SUMMARY AND CONCLUSIONS

6.1 Summary and future directions

6.1.1 Meiotic recombination and sperm aneuploidy

Infertility is a rising health concern affecting 16% of couples in Canada (Bushnik *et al.,* 2012), and approximately 30% of infertile cases are solely due to male factors. With advancements in immunocytogenetic approaches, it has become possible to closely study the meiotic events that are critical for proper sperm production. The results presented in this thesis add further evidence that errors in early meiotic processes may contribute to impaired sperm production. Our first study described in Chapter 2 examined two key events in early meiosis, 1) synapsis and 2) recombination, in cohorts of infertile and fertile men. We found that 16.7% of the OA men (2/12), and 37.5% of the NOA men (3/8) displayed increased rates of synaptic errors when compared to controls (n=14). Moreover, 25% of the OA men (3/12), and 62.5% of the NOA men (5/8) displayed reduced rates of genome-wide recombination. It appears that meiotic defects may be present in some OA men who otherwise show normal spermatogenesis. However, NOA men may experience more frequent, and severe meiotic defects than OA men. XY disomy was observed to be the most frequently elevated aneuploidy in the sperm of infertile men, where it was increased in 50% of OA men (6/12), and 62.5% of NOA men (5/8). When all the infertile men were pooled (n=20), we noticed a negative correlation between the rate of XY disomy in the sperm, and frequency of recombination on the sex chromosomes. Hence, the absence of recombination on the sex chromosomes may be indicative of the level of sex chromosomal aneuploidy in the sperm. Lastly, we are the first to report a negative correlation between the rate of disomy 21 and frequency of recombination on bivalent 21 in infertile men. As a lack of recombination on

bivalent 21 in oocytes has been implicated in trisomic 21 cases, our findings warrant further research into whether this pattern also applies to spermatocytes of infertile men. Overall, we speculate that infertile men displaying an absence of recombination on chromosome 21 or the sex chromosomes may be at increased risks of producing aneuploid sperm, and possibly chromosomally abnormal conceptions following ICSI.

With regards to chromosome-specific recombination frequencies, 41.7% of the OA men (5/12), and 50% of NOA men (4/8) showed alterations on at least one of the chromosomes studied (13, 18, and 21). Thus, our study in Chapter 3 was interested in whether the distribution of crossovers along these chromosomes may also be altered in infertile men. We observed changed crossover distributions in 41.7% of OA men (5/12), and 50% of NOA men. We most frequently noted an increase in crossover formation near the centromere, and decrease in crossover formation at the subtelomeres. One infertile man also showed an increase in crossovers at the vicinity of the telomeres. Strikingly these three patterns of crossover distribution have been shown to likely lead to chromosome missegregation in yeast and mice (Blitzblau *et al*., 2007). Hence, the change in crossover distribution may be another mechanism that results in the increased production of aneuploid sperm seen in infertile men. Notably, all of the NOA men in our study had altered crossover frequencies on the same chromosome where they displayed altered crossover distribution. We postulate that these two types of meiotic defects may synergistically disturb chromosome segregation, and lead to the higher level of aneuploid sperm in NOA men when compared to OA men and controls (Kirkpatrick *et al*., 2007; Templado *et al*., 2013).

Moving forward, we plan to investigate the relationship between crossover distribution and sperm aneuploidy in hopes of elucidating whether spermatocytes with

abnormal crossover localization are arrested at meiotic checkpoints, or progress through meiosis to potentially give rise to aneuploid sperm. We also aim to correlate different patterns of altered crossover distribution, such as increases in crossovers near the centromere, with sperm disomy rates for the corresponding chromosome in infertile men. Such work would be useful for discovering novel mechanisms for chromosome segregation, meiotic arrest, and infertility in humans.

6.1.2 Telomere homeostasis and recombination

In Chapter 4, we aimed to elucidate the underlying mechanisms behind the recombination errors we observed in infertile men. As telomeres play an essential role in chromosome synapsis and recombination, we sought to determine if telomere instability, caused by the improper association of telomeres to telomerase (TERT), may be linked to recombination errors in men. We found that the levels of telomere-associated TERT in pachytene cells were decreased in OA men (n=2) compared to controls (n=4). Further correlation of the percentage of telomere-bound TERT, and frequency of genome-wide recombination in pachytene cells showed a moderate positive correlation. However, the correlation was not statistically significant likely due to the small sample size. Nonetheless, our findings suggest that deficient levels of telomere-associated TERT may possibly contribute to recombination errors and male infertility.

The limitations to our current study include the small sample size, and the inability to study telomere homeostasis in NOA men with our current protocol. Our future studies can build on the preliminary findings presented in this thesis, by examining the relationship between telomeric protein homeostasis, and crossover distribution in addition to frequency in a larger group of NOA and OA men. Furthermore, it will be useful to examine telomere

homeostasis during the different stages of prophase I, in which telomeres are actively interacting with the nuclear membrane to facilitate chromosome synapsis and recombination. Such a study would be useful for elucidating the role of telomeres in crossover formation, and provide further insight for identifying genes that may be implicated in male infertility.

6.1.3 The fate of sex chromosomal mosaicism in the testes

In Chapter 5, we investigated the meiotic behaviour and sperm aneuploidy rate of an azoospermic man with a 45,X/46,XY karyotype. Despite a 50:50 somatic mosaicism in the patient, we found a surprisingly low level of 45,X pachytene cells (25%). Assuming that the chromosome constitution of the germ cells is identical to that of the somatic cells, this result suggests that half of the 45,X germ cells are arrested prior to meiosis. Interestingly, only 63% of the pachytene cells showed normal pairing between the X and Y chromosomes, whereas 12% of the cells displayed unpaired sex chromosomes (X+Y). These X+Y cells, along with 45,X cells, showed abnormal transcriptional silencing of the pseudoautosomal region (PAR). As demonstrated in mouse studies, the vast majority of cells with unpaired chromosomes are likely arrested at the pachytene checkpoint (McElreavey and Cortes, 2001). Most importantly, although the patient's testicular sperm showed increased levels of aneuploidy, the majority were of normal constitution. However, the X:Y sperm ratio was significantly increased, possibly suggesting that some 45,X cells completed meiosis to give rise to X-bearing sperm. Our findings provide insight into the fate of mosaic germ cells in meiosis, and support the theory that checkpoints ensure the favorable production of sperm with normal chromosomal constitution despite an individual's abnormal karyotype.

Although there has been reports of sperm aneuploidy rates in infertile men with other types of sex chromosomal abnormalities, information on meiotic analyses is scarce. Future

meiotic studies in these individuals can improve our knowledge of how abnormal sex chromosomes behave during meiosis, and possibly identify new mechanisms for meiotic arrest, or chromosome missegregation. For example, this study surprisingly found a high frequency of unpaired sex chromosomes in otherwise normal 46.XY cells in the mosaic individual. This phenomenon has also been reported in mosaic men with lower levels of 45,X/46,XY, and even 47,XYY. We postulate that a mosaic somatic environment may have intercellular effects on chromosome behavior in germ cells during meiosis due to physical, hormonal, or epigenetic factors.

6.2 Conclusion

In summary, we observed meiotic defects in the form of synaptic errors, reduced levels of recombination, and changes in the distribution of crossovers in infertile men with idiopathic infertility. These impairments are implicated in the majority of infertile men with NOA, suggesting that meiotic errors may be contributing to spermatogenic arrest in this population. Moreover, smaller chromosomes that usually only experience one crossover event may be more likely to be disrupted; we found that reduced recombination on the sex chromosomes and bivalent 21 may be associated with higher rates of sperm disomy for the affected chromosome in infertile men. A possible explanation for the recombination errors frequently seen in infertile men is the perturbation of telomere homeostasis during meiosis. Spermatocytes of infertile men are more likely to have deficient association between the telomeres and its structural protein, thus potentially causing compromised telomere function. Aside from infertile men with normal karyotypes, infertile men with mosaic sex chromosomes also show substantial defects during meiosis. Our results revealed that the chromosome constitution of germ cells may not be similar to that in somatic cells, possibly

due to germ cell arrest prior to meiosis. Despite a mosaic karyotype, infertile men may nevertheless produce sperm with mostly normal constitution, and safely undergo ICSI to father biological children. Taking into account the data from this thesis, it is clear that future meiotic studies are warranted in order to reaffirm our findings in larger sample sizes, as well as address the novel postulations that have arisen from our work.

