Joint Biotechnology Master Program

Palestine Polytechnic University
Deanship of Graduate Studies and Scientific Research

Bethlehem University
Faculty of Science

A Truncating *MEIOB* Homozygous Frame-shift Mutation Contributes in a Critical Way to Non-Obstructive Azoospermia in Five Palestinian-Arab Infertile Males

By

Islam Issa Abu Allan

In Partial Fulfillment of the Requirements for the Degree

Master of Biotechnology

September, 2020

Biotechnology Master Program

The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

"A Truncating *MEIOB* Homozygous Frame-shift Mutation Contributes in a Critical Way to Non-Obstructive Azoospermia in Five Palestinian-Arab Infertile Males"

by

Islam Issa Abu Allan

A thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology.

Graduate Advisory Committee:

Committee Member (Student's Supervisor)	15-2-2021 Date
Dr. Hashem Shahin, Bethlehem University	
Committee Member (Internal Examiner)	16.2.2021 Date
Dr. Omar Darissa, Bethlehem University	
Committee Member (External Examiner)	7.2.2021 Date
Dr. Saad Allahham, An-Najah National University	

Approved for the Faculties

Dean of Graduate Studies and Scientific Research	Dean of Faculty of Science
Palestine Polytechnic University	Bethlehem University
Date	17-2-2021 Date

"A Truncating *MEIOB* Homozygous Frame-shift Mutation Contributes in a Critical Way to Non-Obstructive Azoospermia in Five Palestinian-Arab Infertile Males"

by Islam Issa Abu Allan

ABSTRACT

Up to 12% of couples who are trying to get a successful pregnancy will fail after a year of attempts due to infertility. The contribution of male factor accounts for 20-30% of infertility cases. Although there is a growing list of diagnostic tools that successfully identify the etiology of male infertility, yet, 40% of cases are of unknown etiology, and most of them are expected to be due to genetic factors alterations. MEIOB gene encodes for a single-strand DNA binding protein which is together with SPATA22 and RPA are involved in proper chromosomal synapsis, crossover formation, and DNA double-strand break repair. MEIOB null mice showed sterility in both sexes. Herein, using direct Sanger sequencing technique we screened for MEIOB mutations in eighty two Palestinian Arab males with non-obstructive azoospermia, two females with ovarian dysgenesis, and two hundred fertile males as a control. All infertile males underwent Y-chromosome microdeletion assessment. We identified a homozygous frame-shift deletion mutation that leads to a premature stop codon (1,889,376 del G MEIOB S366fs*12) in five males, and interestingly in the two infertile females, and this mutation was not detected in any of the healthy controls. This mutation is predicted to produce a truncated MEIOB proteins which lacks the conserved C'-terminal DNA binding domain and may lead to meiotic arrest and thus, infertility.

"A Truncating *MEIOB* Homozygous Frame-shift Mutation Contributes in a Critical Way to Non-Obstructive Azoospermia in Five Palestinian-Arab Infertile Males"

الملخص

إنّ ما يقارب 12% من الأزواج الذين يحاولون تحقيق حمل ناجح سوف يفشلون بعد سنة كاملة من المحاولات بسبب العقم. تساهم العوامل الذكرية في 20-30% من الحالات. وبالرغم من وجود قائمة متزايدة من أدوات التشخيص التي تحدد بنجاح مسببات العقم عند الذكور، إلا أنّ 40% من الحالات تعود لأسباب غير معروفة ومن المتوقع أن يكون معظمها تغيرات في العوامل الجينية. يشفر جين MEIOB بروتين رابط للسلسلة الأحادية للحمض النووي والذي بالتعاون مع بروتين SPATA22 وبروتين RPA يساهم في تشابك الكروموسومات بشكل سليم، تشكل عملية العبور، وإصلاح عملية قطع سلسلتي الحمض النووي أثناء عملية الإنقسام المنصف. كما أن إزالة هذا الجين من الفئران أدى إلى العقم في كلا الجنسين. هنا في هذه الدراسة، قمنا باستخدام طريقة سانجر لقراءة التسلسل النيوكليوتيدي للجين للبحث في وجود أية طفرات في اثنين وثمانين من الذكور العرب الفلسطينيين الذين يعانون من انعدام أو نقص عدد الحيوانات المنوية (العقم)، واثنتان من الإناث اللواتي يعانن من خلل في المبايض وهما قريبتين لأحد الذكور العقيمين، ومئتي ذكراً غير عقيمين. خضع جميع الذكور العقيمين أيضاً لفحص طفرات الحذف على كروموسوم Y. لقد تمكنّا من الكشف عن طفرة حذف متنحية تؤدي إلى إزاحة إطار القراءة وبالتالي كودون توقف مبكر (1,889,376 del G MEIOB S366fs*12) في خمسة ذكور عقيمين، ومن المثير للاهتمام أن الأنثيين العقيمتين مصابتان بهذه الطفرة أيضاً. أما في الذكور غير العقيمين فلم يكن أحداً منهم مصاباً بهذه الطفرة. من المتوقع أن هذه الطفرة تؤدي إلى بروتين غير كامل مقطوع في جزء مهم للربط مع سلاسل الحمض النووي وهو C' -terminal مؤدّياً إلى توقف عملية الانقسام المنصف وبالتالي حدوث العقم.

DECLARATION

I declare that the Master Thesis entitled "dissertation title" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: _____"Islam Issa Abu Allan"____

Date_____

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for the joint master degree in biotechnology at Palestine Polytechnic University and Bethlehem University, I agree that the library shall make it available to borrowers under rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from, reproduction, or publication of this thesis may be granted by my main supervisor, or in [his/her] absence, by the Dean of Higher Studies when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature: _____"Islam Issa Abu Allan"_____

Date_____

Dedication

This thesis work is wholeheartedly dedicated to my family. With a special feeling of gratitude to my beloved parents, Issa Abu Allan and Julia Tavger. To my grandparents Leonid Tavger and Lyudmila Tavger. They all have been my source of inspiration, encouragement, support, and push for tenacity. To my siblings Katreen, Abeer, and Obaida Abu Allan who have been always by my side.

I dedicate this thesis work to my best friend Lama Juneidy and thank her for being my lab partner, who worked with me on this project and other existing research projects that were running in the lab. I express my deepest gratitude for her help, constant support, and for being my best friend.

Acknowledgment

I would like to express my deepest appreciation to my supervisor Dr. Hashem Shahin, for his great supervision, guidance, scientific feedback, and kind advice throughout my masters and even before. It was a real privilege and honor for me to be one of his students, to start my molecular laboratory practical experience at his lab, and to share of his scientific knowledge and great human qualities.

Besides my supervisor, I would like to thank the rest of my thesis committee: Dr. Omar Dar Issa, and Dr. Saad Allaham for their insightful comments and suggestions.

I would like also to express my sincere gratitude to all my professors (Dr. Muneef Ayyash, Prof. Mazin Qumsiyeh, Dr. Ghassan Handal, Dr. Mousa Hindiyeh, Dr. Omar Issa, Prof. Moien Kanaan, Dr. Robin Abu Ghazaleh, Dr. Yaqoub Ashhab, Dr. Rami Arafeh, Dr. Fouad Zahdeh, and Dr. Areej Al khatib), who were more than generous with their knowledge, expertise, and precious time throughout master's program.

I would like to deeply thank Dr. Iyad Afaneh and all his team at Palestinian-European Fertilization Clinic and Dr. Sahar Araj for their cooperation to our work by facilitating the process of meeting with all infertile individuals recruited to this study. And of course, I owe my gratitude to all participating patients who made it possible to conduct our study. Special thanks to my lab colleagues for their assistance and support. Thanks to Rua' Thawabteh, Amal Abu Rayyan, Reena Saed, Christina Canavati, and Grace Rabie'.

Abbreviations:

AIS	Androgen Insensitivity Syndrome
AnP	Antarctic Phosphatase
AR	Androgen Receptor
AUKC	Aurora Kinase C
AZF	Azoospermia Factor
CAVD	Congenital Absence of the Vas Deferens
CBAVD	Congenital Bilateral Absence of the Vas Deferens
Cen	Centromere
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CGH	Comparative Genomic Hybridization
CHH	Congenital Hypogonadotropic Hypogonadism
CNV	Copy Number Variation
dHJ	Double-Holliday Junction
D-loop	Displacement loop
DMC1	DNA Meiotic Recombinase 1
DNA	Deoxyribonucleic acid
dpc	days post-coitum
dpp	days post-partum
DSBs	Double Strands Breaks
dsDNA	double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
Exo I	Exonuclease I
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropic Releasing Hormone
ICMART	International Committee for Monitoring Assisted Reproductive Technology
ICSI	Intra-Cytoplasmic Sperms Injection

KEGG SSDB	Sequence Similarity Data Base of the Kyoto Encyclopedia of Genes and Genomes
LH	Luteinizing Hormone
MAIS	Mild Androgen Insensitivity Syndrome
MEIOB	Meiosis-specific with OB-domain
MMAF	Morphological Abnormalities of the Sperm Flagella
NAHR	Non-allelic Homologous Recombination
NGS	Next Generation Sequencing
NOA	Non-Obstructive Azoospermia
PAIS	Partial Androgen Insensitivity Syndrome
PAR	Pseudo-autosomal Region
PCD	Primary Ciliary Dyskinesia
PCR	Polymerase Chain Reaction
PGD	Preimplantation Genetic Diagnosis
RAD51	Recombinase Radiation Sensitive 51
RBCs	Red Blood Cells
RPA	Replication Protein A
rpm	Rounds per minute
S	Serine
SC	Synaptonemal Complex
SCOS	Sertoli Cell Only Syndrome
SDSA	Synthesis-dependent strand annealing
SDS	Sodium Dodecyl Sulfate
SGA	Spermatogenic Arrest
SNPs	Single Nucleotide Polymorphisms
SPATA22	Spermatogenesis-associated protein 22
SRY	Sex-determining Region of Y-Chromosome
SSCs	Spermatogonial Stem Cells
ssDNA	Single-strand DNA

TAE	Tris-acetate-EDTA
TESE	Testicular Sperm Extraction
TEX14	Testis-expressed gene 14
UV	Ultra Violet
WBCs	White Blood Cells
WGA	Whole Genome Analysis
WHO	World Health Organization
ZFX	Zinc-Finger Protein X-Linked
ZFY	Zinc-Finger Protein Y-Linked

List of Figures:

List of Tables:

Table of Content:

CHAPTER 1

Introduction

Infertility is a reproductive system disease which affects about 8-12% of reproductive-aged couples, who are trying for more than twelve months to achieve a successful clinical pregnancy but fail (Inhorn and Patrizio, 2015). It is considered a global health issue since it affects the suffering couples emotionally, financially, and socially (Greil, Slauson-Blevins and McQuillan, 2010). Male factors account for about 20-30% of infertility cases (Agarwal *et al.*, 2015), with the most severe form called azoospermia, which is characterized by the absence of spermatozoa in the semen (Ferlin, Dipresa and Foresta, 2019). Although 15-20% of severe cases are due to identifiable genetic causes (Ferlin, Dipresa and Foresta, 2019), 50% of the cases are considered idiopathic, and genetic factors that are not figured out yet may contribute to large proportion of these cases (Krausz and Consultant, 2011).

Several genetic tests are currently available to identify the aetiology of male infertility, yet, they are limited and include karyotype analysis, screening for azoospemia factor (AZF) microdeletions, screening for some candidate single gene mutations such as CFTR gene (Krausz and Riera-Escamilla, 2018). Moreover, patients who have non-obstructive azoospermia usually undergo invasive clinical processes such as testicular sperms extraction (TESE) surgeries and/or testicular biopsies, which could be useless if the patient has zero sperms (Gershoni *et al.*, 2019). Thus, it is important to reveal the unidentified genetic factors and confirm their role in spermatogenesis process, to help in improving and developing non-invasive diagnostic tests especially in these cases with genetic mutations that cause complete spermatogenesis impairment; i.e., no need to perform invasive surgeries to search for sperms if the case has a pathogenic mutation in a genetic factor which is known to cause azoospermia with complete absence of sperms in the semen, and could be easily detected.

As a part of studying genetic aetiology of male infertility in the Palestinian population, we have applied direct Sanger sequencing to screen for MEIOB gene mutations in eighty two infertile Palestinian-Arab males suffering from oligospermia, severe oligospermia, or non-obstructive azoospermia (NOA), and two additional females diagnosed with ovarian dysgenesis and who are relatives of one azoospermic male. We identified a homozygous recessive probably causative frame-shift mutation in exon 12 of MEIOB gene in five azoospermic males from four consanguineous families, and interestingly in two related females with ovarian dysgenesis. This is actually not surprising, since MEIOB encodes for meiosis-specific with OB-domain protein which is a highly conserved single-strand DNA binding protein (Luo *et al.*, 2013; Souquet *et al.*, 2013).

MEIOB is involved in meiotic double-strand breaks repair, chromosomal second-end capture, crossover formation, and appropriate chromosomal synapsis during meiosis. Lack of MEIOB in mice led to sterility in both male and female mice due to meiotic arrest (Luo *et al.*, 2013) (Souquet *et al.*, 2013), and this phenotype in mice is actually consistent with our findings. In our study, a recessive homozygous deletion of G on 1889376 of chromosome 16 (NM_001163560; Chr16: 1889376 del G MEIOB, exon 12 – S366fs*) was identified in five male individuals diagnosed with NOA and two female individuals diagnosed with ovarian dysgenesis. This mutation resulted in a premature stop codon, and is predicted to cause a truncation in the C'-terminal DNA binding domain of the MEIOB protein since the C'-terminal part of MEIOB locates between 294 and 450 including SPATA22-binding fold. Therefore, it is expected that this truncation may affect the interaction between MEIOB and its co-factor SPATA-22, and thus, crossover formation insufficiency. Same and different mutations in MEIOB gene were reported in three recent studies, and all of them were found in infertile individuals of Arab origins (Gershoni *et al.*, 2017, 2019; Caburet *et al.*, 2019).

The exact same MEIOB gene mutation as reported in our study, was implicated in two Israeli-Arab brothers by Gershoni and colleagues at Weizmann Institute of Science (Gershoni *et al.*, 2019), guiding us to think that this mutation could be an Arab-founder

mutation. The Strength of our data is manifested in finding this mutation in five families with one family in which the affected male has two infertile female relatives with ovarian dysgenesis who are affected as well. Our results add valuable data together with other recent researches to prove the role of MEIOB in male infertility (i.e., NOA) and may be, in female infertility as well. For the latter, more data is required and will be further studied by the following master students.

CHAPTER 2

Male Infertility

2.1 Infertility Definition and Epidemiology

According to International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO), infertility is defined as a kind of reproductive system disease in which the sexually active, non-contracepting couple is not able to achieve clinical pregnancy in twelve months or more (Zegers-Hochschild *et al.*, 2009).

Infertility is considered a global health issue since it has serious relational, social, medical, financial, and emotional effects worldwide (Greil, Slauson-Blevins and McQuillan, 2010). It is estimated that infertility affects 8-12% of reproductive-aged couples around the world with some regions reaching approximately 30% including sub-Saharan Africa, Middle East and North Africa, South Asia and Central Asia, and Eastern and Central Europe (Inhorn and Patrizio, 2015). WHO states that about 50-80 million individuals around the world struggle with infertility (Rutstein and Shah, 2004).

Several large scale studies have showed that female factors account for 50% of all infertility cases, however, infertility due to male factors account for about 20-30%, and 20-30% of cases are due to contribution of both male and female factors (Sharlip *et al.*, 2002; Agarwal *et al.*, 2015). Interestingly, meta-analysis studies recently showed that 20-70% of infertility cases are due to male factors (Agarwal *et al.*, 2015), although they may not accurately represent the prevalence of this complication around the globe. This is mainly due to lack of precise comprehensive statistical methods that include all bias, cultural constraints, and data collection heterogeneity with less

available data in regions like developing countries (Babakhanzadeh *et al.*, 2020). Refer to (Figure 2.1) to see the distribution of percentages of infertility due to male factors around the globe (Agarwal *et al.*, 2015).

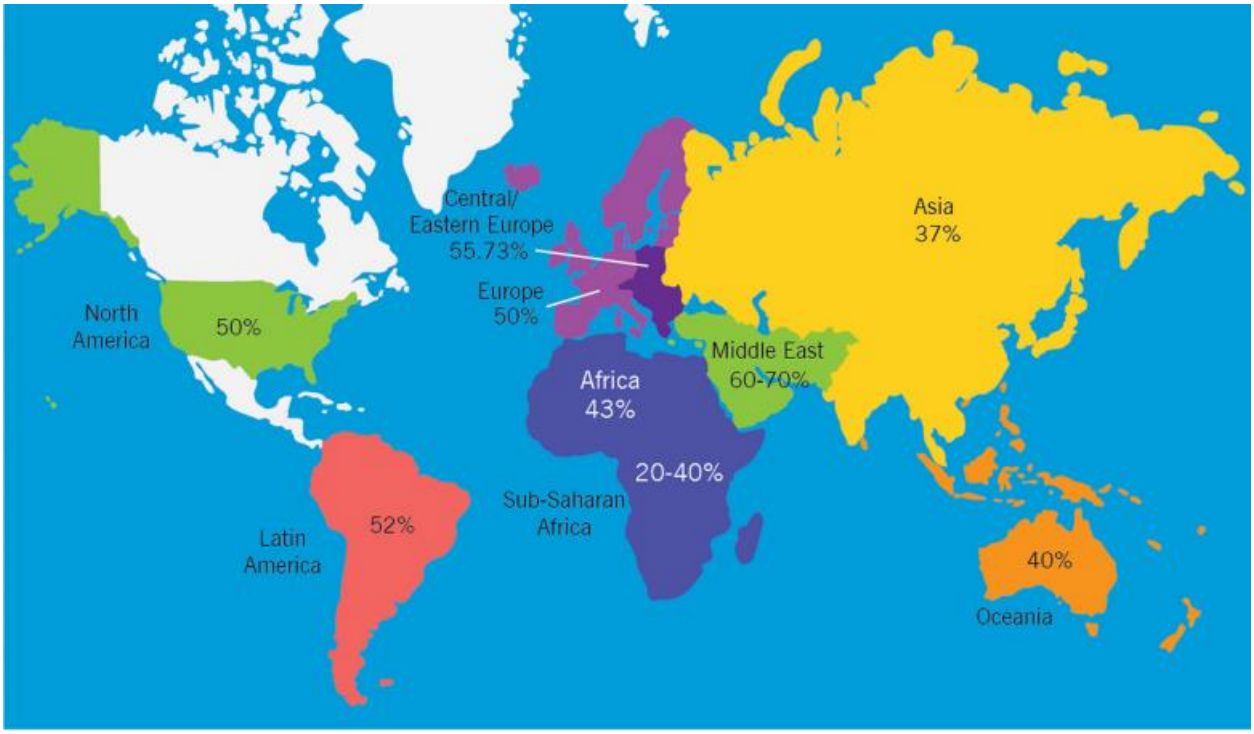


Figure 2.1: World map representing percentages of infertility due to involvement of male factors in the indicated studied regions around the globe (Agarwal *et al.*, 2015).

2.2 Aetiology of Male Infertility

The aetiology of male infertility is traditionally classified to three categories: pre-testicular, testicular, or post-testicular level. More recently, it is suggested to clinically categorize the aetiology of male infertility in terms of quantitative spermatogenesis, qualitative spermatogenesis, ductal obstruction, and hypothalamic-pituitary-gonadal axis function (Tournaye, Krausz and Oates, 2017). The most common aetiology of male infertility is due to primary testicular failure, and it accounts for about 75% of male infertility cases (Krausz, 2011). The second most common aetiology of male infertility is due to ductal obstruction, followed by secondary testicular failure (hypothalamic-pituitary-gonadal axis alterations), and then, the least common aetiology which is the disturbance in spermatogenic quality (Tournaye, Krausz and Oates, 2017). Interestingly, about

15-20% of severe male infertility cases are diagnosed to have identifiable genetic cause (Ferlin, Dipresa and Foresta, 2019). However, male infertility in about 50% of male infertility cases is still of unknown aetiology, and in this case it is considered as "idiopathic infertility", which most probably will be due to involvement of other genetic factors that have not been figured out yet (Krausz, 2011).

2.2.1 Quantitative Spermatogenic Impairment

Quantitative impairment of spermatogenesis affects the production and the number of produced sperms. It can appear in different seminal phenotypes according to semen analyses. The term normozoospermia or normospermia is used when the number of sperms in the ejaculate is more than 15 million/ mL (which is the normal state), cryptozoospermia when the number of produced sperms is less than one million/ mL, oligozoospermia when the number is less than 15 million/ mL, and azoospermia when there are no sperms in the ejaculate (Grimes and Lopez, 2007; Krausz and Riera-Escamilla, 2018). Quantitative spermatogenesis defects can occur due to the following:

2.2.1.1 Chromosomal Anomalies

Structural and numerical chromosomal anomalies lead to spermatogenic defects by interfering with chromosomes synapsis during meiosis or by affecting the gene expression in case of deletions, duplications, or breakpoints that cause chromosomal rearrangements which lead to removal or disturbance of promoters, or even the sequence of genes involved in spermatogenesis process (Sun *et al.*, 2007; Harewood and Fraser, 2014). About 15% of infertile males who are diagnosed with NOA and about 4% of those who are diagnosed with moderate oligospermia (< 10 million/ mL) have abnormal karyotype (chromosomal anomalies) (Jungwirth *et al.*, 2012; Krausz and Riera-escamilla, 2018).

Klinefelter syndrome (47, XXY or mosaic 46, XY/ 47, XXY) is considered to be the most common form of chromosomal anomalies that exists in non-obstructive azoospermia males. In the general population, it occurs in a frequency of 1 in 600, while in non-obstructive azoospermia males it occurs in a frequency of 1 in 7 (Punab *et al.*, 2017). More than 90% of individuals diagnosed with Klinefelter syndrome are azoospermic, however, the remaining have either severe oligospermia (< 5 million/ mL) or cryptospermia especially in mosaic variant of Klinefelter

syndrome (Krausz and Riera-Escamilla, 2018). The most frequent chromosomal anomalies in oligospermic males are either inversions or translocations, for example, de la Chapelle syndrome (46, XX male syndrome) in which most cases are due to SRY translocation on X-chromosome (80-90% of the cases) (Krausz and Riera-Escamilla, 2018). The remaining instances of this syndrome are due to activation of some other genes (i.e., SRY downstream genes) that are also required for testes development (Skaletsky *et al.*, 2003). It occurs in a frequency of 1 in 20,000 newborn males. All individuals with de la Chapelle syndrome are totally azoospermic due to absence of AZF region of Y-chromosome (Vorona *et al.*, 2007).

2.2.1.2 Submicroscopic Chromosomal Anomalies

2.2.1.2.1 Y Chromosome Microdeletions

In 1976, Tiepolo and Zuffardi have predicted that on the long arm of Y chromosome there are many genes essentially involved in spermatogenesis process. They predicted and proposed the azoospermia factor (AZF) by studying several azoospermia cases in which they observed de novo microdeletions on the long arm of Y chromosome (Tiepolo and Zuffardi, 1976). Then, these AZF regions on Y chromosome were studied at the molecular level by Vogt et al. and Skaletsky et al. (Vog *et al.*, 1996; Skaletsky *et al.*, 2003). After this, Y chromosome became an important target in male infertility. The boundaries of AZF regions are characterized by the presence of repeated homologous sequences which are through non-allelic homologous recombination (NAHR) highly predisposed to duplications or deletions, and it is also responsible for Y chromosome-linked copy number variations (CNVs), for example, removal of DNA segments that can reach about 0.8 Mb to 7.7 Mb occurs frequently on the five Yq fragile sites (Krausz *et al.*, 2014; Krausz and Casamonti, 2017).

Microdeletions are clinically classified to AZFa, AZFb, AZFbc, and AZFc (Krausz *et al.*, 2014; Krausz and Casamonti, 2017). All these regions are enriched with genes involved in testis development and function, and also in spermatogenesis process and most of them are expressed in the testis (Krausz and Casamonti, 2017). Refer to Figure 2.2 to see the locations of microdeletions including AZFa, AZFb, AZFbc, AZFc, and gr/gr (which is discussed in the next section below) on different regions on Y chromosome and the effect of each microdeletion (i.e., semen phenotype and testis histology) (Krausz and Riera-Escamilla, 2018).

Y-chromosome microdeletions are considered to be the second most frequent molecular genetic cause of idiopathic NOA and severe oligospermia, with higher incidence in azoospermic males (Krausz *et al.*, 2014). In general population, the frequency of AZF microdeletions is really rare, i.e. 1 in 4000. But its frequency significantly increases in infertile males to reach 5-10% in males with non-obstructive azoospermia and 2-5% in males with severe oligospermia, especially those who have less than 2 million sperms/ mL (Krausz *et al.*, 2014; Krausz and Casamonti, 2017; Krausz and Riera-Escamilla, 2018).

The majority of these microdeletions are de novo and occur during meiosis process in the gametes of affected individual's father. But some rare naturally transmitted cases are reported as well such as partial AZFa, partial AZFb, and complete AZFc (Krausz *et al.*, 2014). This transmission is not really surprising since these microdeletions could occur in oligospermic cases which could be compatible with natural pregnancies, especially when the female partner is strongly fertile (Krausz and Riera-Escamilla, 2018).

It is very important to test for the kind of AZF microdeletion to decide patient's candidacy to do Testicular Sperm Extraction (TESE) or not, since complete deletions of the AZFa and AZFb regions lead to azoospermia, and thus, a zero chance of getting sperms by doing testis biopsies (Krausz and Casamonti, 2017; Krausz and Riera-Escamilla, 2018). For example, those who have AZF microdeletions and still have the ability to produce sperms would be able to have biological children by extracting their sperms via TESE and then applying Intracytoplasmic Sperms Injection (ICSI). In this case, the deletion will be obligatory transmitted to their male offspring and consequently, they will have similar phenotype as well (spermatogenesis insufficiency). In some cases like in men who have AZFc microdeletion, it has been reported that they develop reduction in sperms production over time, and it is highly suggested to have a kind of cryopreservation for their sperms during their early adulthood for future fertility needs (Krausz *et al.*, 2014).

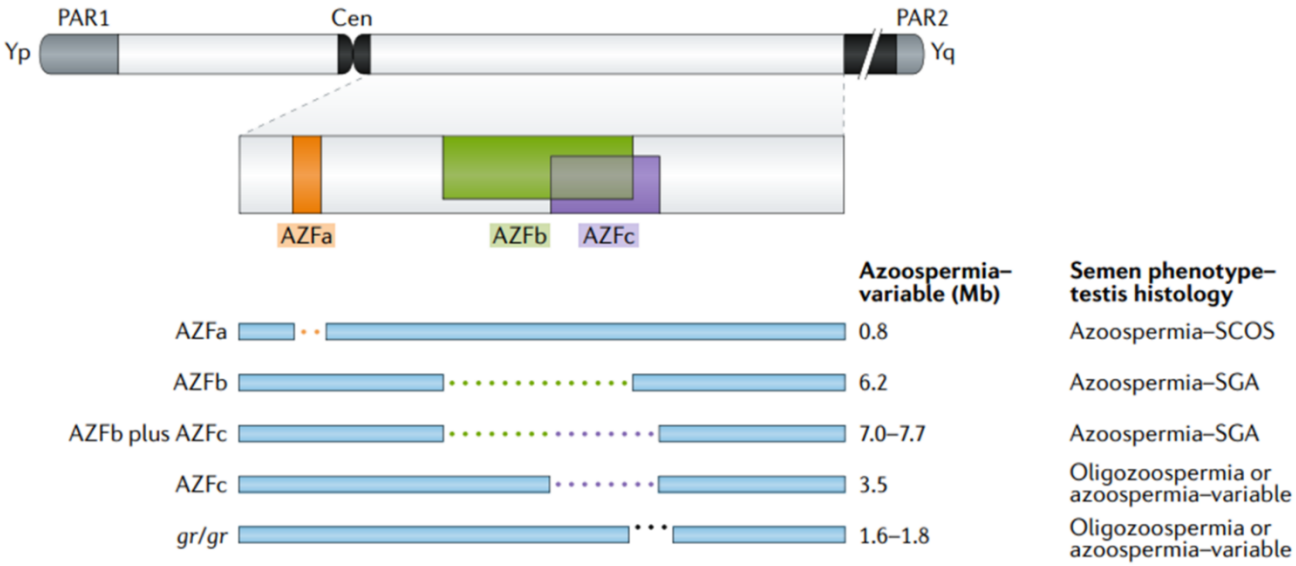


Figure 2.2: Represents Y-Chromosome and different kinds of microdeletions (AZF deletions and gr/gr deletions), their size (Mb), and the resulting phenotype (i.e., semen phenotype and testis histology). PAR stands for Pseudoautosomal Region. SGA stands for spermatogenic arrest. SCOS stands for Sertoli-cell-only syndrome. Cen stands for Centromere (Krausz and Riera-Escamilla, 2018).

2.2.1.2.2 gr/gr Deletion

AZFc region on Yq is very rich with hotspots for NAHR making it the most dynamic region on Y chromosome for partial deletions or duplications of AZFc genes. Among them, the most common and clinically relevant is the gr/gr deletion (partial AZFc deletion) (Repping *et al.*, 2003). This deletion removes half of AZFc region, and consequently, removing many critical genes and transcription units in this region which are involved in spermatogenesis process such as BPY2, DAZ, PRY, CDY1, RBMY, CSPG4LY, and many other genes referred by Repping and colleagues (Repping *et al.*, 2003). However, in some cases the phenotype resulted from gr/gr microdeletion may not lead to spermatogenesis impairment, and this is due to differences in Y-chromosome haplogroups between ethnic groups, for example, Yang and colleagues stated that in Chinese population there are no negative consequences of gr/gr microdeletions on spermatogenesis (Yang *et al.*, 2010).

1.2.1.3 Ductal Obstruction

To achieve a successful fertilization, it is not enough to have sperms of a good quality and quantity, but also to be successfully delivered via all parts of male proximal and distal urogenital tract (i.e., to pass intra-testicular, efferent duct, epididymis, vas deferens, ejaculatory duct, and urethra). Any interference with sperms transportation via this transportation system and any of its anatomical or neurological integrity is referred as ductal obstruction (Tournaye, Krausz and Oates, 2017).

Alterations in this transportation system cause impaired spermatogenesis, but testis histology appears normal. Congenital bilateral absence of the vas deferens (CBAVD) with normal renal anatomy is considered to be the only known congenital form of ductal obstruction with known genetic causes (Tournaye, Krausz and Oates, 2017; Krausz and Riera-Escamilla, 2018). However, most cases of ductal obstruction (approx. 80%) are secondary to recessive mutations in the gene called cystic fibrosis transmembrane conductance regulator (CFTR) gene which encodes for CFTR protein that is involved in chloride conduction across the membranes of epithelial cells. Ductal obstructions caused by these mutations are considered to be kind of genital forms of cystic fibrosis (Casals *et al.*, 2000) (Krausz and Riera-Escamilla, 2018). This disease leads to seminal vesicles agenesis and epididymis malformations. The severity of the disease depends on the nature of the inherited mutations, i.e., inheritance of two severe copies of the mutation leads to cystic fibrosis, however, inheritance of one mild and one severe copies or two mild copies leads to a less severe condition of cystic fibrosis such as CAVD (Estivill, Bancells and Ramos, 1997; Weiske *et al.*, 2000).

There is a panel of the most common mutations (about 30-50 mutations) in CTFR gene for different ethnicities, and they are routinely screened for suspected cases (de Souza *et al.*, 2018). It is necessary for any male who has CBAVD and planning to undergo TESE–ICSI to test for the CFTR mutations before the process of sperms harvesting, since there is a high risk of transmitting the mutation to his offspring. Given that the carrier frequency of CFTR mutations is high in some populations such as Europeans, it is highly recommended to test for the mutation in the partner as well and if both are carriers, then preimplantation genetic diagnosis (PGD) will be the choice for further steps (Krausz and Riera-Escamilla, 2018).

2.2.1.4 Monogenic Causes (Androgen Receptor Gene Mutations)

Testing for Androgen Receptor (AR) gene mutations in patients with quantitative spermatogenic impairment is the only introduced monogenic screening test in the current clinical practices since there are more than 1000 identified mutations in AR gene (Gottlieb *et al.*, 2012). AR gene locates on the X-chromosome and encodes for a protein called androgen receptor. It is required for appropriate response of the body to androgen hormones such as testosterone (Bennett *et al.*, 2010). When a certain androgen protein binds to its receptor (androgen receptor), the resulted complex binds the DNA and regulates the expression of androgen responsive genes that are responsible to direct the development of male and female features, such as hair growth, sex drive, and many other features (Bennett *et al.*, 2010).

There is a range of phenotypes resulting from different AR gene mutations, such as Androgen Insensitivity Syndrome (AIS) in which the individual genetically is a male (XY) with some or all female physical traits. Partial Androgen Insensitivity Syndrome (PAIS) resulting in an undervirilized male phenotype is another phenotype, and Mild Androgen Insensitivity Syndrome (MAIS) in which males have normal genital phenotypes with impaired spermatogenesis (Hughes *et al.*, 2012). Accordingly, if any PAIS or MAIS is suspected, it is highly recommended to screen for AR mutations since the frequency of AR mutations in PAIS is about 41% (Gottlieb *et al.*, 2012). Refer to the section "spermatogenesis" below to see more genes integrated in spermatogenesis process in which any pathogenic mutation could lead to spermatogenesis impairment.

2.2.1.5 Hypothalamic-pituitary-axis Disturbances

For appropriate male sexual development and function, gonadotropic releasing hormone (GnRH) and so testosterone have to be regularly provided in the right concentrations. This is achieved by what is called male reproductive hormone axis or hypothalamic-pituitary-gonadal axis, which is composed of hypothalamic gland, pituitary gland, and testicular gland. Thus, any defect in the regulation of these hormones will affect reproductive function (Corradi, Corradi and Greene, 2016). For example, low follicle-stimulating hormone (FSH) and low luteinizing hormone (LH) levels are associated with testis function deficiency. These hormonal regulation defects could be congenital or acquired. Congenital Hypogonadotropic Hypogonadism (CHH) is an example of

hypothalamic-pituitary axis defect. CHH is a rare disorder with clinical and genetic heterogeneity, and to date, there are more than 25 identified causative genes (Boehm *et al.*, 2015).

CHH is characterized by delayed or absent puberty, sparse or absent body hair, low testicular volume, cryptorchidism, gynaecomatia and other symptoms. In some other cases, the only phenotype is reduction in spermatogenesis with mild hypoandrogenism. It can also be associated with some other developmental anomalies like hearing and ear impairment, dental agenesis, renal agenesis, cleft lip and palate anomalies, and skeletal anomalies (Boehm *et al.*, 2015). CHH is involved in other syndromes like Kallmann, Gordon Holmes, Waardenburg syndromes, and many others (Boehm *et al.*, 2015).

Mutation in genes including FGFR1, PROKR2, GNRHR, KAL1, TAC3, KISS1, and many others have been reported to be involved in CHH. CHH caused by mutations in the mentioned genes could be reserved using testosterone therapy (Krausz and Riera-Escamilla, 2018). Consequently, it is important to screen for these mutations in patients with symptoms, especially if they are planning to have children.

2.2.2 Qualitative Spermatogenic Alterations

Spermatogenic alterations are not limited to quantitative disturbances, but could be due to qualitative alterations as well. What is meant by qualitative alterations is any alteration that can affect morphology, motility, and sperms functionality including chromatin DNA integrity. To date, many recurrent gene mutations have been reported to cause such alterations. For example, macrozoospermia is a rare condition in which the sperms are large-headed, irregular, and multi-tailed (multi-flagellated) with each tail arising form its own basal plate (Nistal, Paniagua and Herruzo, 1978). The only validated genetic cause of this condition is different mutations in the gene called Aurora Kinase C (AURKC) gene, and it is highly recommended to test for these mutations in those who have macrozoospermia to decide the further clinical steps (Dieterich *et al.*, 2007; Coutton *et al.*, 2015).

Another example of qualitative spermatogenic alterations is a very rare (with an incidence of 0.1%) condition known as globozoospermia. It is characterized by round-headed spermatozoa that lack the acrosome, and thus, inability to fertilize the oocyte (Coutton *et al.*, 2015; Ray *et al.*, 2017).

There are more than 50 mice models (i.e., > 50 different gene mutations) in the Mouse Genome Informatics database with globozoospermia indicating the multitude of proteins involved in the formation of the acrosome (Coutton *et al.*, 2015), however, targeted sequencing identified only few mutations that cause globozoospermia in human orthologues, such as mutations in DPY19L2 gene, especially those deletions that occur due to NAHR and it is considered to be the most common and validated genetic defect detected in globozoospermia patients (Coutton *et al.*, 2015; Ray *et al.*, 2017). Mutations in PICK1, SPATA16, and ZPBP1 have been also reported to cause globozoospermia in humans (Ray *et al.*, 2017; Krausz and Riera-Escamilla, 2018).

Multiple morphological abnormalities of the sperm flagella (MMAF) is another example of qualitative defects. It is characterized by asthenozoospermia (i.e., reduced sperm motility) resulting from a combination of many flagellar morphological deficiencies including the absence of the flagella, bent, short, irregular width, coiled, or angular flagella (Khelifa *et al.*, 2014). Khelifa and colleagues reported that about 28% of the MMAF cases have a mutation in the DNAH1 gene. It encodes for axonemal Inner Dynein Heavy arm Chain which is highly expressed in males' testis. Mutations that affect the expression of this chain leads to axonemal disorganizations, and so, flagellar abnormalities in the affected sperm (Khelifa *et al.*, 2014). Primary Ciliary Dyskinesia (PCD) is another qualitative spermatogenic abnormality and defined as a multi-systemic genetic autosomic heterogeneous recessive disorder of motile cilia and flagella. It is characterized by chronic respiratory infections, respiratory distress, chronic otosinopulmonary disease, situs inversus in which organs develop on the opposite site of the body due to dysfunction of the motile embryonic node cilia (about 50% of the cases), and asthenozoospermia due to flagellar defects (Coutton *et al.*, 2015; Knowles, Zariwala and Leigh, 2016). There are more than 26 gene mutations involved in PCD and affect the axonemal ultrastructure and sperm phenotype in humans including DNAH5 and DNAI1 genes that are responsible for 30% of the PCD cases (Coutton *et al.*, 2015).

2.2.3 Idiopathic Male infertility

Although there are highly advanced methods used to diagnose males infertility, 40-50% of male infertility cases are still of unknown aetiology after a complete diagnostic workup including genetic tests, and in this case it is defined as idiopathic male infertility (Krausz, 2011; Krausz and Riera-Escamilla, 2018). Since there are probably more than 2000 genes involved in

spermatogenesis process, and only a small part of them is identified and reported to cause male infertility, this guides us to think that the causes of idiopathic male infertility are most probably due to mutations in these genes that may act directly or via gene-environment interactions (Hochstenbach and Hackstein, 2000; Krausz, 2011).

Many Single Nucleotide Polymorphisms (SNPs) associated with male infertility have been reported in several studies, however, many of them still of weak association and will never solely explain individuals cause of infertility and require further investigations. In addition to this, repeating/ replicating some initial findings failed to give the same results telling us the importance of further studies of other factors such as other gene variants, epigenetic factors, environmental factors, hormonal changes, infections and others that could play any pathogenic role (Tüttelmann *et al.*, 2007; Krausz, 2011).

High-throughput genomic platforms such as large-scale Whole Genome Analysis (WGA) made a big advancement in identifying new genetic factors and succeeded in reporting the pathogenicity of some SNPs as risk factors for male infertility, however, larger study population is still lacking for data confirmation (Krausz, 2011). Other genomic platforms including Comparative Genomic Hybridization (CGH), SNPs arrays, and Next Generation Sequencing (NGS) are used to identify the unknown genetic factors involved in spermatogenesis process (Krausz and Riera-Escamilla, 2018).