BIBLIOGRAPHY

Aarabi M, Modarressi MH, Soltanghoraee H, Behjati R, Amirjannati N, Akhondi MM. Testicular expression of synaptonemal complex protein 3 (SYCP3) messenger ribonucleic acid in 110 patients with nonobstructive azoospermia. Fertil Steril. 2006;86:325-331.

Ahmed, Shawn, and Jonathan Hodgkin. "MRT-2 checkpoint protein is required for germline immortality and telomere replication in C. elegans."Nature 403.6766 (2000): 159-164.

Allshire R. Cell division Guardian spirit blesses meiosis. Nature. 2004;427:495-497.

Amann RP. The Cycle of the Seminiferous Epithelium in Humans: A Need to Revisit? J Androl. 2008;29:469-487.

Agarwal A, Bertolla RP, Samanta L. (2016) Sperm Proteomics: potential impact on male infertility treatment. 13(3):285-96.

Arnedo N, Nogués C, Bosch M, Templado C. Mitotic and meiotic behaviour of a naturally transmitted ring Y chromosome: reproductive risk evaluation. Hum Reprod. 2005; 2004;20:462-468.

Ayoub N, Richler C, Wahrman J. Xist RNA is associated with the transcriptionally inactive XY body in mammalian male meiosis. Chromosoma. 1997;106:1-10.

Baarends WM, Roest HP, Grootegoed JA. The ubiquitin system in gametogenesis. Mol Cell Endocrinol. 1999;151:5-16.

Baarends WM, Wassenaar E, Roald van der Laan, et al. Silencing of Unpaired Chromatin and Histone H2A Ubiquitination in Mammalian Meiosis. Mol Cell Biol. 2005;25:1041-1053.

Bachtrog D. Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. Nature reviews.Genetics. 2013;14:113-124.

Badge RM, Yardley J, Jeffreys AJ, Armour JA. Crossover breakpoint mapping identifies a subtelomeric hotspot for male meiotic recombination. Hum Mol Genet. 2000;9:1239-1244.

Bao J, Bedford MT. (2016) Epigenetic regulation of the histone to protamine transition during spermiogenesis. Reproduction. 151(5):R55-70

Barlow AL, Hulten MA. (1998) Crossing over analysis at pachytene in man. Eur J Hum Genet., 6, 350-358.

Barton AB, Su Y, Lamb J, Barber D, Kaback DB. A function of subtelomeric DNA is Saccharomyces cerevisiae. Genetics 2003; 165:929–934.

Bass, H. W., Riera-Lizarazu, O., Ananiev, E. V., Bordoli, S. J., Rines, H. W., Phillips, R. L., Sedat, J. W., Agard, D. A. & Cande, W. Z. (2000) J. Cell Sci.
113, 1033–1042.\

Baudat F, Imai Y, de Massy B. Meiotic recombination in mammals: localization and regulation. Nature reviews.Genetics. 2013;14:794.

Bellani MA, Boateng KA, McLeod D, Camerini-Otero RD. The Expression Profile of the Major Mouse SPO11 Isoforms Indicates that SPO11β Introduces Double Strand Breaks and Suggests that SPO11α Has an Additional Role in Prophase in both Spermatocytes and Oocytes. Mol Cell Biol. 2010;30:4391.

Bernardini L, Gianaroli L, Fortini D, et al. Frequency of hyper-, hypohaploidy and diploidy in ejaculate, epididymal and testicular germ cells of infertile patients. Hum Reprod. 2000;15:2165-2172.

Berookhim BM, Schlegel PN. Azoospermia due to spermatogenic failure. Urol Clin North Am. 2014;41:97.

Blanco J, Egozcue J, Vidal F. Meiotic behaviour of the sex chromosomes in three patients with sex chromosome anomalies (47,XXY, mosaic 46,XY/47,XXY and 47,XYY) assessed by fluorescence in-situ hybridization. HUMAN REPRODUCTION. 2001;16:887-892.

Blitzblau HG, Bell SP, Bell GW, Rodriguez J, Hochwagen A. Mapping of Meiotic Single-Stranded DNA Reveals Double-Strand-Break Hotspots near Centromeres and Telomeres. Current Biology. 2007;17:2003-2012.

Blouin JL, Christie DH, Gos A, et al. A new dinucleotide repeat polymorphism at the telomere of chromosome 21q reveals a significant difference between male and female rates of recombination. Am J Hum Genet. 1995;57:388-394.

Bofinger MK, Needham DF, Saldana LR, Sosnowski JP, Blough RI. 45,X/46,X,r(Y) karyotype transmitted by father to son after intracytoplasmic sperm injection for oligospermia. A case report. J Reprod Med. 1999;44:645.

Borde V, de Massy B. Programmed induction of DNA double strand breaks during meiosis: setting up communication between DNA and the chromosome structure. Curr Opin Genet Dev. 2013;23:147-155.

Borner GV, Kleckner N, Hunter N. (2004) Crossover/noncrossover differentiation, synatonemal complex
formation, and regulatory surveillance at the lepotene/zygotene transition of meiosis. Cell., 117, 29-45.

Brosman SA. Mixed gonadal dysgenesis. J Urol. 1979;121:344.

Buch B, Galán JJ, Lara M, Real LM, Martínez-Moya M, Ruiz A. Absence of de novo Y-chromosome microdeletions in male children conceived through intracytoplasmic sperm injection. Fertil Steril. 2004;82:1679-1680.

Bugge M, Collins A, Petersen MB, et al. Non-disjunction of chromosome 18. Hum Mol Genet. 1998;7:661-669.

Bugge M, Collins A, Hertz JM, et al. Non-disjunction of chromosome 13. Hum Mol Genet. 2007;16:2004-2010.

Bukulmez O. Genetic aspects of male infertility. In: 2012th ed. New York, NY: Springer New York; 2012:171-189.

Burgoyne PS, Mahadevaiah SK, Sutcliffe MJ, Palmer SJ. Fertility in mice requires X-Y pairing and a Y-chromosomal "Spermiogenesis" gene mapping to the long arm. Cell. 1992;71:391-398.

Burrello N, Calogero AE, De Palma A, et al. Chromosome analysis of epididymal and testicular spermatozoa in patients with azoospermia. European Journal of Human Genetics. 2002;10:362-366.

Burrello N, Vicari E, Calogero AE. (2005) Chromosome abnormalities in spermatozoa of patients with azoospermia and normal somatic karyotype. Cytogenet Genome Res., 111, 363-365.

Bushnik T, Cook JL, Yuzpe AA, Tough S, Collins J. Estimating the prevalence of infertility in Canada. Human Reproduction. 2012;27:738-746.

Chang HJ, Clark RD, Bachman H. The phenotype of 45,X/46,XY mosaicism: an analysis of 92 prenatally diagnosed cases. Am J Hum Genet. 1990;46:156-167.

Chatziparasidou A, Christoforidis N, Samolada G, Nijs M. Sperm aneuploidy in infertile male patients: a systematic review of the literature. Andrologia. 2015;47:847-860.

Checchi PM, Engebrecht J. Heteromorphic sex chromosomes: Navigating meiosis without a homologous partner. Mol Reprod Dev. 2011;78:623-632.

Cherry SM, Adelman CA, Theunissen JW, Hassold TJ, Hunt PA, Petrini JHJ. The Mre11 Complex Influences DNA Repair, Synapsis, and Crossing Over in Murine Meiosis. Current Biology. 2007;17:373-378.

Chikashige Y, Haraguchi T, Hiraoka Y. Another way to move chromosomes. Chromosoma. 2007;116:497-505.