Environmental factors and lifestyle could also contribute to idiopathic male infertility, for example, environmental exposures (e.g. chemical exposure, radiations, prolonged exposure to high temperatures, polluted air) together with genetic factors and lifestyle (e.g. malnutrition, certain drugs) (Juul *et al.*, 2014; Babakhanzadeh *et al.*, 2020) are required to ensure the development of fetal testis appropriately, and any aberration may lead to testicular dysgenesis and so testicular dysgenesis syndrome (Juul *et al.*, 2014).

2.2.4 Epigenetic Factors

Mature spermatozoa are characterized by their specialized epigenome, which facilitates their unique function to protect and deliver paternal genome safely in a highly compacted form to integrate with maternal genome in the egg. Male germ cells contain special kind of nuclear proteins

in their nuclei called protamines, which replace 85%-95% of histones in a step-wise process of nuclear reorganization and condensation (histone-to-protamine transition) during post meiotic maturation (Jenkins and Carrell, 2012). Epigenetic signals including histone-tail modifications, DNA methylation, programmatic histones retentions after protamination process, and DNA demethylation intermediates formation are all crucial to sustain a proper sperm genome function (Jenkins and Carrell, 2012; Boissonnas, Jouannet and Jammes, 2013). Any abnormal alteration in one of these epigenetic marks is associated with spermatogenesis process disruption and in turn, decrease in male fertility efficacy, embryo quality, and inefficient pregnancy (Oakes *et al.*, 2007; Jenkins and Carrell, 2012).

Many studies demonstrated the relationship between epigenetic marks and male fertility in mice using the demethylating agents such as 5-aza-2'-deoxycytidine, which is a clinically useful cytidine analogue that incorporates only with the DNA leading to decrease the methylation levels on it (Kelly, Li and Trasler, 2003). In 1996, Doerksen and Trasler evaluated the effect of administrating 5-Azacytidine on the development and functioning of paternal germ cells. They found that administrating 5-azacytidine resulted in reduction in males reproductive organ weight, reduction in the quantity of condensed spermatids and spermatocytes in the testes and epididymides, and impaired development of preimplantation (Doerksen and Trasler, 1996). Another study was conducted by Kelly and colleagues in 2003. They analyzed the effect of using 5-aza-2'-deoxycytidine on mice spermatogenesis development and they showed that administrating 5-aza-2'-deoxycytidine on paternal germ cells led to negative interference with their development without affecting the general health status of targeted mice, reduction in their sperm functions, and reduced fertility (Kelly, Li and Trasler, 2003).

Thus, alterations in DNA methylation levels and other epigenetic marks are associated with histone/ protamine disequilibrium and RNA retention, and accordingly, with spermatogenesis development and male's fertility (Boissonnas, Jouannet and Jammes, 2013).

CHAPTER 3

Meiosis

3.1 Meiosis and Meiotic Recombination

Meiosis is a process of reductive division in which haploid gamete cells (sperms and eggs) are generated from their diploid precursors in two rounds; one round of replication and then two rounds of chromosome segregation. The first division is called meiosis I, in which homologous chromosomes segregate. Whereas in the second division, meiosis II, sister chromatids segregate. During early prophase stages (leptotene and zygotene), each chromosome recognizes its homologue pairing partner and aligns along its length. Once this happens, a complex of proteins called Synaptonemal Complex (SC) (which is composed of SC central element proteins 1 (SYCE1), SYCE2, SC protein 1 (SYCP), SYCP2, SYCP3 and testis-expressed gene 12 (TEX12) in mammals) forms during the synapsis process at zygotene stage (Roeder, 1997; Costa and Cooke, 2007). It strengthens and maintains homologous chromosomes in a close juxtaposition and promotes crossover recombination factors recruitment. A full synapsis is accomplished at pachytene stage (Roeder, 1997).

Meiotic crossover recombination occurs between DNA molecules of the aligned homologs. It is required to form new allelic recombination which leads to genetic diversity, but also to ensure appropriate and accurate segregation of the chromosomes at meiosis I, since crossover creates mechanical tension between homologous chromosomes to ensure their alignment during metaphase stage leading to proper attachment of the spindle to them (Roeder, 1997).

Meiotic crossover recombination is initiated right after the formation of DNA double strands breaks (DSB), which have been observed to occur at certain hotspots (Bullard *et al.*, 1996). Several proteins together with the endonuclease called Meiotic recombination protein SPO11 catalyze crossover process through topoisomerase-like reaction and initiate the DSBs to form a transient, covalent protein-DNA intermediate (Martini *et al.*, 2006) (Ribeiro *et al.*, 2016).

After formation of DSBs, SPO11 is removed and DSBs are nucleolytically resected to generate 3’ single-strand DNA (ssDNA) overhanged tails, which are considered to be the substrate for

initiating homologous recombination (Sun, Treco and Szostak, 1991). Replication protein A (RPA) complex assembles and coats these resected 3' ssDNA overhangs to protect them from degradation, and to remove the inhibitory secondary structures that affect the recruitment of required proteins like recombinases (Wang and Haber, 2004). By this, RPA facilitates the binding of the next proteins such as recombinase radiation sensitive 51 (RAD51) which displaces RPA, and recombinase dosage repressor of Mck1 (DMC1) to form continues nucleoprotein filaments on 3' ssDNA overhangs. These filaments are critical to facilitate the search for a complimentary sequence within a homologous chromosome to catalyze the formation of single end strand invasions (Eggler, Inman and Cox, 2002). Single end strand invasion leads to formation of displacement loop (D loop) recombination intermediate. If the second end strand of the original double strand break also participates in homologue invasion, a double-Holliday Junction (dHJ) recombination intermediate is formed. And here, at this stage, the role of Meiob protein comes; Meiob together with its cofactor protein called Spermatogenesis-associated protein 22 (SPATA22) form a complex with RPA, and coats the D-loop and the second end strand to help in capturing the second end strand and so, the formation of dHJ (Bishop and Zickler, 2004; Luo *et al.*, 2013; Xu *et al.*, 2017). In which DNA synthesis of both involved ends continues and fills the space resulted from strands resection, after then, these two resected ends ligate together leading to dHJ formation (Bishop and Zickler, 2004). And then, these dHJs are removed by what is called dissolution or resolution pathway. Dissolution, in which DNA helicase and IA topoisomerase are involved to catalyze decatenation of these dHJs to generate non-crossover chromosome products. This is important for example in somatic cells to prevent sister chromatid exchanges (Bishop and Zickler, 2004).

However, in dHJ resolution, several types of structure-selective endonucleases that are called resolvases generate either crossover or non-crossover chromosomes via canonical or non-canonical cleavage mechanisms (Wyatt and West, 2014). Canonical resolvases cleave dHJs in a symmetrical way around helical axes, leading to production of nicked DNA duplexes that will ligate immediately generating crossover products (Wyatt and West, 2014).

In the other way, non-canonical resolvases cleave dHJs asymmetrically generating flapped DNA duplexes which will undergo further processing to be ready to ligation and formation of non-

crossover products (Wyatt and West, 2014). For more illustration follow (Figure 3.1) below. However, dHJ may not be generated, and instead, the D-loop dissociates and the invading end rather engages with the opposite strand of the original break leading to the formation of non-crossover products, the same as in mitotic synthesis-dependent strand annealing. Or, this D-loop intermediate could generate crossover products that are interference-independent upon catalysis of many enzymes such as mutagenesis sensitive 81 and other enzymes (Bishop and Zickler, 2004). The latter is not shown in the figure below.

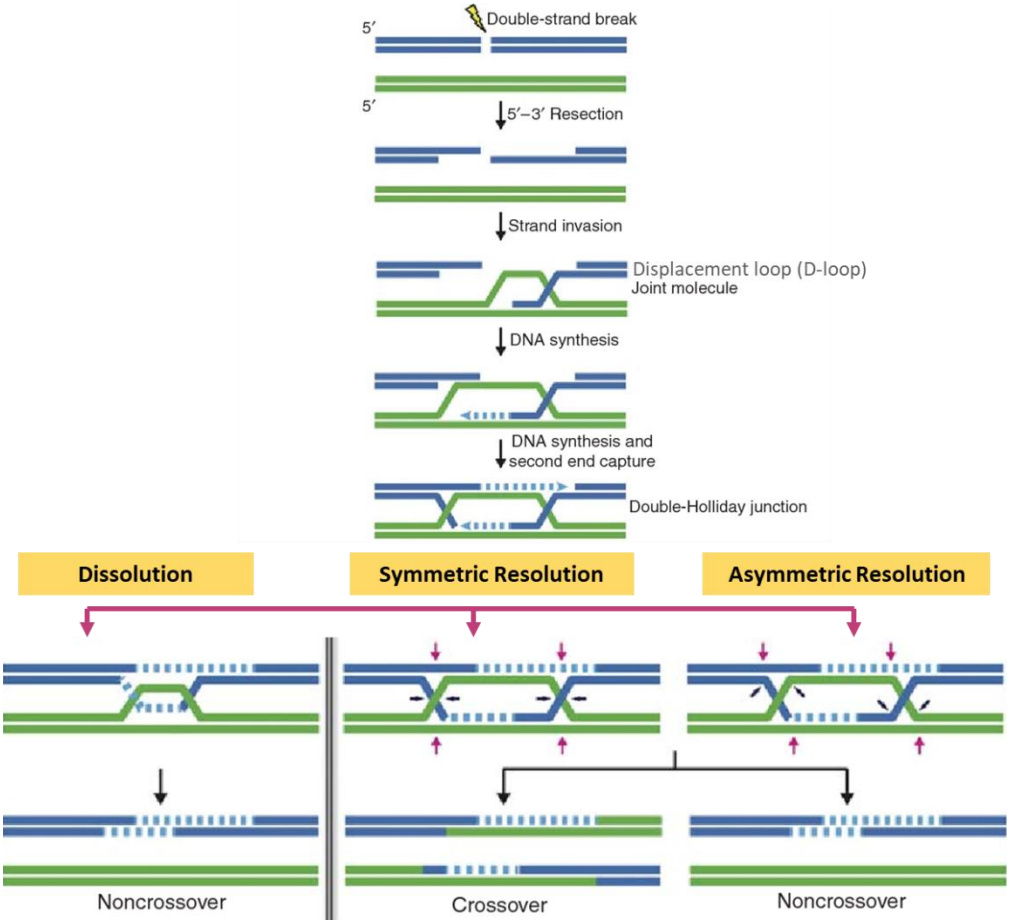


Figure 3.1: Represents the formation of double-Holliday Junctions (dHJs) and their dissolution and resolution pathways to generate crossover and non-crossover chromosome products. After double-strand break and resection of their 5’ ends, 3’ overhanged tails are generated. Single end strand invasion then occur leading to formation of D-loop intermediate. If the second end also associates with the invasion process of the homologue, dHJ is generated, which will form

crossover and non-crossover products according to the followed pathway (i.e., dissolution or resolution) (Wyatt and West, 2014).

3.2 Spermatogenesis Process

Spermatogenesis is defined by a process of production of haploid mature spermatozoa from their diploid progenitors. It occurs in seminiferous tubules of male reproductive system with duration of 74-days. Spermatogenesis process goes through three main phases, the proliferation of spermatogonia, followed by spermatocytes meiotic division, and then followed by the process called spermiogenesis, which is defined by the sum of morphological and nuclear content changes of spermatids to produce flagellated functional mature spermatozoa (Figure 3.2). The whole process of sperm production and so male fertility is maintained through complex cellular and molecular interactions at each of these phases (Potter and DeFalco, 2017).

Spermatogonial stem cells (SSCs) which are undifferentiated spermatogonia defined by their ability to maintain self-renewal and to generate several types of committed undifferentiated spermatogonia such as paired spermatogonia (A_{paired}) and aligned spermatogonia ($A_{aligned}$) (Yoshida *et al.*, 2006), which in turn, differentiate into differentiated types A1, A2, A3, A4, and type B spermatogonia with a duration of 16 days. Then, type B spermatogonia are differentiated into primary spermatocytes within sixteen additional days. Several markers and transcription factors that are required for differentiation and proliferation regulation are expressed in these cells (Cannarella, Condorelli, Duca, La Vignera, *et al.*, 2019). These markers include PAX7, PLZF, SALL4, NANOS1, NANOS2, NANOS3, SOHLH1, DMRT1, and many others. Refer to (Figure 3.2) for more details about involved genes and at which stage they are expressed.

Primary spermatocytes then differentiate into secondary spermatocytes within sixteen days through the first round of meiotic division. At this stage of prophase I, Polo-like kinase 4 (PLK-4) is expressed to regulate centrioles duplication to ensure proper chromosomes segregation (Pawlowski and Cande, 2005) and human augmin complex (HAUS) is also expressed at this stage to stabilize kinetochore microtubules and so centrosome and spindle integrity (Lawo *et al.*, 2009). DSBs, homologous chromosomes pairing, and meiotic recombination via crossover also happen at this stage (discussed in details previously) (Pawlowski and Cande, 2005). SPO11 protein as mentioned previously is involved in DSBs initiation (Martini *et al.*, 2006) (Robert *et al.*, 2016).

The ssDNA is then coated by RPA complex (RPA1, RPA2, and RPA3) to protect it from degradation (Bochkareva *et al.*, 2002) (Wang and Haber, 2004). Subsequently to DSBs, early in leptotene stage, SC is formed along each homologue. And these DSBs are repaired to create the crossover. Many proteins are also involved in meiotic recombination including MEIOB, SPATA 22, RAD51, DMC1, TEX11, TEX15, MLH1, MLH3, SYCE1, and many others indicated in (Figure 3.2) (Cannarella, Condorelli, Duca, La Vignera, *et al.*, 2019).

After then, segregation process occurs; homologous chromosomes separation after meiotic recombination is completed resulting in daughter cells division. However, divided daughter cells stay attached through intercellular bridges which become localized by a testis-specific protein called testis-expressed gene 14 (TEX14). This protein remains expressed and localized there until the differentiating spermatogonia become mature spermatozoa (Greenbaum *et al.*, 2006). Then, within few hours, secondary spermatocytes undergo the second round of meiotic division in which sister chromatids segregate resulting in haploid round spermatids. And again, the intercellular bridges are also built at this stage with TEX14 expression and localization (Greenbaum *et al.*, 2006).

Haploid round spermatids then undergo a process called spermiogenesis, in which several morphological and nuclear changes happen to differentiate into mature functional flagellated spermatozoa. These changes include reorganization and compaction of the DNA, spherical spermatids elongation, cytoplasm expulsion, acrosome cap generation, mitochondrial rearrangements, and flagellum formation (O'Donnell, 2014). This process involves DPY19L2, SIRT1, SEPT12, DNAI1, DNAI2, DXY1C1, HEATR2, CCDC39, DNAAF1, DNAAF2, DNAH5, DNAH6, and many others. Yet, molecular mechanisms are not well understood in spermiogenesis (Cannarella, Condorelli, Duca, La Vignera, *et al.*, 2019).

Any significant mutation that leads to the absence or impairment of any factor in any stage of spermatogenesis might cause spermatogenesis failure, and so, male infertility (Cannarella, Condorelli, Duca, La Vignera, *et al.*, 2019). For more information refer to (Cannarella, Condorelli, Duca, La, *et al.*, 2019) review, in which they provide sixty candidate genes that have been proven to contribute to spermatogenic failure.

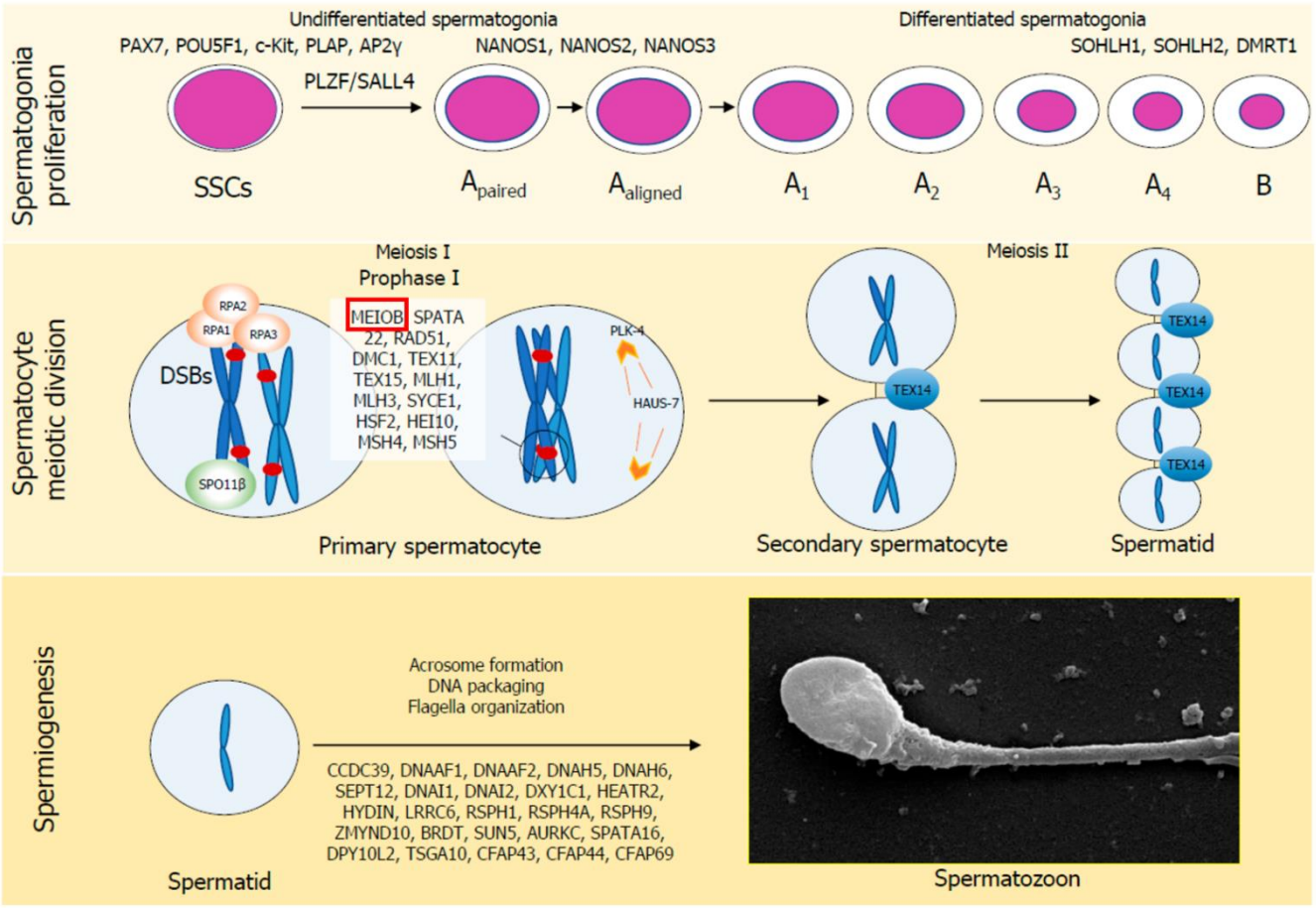


Figure 3.2: Illustration of the three main phases of human spermatogenesis process: spermatogonia proliferation, spermatocytes meiotic division and spermiogenesis showing involved molecular factors in each phase (Cannarella, Condorelli, Duca, La Vignera, *et al.*, 2019).

CHAPTER 4

MEIOB Gene

4.1 Human and Mice MEIOB Gene Representations and Chromosomal Locations

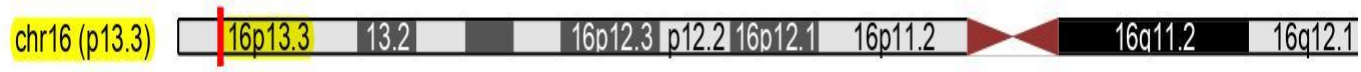
MEIOB gene encodes for Meiosis Specific with OB-Fold (MEIOB) protein. It is a single-strand DNA binding protein and involved in DNA DSBs repair, formation of crossovers, appropriate and complete synapsis, stabilize some recombinases required during meiotic recombination, and also to maintain a proper number of some recombinases like RAD51 recombinase and DNA Meiotic Recombinase 1 (DMC1) right after zygotene stage. It also shows in-vitro single-stranded DNA 3'-exonuclease activity (Luo *et al.*, 2013; Souquet *et al.*, 2013). The functions are discussed in details in other section below. MEIOB gene is composed of fourteen 14 exons. In humans, MEIOB locates on chromosome 16p13.3 (chr16:1,883,984-1,922,179) with a size of 38,196 bp, and the coding exons are 13, while in mice, Meiob locates on chromosome 17qA3.3 (chr17:24,804,311-24,839,787) with a size of 35,477 bp, and the coding exons are 13 as well (Souquet *et al.*, 2013). Look at (Figure 4.1) below to see the representation of human and mice MEIOB gene from UCSC genome browser and their chromosomal locations.

4.2 MEIOB Protein Structure

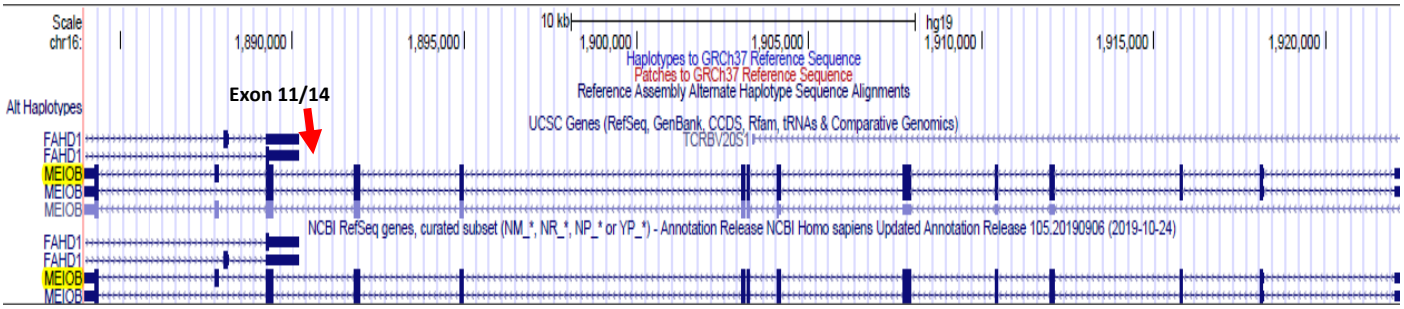
Souquet and colleagues have used InterProScan Search to determine the structure of MEIOB protein. They found out that MEIOB is composed of three oligonucleotide/oligosaccharide-binding OB fold domains, which are known to bind single-stranded DNA in eukaryotes and prokaryotes. These OB domains are homologous to OB domains of Replication Protein A1 (RPA1) (Souquet *et al.*, 2013). MEIOB resembles RPA1 in that it has two OB-folds and one long C'-terminal OB-fold. Using the Sequence Similarity Data Base of the Kyoto Encyclopedia of Genes and Genomes (KEGG SSDB), Ribeiro and colleagues identified a 30 amino acids motif inserted in this C-terminal OB-domain in humans, and it contains a putative zinc ion-binding domain in both MEIOB and RPA1. This motif appears in both MEIOB and RPA1 as a double zinc ribbon, and the difference between these two homologs is the number of amino acid residues exist between cysteines in this motif. The other difference is the persistence of the N-terminal part of the protein; the N-terminal domain exists in RPA1 but not in MEIOB (Ribeiro *et al.*, 2016). This well

conserved N-terminal domain part of the RPA1 performs many specific interactions with other proteins like DNA polymerase ɑ (Kim, Stigger and Lee, 1996). However, MEIOB developed other different specific interactions in the absence of this N-terminal domain part of the protein, and a part of explanation for why RPA1 was not able to compensate the absence of Meiob in Meiob $^{-/-}$ mutated mice is due to this difference; both proteins have their own specific interactions and both are required during meiosis (Luo *et al.*, 2013; Souquet *et al.*, 2013). To make it easier, refer to (Figure 4.2) to see a simplified schematic representation of MEIOB domains structure and Stereo ribbon presentation of predicted MEIOB structure model.

(a)



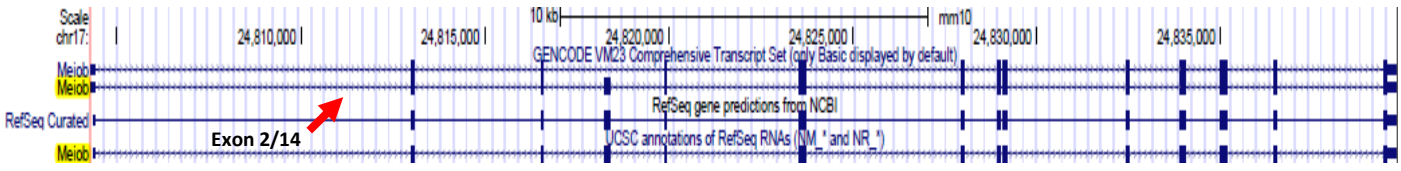
(b)



UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly

(c)

(d)



UCSC Genome Browser on Mouse Dec. 2011 (GRCm38/mm10) Assembly

Figure 4.1: Human and mice MEIOB gene representations and chromosomal location from UCSC genome browser. (a) Represents chromosome 16 of humans and the red vertical block is the location of MEIOB gene (RefSeq Accession: NM_001163560) at position p13.3 of chromosome

16. (b) Representation of human MEIOB gene, exons are the vertical blue blocks and the in-between lines are the introns. The used assembly is Human Feb. 2009 (GRCh37/hg19). (c) Represents chromosome 17 of mice and the red vertical block is the location of MEIOB gene (from RefSeq Accession: NM_029197) at position qA3.3 of chromosome 17. (d) Representation of mice Meiob gene, exons are the vertical blue blocks and the in-between lines are the introns. The used assembly is Mouse Dec. 2011 (GRCm38/mm10).

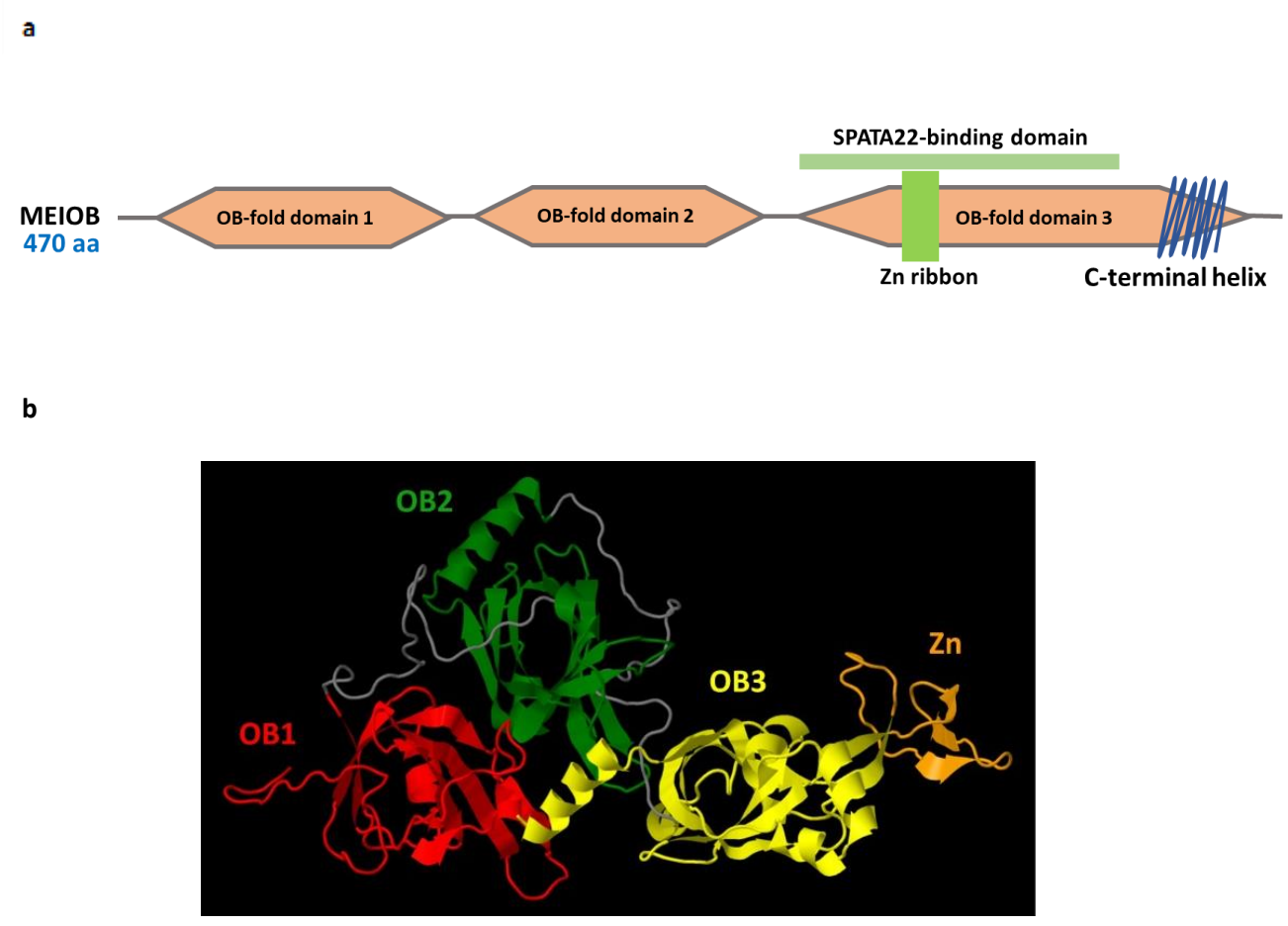


Figure 4.2: MEIOB protein domains structures. (a) Simplified schematic illustration of MEIOB subunits domains. Pinky blocks represent the three folded domains and the unfolded domains are shown as grey linkers between the blocks. The green small block on domain 3 represents zinc ion-binding domain. (b) Illustrates Stereo ribbon presentation of the predicted MEIOB protein

structure. Red, green, and yellow colors represent the folded domains of MEIOB. The grey tiny linkers between them are the unfolded domains, and the zinc ion-binding domain is represented in orange color (Ribeiro *et al.*, 2016).

4.3 Evolution of MEIOB

Long time ago, the only identified paralog of RPA in mammals was RPA4, which is considered to be one member of RPA2 family. In 2013, both Souguet's and Luo's groups discovered MEIOB and identified it as a new paralog of RPA1 in metazoans (Luo *et al.*, 2013; Souquet *et al.*, 2013). Using tBlastn with the full amino acids sequence length or short conserved motifs, Souquet and colleagues identified MEIOB orthologs almost in all metazoans' genomes but not in *Nematoda and drosophila* . A high degree of conservation was achieved between various Meiob homologs in vertebrates using full length amino acid sequences multiple alignment, for example, the identity between human and mouse Meiob homologs is of 85% and the similarity is of 91%. A lower degree of conservation was retrieved when compared with invertebrates (Souquet *et al.*, 2013). In drosophila, MEIOB ortholog is called Hold'em (HDM) and is not specific for meiosis (Joyce, Tanneti and McKim, 2009).

Although Souquet's group did not achieve any Meiob orthologs in the genomes of fungi and plants, Ribeiro and colleagues retrieved Meiob sequence similarities between metazoan and some fungal genomes like zygomycotan fungi called *Mortierella verticillata*, and ascomycetes like *Neurospora crassa* telling us that the evolution of Meiob was even earlier than it was thought (Ribeiro *et al.*, 2016). Souquet and colleagues identified the presence of MEIOB homolog in Capsasporidae, a unicellular eukaryote called *Capsaspora owczarzaki* indicating that the evolution of MEIOB from RPA1 is about 600 million years ago and was by duplication event before metazoans appearance, and then, multicellularity led to MEIOB evolution (Souquet *et al.*, 2013). Since Meiob homologs were retrieved from *C. owczarzaki*, some fungal species and almost all metazoans, then, the emergence of Meiob most probably appeared during the evolution of Opisthokonta group (previously called metazoan/fungi group). The reason why some Meiob homologs were not identified in the genome of some fungal species like *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* is that the ancestral Meiob gene was lost during species

evolution. This is somehow common with many other meiotic genes (Ribeiro *et al.*, 2016). To see the phylogenetic relationship between RPA and MEIOB homologs refer to (Figure 4.3) below.

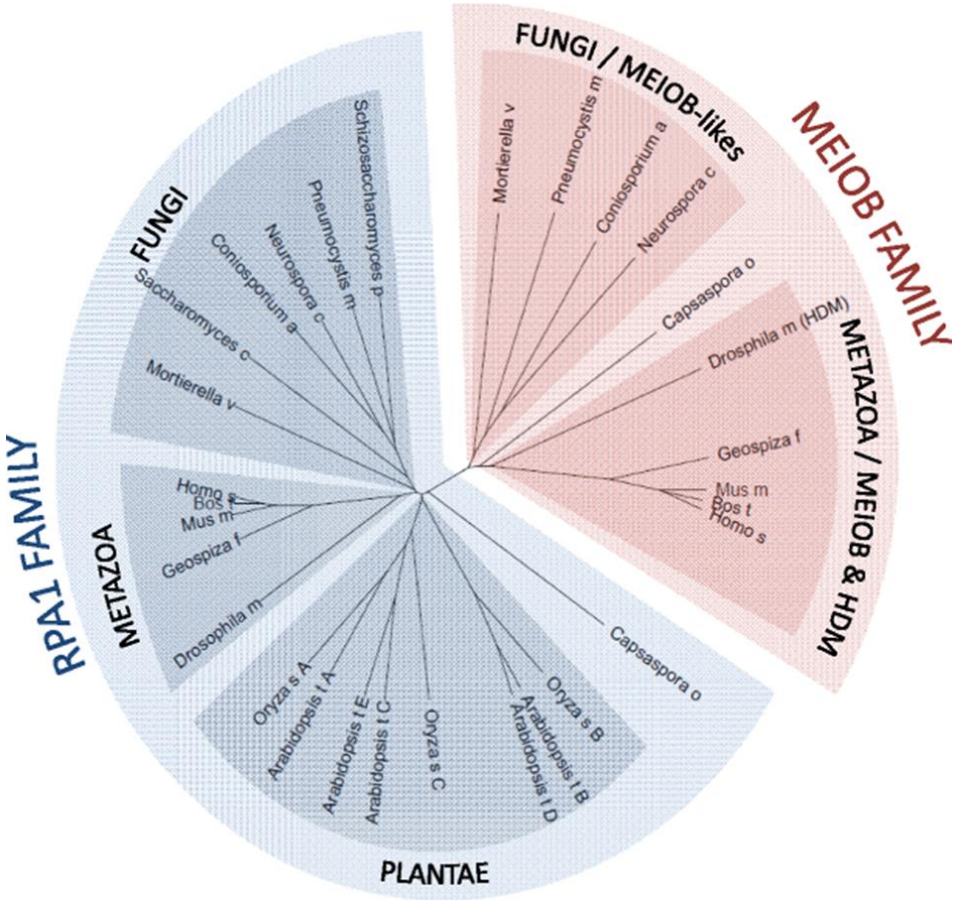


Figure 4.3: Phylogenetic relationship between RPA and MEIOB homologs represented from different families according to the full-length amino acids sequence alignments of these two proteins. Multiple sequence alignment was processed using Clustal Omega, and the visualization of the obtained phylogenetic tree was with Archaeopteryx Version 0.9901 beta (Ribeiro *et al.*, 2016).

4.4 Putative Roles of MEIOB

Luo and colleagues performed systematical proteomics screening for novel meiosis-specific chromatin-associated proteins in mice, and they identified MEIOB in mice testes (Luo *et al.*, 2013). At the same timeframe, Souquet's group also identified MEIOB as a protein which is specifically implicated in homologous recombination during meiosis based on differential

expression of Meiob gene between male embryonic germ cells and female embryonic germ cells. Only female germ cells entered meiosis (Souquet *et al.*, 2013). Both of these groups found that homologous deletion of Meiob (Meiob$^{-/-}$) in male and female mice caused sterility, due to meiotic arrest at zygotene/pachytene-like stage in prophase I of meiosis. This deletion caused impairment of DNA double strand break repair and homologous chromosome synapsis in Meiob$^{-/-}$ meiocytes. Meiob co-localizes together with RPA on chromosomal axes in these early meiotic germ cells (i.e., from leptotene to pachytene stages) (Luo *et al.*, 2013; Souquet *et al.*, 2013).

Although deletion or lack of DMC1 led to the impairment of strand invasion of the homologous sequence, but this did not affect the recruitment of MEIOB to meiotic chromosomes, indicating that MEIOB could be required at early stages on the ssDNA after DSBs formation. However, it could also play a role in later stages during strand invasion with other joint molecules since it is given that MEIOB was existed until the pachytene stage (Luo *et al.*, 2013) (Souquet *et al.*, 2013). The deletion of Meiob led to destabilization of DMC1 and RAD51 recombinases; these recombinases were loaded on chromosomes axes but they were not maintained even though RPA was there. Telling us that MEIOB is unnecessary for the initial recruitment of recombinases, but is required to maintain a proper number of recombinases (RAD51 and DMC1) beyond the zygotene stage (Souquet *et al.*, 2013).

Interestingly, in a similar study conducted by Ishishita et al., they reported that meiosis was arrested at the zygotene-like stage in the spermatocytes of rats lacking functional SPATA22 protein which is a MEIOB protein partner. The number of RPA foci was normally maintained during leptotene and zygotene like stages (Ishishita, Matsuda and Kitada, 2014). However, the number of RAD51 foci was not normally maintained; it was initially normal but then started to decline from the leptotene-like stage. These results suggest that MEIOB and its partner SPATA22 are mandatory for the stabilization but not for the loading of recombinases like RAD51 to chromosomes axes during meiosis (Ishishita, Matsuda and Kitada, 2014).

Using oligonucleotide pull-down assays, Souquet et al. investigated the binding affinity of MEIOB OB-domains to ssDNA and double stranded DNA (dsDNA). MEIOB had greater retention on ssDNA than dsDNA (Souquet *et al.*, 2013). Lou et al. have also investigated that truncated MEIOB with the second OB-domain binds to ssDNA but not to dsDNA. In addition, MEIOB exhibits enzymatic 3'-5' exonuclease activity that is specific for ssDNA and that depends on Mg^{2+}. They found that truncated MEIOB strongly binds ssDNA of 18 nt, and weakly binds shorter oligonucleotides of 5-15 nt in vitro, indicating that the stronger MEIOB binds to ssDNA, the greater exonuclease activity it possesses (Luo *et al.*, 2013).

They also tested the specificity of this enzymatic activity by creating point and truncation mutations that are supposed to affect the nuclease activity in MEIOB constructs, as a result, the enzymatic activity was reduced and the ssDNA binding affinity was affected as well (Luo *et al.*, 2013).

4.5 Proposed Model of MEIOB and its Cofactor SPATA22 Functioning During Meiotic Recombination

Luo and colleagues proposed that during mammalian meiotic recombination, MEIOB together with its cofactor SPATA22 mediates second-end capture which is a preliminary step for the dHJ, and this is achieved right after strand invasion through interaction with RPA proteins, which coat the resected second end and the D-loop. Thus, the interaction between MEIOB-SPATA22 with RPA forms a complex that coats the ssDNA of the second-end and the D-loop as well leading to a physical connection between RPA-coated D-loop and the second broken end. Refer to (Figure 4.4) for better illustration (Luo *et al.*, 2013).

The first-end starts DNA synthesis right after strand invasion to extend the D-loop to the second end. When the second end is captured, the first-end ligates to the resected strand of the second-end. The synthesis of the first end-primed DNA can continue even beyond the end point of the second-end resected strand, and this leads to the formation of the 3'- ssDNA flap right after annealing of the single-stranded second-end with the D-loop or after synthesis dependent strand annealing (SDSA). Thus, after annealing of the second-end, MEIOB may digest and remove the 3'- ssDNA flap of the first- end in SDSA and dHJ pathways since MEIOB exposes exonuclease activity. For more illustration refer to (Figure 4.4) (Luo *et al.*, 2013).