Chua PR, Roeder GS. Tam1, a telomere-associated meiotic protein, functions in chromosome synapsis and crossover interference. Genes & Dev. 1997;11:1786–1800.

Codina-Pascual M, Oliver-Bonet M, Navarro J, Campillo M, Garcia F, Egozcue S, Abad C, Egozcue J, Benet J. (2005) Synapsis and meiotic recombination analyses: MLH1 focus in the XY as an indicator. Hum Reprod., 8, 2133-2139.

Codina-Pascual M, Campillo M, Kraus J, Speicher MR, Egozcue J, Navarro J, Benet J. (2006) Crossover frequency
and synaptonemal complex length: their variability and effects on human male meiosis. Mol Hum Reprod., 12, 123-
133.

Colomé-Tatché M, Cortijo S, Wardenaar R, et al. Features of the Arabidopsis recombination landscape resulting from the combined loss of sequence variation and DNA methylation. Proc Natl Acad Sci U S A. 2012;109:16240-16245.

Conrad MN, Lee C, Chao G, et al. Rapid Telomere Movement in Meiotic Prophase Is Promoted By NDJ1, MPS3, and CSM4 and Is Modulated by Recombination. Cell. 2008;133:1175-1187.

Cooper TG, Noonan E, von Eckardstein S, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update. 2010;16:231-245.

Crackower MA, Kolas NK, Noguchi J, et al. Essential Role of Fkbp6 in Male Fertility and Homologous Chromosome Pairing in Meiosis. Science. 2003;300:1291-1295.

Cram DS, Ma K, Bhasin S, et al. Y chromosome analysis of infertile men and their sons conceived through intracytoplasmic sperm injection: vertical transmission of deletions and rarity of de novo deletions. Fertil Steril. 2000;74:909-915.

Cromie GA, Smith GR. Branching out: meiotic recombination and its regulation. Trends Cell Biol. 2007;17:448-455.

Crosignani PG, Rubin B. (1996) The ESHRE Capri Workshop. Guidelines to the prevalence, diagnosis, treatment
and management of infertility. Hum Reprod., 11, 1775-1807.

Dernburg AF, McDonald K, Moulder G, Barstead R, Dresser M, Villeneuve AM. Meiotic Recombination in C. elegans Initiates by a Conserved Mechanism and Is Dispensable for Homologous Chromosome Synapsis. Cell. 1998;94:387-398.

Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. Hum Reprod Update. 2004;10:19-28.

de Lange, Titia. "Ending up with the right partner." Nature 392.6678 (1998): 753-754.

Dilworth DD, McCarrey JR. Expression of Xist in mouse germ cells correlates with X-chromosome inactivation. Nat Genet. 1992;2:200-203.

Dimitriadis F, Tsiampali C, Chaliasos N, Tsounapi P, Takenaka A, Sofikitis N. The Sertoli cell as the orchestra conductor of spermatogenesis: spermatogenic cells dance to the tune of testosterone. Hormones (Athens, Greece). 2015;14:479.

Do AT, Brooks JT, Le Neveu MK, LaRocque JR. Double-strand break repair assays determine pathway choice and structure of gene conversion events in Drosophila melanogaster. G3 (Bethesda, Md.). 2014;4:425-432.

Egozcue S, Vendrell JM, Garcia F, Veiga A, Aran B, Barri PN, Egozcue J. Increased incidence of meiotic anomalies in oligoasthenozoospermic males preselected for intracytoplasmic sperm injection. J Assist Reprod Genet. 2000;17:307-309.

Egozcue S, Egozcue J, Blanco J, Anton E, Sarrate Z, Vidal F. Genetic Analysis of Sperm and Implications of Severe Male Infertility—A Review. Placenta. 2003;24:S62-S65.

Eichenlaub-Ritter U. Oocyte ageing and its cellular basis. Int J Dev Biol. 2012;56:841.

Elborn JS. Cystic fibrosis. The Lancet. 2016.

Ercelen N, Turtar E, Gultomruk M, et al. Successful preimplantation genetic aneuploidy screening in Turkish patients. Genetics and molecular research : GMR. 2011;10:4093.

Ewen-Campen B, Schwager EE, Extavour CGM. The molecular machinery of germ line specification. Mol Reprod Dev. 2010;77:3-18.

Feng C, Wang L, Dong M, Huang H. Assisted reproductive technology may increase clinical mutation detection in male offspring. Fertil Steril. 2008;90:92-96.

Ferguson KA, Wong EC, Chow V, Nigro M, Ma S. Abnormal meiotic recombination in infertile men and its association with sperm aneuploidy. Hum Mol Genet. 2007;16:2870-2879.

Ferguson KA, Chow V, Ma S. Silencing of unpaired meiotic chromosomes and altered recombination patterns in an azoospermic carrier of a t(8;13) reciprocal translocation. Hum Reprod. 2008;23:988-995.

Ferguson, K. A., Leung, S., Jiang, D., & Ma, S. (2009). Distribution of MLH1 foci and inter-focal distances in spermatocytes of infertile men. *Human reproduction*, *24*(6), 1313-1321.

Ferfouri F, Boitrelle F, Ghout I, Albert M, Molina Gomes D, Wainer R, Bailly M, Selva J, Vialard F. A genome-wide DNA methylation study in azoospermia. Andrology. 2013;1:815-821.

Flannigan RK, Chow V, Ma S, Yuzpe A. 45,X/46,XY mixed gonadal dysgenesis: A case of successful sperm extraction. Canadian Urological Association journal. 2014;8:E108.

Fragouli E, Lenzi M, Ross R, Katz-Jaffe M, Schoolcraft WB, Wells D. Comprehensive molecular cytogenetic analysis of the human blastocyst stage. Human Reproduction. 2008;23:2596-2608.

Fragouli E, Alfarawati S, Goodall N, Sánchez-García JF, Colls P, Wells D. The cytogenetics of polar bodies: insights into female meiosis and the diagnosis of aneuploidy. Mol Hum Reprod. 2011;17:286-295.

Fraune J, Schramm S, Alsheimer M, Benavente R. The mammalian synaptonemal complex: protein components, assembly and role in meiotic recombination. Exp Cell Res. 2012;318:1340.

Fujisawa M, Tanaka H, Tatsumi N, Okada H, Arakawa S, Kamidono S. Telomerase activity in the testis of infertile patients with selected causes. Hum
Reprod 1998;13:1476–9.

Gabriel AS, et al. Array comparative genomic hybridisation on first polar bodies suggests that nondisjunction is not the predominant mechanism leading to aneuploidy in humans. J. Med. Genet. 2011; 48:433–437.

Gantt PA, Byrd JR, Greenblatt RB, McDonough PG. A clinical and cytogenetic study of fifteen patients with 45,X/46XY gonadal dysgenesis. Fertil Steril. 1980;34:216.

Gazvani R, Lewis-Jones I. Cystic fibrosis screening in assisted reproduction. Curr Opin Obstet Gynecol. 2006;18:268-272.

Geraedts J, et al. Polar body array CGH for prediction of the status of the corresponding oocyte. Part I: clinical results. Hum. Reprod. 2011; 26:3173–3180.

Ghasemzadeh-Hasankolaei M, Sedighi-Gilani M, Eslaminejad M. Induction of Ram Bone Marrow Mesenchymal Stem Cells into Germ Cell Lineage using Transforming Growth Factor-β Superfamily Growth Factors. Reproduction in Domestic Animals. 2014;49:588-598.

Gilbert SF. Developmental Biology. Tenth ed. Sunderland, MA, USA: Sinauer Associates, Inc. Publishers; 2014.

Gorbsky GJ. The spindle checkpoint and chromosome segregation in meiosis. FEBS Journal. 2015;282:2458-2474.

Gutiérrez-Mateo C, Colls P, Sánchez-García J, et al. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. Fertil Steril. 2011;95:953-958.

Hall HE, Chan ER, Collins A, et al. The origin of trisomy 13. American Journal of Medical Genetics Part A. 2007;143A:2242-2248.