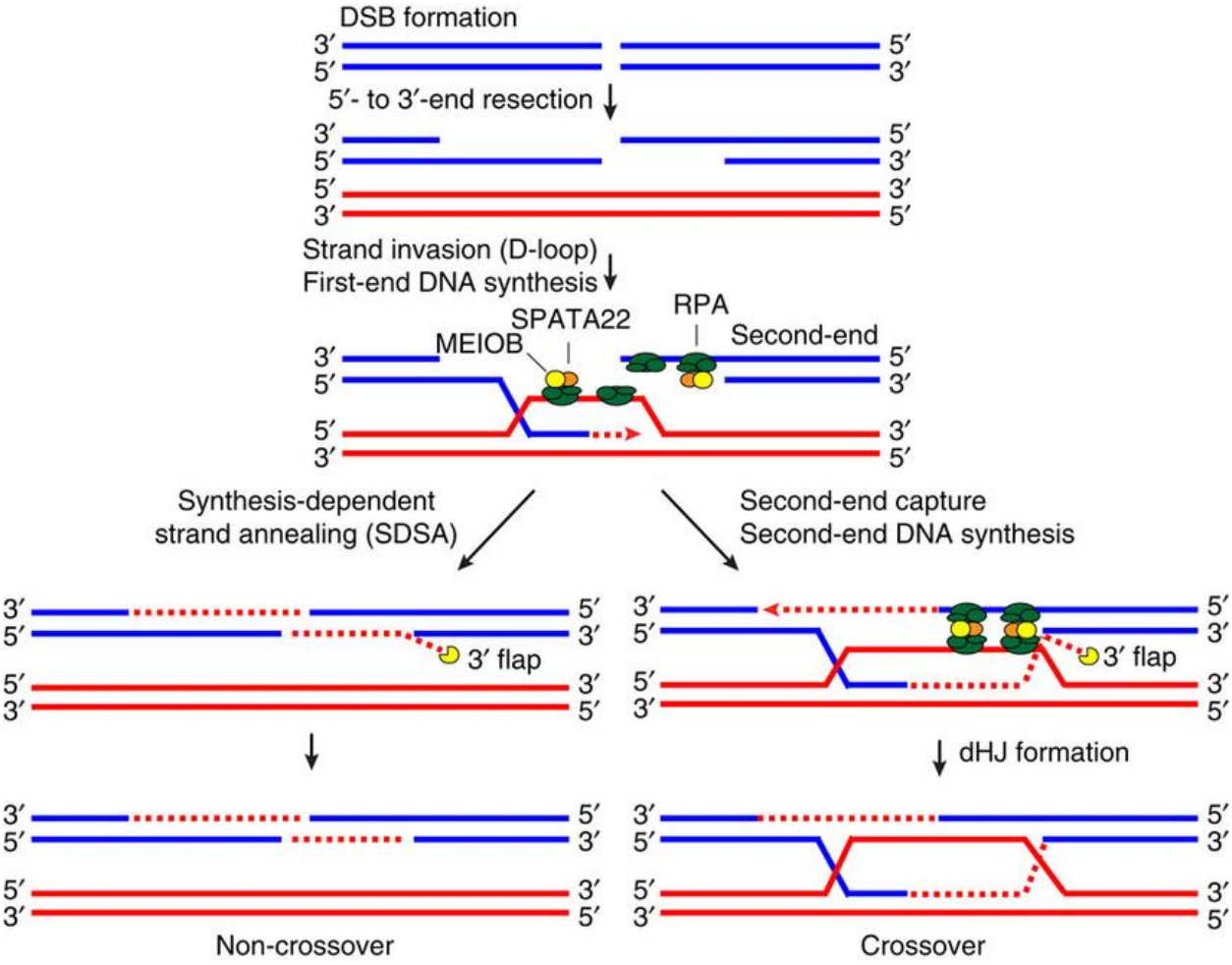


Figure 4.4: Basic steps of meiotic recombination and the proposed role of MEIOB and its cofactor SPATA22 in meiotic recombination (Luo *et al.*, 2013).

4.6 MEIOB gene expression and conservation

According to transcriptome analysis in mice, Meiob gene is specifically expressed early in meiotic germ cells. It was detected in postnatal testis, liver, and in fetal ovaries as well. In testis, the expression of Meiob was first detected at 10 days post-partum (dpp), reached the maximum at 20 dpp, and after then, maintained throughout adult's life, whereas the expression in ovaries was firstly detected at 12.5 days post coitum (dpc), and peaked at 15.5 dpc. Then, the expression started to decrease until it became undetectable in post-natal life (Luo *et al.*, 2013; Souquet *et al.*, 2013). At the developmental stages, the expression of Meiob was only detected early in meiosis in prophase I; 4n-fraction containing leptotene, zygotene and few pachytenes spermatocytes. There

was no expression during later stages of meiosis (Luo *et al.*, 2013). Look to Figure 4.5 below to see the expression of Meiob in the top 10 tissues out of 499 tested tissues in mice.

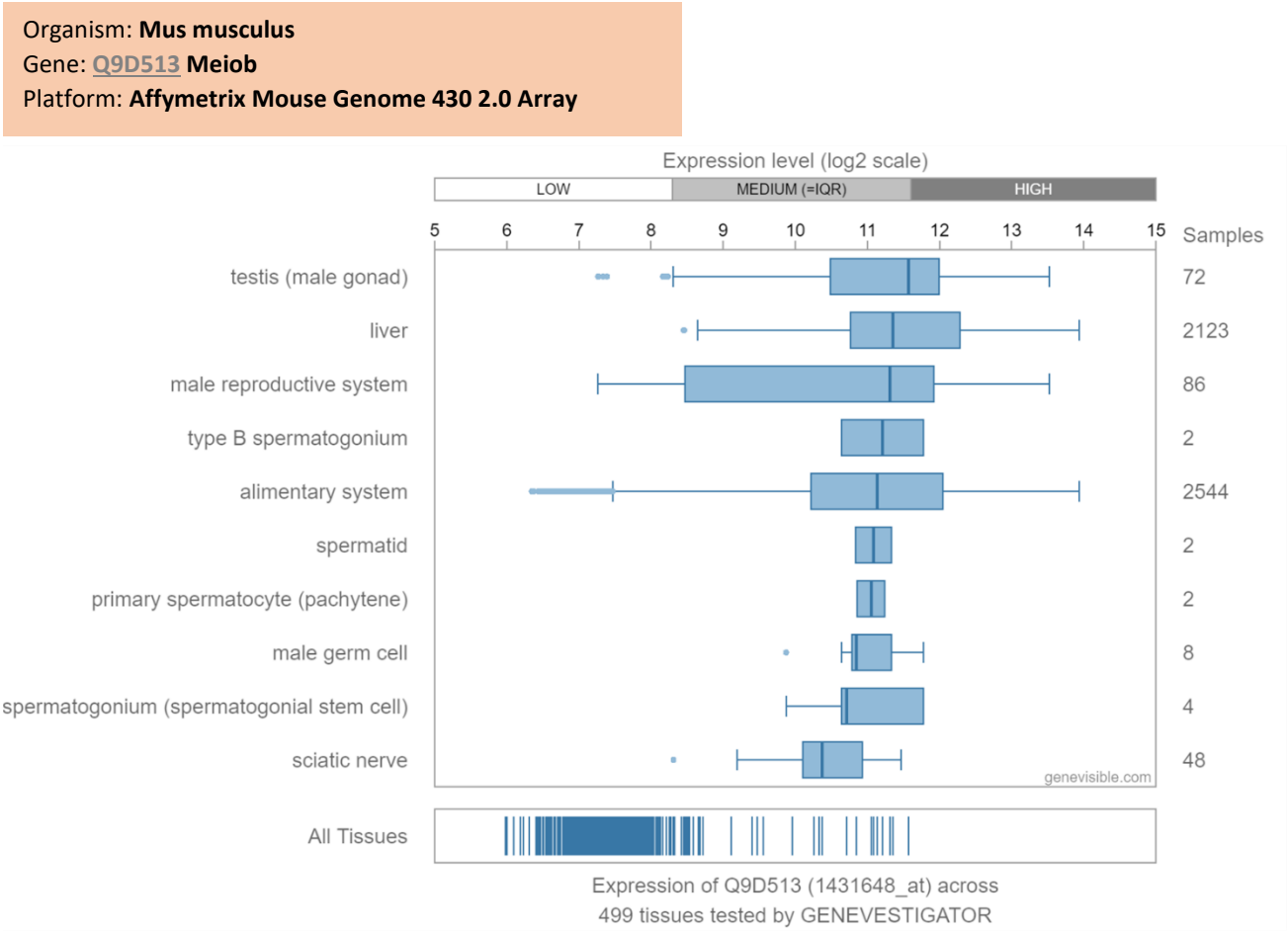


Figure 4.5: Meiob gene expression in the top 10 tissues out of 499 tissues tested by GENEVESTIGATOR. For online visualization follow this link: https://genevisible.com/tissues/MM/UniProt/Q9D513

In fetal gonads of humans, MEIOB gene expression starts at the 14th week after fertilization, whereas the expression of MEIOB in adult's life is restricted to the testis (Souquet *et al.*, 2013). Gershoni and colleagues analyzed the expression of MEIOB gene in 53 tissues kinds for 544 adult human donors, and they found that the highest expression was observed in the testis (Gershoni *et al.*, 2017). Look to Figure 4.6 below to see the expression

of MEIOB in the top 10 tissues out of 534 tested tissues in humans.

Organism: **Homo sapiens**
Gene: Q8N635 **MEIOB**
Platform: **Affymetrix Human Genome U133 Plus 2.0**

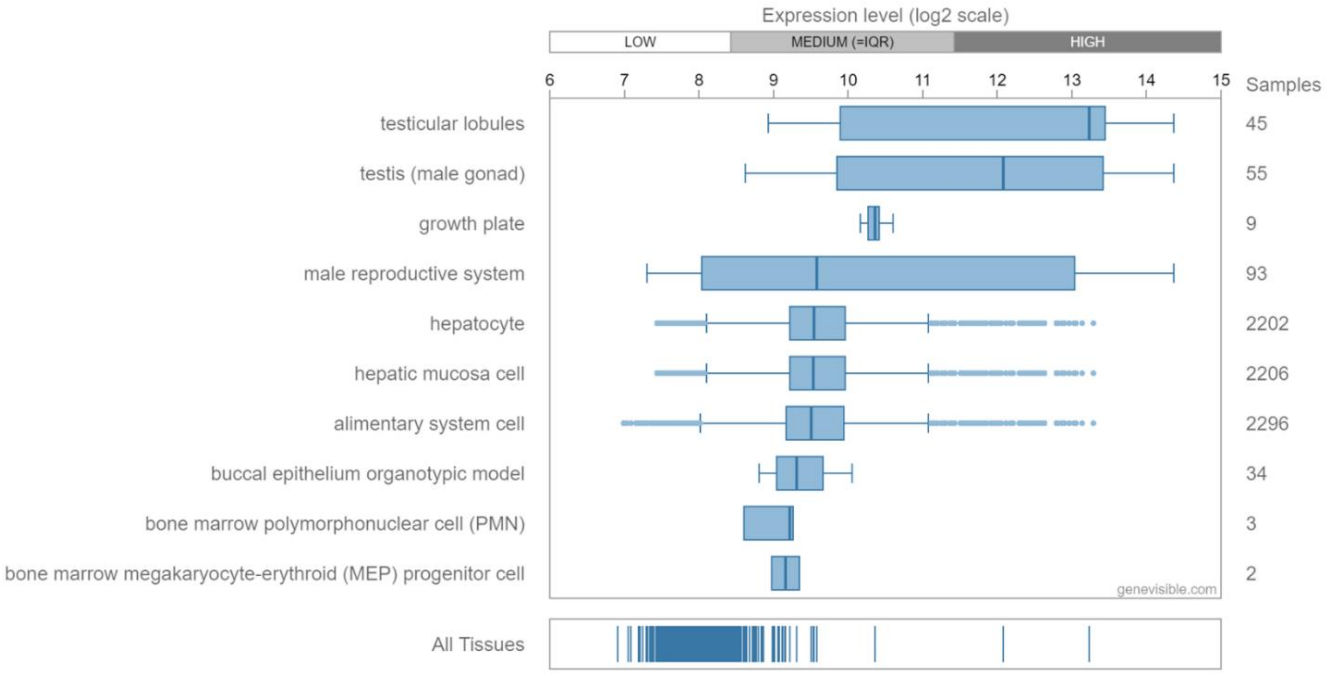


Figure 4.6: Expression of MEIOB gene in the top 10 tissues out of 534 human tested tissues by GENEVESTIGATOR. For online visualization follow this link: https://genevisible.com/tissues/HS/UniProt/Q8N635

4.7 MEIOB and Human Infertility

Recently, several interesting studies have confirmed the role of MEIOB gene in male and female infertility. In 2017, Gershoni and colleagues identified a novel deleterious asparagine-to-isoleucine nonsynonymous mutation in MEIOB gene (NM_001163560:c.A191T:p.N64I). This mutation locates in a conserved DNA binding domain of the MEIOB and this amino acids change may affect the structure of the protein. The patients with this mutation were suffering from NOA with a complete testicular meiotic maturation arrest. Moreover, they revealed a low number of spermatocytes with X-Y chromosomes (sex-body) in proximity to each other, indicating incomplete synapsis (Gershoni *et al.*, 2017) and, it is the same phenotype observed in MEIOB-

null mice; sex-body was absent at pachytene-like spermatocytes and meiosis was arrested (Luo *et al.*, 2013; Souquet *et al.*, 2013).

In 2019, Gershoni and colleagues identified another novel frame-shift mutation in MEIOB gene (NM_001163560; exon12: c.1098delC: p.S366fs) in two infertile brothers diagnosed with NOA with abnormal testicular spermatocyte maturation arrest, and histologically their testes were devoid from spermatids. This frame-shift causes a truncation in the C terminal region of the protein, which is considered to be one of the three conserved DNA binding domains and, thus, the function of the protein might be seriously affected (Gershoni *et al.*, 2019). The same mutation in MEIOB gene was identified in our work in both infertile males and females.

In 2019, Caburet and colleagues also identified a new mutation in MEIOB gene in two female sisters who were diagnosed with primary ovarian insufficiency. They found a homozygous variant in the last base of exon 12, and it was predicted to affect the pre-mRNA splicing leading to exon 12 skipping. Skipping of exon 12 led to a truncation in the C terminal region of the protein which strongly affects the structure of the protein and its interaction with its cofactor SPATA22 and so preventing its recruitment to DSBs. These defects are expected to deplete the follicular stock and cause meiotic defects in ovaries (Caburet *et al.*, 2019). The same phenotype was previously observed in Meiob-null female mice (Luo *et al.*, 2013) (Souquet *et al.*, 2013). In our study we identified a homozygous frame-shift mutation in MEIOB gene in exon 12 (1,889,376 del G MEIOB S366fs*12) in males diagnosed with NOA and other two female members diagnosed with ovarian dysgenesis. All these recent results confirm the previous results conducted in mice and indicate the importance of MEIOB in meiosis and so in both male and female fertility in humans as well.

CHAPTER 5

Materials and Methods

5.1 Materials (Consumables and Equipment)

5.1.1 List of Buffers, Solutions, and Gels

Table 5.1: List of required buffers, solutions, and gels including their compositions

Buffers and Gels	Composition
1X Red Blood Cells (RBCs) lysis buffer	155 mM NH_4Cl 100 M NH_4HCO_3 0.1 mM EDTA (pH=7.4)
1X White Blood Cells (WBCs)/ DNA lysis buffer	50 mM Tris HCL (pH = 7.5) 100 mM NaCl, 1 mM EDTA (pH=8.0)
5X Loading dye	0.25% Bromophenol blue 0.25% Xylene Cyanol FF 30% Glycerol in water
50X TAE (Tris/ Acetic Acid/ EDTA) buffer (Running buffer), pH=8.0	2M Tris (pH 8.0) 1M Acetic acid 0.05M EDTA
6N NaCl Solution	Sodium chloride dH_2O
Agarose gel	Agarose 1X TAE buffer 0.01% ethidium bromide

5.1.2 List of Reagents and Kits

Table 5.2: List of required reagents and kits including their specifications

Reagent	Manufacturer/ Company	Product Specifications
Proteinase K (5 mg/ mL)	aMReSCO®	Cat. No. E195
20% Sodium Dodecyl Sulfate (SDS)	aMReSCO®	Cat. No. 083754-500ml
Agarose	aMReSCO®	CAS. No. 9012-36-6
10X polymerase buffer	Eisenberg Bros	Cat. No. JMR-420
Q-Solution	QIAGEN	
0.02% Sodium Azide	Sigma Aldrich	Cat. No. S2002
Nucleotides dNTPs (2.5 mM)	TAMAR	Cat. No. R0181,4X0.25mM
DNA Oligonucleotide primers (for details refer to tables below)	Hylabs	
Super Therm Taq DNA polymerase	Eisenberg Bros	Cat. No. JMR-801
100bp DNA ladder H3 RTU (size: 54 µg/ 500 µL)	GeneDireX®	Cat. No. DM003-R500
GoTaq® Green Master Mix (100 reactions)	Promega	Cat. No. M7122
Ultra-Pure Water (DNase and RNase-free)	Biological Industries (BI)	Cat. No. 01-866-1A
Exonuclease I (20,000 units/ml)	BioLabs	Cat. No. M0293L
Antarctic Phosphatase AnP (5,000 units/mL)	BioLabs	Cat. No. M0289L
BDX64 (BigDye® Enhancing Buffer) (2 x1.25 ml)	MCLAB	Cat. No. BDX-100
BigDye Terminator 5X Sequencing Buffer	MCLAB	Cat. No. SBUF-100
BigDyeTM Terminators v1.1 Cycle Sequencing Reaction Kit	Applied Biosystems	Cat. No. 4337451-100

5.1.3 List of Equipment

Table 5.3: List of required equipment (devices) and their specifications

Device/ Instrument	Manufacturer/ Company	Product Specifications
NanoPhotometer® N60 touch	Implen	N60 Touch
Thermal Cycler/ PCR machine	Applied Biosystems	GeneAmp® PCR System 9700
Sub-Cell GT Horizontal Electrophoresis System, 15 x 10 cm tray/ Gel electrophoresis apparatus	BioRad	SUB-CELL® GT Cat. No. 1704401
PowerPac™ Basic Power Supply/ Gel electrophoresis power supply	BioRad	Power PAC 300 Cat. No. 1645050
Molecular Imager, Gel DOC™ XR+ Imaging System/ Gel documentation system	BioRad	Cat. No. 1708195
ABI 3130XL Genetic Analyzer/ Sanger sequencing machine	Applied Biosystems	ABI 3130XL Genetic Analyzer

5.2 Methods

5.2.1 Samples Recruitment

In collaboration with Palestine European Fertility Center and some local fertility clinics, eighty two male members from different parts of West Bank (most of them are from Hebron and Bethlehem) diagnosed either with oligospermia, severe oligospermia or non-obstructive azoospermia according to their physician diagnosis were recruited to this study. Some of them were with a history of having relatives with the same condition and some were sporadic with no history of infertility in their family. An interesting family with many female members diagnosed with ovarian dysgenesis which has other relative male members diagnosed with non-obstructive azoospermia was also recruited to this study. In addition to them, twenty other individuals such as patients' spouses and parents were sampled for segregation purposes. All these patients

were interviewed at Palestine European Fertility Center, and asked to sign an informed consent form provided by Bethlehem University Institutional Review Board (consent forms of those individuals who are under the 18 years were signed by their parents). Afterwards, each of them filled a questionnaire with some personal information, some information regarding their sperm production state, karyotyping results if exist, any previously done genetic tests, Testicular Fine Needle Aspiration (TFNA) results, and if they have any history of physical obstruction (obstructive azoospermia) of the post testicular genital tracts (refer to Appendix I for the complete questionnaire form). Family pedigree was drawn for each participant and about 10-mL blood was withdrawn from each and collected in EDTA vacutainer blood tube.