Hall HE, Surti U, Hoffner L, Shirley S, Feingold E, Hassold T. The origin of trisomy 22: Evidence for acrocentric chromosome-specific patterns of nondisjunction. American Journal of Medical Genetics Part A. 2007;143A:2249-2255.

Handel MA. The XY body: a specialized meiotic chromatin domain. Experimental Cell Research. United States: Elsevier Inc; 2004;296:57-63.

Hann MC, Lau PE, Tempest HG. Meiotic recombination and male infertility: from basic science to clinical reality? Asian J Androl. 2011;13:212-218.

Hansen M, Bower C, Milne E, de Klerk N, Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects--a systematic review. Hum Reprod. 2005; 2004;20:328-338.

Hassold T, Abruzzo M, Adkins K, et al. Human aneuploidy: incidence, origin, and etiology. Environ Mol Mutagen. 1996;28:167-175.

Hassold T, Hall H, Hunt P. The origin of human aneuploidy: where we have been, where we are going. Hum Mol Genet. 2007;16 Spec No. 2:R203-R208.

Hauf S, Watanabe Y. Kinetochore Orientation in Mitosis and Meiosis. Cell. United States: Elsevier Inc; 2004;119:317-327.

Heyting C, Moens PB, van Raamsdonk W, Dietrich AJ, Vink AC, Redeker EJ. Identification of two major components of the lateral elements of synaptonemal complexes of the rat. Eur J Cell Biol. 1987;43:148.

Higgins JD, Perry RM, Barakate A, et al. Spatiotemporal Asymmetry of the Meiotic Program Underlies the Predominantly Distal Distribution of Meiotic Crossovers in Barley. Plant Cell. 2012;24:4096-4109.

Hindryckx A, Peeraer K, Debrock S, et al. Has the Prevalence of Congenital Abnormalities after Intracytoplasmic Sperm Injection Increased? The Leuven Data 1994-2000 and a Review of the Literature. Gynecol Obstet Invest. 2010;70:11-22.

Hoffmann ER, Borts RH. Meiotic recombination intermediates and mismatch repair proteins. Cytogenetic and Genome Research. 2004;107:232-248.

Hunt PA, Hassold TJ. Sex Matters in Meiosis. Science. 2002;296:2181-2183.

Hunt P, Hassold T. To err (meiotically) is human: the genesis of human aneuploidy. Nature Reviews Genetics. 2001;2:280-291.

Hunter N, Borts RH. Mlh1 is unique among mismatch repair proteins in its ability to promote crossing-over during meiosis. Genes Dev. 1997;11:1573-1582.

Hunter N, Kleckner N. The single-end invasion: an asymmetric intermediate at the double-strand break to double-holliday junction transition of meiotic recombination. Cell. 2001;106:59.

Hulten M, Eliasson R, Tillinger KG. (1970) Low chiasma count and other meiotic irregularities in two infertile 46,XY men with spermatogenic arrest. Hereditas, 65, 285-290.

Iersel v, M., Heyting C, Meuwissen RLJ, Offenberg HH, Dietrich AJJ, Riesewijk A. A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes. EMBO J. 1992;11:5091-5100.

Ioannou D, Tempest HG. Meiotic Nondisjunction: Insights into the Origin and Significance of Aneuploidy in Human Spermatozoa. Adv Exp Med Biol. 2015;868:1.

JACOBS P, DALTON P, JAMES R, et al. Turner syndrome: a cytogenetic and molecular study. Ann Hum Genet. 1997;61:471-483.

James RS, Jacobs PA. Molecular studies of the aetiology of trisomy 8 in spontaneous abortions and the liveborn population. Hum Genet. 1996;97:283-286.

Jaramillo-Lambert A, Engebrecht J. A Single Unpaired and Transcriptionally Silenced X Chromosome Locally Precludes Checkpoint Signaling in the Caenorhabditis elegans Germ Line. Genetics. 2010;184:613-628.

Jia L, Kim S, Yu H. Tracking spindle checkpoint signals from kinetochores to APC/C. Trends Biochem Sci. 2013;38:302.

Johansson MM, Geystelen AV, Maarten H D Larmuseau, et al. Microarray Analysis of Copy Number Variants on the Human Y Chromosome Reveals Novel and Frequent Duplications Overrepresented in Specific Haplogroups: e0137223. PLoS One. 2015;10.

Johnson MD. Genetic risks of intracytoplasmic sperm injection in the treatment of male infertility: recommendations for genetic counseling and screening. Fertil Steril. 1998;70:397-411.

Joseph I, Lustig A. Telomeres in meiotic recombination: The Yeast Side Story. Cellular and Molecular Life Sciences. 2007;64:125-130.

Jorgez CJ, Weedin JW, Sahin A, et al. Y-chromosome microdeletions are not associated with SHOX haploinsufficiency. Hum Reprod. 2014;29:1113.

Karadima G, Bugge M, Nicolaidis P, Vassilopoulos D, Avramopoulos D, Grigoriadou M, Albrecht B, Passarge E, Anneren G, Blennow E, Clausen N, Galla-Voumvouraki A, Tsezou A,
Kitsiou-Tzeli S, Hahnemann JM, Hertz JM, Houge G, Kuklik M, Macek M, Lacombe D, Miller K, Moncla A, Lopez Pajares I, Patsalis PC, Petersen MP et al. (1998) Origin of nondisjunciton in trisomy 8 and trisomy 8 mosaicism. Eur J Hum Genet., 6, 432-438.

Kauppi L, Barchi M, Baudat F, Romanienko P, Keeney S, Jasin M. Distinct Properties of the XY Pseudoautosomal Region Crucial for Male Meiosis. Science. 2011;331:916-920.

Kauppi L, Barchi M, Lange J, Baudat F, Jasin M, Keeney S. Numerical constraints and feedback control of double-strand breaks in mouse meiosis. Genes Dev. 2013;27:873-886.

Keefe DL, Blasco MA, Liu L. Requirement of functional telomeres for metaphase chromosome alignments and integrity of meiotic spindles. EMBO Rep. 2002;3:230-234.

Keeney S, Giroux CN, Kleckner N. Meiosis-Specific DNA Double-Strand Breaks Are Catalyzed by Spo11, a Member of a Widely Conserved Protein Family. Cell. 1997;88:375-384.

Kent-First M, Muallem A, Shultz J, et al. Defining regions of the Y-chromosome responsible for male infertility and identification of a fourth AZF region (AZFd) by Y-chromosome microdeletion detection. Mol Reprod Dev. 1999;53:27-41.

Kent-First MG, Kol S, Muallem A, et al. The Incidence and Possible Relevance of Y-Linked Microdeletions in Babies Born After Intracytoplasmic Sperm Injection and Their Infertile Fathers. J Urol. 1998;159:609-610.

Khalil AM, Boyar FZ, Driscoll DJ, Gartler SM. Dynamic Histone Modifications Mark Sex Chromosome Inactivation and Reactivation during Mammalian Spermatogenesis. Proc Natl Acad Sci U S A. 2004;101:16583-16587.

Kimmins S, Crosio C, Kotaja N, et al. Differential Functions of the Aurora-B and Aurora-C Kinases in Mammalian Spermatogenesis. Molecular Endocrinology. 2007; 2006;21:726-739.

Kirkpatrick G, Ferguson KA, Gao H, et al. A comparison of sperm aneuploidy rates between infertile men with normal and abnormal karyotypes. Human Reproduction. 2008;23:1679-1683.

Kirkpatrick G, Ren H, Liehr T, Chow V, Ma S. Meiotic and sperm aneuploidy studies in three carriers of Robertsonian translocations and small supernumerary marker chromosomes. Fertil Steril. 2015;103:1162.

Kirkpatrick G, Chow V, Ma S. Meiotic recombination, synapsis, meiotic inactivation and sperm aneuploidy in a chromosome 1 inversion carrier. Reproductive biomedicine online. 2012;24:91-100.

Kneitz B, Cohen PE, Avdievich E, et al. MutS homolog 4 localization to meiotic chromosomes is required for chromosome pairing during meiosis in male and female mice. Genes Dev. 2000;14:1085-1097.

Kniewel R, Keeney S. Histone methylation sets the stage for meiotic DNA breaks. EMBO J. 2009;28:81-83.

Komori S, Kato H, Kobayashi S, Koyama K, Isojima S. Transmission of Y chromosomal microdeletions from father to son through intracytoplasmic sperm injection. J Hum Genet. 2002;47:465-468.

Krausz C, Hoefsloot L, Simoni M, Tüttelmann F, European Molecular Genetics Quality Network, European Academy of Andrology. EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013. Andrology. 2014;2:5-19.

Krausz C, Degl'Innocenti S. Y chromosome and male infertility: update, 2006. Frontiers in bioscience : a journal and virtual library. 2006;11:3049.

Kühnert B, Gromoll J, Kostova E, et al. Case report: natural transmission of an AZFc Y-chromosomal microdeletion from father to his sons. Hum Reprod. 2004;19:886-888.

Kumar N, Singh AK. Trends of male factor infertility, an important cause of infertility: A review of literature. Journal of human reproductive sciences. 2015;8:191-196.

Kurdzo EL, Dawson DS. Centromere pairing – tethering partner chromosomes in meiosis I. FEBS Journal. 2015;282:2445-2457.

Lahn BT, Page DC. Functional Coherence of the Human Y Chromosome. Science. 1997;278:675-680.

Lam I, Keeney S. Mechanism and regulation of meiotic recombination initiation. Cold Spring Harbor perspectives in biology. 2015;7:a016634.

Lamb NE, Freeman SB, Savage-Austin A, Pettay D, Taft L, Hershey J, Gu Y, Shen J, Saker D, May KM, Avramopoulos D, Petersen MB, Hallberg A, Mikkelsen M, Hassold TJ, Sherman SL. (1996) Susceptible chiasmate configurations of chromosome 21 predispose to non-disjunction in both maternal meiosis I and meiosis II. Nat Genet., 14, 400-405.

Lamb NE, Sherman SL, Hassold TJ. (2005) Effect of meiotic recombination on the production of aneuploid gametes
in humans. Cytogenet Genome Res., 111, 250-255.

Laurie DA, Hultén MA. Further studies on chiasma distribution and interference in the human male. Ann Hum Genet. 1985;49:203-214.

Layman LC, Tho SPT, Clark AD, Kulharya A, McDonough PG. Phenotypic spectrum of 45,X/46,XY males with a ring Y chromosome and bilaterally descended testes. Fertil Steril. 2009;91:791-797.

Lee SH, Ahn SY, Lee KW, Kwack K, Jun HS, Cha KY. Intracytoplasmic sperm injection my lead to vertical transmission, expansion, and de novo occurrence of Y-chromosome microdeletions in male fetuses. Fertil Steril. 2006;85:1512-5.

Lehmann R, Richardson BE. Mechanisms guiding primordial germ cell migration: strategies from different organisms. Nature Reviews Molecular Cell Biology. 2010;11:37-49.

Lie RT, Lyngstadaas A, Ørstavik KH, Bakketeig LS, Jacobsen G, Tanbo T. Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods; a meta-analysis. Int J Epidemiol. 2005; 2004;34:696-701.

Liu L, Franco S, Spyropoulos B, Moens PB, Blasco MA, Keefe DL. Irregular Telomeres Impair Meiotic Synapsis and Recombination in Mice. Proc Natl Acad Sci U S A. 2004;101:6496-6501.

Lo Giacco D, Chianese C, Sanchez-Curbelo J, et al. Clinical relevance of Y-linked CNV screening in male infertility: new insights based on the 8-year experience of a diagnostic genetic laboratory. EUROPEAN JOURNAL OF HUMAN GENETICS. 2014;22:754-761.

Loft A, Petersen K, Erb K, et al. A Danish national cohort of 730 infants born after intracytoplasmic sperm injection (ICSI) 1994-1997. Hum Reprod. 1999;14:2143.

Lukasa T, Fryns JP, Den Berghe HV. Gonadoblastoma and Y-Chromosome Fluorescence. Obstet Gynecol Surv. 1986;41:712-713.

Lunardi J, Zahi M, Sèle B, et al. Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. Nat Genet. 2007;39:661-665.

Lynn A, Koehler KE, Judis L, et al. Covariation of Synaptonemal Complex Length and Mammalian Meiotic Exchange Rates. Science. 2002;296:2222-2225.

Ma S, Ferguson KA, Arsovska S, Moens P, Chow V. (2006a) Reduced recombination associated with the production of aneuploid sperm in an infertile man: a case report. Hum Reprod., 21, 980-985.

Ma S, Arsovska S, Moens P, Nigro M, Chow V. (2006b) Analysis of early meiotic events and aneuploidy in nonobstructive azoospermic men: a preliminary report. Fertil Steril., 85, 646-652.

Macaulay M, Copenhaver GP, Henderson IR, et al. Epigenetic Remodeling of Meiotic Crossover Frequency in Arabidopsis thaliana DNA Methyltransferase Mutants. Plos Genetics. 2012;8:e1002844.

MacDonald M, Hassold T, Harvey J, Wang LH, Morton NE, Jacobs P. The origin of 47,XXY and 47,XXX aneuploidy: heterogeneous mechanisms and role of aberrant recombination. Hum Mol Genet. 1994;3:1365-1371.

Magli MC, Gianaroli L, Ferraretti AP. Chromosomal abnormalities in embryos. Mol Cell Endocrinol. 2001;183:S29-S34.

Mariano Eliécer Acosta Lobo, Neil Aldrin Vásquez Araque, Luis Fernando Londoño Franco. X chromosome inactivation in mammalian embryonic development/Inactivación del cromosoma X en el desarrollo embrionario mamífero/Inativação do cromossomo X no desenvolvimento embrionário dos mamíferos. CES Medicina Veterinaria y Zootecnia. 2013;8:108.

Marston AL. Shugoshins: tension-sensitive pericentromeric adaptors safeguarding chromosome segregation. Mol Cell Biol. 2015; 2014;35:634-648.

Martin RH. Meiotic recombination and errors during spermatogenesis. In: New York, NY: Springer New York; 2010:357-365.

Martin, R.H. (2006) Meiotic chromosome abnormalities in human spermatogenesis. Reprod Toxic 22:142–147.

Martin LJ. Cell interactions and genetic regulation that contribute to testicular Leydig cell development and differentiation: LEYDIGCELLDEVELOPMENTANDDIFFERENTIATION. Mol Reprod Dev. 2016;83:470-487.

Martin RH, Ko E, Rademaker A. Distribution of aneuploidy in human gametes: comparison between human sperm and oocytes. Am J Med Genet. 1991;39:321.

McElreavey K, Cortes LS. X-Y Translocations and Sex Differentiation. Semin Reprod Med. 2001;19:133-140.

McLachlan RI, Rajpert-De Meyts E, Hoei-Hansen CE, de Kretser DM, Skakkebaek NE. Histological evaluation of the human testis--approaches to optimizing the clinical value of the assessment: mini review. Hum Reprod. 2007; 2006;22:2-16.

Melamed-Bessudo C, Levy AA. Deficiency in DNA methylation increases meiotic crossover rates in euchromatic but not in heterochromatic regions in Arabidopsis. Proceedings of the National Academy of Sciences. 2012;109:E981-E988.

Mezard C, Jahns M, Grelon M. Where to cross? New insights into the location of meiotic crossovers. TRENDS IN GENETICS. 2015;31:393-401.

Micic M, Micic S, Diklic V. (1982) Low chiasma frequency as an aetiological factor in male infertility. Clin Genet., 22, 266-269.

Milazzo J, Rives N, Mousset-Simeon N, Mace B. Chromosome constitution and apoptosis of immature germ cells present in sperm of two 47,XYY infertile males. HUMAN REPRODUCTION. 2006;21:1749-1758.