5.2.2 Total Genomic DNA Extraction from Blood by Salting-out Technique

Approximately 10-mL of blood was collected from each participant in 10-mL EDTA vacutainer blood tube. The blood was transferred to 50-mL falcon conical centrifuge tube. The tube with blood then filled with 45-mL of Red Blood Cells (RBCs) lysis buffer for optimal lysis of erythrocytes with minimal effect on lymphocytes. The tubes were kept on ice for about 20-30 minutes with gentle shaking in between. Then, the tubes were centrifuged at 2000 rpm for 12 minutes at 4° C. After centrifugation, the supernatant was carefully discarded; the pellet was re-suspended in 15-mL RBCs lysis buffer and centrifuged again at the same parameters as in the first centrifugation step. The supernatant was discarded and the pellet washed with 5-mL RBCs lysis buffer and then re-suspended in a mix of 3 mL of 1X White Blood Cells (WBCs) lysis buffer, 100-µL of 20% sodium dodecyl sulfate (SDS) and 100-µL of 5 mg/ mL proteinase K. Then, the tubes were incubated with continuous shaking overnight at 37 °C in an incubation shaker. After that, 3-mL of 6N NaCl was added, vortexed strongly to get foamy homogeneous solution and centrifuged at 3000 rpm for 20 minutes at 25 °C. Then, the supernatant was transferred to a new 15-mL falcon tube and centrifuged at the same parameters as in the step before, and again, the supernatant was transferred to the new tube and cold absolute ethanol was added (twice the volume of the supernatant; ~ 8 mL). The tube was then gently inverted until the DNA strands were

precipitate and became visible as white thin network of fibers, which then was fished using end sealed hooked Pasteur pipettes and was washed in 70% ethanol. The DNA on Pasteur pipettes was then air dried for about 2 minutes, transferred to a screw cap micro-tube that contains (200-600) µL of 0.02% sodium azide depending on the amount of precipitated DNA to dissolve the DNA and preserve it from bacterial and fungal contaminations.

5.2.3 Genotyping

5.2.3.1 Primers Design

Standard Y-chromosome microdeletion primers for (ZFY, SRY, sY254, sY127, sY134, sY84, sY255, sY86) markers were chosen according to European Academy of Andrology and the European Molecular Genetics Quality Network (Krausz *et al.*, 2014), and directly ordered from the hylabs (refer to the **Table 5.4** below to see primers' sequences). MEIOB primers were designed using Primer3 (v. 0.4.0) software (http://frodo.wi.mit.edu/primer3/) and ordered directly from the hylabs as well (refer to the **Table 5.5** below to see the primers sequences).

5.2.3.2 Y- Chromosome Microdeletion Primers

Table 5.4 Y-Chromosome microdeletion set of primers

No.	Oligo Name	Sequence 5' - 3'	Length (bases)	Product size (bp)
1	**ZFY-F**	ACC RCT GTA CTG ACT GTG ATT ACA C	25	495
2	**ZFY-R**	GCA CYT CTT TGG TAT CYG AGA AAG T	25	
3	**SRY-F**	GAA TAT TCC CGC TCT CCG GA	20	472
4	**SRY-R**	GCT GGT GCT CCA TTC TTG AG	20	
5	**sY86-F**	GTG ACA CAC AGA CTA TGC TTC	21	318
6	**sY86-R**	ACA CAC AGA GGG ACA ACC CT	20	

No.	Oligo Name	Sequence 5' - 3'	Length (bases)	Product size (bp)
7	sY127-F	GGC TCA CAA ACG AAA AGA AA	20	274
8	sY127-R	CTG CAG GCA GTA ATA AGG GA	20	
9	sY254-F	GGG TGT TAC CAG AAG GCA AA	20	400
10	sY254-R	GAA CCG TAT CTA CCA AAG CAG C	22	
11	sY84-F	AGA AGG GTC TGA AAG CAG GT	20	326
12	sY84-R	GCC TAC TAC CTG GAG GCT TC	20	
13	sY134-F	GTC TGC CTC ACC ATA AAA CG	20	301
14	sY134-R	ACC ACT GCC AAA ACT TTC AA	20	
15	sY255-F	GTT ACA GGA TTC GGC GTG AT	20	123
16	sY255-R	CTC GTC ATG TGC AGC CAC	18	

5.2.3.3 MEIOB Gene Primers

Table 5.5 MEIOB Gene Primers

MEIOB Exon No.	Oligo Sequence	Annealing Temp. (°C)	Length (bases)	Product size (bp)
Exon1	Non Coding	---	---	---
Exon2-F	TTG CCA ATT ATA AAA GCA TGT G	57.8	22	919
Exon2-R	TCC AAA GCA GTA AAC TAT CCA GTG	59.7	24	
Exon3-F	CAG AAA TGT TTG TTA GAG CCA GT	57.6	23	305
Exon3-R	CTT GAC CAT CCA ATA GAG AGC A	59.3	22	
Exon4-F	GGC AGA GAA GCG ATT GAA AC	60.0	20	242

Exon4-R	CCA CAG GAA TAA CAC TAT GAT GGA	60.1	24	
Exon5-F	TTG AAA TAG AAA CAG AAC AC	47.7	20	333
Exon5-R	ATG TTA TAT GTT GAT GAT GG	47.9	20	
Exon6-F	TGT AGT TTA AAT GTG GAC CTG GAA	59.8	24	353
Exon6-R	TCC ATC CCA AAC ACA GAT CA	59.9	20	
Exon7-F	TTT GGT GTT TAA AAG GTG AAC A	57.3	22	300
Exon7-R	TCC TCT CTT GAT ATG AAG TCC ATC	58.7	24	
Exon8 + 9-F	CCA GCC ACT GAA GCT TTC TT	59.6	20	372
Exon8 + 9 -R	TCA TTT CCA AAG GGA TAA AAG G	59.3	22	
Exon10-F	CAA ACC TTT ACA GGC CCA AT	58.9	20	201
Exon10-R	TTT GCT TTT TAC TTA TTA TGC ATT TTC	58.5	27	
Exon11-F	TTT CCA TTC GGA TGT AAC CTT	58.4	21	257
Exon11-R	TGA ATT TGC TAA AAG TGA CAC G	58.0	22	
Exon12-F	AAT TTG TAG CTT AGG GCA TCA CA	60.0	23	293
Exon12-R	TGG GAA AAA GCA TTC ACT TTG	60.1	21	
Exon13-F	TCG AAT TTA TCT AGG TTT TTC TTA TGG	59.4	27	235
Exon13-R	GTC AAA AAT GAA TTT TCT TTG TAA ACT	57.7	27	
Exon14-F	TCA TTG CAG TTC TCT ATT CGT GA	59.9	23	308
Exon14-R	CAA AAT GCA ATT TTC CCC ATA	59.7	21	

5.2.3.4 Y- Chromosome Microdeletion Polymerase Chain Reaction (PCR)

For Y-chromosome microdeletion test, GoTaq Green Master Mix that contains GoTaq® DNA polymerase in a reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$ was used. Forward and reverse primers, 100 ng/ µL sample DNA template and DNase RNase free water were added to reach a total of 12.5 µL reaction volume as the following.

Table 5.6 Y-Chromosome microdeletion PCR reaction

Material	For 1X (µL)
GoTaq Green Master Mix	6.25
Forward primer	0.5
Reverse primer	0.5
DNase RNase free H_2O	4.75
DNA template	0.5

This reaction was carried for every single sample recruited to this study as a first step to exclude any sample that has Y-chromosome microdeletion. In parallel to each reaction, a female sample was processed as a negative control for DNA contamination during the whole procedure. The internal PCR control in this test was zinc-finger protein X-linked (ZFX) and zinc-finger protein Y-linked (ZFY) genes, and that's because the primers of this region amplify a unique fragment that exists in both male and female DNA respectively. As a positive control, DNA sample was taken from a man who has normal spermatogenesis and has no Y-chromosome microdeletion. Nuclease free water was served as no template control (NTC); all reaction components are there but the water was used instead of the DNA template as a control for the contamination as well.

Polymerase chain reaction for these markers was carried using GeneAmp® PCR System 9700, and the PCR program for (ZFY, SRY, sY254, sY127, sY134) is illustrated in **Table 5.7** below:

Table 5.7 PCR program for (ZFY, SRY, sY254, sY127, sY134)

Phase	Temperature	Time (minutes)	No. Cycles
Initial Denaturation	95.0 °C	15:00	
Denaturation	95.0 °C	00:30	**35 cycles**
Annealing	**57.0 °C**	01:30	
Extension	72.0 °C	01:00	
Final Extension	72.0 °C	10:00	
Final Hold	04.0 °C	∞	

For the rest of the markers (sY 84, sY 255, sY 86), the previous program did not fit perfectly since they showed many unspecific bindings, thus, optimization was carried out to minimize unspecific bindings using the following touch down PCR program:

Table 5.8 Touch down PCR program for (sY84, sY255, sY86)

Phase	Temperature	Time (minutes)	No. Cycles
Initial Denaturation	94.0 °C	15:00	
Denaturation	94.0 °C	00:30	**3 cycles**
Annealing	**65.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**3 cycles**
Annealing	**63.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**3 cycles**
Annealing	**61.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**3 cycles**

Annealing	**59.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**35 cycles**
Annealing	**57.0 °C**	00:30	
Extension	72.0 °C	00:30	
Final Extension	72.0 °C	05:00	
Final Hold	04.0 °C	∞	

5.2.3.5 Agarose Gel Electrophoresis for Amplified Y-Chromosome Microdeletion PCR Products

The percentage of the agarose gel depends on the molecular weight of the amplified product. For this amplification reaction, 1.5% agarose gel was prepared by dissolving 1.5 g of agarose powder in 100-mL 1X TAE buffer and boiling it for about 2 minutes in the microwave. Then, the solution was cooled and about 4-µL of ethidium bromide (EtBr) was added. The solution was then poured into the appropriate gel tray with combs and allowed to dry until the gel became milky in color. The gel was transferred to the gel electrophoresis container filled with 1X TAE running buffer. Then, 5-µL of 100 bp DNA ladder, 10-µL of each sample were loaded to the wells of the gel, and the electrophoresis was run for about 20-30 minutes at 120 Volt. For DNA visualization, Molecular Imager, Gel DOC™ XR+ Imaging System (Gel documentation system) was used. Any sample with microdeletion was excluded and did not screened for the deletion mutation in MEIOB gene in the following step.

5.2.3.6 MEIOB Polymerase Chain Reaction

All samples that did not have Y-chromosome microdeletion were screened for MEIOB mutations. As it was mentioned before, MEIOB is composed of fourteen exons, the first exon is a non-coding, while the rest are coding. According to the

previous studies, the mutation was detected in exon number 12. In this study, primers were designed for every single exon (refer to **Table 5.5**). For MEIOB amplification reaction, 10X polymerase buffer, Q-Solution, 2.5 mM dNTPs, forward and reverse oligonucleotide primers, Super Therm Taq DNA polymerase, DNase RNase free H_2O, and DNA template for a total of 25-µL volume reaction. This recipe was used for one reaction 1X; multiplied to the number of needed reactions as illustrated in **Table 5.9** below. For each reaction, No Template Control was used (nuclease free water was used instead of the DNA template).

Table 5.9 MEIOB PCR reaction

Material	For 1X (µL)
10X polymerase buffer	2.50
Q-Solution	5.00
2.5 mM dNTPs	2.00
F - primer	0.50
R - primer	0.50
Super Therm Taq DNA polymerase	0.25
DNase RNase free H_2O	13.25
DNA template	1.00

Touch down PCR program was used for each set of primers. For (Exon 7, Exon 10, Exon 11, and Exon 13), touch down 55 °C was used. For the rest of the primers, touch down 60 °C was used. Refer to the **Tables 5.10 and 5.11** below for PCR program illustrations:

Table 5.10 Touch down 55 °C PCR program for (Exon 7, 10, 11, and 13)

Phase	Temperature	Time (minutes)	No. Cycles
Initial Denaturation	94.0 °C	05:00	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**63.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**61.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**59.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**57.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**35 cycles**
Annealing	**55.0 °C**	00:30	
Extension	72.0 °C	00:30	
Final Extension	72.0 °C	07:00	
Final Hold	04.0 °C	∞	

Table 5.11 Touch dwon 60° C PCR pragramme for (Exon 2, 3, 4, 5, 6, 8, 9, 12, 13, and 14)

Phase	Temperature	Time (minutes)	No. Cycles
Initial Denaturation	94.0 °C	05:00	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**68.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**66.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**64.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**2 cycles**
Annealing	**62.0 °C**	00:30	
Extension	72.0 °C	00:30	
Denaturation	94.0 °C	00:30	**35 cycles**
Annealing	**60.0 °C**	00:30	
Extension	72.0 °C	00:30	
Final Extension	72.0 °C	07:00	
Final Hold	04.0 °C	∞	

5.2.3.7 Agarose Gel electrophoresis for Amplified MEIOB PCR Products

For MEIOB amplification products, 1.5% agarose gel was prepared (exactly the same as illustrated in Y-Chromosome microdeletion agarose gel electrophoresis (section 5.2.3.5). For samples loading, 4-µL of 5X loading dye was well mixed with 4-µL of PCR product and then, the whole mixture was loaded into the gel. Electrophoresis was run in 1X TAE running buffer at 120V for about 25-30 minutes. And then, the DNA

was visualized using gel imaging documentation system as previously mentioned and illustrated.

5.2.3.8 Enzymatic Cleanup of MEIOB PCR Products

After making sure that the amplification of all MEIOB exons was successful with clear single bands on the gel, enzymatic PCR cleanup was performed using Exonuclease I (Exo I) and Antarctic Phosphatase (AnP). Exo I was added to degrade the residual PCR primers. AnP was then added to dephosphorylate the remaining dNTPs. After then, PCR product was added and the reaction was completed using DNase RNase free H_2O to reach a total volume of 7-µL in the following manner (**Table 5.12**):

Table 5.12 Enzymatic cleanup reaction of MEIOB PCR products

Material	For 1X (µL)
Exonuclease I (Exo I)	0.25
Antarctic Phosphatase (AnP)	0.25
DNase RNase free H_2O	1.50
PCR product	5.00

Then, the reaction was carried out using thermal cycler machine according to the PCR cleanup program showed in the (**Table 5.13**) below.

Table 5.13 MEIOB PCR cleanup program

Phase	Temperature	Time of incubation (mins)
Enzymes activation	37 °C	30:00
Enzymes inactivation	80 °C	20:00
Hold in cold	4.0 °C	∞

5.2.3.9 Sequencing of Purified MEIOB PCR Products

For the sequencing, direct Sanger sequencing method was performed using BigDye™ Terminator V1.1 Cycle Sequencing Reaction Kit. 5X sequencing buffer, BigDye terminator, forward or reverse primer, 64X buffer- BigDye® enhancing buffer (to enhance the activity of the polymerase, to reduce the extension time from 4 to 1 minute, and to result in even peak distribution), PCR clean product, and DNase RNase free H_2O were mixed to reach a total of 16-µL reaction volume (1X) multiplied by the number of needed reactions as shown in **Table 5.14**.

Table 5.14 Sequencing reaction of MEIOB purified PCR products

Material	For 1X (µL)
BDX64 (BigDye® Enhancing Buffer)	0.75
BigDye® Terminator 5X Sequencing Buffer	1.50
BigDye Terminator	0.40
F or R primer	1.00
PCR clean product	2.00
DNase RNase free water	10.35

The sequencing reaction was carried out using thermal cycler machine using the following program:

Table 5.15 Cycle-sequencing of MEIOB program

Phase	Temperature	Time (minutes)	No. Cycles
Initial Denaturation	96.0 °C	03:00	
Denaturation	96.0 °C	00:10	**30 cycles**
Annealing	**50.0 °C**	0:05	
Extension	60.0 °C	02:00	
Final Hold	04.0 °C	∞	

After then, the cycle-sequenced products were taken for the next step to purify and prepare for the capillary electrophoresis (sequencing in the ABI 3130XL Genetic Analyzer).

5.2.3.10 Sequence Clean Using EDTA/Ethanol Precipitation of Cycle-Sequenced Products and Capillary Electrophoresis

EDTA/Ethanol precipitation method was used to purify and clean Cycle-Sequenced products. EDTA helps to remove any unincorporated dyes and it helps to stabilize extension products during precipitation. To each well that contains 16-µL reaction volume, 12.5-µL of 0.125 Molar EDTA and 100-µL 99.8% cold Ethanol were added, well mixed and incubated at -20 °C for 30 minutes. After then, centrifuged at 3800 rpm for 30 minutes at 4 °C. The supernatant was then discarded and 60-µL of 70% Ethanol was added to wash the precipitated DNA, centrifuged again at 3800 rpm for 20 minutes at 4 °C. Then, the supernatant was totally discarded and the PCR strips containing the pellets were turned carefully upside down on a tissue paper and spun down at 500 rpm for one minute at 4 °C. To make sure that the ethanol is completely discarded, the pellet was dried by putting the opened strips on a 95 °C heated hotplate for 5 minutes. After drying, to each well 10-µL of Hi-Di Formamide was added, well vortexed and then heated on the hotplate for 5 minutes at 95 °C for the denaturation of DNA strands. And then, the samples were immediately moved to ice to prevent the renaturation of DNA strands for 5 minutes and loaded onto the 96-well optical reaction plate. Finally, the samples in the plate were run on ABI 3130XL Genetic Analyzer for capillary electrophoresis.

5.2.3.11 Analysis of Sequencing Data

FinchTV program was used to open the DNA chromatogram files and display the DNA sequence in a graph (electropherogram) to make it easier to analyze the DNA sequences.

5.2.3.11 Healthy Fertile Controls

Two hundreds fertile Palestinian males (each at least has one child) were recruited to this study as a control. DNA was extracted from their blood and then screened for the mutation using the same primers set following the previously mentioned protocol.

CHAPTER 6

Results

6.1 Family Pedigrees of Affected Individuals

A hundred and four (104) individuals were recruited for this study. Eighty two (82) out of them were males diagnosed with oligospermia, severe oligospermia, or azoospermia. Six (6) male members tested positive for the Y-chromosome microdeletion assessment, and so, excluded from the study. One azoospermic male was also excluded since he was diagnosed with Klinefelter syndrome (47, XXY). Two (2) were females diagnosed with ovarian dysgenesis. And, the rest twenty (20) were parents of affected individuals, spouses and other relatives who agreed to take part in this study even though they did not fit the criteria of our study. Refer to the schematic representation below (Figure 6.1). For the family pedigrees, every sampled individual was asked to fill a questionnaire (Appendix I) as previously mentioned in the section of sample recruitment (5.2.1) before. The presented family pedigrees are only for those individuals in whom the mutation was detected (Figure 6.2).

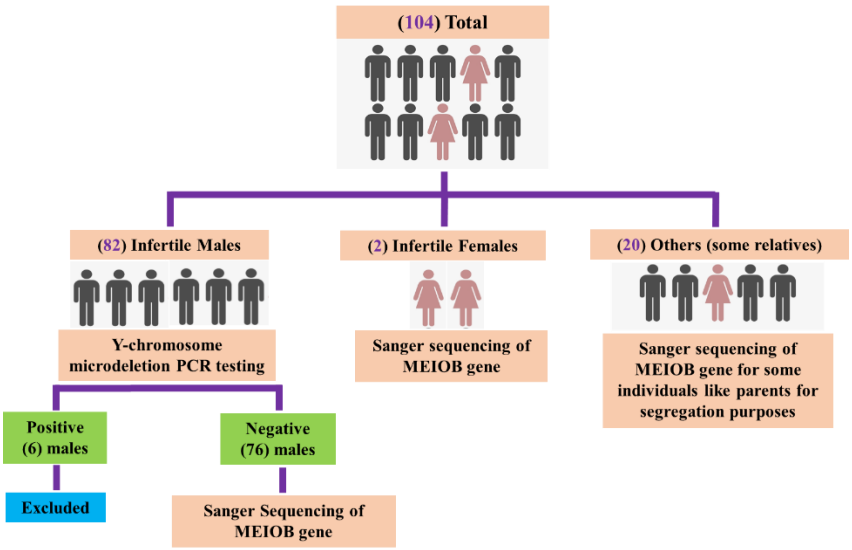


Figure 6.1: Schematic representation illustrating the kind and number of sampled individuals and the workflow after sampling.