Minor A, Wong EC, Harmer K, Ma S. Molecular and cytogenetic investigation of Y chromosome deletions over three generations facilitated by intracytoplasmic sperm injection. Prenat Diagn. 2007;27:743-747.

Minor A, Chow V, Ma S. Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with obstructive azoospermia and undergoing vasectomy reversal. Reproduction (Cambridge, England). 2011;141:749-757.

Mira-Bontenbal H, Gribnau J. New Xist-Interacting Proteins in X-Chromosome Inactivation. Current Biology. 2016;26:R338-R342.

Mirouze M, Lieberman-Lazarovich M, Aversano R, et al. Loss of DNA methylation affects the recombination landscape in Arabidopsis. Proc Natl Acad Sci U S A. 2012;109:5880-5885.

Miyamoto T, Hasuike S, Yogev L, et al. Azoospermia in patients heterozygous for a mutation in SYCP3. The Lancet. 2003;362:1714-1719.

Moosani N, Pattinson HA, Carter MD, Cox DM, Rademaker AW, Martin RH. (1995) Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescent in-situ hybridization. Fertil Steril., 64, 811-817.

Moses MJ. The Relation between the Axial Complex of Meiotic Prophase Chromosomes and Chromosome Pairing in a Salamander (Plethodon cinereus). J Cell Biol. 1958;4:633-638.

Moses MJ. Chromosomal Structures in Crayfish Spermatocytes. J Cell Biol. 1956;2:215-218.

Moussaif N, Haddad NE, Iraqi N, Gaouzi A. 45,X/46,XY mosaicisme: Report of five cases and clinical review. Ann Endocrinol. 2011;72:239-243.

Mroz K, Hassold TJ, Hunt PA. Meiotic aneuploidy in the XXY mouse: evidence that a compromised testicular environment increases the incidence of meiotic errors. Hum Reprod. 1999;14:1151-1156.

Munné S, Wells D, Cohen J. Technology requirements for preimplantation genetic diagnosis to improve assisted reproduction outcomes. Fertil Steril. 2010;94:408-430.

Murray KS, James A, McGeady JB, Reed ML, Kuang WW, Nangia AK. The effect of the new 2010 World Health Organization criteria for semen analyses on male infertility. Fertil Steril. 2012;98:1428-1431.

Nagaoka SI, Hassold TJ, Hunt PA. Human aneuploidy: mechanisms and new insights into an age-old problem. Nature reviews.Genetics. 2012;13:493-504.

Nagvenkar P, Zaveri K, Hinduja I. Comparison of the sperm aneuploidy rate in severe oligozoospermic and oligozoospermic men and its relation to intracytoplasmic sperm injection outcome. Fertil Steril. 2005;84:925-931.

Nambiar M, Smith GR. Repression of harmful meiotic recombination in centromeric regions. Seminars in Cell and Developmental Biology. 2016;54:188-197.

Namekawa SH, Park PJ, Zhang L, et al. Postmeiotic Sex Chromatin in the Male Germline of Mice. Current Biology. 2006;16:660-667.

Naranjo T. Finding the correct partner: the meiotic courtship. Scientifica. 2012;2012:509073.

Navarro-Costa P, Goncalves J, Plancha C. The AZFc region of the Y chromosome: at the crossroads between genetic diversity and male infertility. Hum Reprod Update. 2010;16:525-542.

Newberg MT, Francisco RG, Pang M, Brugo S, Doncel GF, Acosta AA, Hoegerman SF, Kearns WG. Cytogenetics of somatic cells and sperm from a 46,XY/45,X mosaic male with moderate oligoasthenoteratozoospermia. Fertil Steril. 1998;69:146-148.

Nimmo, Elaine R., et al. "Defective meiosis in telomere-silencing mutants of Schizosaccharomyces pombe." Nature 392.6678 (1998): 825-828.

Oates RD, Silber S, Brown LG, Page DC. Clinical characterization of 42 oligospermic or azoospermic men with microdeletion of the AZFc region of the Y chromosome, and of 18 children conceived via ICSI. Hum Reprod. 2002;17:2813-2824.

Obradors A, Rius M, Daina G, Ramos L, Benet J, Navarro J. Whole-Chromosome Aneuploidy Analysis in Human Oocytes: Focus on Comparative Genomic Hybridization. Cytogenetic and Genome Research. 2011;133:119-126.

Offenberg HH, Schalk JA, Meuwissen RL, et al. SCP2: a major protein component of the axial elements of synaptonemal complexes of the rat. Nucleic Acids Res. 1998;26:2572-2579.

O'Flynn O'Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: A review. Fertil Steril. 2010;93:1-12.

Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T, Saitou M. A Signaling Principle for the Specification of the Germ Cell Lineage in Mice. Cell. 2009;137:571-584.

Oliva R. Protamines and male infertility. Hum Reprod Update. 2006;12:417-435.

Oliver TR, Bhise A, Feingold E, Tinker S, Masse N, Sherman SL. Investigation of factors associated with paternal nondisjunction of chromosome 21. American Journal of Medical Genetics Part A. 2009;149A:1685-1690.

Oliver TR, Middlebrooks CD, Tinker SW, et al. An examination of the relationship between hotspots and recombination associated with chromosome 21 nondisjunction. PloS one. 2014;9:e99560.

O'Sullivan JO, Karlseeder J. Telomeres: protecting chromosomes against genome instability. Nat Rev Mol Cell Biol 2010;11:171–81.

Pacchierotti F, Adler I-, Eichenlaub-Ritter U, Mailhes JB. Gender effects on the incidence of aneuploidy in mammalian germ cells. Environ Res. 2007;104:46-69.

Page SL, Hawley RS. Chromosome Choreography: The Meiotic Ballet. Science. 2003;301:785-789.

Pan J, Neale MJ, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. Nature. 2005;436:1053-1057.

Paoloni-Giacobino A, Morris MA, Dahoun SP. Prenatal supernumerary r(16) chromosome characterized by multiprobe FISH with normal pregnancy outcome. Prenat Diagn. 1998;18:751.

Payer B. Developmental regulation of X-chromosome inactivation. Semin Cell Dev Biol. 2016.

Pellestor F, Andréo B, Anahory T, Hamamah S. The occurrence of aneuploidy in human: lessons from the cytogenetic studies of human oocytes. European Journal of Medical Genetics. 2006;49:103-116.

Peoples-Holst TL, Burgess SM. Multiple branches of the meiotic recombination pathway contribute independently to homolog pairing and stable juxtaposition during meiosis in budding yeast. Genes Dev. 2005;19:863-874.

Pratto F, Brick K, Khil P, Smagulova F, Petukhova GV, Camerini-Otero RD. Recombination initiation maps of individual human genomes. Science. 2014;346:1256442-1256442.

Ravindranath N, Dalal R, Solomon B,DjakiewD, DymM. Loss of telomerase activity during male germ cell differentiation. Endocrinology 1997;138:4026–9.

Reeves A. (2001) MicroMeasure: a new computer program for the collection and analysis of cytogenetic data. Genome, 44, 439-443.

Reig-Viader R, Capilla L, Vila-Cejudo M, et al. Telomere homeostasis is compromised in spermatocytes from patients with idiopathic infertility. Fertil Steril. 2014a;102:728-U434.

Reig-Viader R, Vila-Cejudo M, Vitelli V, Sabat_e M, Giulotto E, Cald_es MG, et al. Telomeric repeat-containing RNA (TERRA) and telomerase are components of telomeres during mammalian gametogenesis. Biol Reprod 2014b; 90:103.

Reig-Viader R, Brie~no-Enríquez MA, Khouriauli L, Toran N, Cabero L, Giulotto E, et al. Telomeric repeat-containing RNA and telomerase in human fetal oocytes. Hum Reprod 2013;28:414–22.

Ren H, Chow V, Ma S. Meiotic behaviour and sperm aneuploidy in an infertile man with a mosaic 45,X/46,XY karyotype. Reproductive biomedicine online. 2015;31:783.

Ren, H., Ferguson, K., Kirkpatrick, G., Vinning, T., Chow, V., & Ma, S. (2016). Altered Crossover Distribution and Frequency in Spermatocytes of Infertile Men with Azoospermia. *PloS one, 11*(6), e0156817.

Riedel CG, Gálová M, Mori S, et al. Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. Nature. 2006;441:53-61.

Riou L, Bastos H, Lassalle B, Coureuil M, Testart J, Boussin FD, et al. The telomerase activity of adult mouse testis resides in the spermatogonial alpha6-integrin-positive side population enriched in germinal stem cells. Endocrinology 2005;146:3926–32.

Robinson WP, Kuchinka BD, Bernasconi F, et al. Maternal meiosis I non-disjunction of chromosome 15: dependence of the maternal age effect on level of recombination. Hum Mol Genet. 1998;7:1011-1019.

Rockmill B, Voelkel-Meiman K, Roeder GS. Centromere-Proximal Crossovers Are Associated With Precocious Separation of Sister Chromatids During Meiosis in Saccharomyces cerevisiae. Genetics. 2006;174:1745-1754.

Rockmill B, Lefrançois P, Voelkel-Meiman K, Oke A, Roeder GS, Fung JC. High throughput sequencing reveals alterations in the recombination signatures with diminishing Spo11 activity. PLoS genetics. 2013;9:e1003932.

Rodrigo L, Rubio C, Mateu E, Simon C, Remoh J, Pellicer A, Gil-Salom M. (2004) Analysis of chromosomal abnormalities in testicular and epididymal spermatozoa from azoospermic ICSI patients by fluorescence in-situ hybridization. Hum Reprod., 19, 118-123.

Rodrigo L, Rubio C, Peinado V, et al. Testicular sperm from patients with obstructive and nonobstructive azoospermia: aneuploidy risk and reproductive prognosis using testicular sperm from fertile donors as control samples. Fertil Steril. 2011;95:1005-1012.

Ross L.O., Rankin, S., Flatters, M., and Dawson, D. 1996. Effects of homology, size and exchange on the meiotic segregation of model chromosomes in Saccharomyces cerevisiae. Genetics 142: 79-89.

Ruan J, He X, Du W, et al. Genetic Variants in TEX15 Gene Conferred Susceptibility to Spermatogenic Failure in the Chinese Han Population. Reproductive Sciences. 2012;19:1190-1196.

Rudak E, Jacobs P, Yanagimachi R. (1978) Direct analysis of the chromosome constitution of human spermatozoa. Nature, 274, 911-913.

Sacristan C, Kops GJPL. Joined at the hip: kinetochores, microtubules, and spindle assembly checkpoint signaling. Trends Cell Biol. 2015;25:21-28.

Sanderson ML, Hassold TJ, Carrell DT. Proteins involved in meiotic recombination: a role in male infertility? Systems biology in reproductive medicine. 2008;54:57-74.

Savage AR, Petersen MB, Pettay D, Taft L, Allran K, Freeman SB, Karadima G, Avramopoulos D, Torfs C,
Mikkelsen M, Hassold TJ, Sherman SL. (1998) Elucidating the mechanisms of paternal non-disjunction of chromosome 21 in humans. Hum Mol Genet., 7, 1221-1227.

Schrader M,M€uller M, Heicappell R, Krause H, Schulze W, Miller K. Telomerase activity and expression of telomerase subunits in the testicular tissue of infertile patients. Fertil Steril 2000;73:706–11.

Scherthan H. Telomeres and meiosis in health and disease: Telomere attachment and clustering during meiosis. Cellular and Molecular Life Sciences. 2007;64:117-124.

Scherthan H. A bouquet makes ends meet. Nature Reviews Molecular Cell Biology. 2001;2:621-627.

Ségurel L, Leffler EM, Przeworski M. The case of the fickle fingers: how the PRDM9 zinc finger protein specifies meiotic recombination hotspots in humans. PLoS biology. 2011;9:e1001211.

Serebrovska ZA, Serebrovskaya TV, Pyle RL, Di Pietro ML. Transmission of male infertility and intracytoplasmic sperm injection (mini-review). Fiziolohichnyĭ zhurnal (Kiev, Ukraine : 1994). 2006;52:110.

Shinohara A, Shinohara M. Roles of RecA homologues Rad51 and Dmc1 during meiotic recombination. Cytogenetic and Genome Research. 2004;107:201-207.

Smagulova F, Gregoretti IV, Brick K, Khil P, Camerini-Otero RD, Petukhova GV. Genome-wide analysis reveals novel molecular features of mouse recombination hotspots. Nature. 2011;472:375-378.

Speed RM. Oocyte development in XO foetuses of man and mouse: the possible role of heterologous X-chromosome pairing in germ cell survival. Chromosoma. 1986;94:115-124.

Staessen C, Platteau P, Van Assche E, et al. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. Hum Reprod. 2004;19:2849-2858.

Steinman K, Ross J, Lai S, Reiss A, Hoeft F. Structural and functional neuroimaging in Klinefelter (47,XXY) syndrome: a review of the literature and preliminary results from a functional magnetic resonance imaging study of language. Developmental disabilities research reviews. 2009;15:295-308.

Stouffs K, Vandermaelen D, Massart A, et al. Array comparative genomic hybridization in male infertility. Human Reproduction. 2012;27:921-929.

Su Y, Barton AB, Kaback DB. Decreased meiotic reciprocal recombination in subtelomeric regions in Saccharomyces cerevisiae. Chromosoma. 2000;109:467-475.

Subramanian VV, Hochwagen A. The meiotic checkpoint network: step-by-step through meiotic prophase. Cold Spring Harbor perspectives in biology. 2014;6:a016675.

Sun F, Oliver-Bonet M, Liehr T, Starke H, Trpkov K, Ko E, Rademaker A, Martin RH. (2005a) Discontinuities and unsynapsed regions in meiotic chromosomes have a cis effect on meiotic recombination patterns in normal human males. Hum Mol Genet., 15, 3013-3018.

Sun, F., Greene, C., Turek, P.J., Ko, E., Rademaker, A. and Martin, R. H. (2005b) Immunofluorescent synaptonemal complex analysis in azoospermic men. Cytogenet Genome Res 111:366–370.

Sun, F., Oliver-Bonet, M., Liehr, T., Starke, H., Ko, E., Rademaker, A., et al. (2004) Human male recombination maps for individual chromosomes. Am J Hum Genet 74:521–531.

Sun, F., Oliver-Bonet, M., Liehr, T., Starke, H., Turek, P., Ko, E., et al. (2006a) Analysis of non-crossover bivalents in pachytene cells from 10 normal men. Hum Reprod 21:2335–2339.

Sun, F., Oliver-Bonet, M., Liehr, T., Starke, H., Turek, P., Ko, E., et al. (2006b) Variation in MLH1 distribution in recombination maps for individual chromosomes from human males. Hum Mol Genet 15:2376–2391.

Sun F, Oliver-Bonet M, Liehr T, Starke H, Ko E, Rademaker A, Martin RH. (2007a) Discontinuities and unsynapsed regions in meiotic chromosomes have a trans effect on meiotic recombination of some chromosomes in human males. Cytogenet Genome Res., 119, 27-32.

Sun, F., Turek, P., Greene, C., Ko, E., Rademaker, A. and Martin, R.H. (2007b) Abnormal progression through meiosis in men with nonobstructive azoospermia. Fertil Steril 87:565–571.

Sun F, Mikhaail-Philips M, Oliver-Bonet M, et al. Reduced meiotic recombination on the XY bivalent is correlated with an increased incidence of sex chromosome aneuploidy in men with non-obstructive azoospermia. Mol Hum Reprod. 2008;14:399-404.

Sutton KA. Molecular mechanisms involved in the differentiation of spermatogenic stem cells. Rev Reprod. 2000;5:93-98.