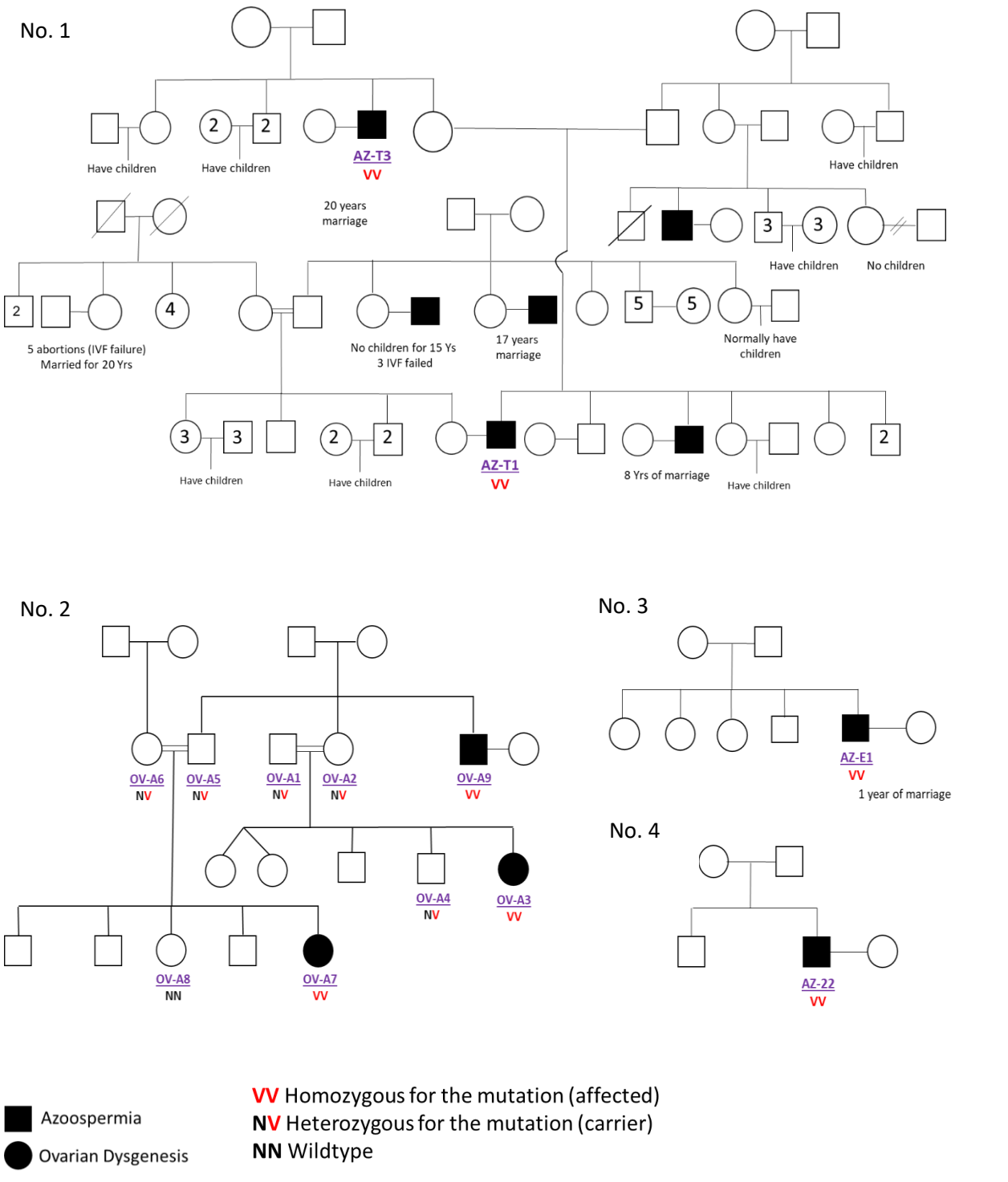
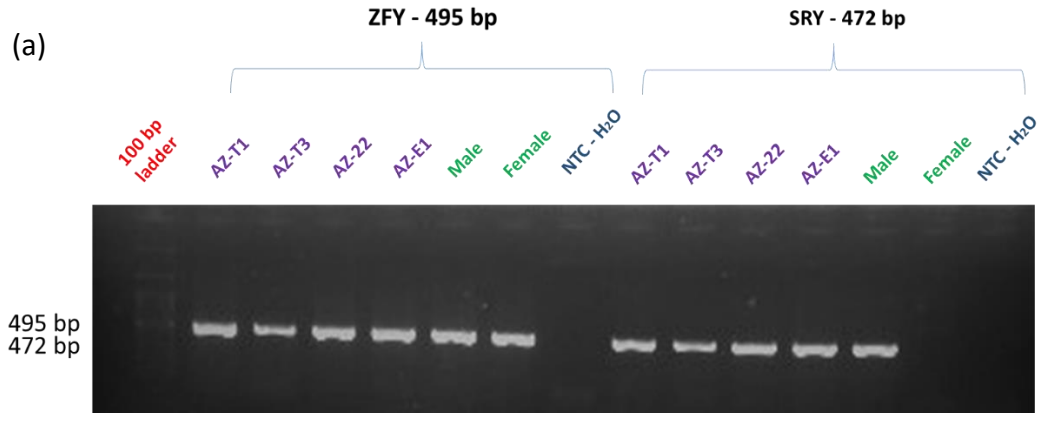


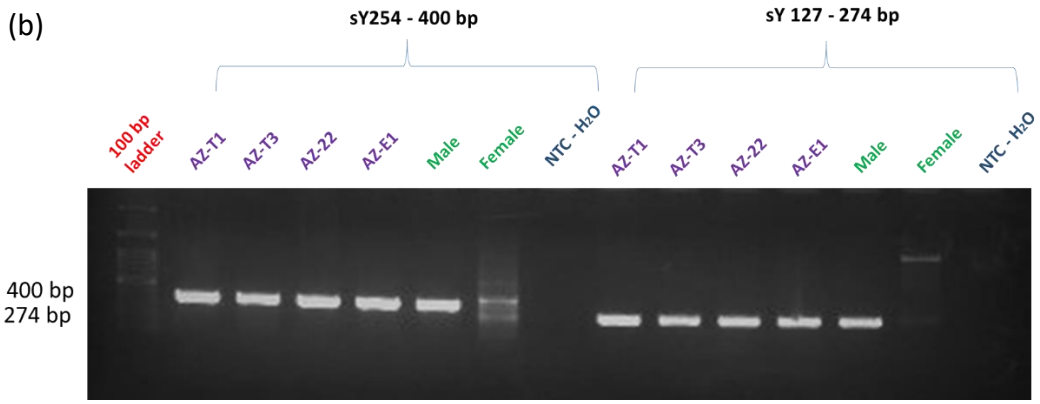
Figure 6.2: Family pedigrees of affected individuals. Coded individuals were sampled and sequenced for the mutation. Under each code, it is indicated whether the sampled individual is affected, carrier or unaffected.

6.2 Y- Chromosome Microdeletion Assessment

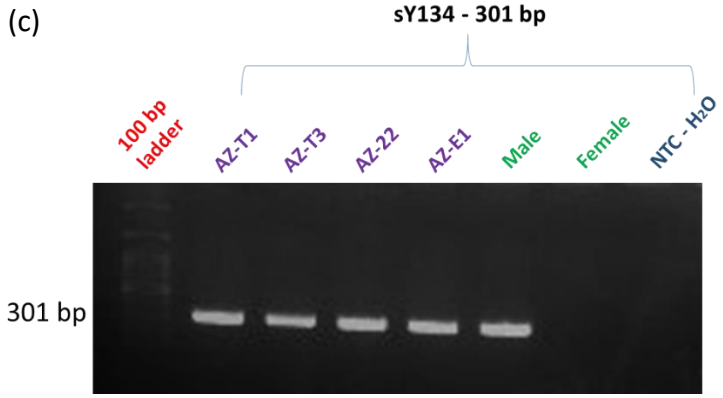
As discussed before, since Klinefelter syndrome and Y-chromosomal microdeletions are considered to be the second most causative genetic factors of male infertility (Krausz *et al.*, 2014), the first exclusion step in our study was to test for the Y-chromosomal microdeletions after making sure that the participated infertile male has a normal karyotyping. However, the karyotyping was not done by our own, but rather, we depended on patient's diagnostic history. One male was excluded due to abnormal karyotyping; he was diagnosed with Klinefelter syndrome. Six other infertile males were positive for Y-chromosomal microdeletion test, and so, excluded from the study; they did not undergo Sanger sequencing of the whole MEIOB gene. For the rest of participated infertile males who were negative for Y-chromosomal microdeletions, they underwent Sanger sequencing of MEIOB gene as shown in the coming section. Y-chromosome microdeletion assessment was not conducted in a multiplex manner in which a multiplex of primers set is amplified in the same reaction tube, but rather, every single gene was amplified in a separated PCR reaction with all required controls (negative control, positive control, and internal control) as shown in the (Figure 6.3) below. The agarose gel electrophoresis of Y-chromosome microdeletion PCR images shown below are only for the individuals tested positive for the mutation in the MEIOB gene.



(b)



(c)



Box 6.1

Annealing Temp.: 57 °C

Sample DNA conc.: 100 ng/ µL

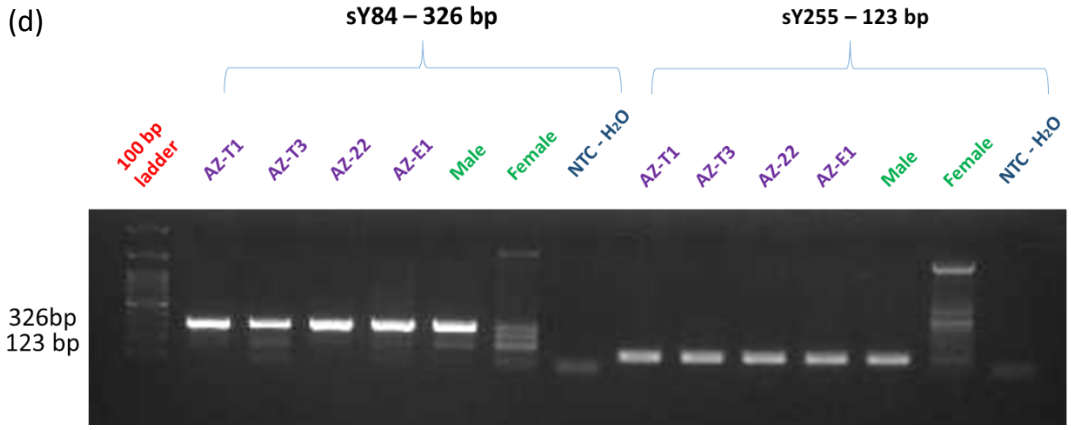
Agarose Gel Conc.: = 1.5%

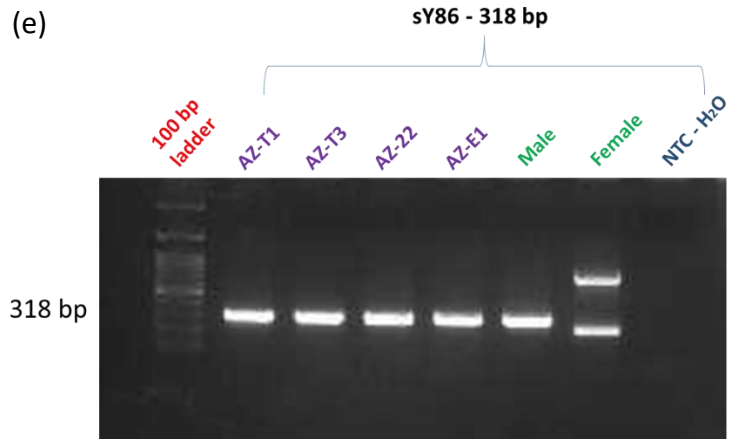
Loading: 10 µL sample

5 µL 100 bp DNA ladder

Electrophoresis: 120 V for 30 mins

(d)





Box 6.2

Annealing Temp.: Touch down 57 °C

Sample DNA conc.: 100 ng/ µL

Agarose Gel Conc.: = 1.5%

Loading: 10 µL sample

5 µL 100 bp DNA ladder

Electrophoresis: 120 V for 30 mins

Figure 6.3: Agarose gel electrophoresis results of Y-chromosome microdeletion PCR. (a) PCR for ZFY marker (495 bp) and SRY (472 bp). (b) PCR for sY254 (400 bp) and sY127 (274 bp). (c) PCR for sY134 (301 bp). All these reactions were conducted at once and loaded on the same gel under same conditions as illustrated in the Box 4.1, but the gel was cropped for illustration reasons. (d) PCR for sY84 (326 bp) and sY255 (123 bp). (e) PCR for sY86 (318 bp). (d) and (e) reactions were performed at once and loaded on the same gel under same conditions as illustrated in the Box 4.2, but the gel was cropped for illustration reasons.

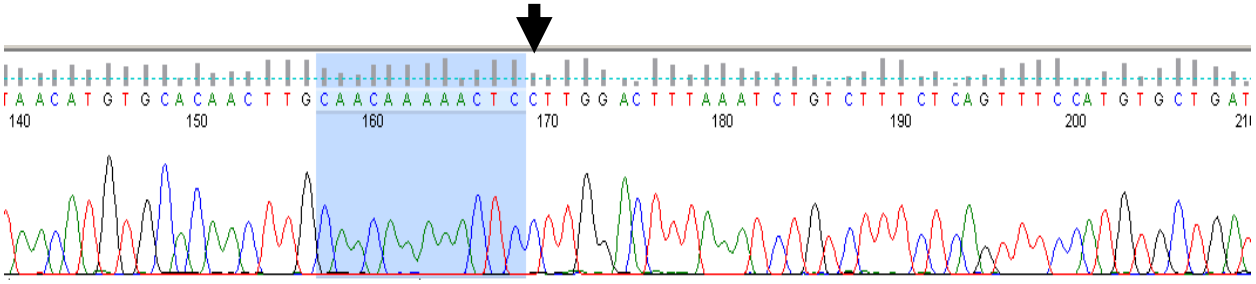
None of the infertile males who have the mutation in MEIOB gene has Y-chromosome microdeletion. All markers were amplified perfectly and no microdeletion was detected. The same for all males who underwent MEIOB gene Sanger sequencing. Touch down PCR program was performed to reduce the unspecific amplification of some markers observed in female samples, however, it was continuously seen. All materials including reagents, primers, female sample were changed to make sure nothing is contaminated, yet, unspecific amplification was observed.

6.3 Sanger Sequencing Results of the Identified MEIOB Mutation

The newly detected mutation in MEIOB gene in azoospermic males is the deletion of G on the position on 1889376 of chromosome 16 (exon 12 of MEIOB gene) which leads to a frameshift at residue number 366, changing the amino acid serine to a stop codon resulting in a premature stop, and in turn, truncation in the conserved C'-terminal DNA binding domain of MEIOB protein. All exons of MEIOB gene were sequenced. Five individuals out of eighty two azoospermic males had this deletion mutation in exon 12 and there was no other mutation detected in other exons. Two of them are relatives (AZ-T1 and AZ-T3), refer to (Figure 6.2) to see their family pedigree (Family No. 1). The other three patients are of unrelated families. Look to (Figure 6.4) to see Sanger sequencing results (electropherograms) of exon 12 compared to wildtype reference sequence. The interesting finding of this study is that one of these azoospermic males has female relatives who are diagnosed with ovarian dysgenesis, refer to (Figure 6.2) to see family pedigree and sampled individuals. We decided to sample them since it is proven that targeted inactivation of MEIOB gene in mice led to infertility in both sexes (Luo *et al.*, 2013). Interestingly, the two females with ovarian dysgenesis (OV-A3 and OV-A7) were homozygous for the same mutation (1889376delG) on MEIOB. To confirm the mutation, segregation within this family was performed. For both females, their parents were heterozygous (carriers) for the deletion mutation which means that this mutation segregates perfectly in a recessive mood and could be a contributor to their ovarian dysgenesis as well. Refer to (Figure 6.4) to see Sanger sequencing results of exon 12 of MEIOB gene for the sampled members of this family. Every sampled individual has a code indicated on the family pedigree and the same code is indicated on the electropherogram of that individual.

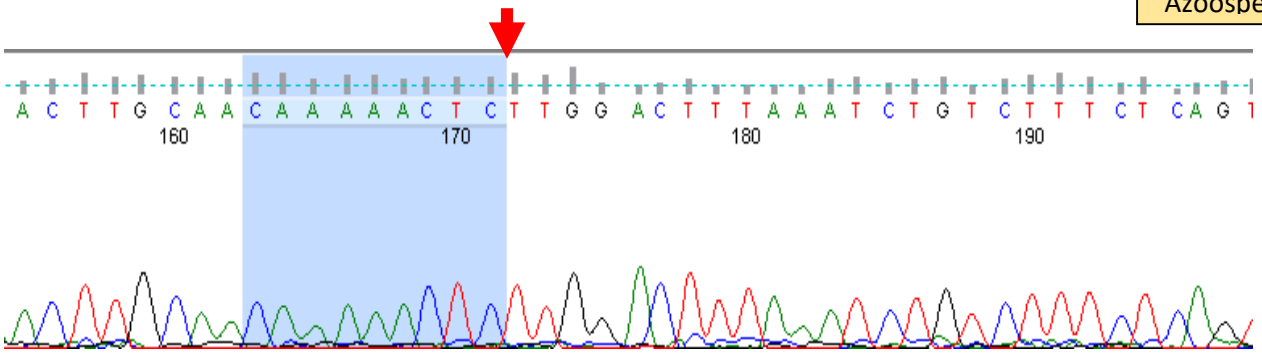
(a) AZ-#1-Forward/ Wildtype (NN)

Fertile



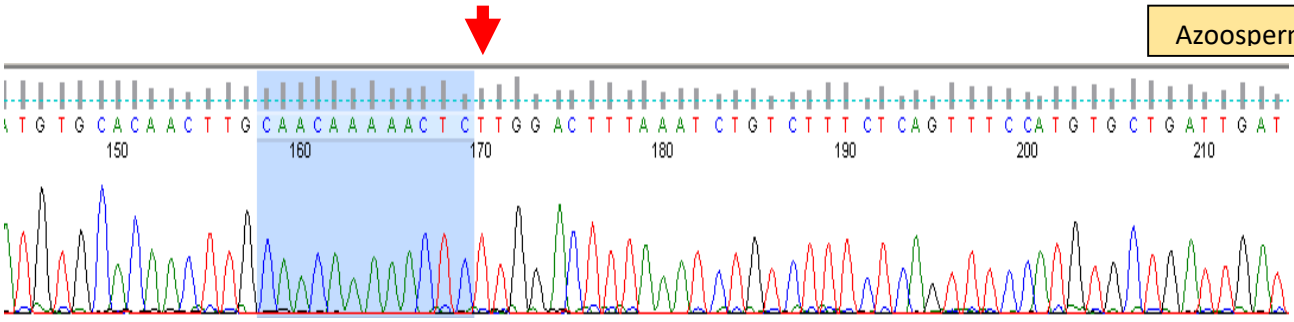
(b) AZ-T1-Forward/ Homozygous for the mutation/ affected (VV)

Azoospermia



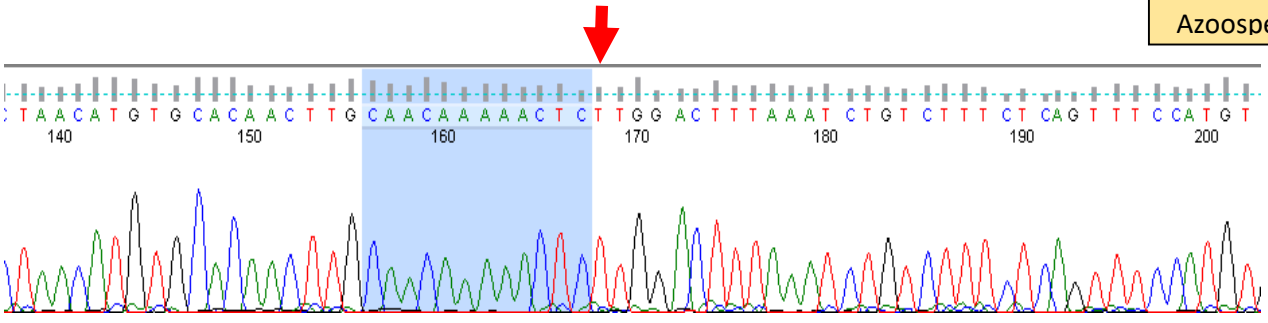
(c) AZ-T3-Forward/ Homozygous for the mutation/ affected (VV)