Tang SS, Gao H, Robinson WP, Ho Yuen B, Ma S. An association between sex chromosomal aneuploidy in sperm and an abortus with 45,X of paternal origin: possible transmission of chromosomal abnormalities through ICSI. Hum Reprod. 2004;19:147-151.

Tease C, Hultén MA. Inter-sex variation in synaptonemal complex lengths largely determine the different recombination rates in male and female germ cells. Cytogenetic and Genome Research. 2004;107:208-215.

Tease C, Hartshorne GM, Hultén MA. Patterns of Meiotic Recombination in Human Fetal Oocytes. The American Journal of Human Genetics. 2002;70:1469-1479.

Telvi L, Lebbar A, Del Pino O, Barbet JP, Chaussain JL. 45,X/46,XY Mosaicism: Report of 27 Cases. Pediatrics. 1999;104:304-308.

Tempest HG. Meiotic recombination errors, the origin of sperm aneuploidy and clinical recommendations. Systems Biology in Reproductive Medicine. 2011;57:93-101.

Templado C, Uroz L, Estop A. New insights on the origin and relevance of aneuploidy in human spermatozoa. Mol Hum Reprod. 2013;19:634.

Templado C, Vidal F, Estop A. Aneuploidy in human spermatozoa. Cytogenet. Genome Res. 2011; 133:91–99.

Terada Y, Luetjens CM, Sutovsky P, Schatten G. Atypical decondensation of the sperm nucleus, delayed replication of the male genome, and sex chromosome positioning following intracytoplasmic human sperm injection (ICSI) into golden hamster eggs: does ICSI itself introduce chromosomal anomalies? Fertil Steril. 2000;74:454-460.

Terasawa M, Ogawa H, Tsukamoto Y, et al. Meiotic Recombination-Related DNA Synthesis and Its Implications for Cross-Over and Non-Cross-Over Recombinant Formation. Proc Natl Acad Sci U S A. 2007;104:5965-5970.

Thilagavathi J, Venkatesh S, Dada R. Telomere length in reproduction. Andrologia. 2013;45:289-304

Thomas NS, Hassold TJ. Aberrant recombination and the origin of Klinefelter syndrome. Hum Reprod Update. 2003;9:309-317.

Topping D, Brown P, Judis L, et al. Synaptic defects at meiosis I and non-obstructive azoospermia. Hum Reprod. 2006;21:3171-3177.

Touati SA, Wassmann K. How oocytes try to get it right: spindle checkpoint control in meiosis. Chromosoma. 2016;125:321.

Treff NR, Levy B, Su J, Northrop LE, Tao X, Scott J,Richard T. SNP microarray-based 24 chromosome aneuploidy screening is significantly more consistent than FISH. Mol Hum Reprod. 2010;16:583-589.

Treff NR, Northrop LE, Kasabwala K, Su J, Levy B, Scott RT. Single nucleotide polymorphism microarray–based concurrent screening of 24-chromosome aneuploidy and unbalanced translocations in preimplantation human embryos. Fertil Steril. 2011;95:1606-1612.e2.

Treff N, Su J, Taylor D, Scott R. Telomere DNA Deficiency Is Associated with Development of Human Embryonic Aneuploidy. PLOS GENETICS. 2011;7:e1002161.

Trelles-Sticken, Edgar, Michael E. Dresser, and Harry Scherthan. "Meiotic telomere protein Ndj1p is required for meiosis-specific telomere distribution, bouquet formation and efficient homologue pairing." The Journal of cell biology 151.1 (2000): 95-106.

Turner, James M. A.. Meiotic sex chromosome inactivation. Development. 2007;134:1823-1831.

Upadhyay RD, Kumar AV, Ganeshan M, Balasinor NH. Tubulobulbar complex: cytoskeletal remodeling to release spermatozoa. Reproductive biology and endocrinology : RB&E. 2012;10:27-27.

Uroz L, Templado C. Meiotic non-disjunction mechanisms in human fertile males. Hum Reprod. 2012;27:1518.

Ushijima1 C, Kumasako Y, Kihaile PE, Hirotsuru K, Utsunomiya T. Andrology. Analysis of chromosomal abnormalities in human spermatozoa using multi-colour fluorescence in-situ hybridization. Human Reproduction. 2000;15:1107.

Vegetti W, Van Assche E, Frias A, et al. Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence in-situ hybridization in infertile men. Hum Reprod. 2000;15:351-365.

Vozdova M, Heracek J, Sobotka V, Rubes J. Testicular sperm aneuploidy in non-obstructive azoospermic patients. Hum Reprod. 2012;27:2233.

Wassmann K. Sister chromatid segregation in meiosis II: Deprotection through phosphorylation. Cell Cycle. 2013;12:1352-1359.

Watanabe Y, Cooper JP, Nurse P. Fission yeast Taz1 protein is required for meiotic telomere clustering and recombination. Nature. 1998;392:828-831.

Wen Q, Cheng CY, Liu Y. Development, function and fate of fetal Leydig cells. Semin Cell Dev Biol. 2016.

Witczak B, Klusek JE, Klusek J. Genetic aspects of male infertility. Medical Studies. 2014; 2015;4:276-279.

Wong EC, Ferguson KA, Chow V, Ma S. Sperm aneuploidy and meiotic sex chromosome configurations in an infertile XYY male. Human Reproduction. 2008; 2007;23:374-378.

World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization; 2010.

Wosnitzer MS. Genetic evaluation of male infertility. Translational andrology and urology. 2014;3:17.

Wu H, Burgess SM. Ndj1, a Telomere-Associated Protein, Promotes Meiotic Recombination in Budding Yeast. Mol Cell Biol. 2006;26:3683-3694.

Yamamoto M. INTERCHROMOSOMAL EFFECTS OF HETEROCHROMATIC DELETIONS ON RECOMBINATION IN DROSOPHILA MELANOGASTER. Genetics. 1979;93:437-448.

Yamamoto A, Hiraoka Y. Monopolar spindle attachment of sister chromatids is ensured by two distinct mechanisms at the first meiotic division in fission yeast. EMBO J. 2003;22:2284-2296.

Yang F, Eckardt S, Leu NA, McLaughlin KJ, Wang PJ. Mouse TEX15 Is Essential for DNA Double-Strand Break Repair and Chromosomal Synapsis during Male Meiosis. J Cell Biol. 2008;180:673-679.

Yang F, Silber S, Leu NA, et al. TEX11 is mutated in infertile men with azoospermia and regulates genome-wide recombination rates in mouse. EMBO Molecular Medicine. 2015;7:1198-1210.

Yashima, Kazuo, et al. "Expression of the RNA component of telomerase during human development and differentiation." Cell Growth and Differentiation-Publication American Association for Cancer Research 9.9 (1998): 805-814.

Yuan L, Liu J, Zhao J, Brundell E, Daneholt B, Höög C. The Murine SCP3 Gene Is Required for Synaptonemal Complex Assembly, Chromosome Synapsis, and Male Fertility. Mol Cell. 2000;5:73-83.

Zalenskaya I, Zalensky A. Telomeres in mammalian male germline cells. In: Vol 218. SAN DIEGO: ELSEVIER ACADEMIC PRESS INC; 2002:37-67.

Zaragoza MV, Millie E, Redline RW, Hassold TJ. Studies of non-disjunction in trisomies 2, 7, 15, and 22: does the parental origin of trisomy influence placental morphology? J Med Genet. 1998;35:924-931.

Zenzes MT, Casper RF. Cytogenetics of human oocytes, zygotes, and embryos after in vitro fertilization. Hum Genet. 1992;88:367-375.

Zheng Y, Li L, Zhou L, et al. Alterations in the frequency of trinucleotide repeat dynamic mutations in offspring conceived through assisted reproductive technology. Hum Reprod. 2013;28:2570.

Zickler D, Kleckner N. Recombination, Pairing, and Synapsis of Homologs during Meiosis. Cold Spring Harbor perspectives in biology. 2015;7:a016626.

APPENDIX I: PATIENT FLOW CHART