Azoospermia

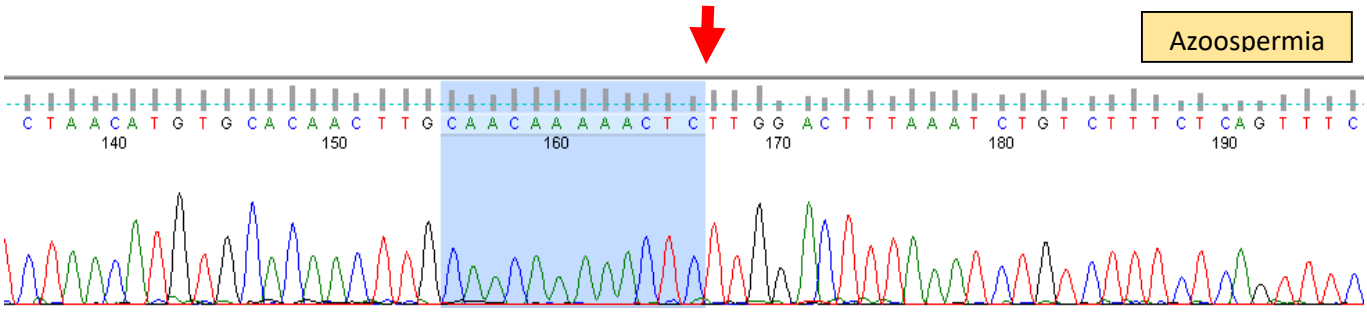


(d) AZ-#22-Forward/ Homozygous for the mutation/ affected (VV)

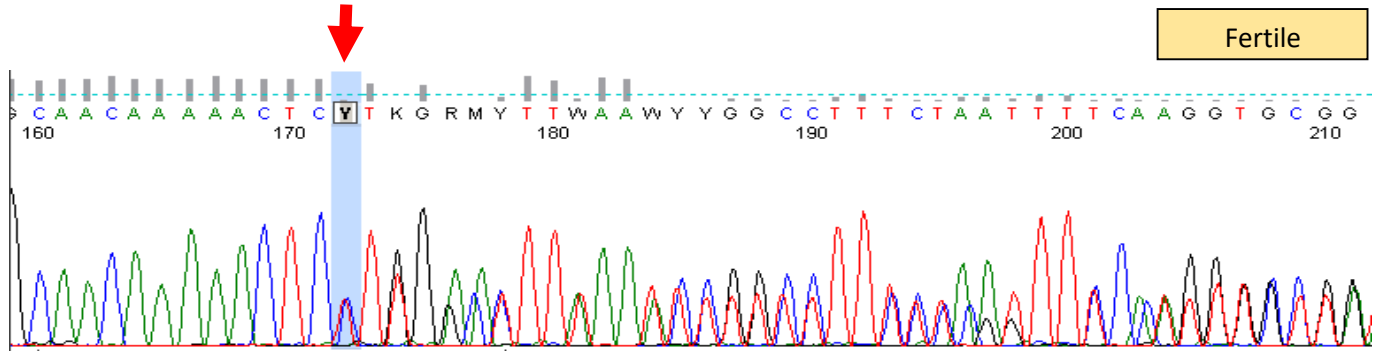
Azoospermia



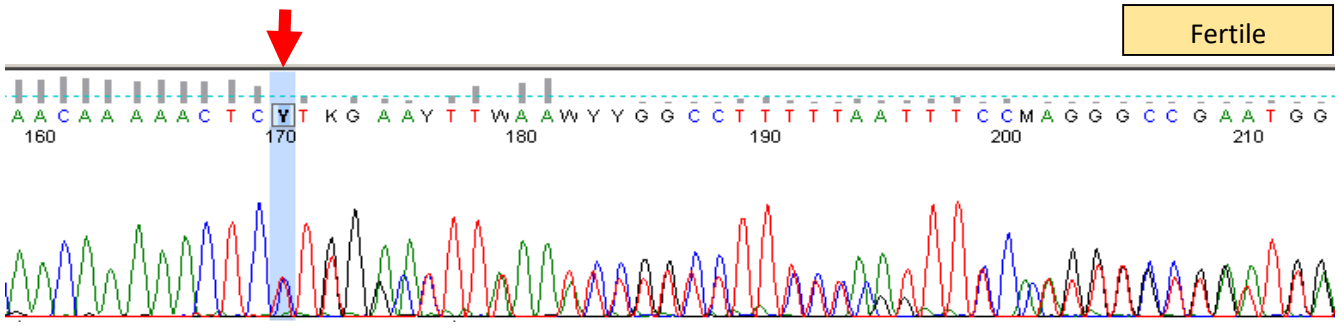
(e) AZ-E1-Forward/ Homozygous for the mutation/ affected (VV)



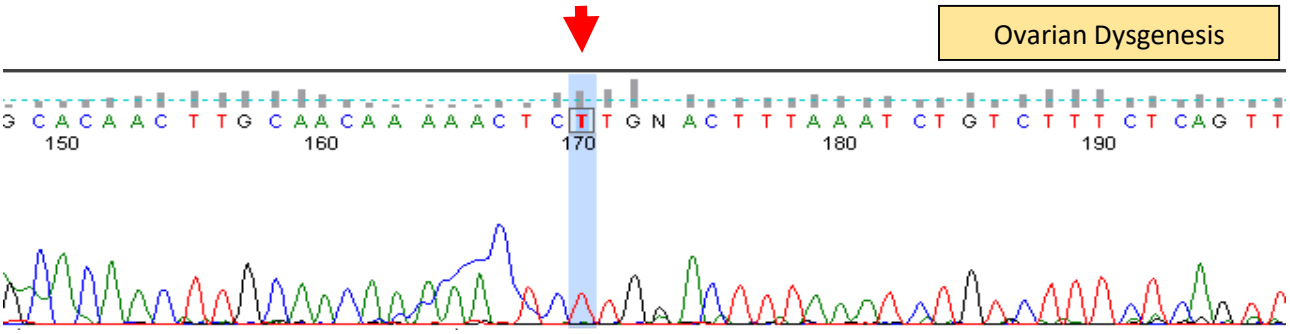
(f) OV-A1-Forward/ Heterozygous for the mutation/ carrier (NV)



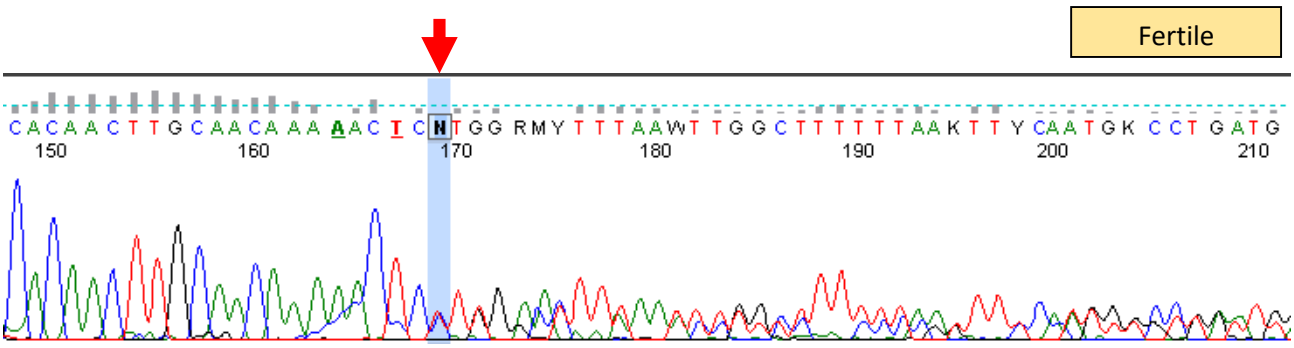
(g) OV-A2-Forward/ Heterozygous for the mutation/ carrier (NV)



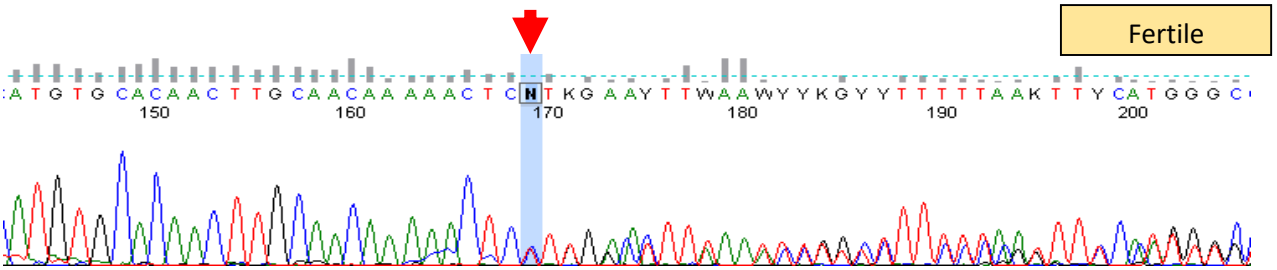
(h) OV-A3-Forward/ Homozygous for the mutation/ affected (VV)



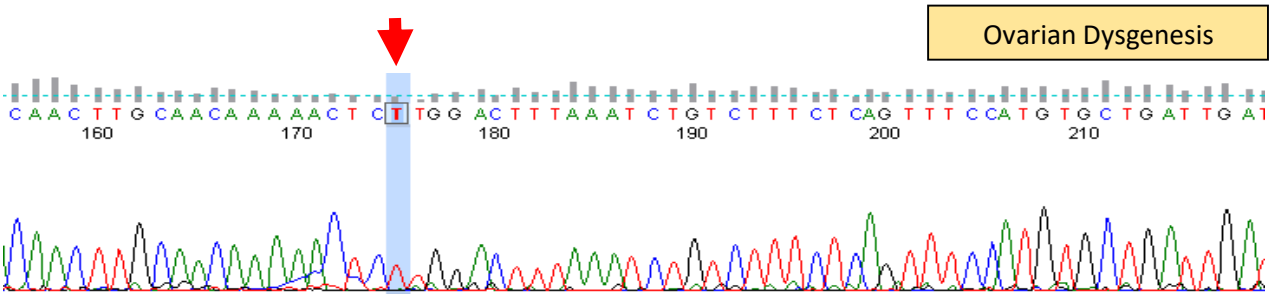
(i) OV-A5-Forward/ Heterozygous for the mutation/ carrier (NV)



(j) OV-A6-Forward/ Heterozygous for the mutation/ carrier (NV)



(k) OV-A7-Forward/ Homozygous for the mutation/ affected (VV)



(l) OV-A9-Forward/ Homozygous for the mutation/ affected (VV)

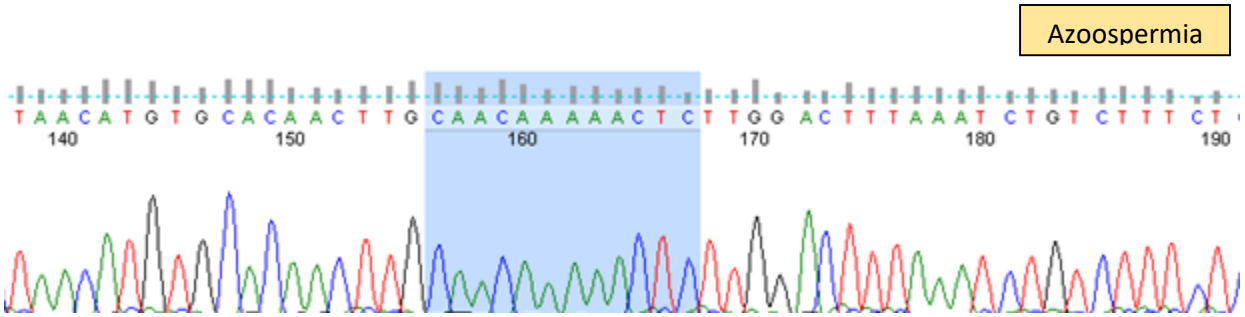


Figure 6.4: Sanger sequencing electropherograms of exon 12 of MEIOB gene. (a) Sanger sequence of exon 12 for a reference wildtype fertile man. (b), (c), (d), and (e) are Sanger sequences results of exon 12 for four males diagnosed with azoospermia and they are all homozygous for the deletion mutation (1889376delG). (h) and (k) are Sanger sequencing results of the two females with ovarian dysgenesis. (f), (g), (i), (j) are parents of the two affected females, and (l) is their affected azoospermic male. The black arrow indicates the position of wildtype (G), while the red arrow indicates the deleted (G). The yellow boxes above each sequence indicates the clinical diagnosis of each individual.

6.4 Fertile Healthy Controls Sanger Sequencing

No mutation was identified in the two hundreds fertile healthy control samples.

CHAPTER 7

Discussion

In our study, we screened for the presence of pathogenic mutations in the gene called MEIOB in eighty two infertile Arab Palestinian males diagnosed either with idiopathic non-obstructive azoospermia, oligospermia, or severe oligospermia. In addition to these 82 samples, two samples of infertile females were included in this study since they are diagnosed with ovarian dysgenesis according to their physician and they were relatives of one azoospermic male. After excluding all individuals with Y-chromosome microdeletions, direct Sanger sequencing was performed to sequence all MEIOB gene coding exons. Homozygous deletion of G on 1889376 of chromosome 16 (NM_001163560; exon12: c.1098delC: p.S366fs*) was revealed in five male individuals diagnosed with NOA and two female individuals diagnosed with ovarian dysgenesis. Segregation analyses were only performed in one consanguineous family and showed that this mutation segregates perfectly in a recessive mode in this family. This mutation was predicted to cause frame-shift which leads to a pre-mature stop codon, and consequently a truncation in the conserved C'-terminal DNA-binding domain of the resulting MEIOB protein. The same mutation was reported in two azoospermic Arab Israeli brothers by Gershoni and colleagues (Gershoni *et al.*, 2019).

According to literature, the unspecific amplification of some Y-chromosome markers in female control sample could be explained by the presence of DNA of male origin in this female sample, i.e., her son's progenitor cells kept circulating in her blood few years after delivery (Rao *et al.*, 2008). Rao and colleagues identified the presence of low-levels of Y-Chromosome mosaicism in thirty one normal females with a male child and in thirteen female cases with abnormal sex chromosomes through nested PCR, however, this was not observed in female members who have female child. They explained this by the presence of male progenitor cells that kept circulating in mother's blood and led to what is called "Microchimerism of Y". Although they used nested PCR, but they also said that this could also happen in conventional PCRs (Rao *et al.*, 2008). So, one possible explanation for our results is that these unspecific bindings in female control are due to microchimerism of Y-chromosome in her blood and maybe we should just replace this woman with one who has only female children or to extract the DNA from other tissue.

Previous studies have shown that Meiob null mice displayed infertility, complete meiotic arrest, and unrepaired DNA strand breaks. Indeed, Meiob null male mice seminiferous tubules were devoid of post-meiotic germ cells, however, female Meiob null mice were completely devoid of oocytes in post-natal developmental stages (Luo *et al.*, 2013) (Souquet *et al.*, 2013), which is so similar to the diagnosis (azoospermic patients with negative TFNA results; i.e., zero sperms) of infertile males who have mutated MEIOB gene in the present study. Interestingly, the two female members recruited to this study and tested positive for the mutation also showed similar phenotypes to Meiob null female mice; they had small ovaries and irregular menstruation which stopped at the age of seventeen. In other recent study, Gershoni and colleagues figured out another pathogenic MEIOB mutation (homozygous missense (N64I) substitution mutation) in four NOA Arab Israeli brothers, and this mutation was predicted to affect a highly conserved part of the DNA binding domain of MEIOB protein. Moreover, Caburet et al. identified a homozygous splicing-altering mutation in the last base of exon 12 resulting in exon 12 skipping and pre-mature termination codon, this led to a truncated protein product in two Arab female individuals who are diagnosed with Primary Ovarian Insufficiency (POI) or what is called premature menopause (Caburet *et al.*, 2019).

Herein, our study provides NOA and ovarian dysgenesis cases with mutated MEIOB gene supporting all previous studies, and increasing a high level of evidence to implicate MEIOB gene as a major contributor to male and female infertility in humans. Taking all these investigations together, and noticing that in all cases the affected individuals were of Arab ethnicity, this raises a big question whether this mutation is an Arab founder mutation or not. May be the currently available data is not enough to answer this question, thus, we recommend recruiting more idiopathic NOA samples from Arab and non-Arab populations and search for MEIOB mutations. We believe by providing more data, we will help in improving non-invasive diagnostic tools and provide better counselling to NOA patients.

CHAPTER 8

Conclusion

Herein our study, as a part of studying genetic bases of male infertility in the Palestinian population, we performed direct Sanger sequencing to screen for MEIOB gene mutations in eighty two infertile Arab-Palestinian males and two additional infertile female members who are relatives of one infertile male. Recessive homozygous deletion of G on 1889376 of chromosome 16 (NM_001163560; Chr16: 1889376 del G MEIOB, exon 12 – S366fs*) was implicated in five male individuals diagnosed with NOA and two female individuals diagnosed with ovarian dysgenesis. This frameshift resulted in a premature stop codon, and is predicted to cause a truncation in the C'-terminal DNA binding domain of the MEIOB protein since the C'-terminal part of MEIOB locates between 294 and 450 including SPATA22-binding fold. Therefore, it is expected that this truncation may affect the interaction between MEIOB and its co-factor SPATA-22, and thus, crossover formation insufficiency. This mutation was only implicated in affected individuals, however, no mutation was detected in the 200 healthy fertile Palestinian controls indicating its pathogenicity.

Other mutations in MEIOB gene were reported in three recent studies, and interestingly, the affected individuals were all from Arab ethnicity, driving us to think that this mutation could be an Arab-founder mutation. Yet, we need to recruit more samples to prove this.

Our results together with all these recent studies add valuable data to prove the role of MEIOB in human male infertility and maybe female infertility as well.

CHAPTER 9

Bibliography

Agarwal, A. *et al.* (2015) 'A unique view on male infertility around the globe', *Reproductive biology and endocrinology*. BioMed Central, 13(1), p. 37.

Babakhanzadeh, E. *et al.* (2020) 'Some of the Factors Involved in Male Infertility: A Prospective Review', *International Journal of General Medicine*. Dove Press, 13, p. 29.

Bennett, N. C. *et al.* (2010) 'Molecular cell biology of androgen receptor signalling', *The international journal of biochemistry & cell biology*. Elsevier, 42(6), pp. 813–827.

Bishop, D. K. and Zickler, D. (2004) 'Early decision: meiotic crossover interference prior to stable strand exchange and synapsis', *Cell*. Elsevier, 117(1), pp. 9–15.

Bochkareva, E. *et al.* (2002) 'Structure of the RPA trimerization core and its role in the multistep DNA-binding mechanism of RPA', *The EMBO journal*. John Wiley & Sons, Ltd, 21(7), pp. 1855–1863.

Boehm, U. *et al.* (2015) 'Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism—pathogenesis, diagnosis and treatment', *Nature Reviews Endocrinology*. Nature Publishing Group, 11(9), p. 547.

Boissonnas, C. C., Jouannet, P. and Jammes, H. (2013) 'Epigenetic disorders and male subfertility', *Fertility and sterility*. Elsevier, 99(3), pp. 624–631.

Bullard, S. A. *et al.* (1996) 'Double strand breaks at the HIS2 recombination hot spot in Saccharomyces cerevisiae', *Proceedings of the National Academy of Sciences*. National Acad Sciences, 93(23), pp. 13054–13059.

Caburet, S. *et al.* (2019) 'A truncating MEIOB mutation responsible for familial primary ovarian insufficiency abolishes its interaction with its partner SPATA22 and their recruitment to DNA double-strand breaks', *EBioMedicine*. Elsevier, 42, pp. 524–531.

Cannarella, R., Condorelli, R. A., Duca, Y., La, S., *et al.* (2019) 'New insights into the genetics of spermatogenic failure : a review of the literature', *Human Genetics*. Springer Berlin Heidelberg, 0(0), p. 0. doi: 10.1007/s00439-019-01974-1.

Cannarella, R., Condorelli, R. A., Duca, Y., La Vignera, S., *et al.* (2019) 'New insights into the genetics of spermatogenic failure: a review of the literature', *Human genetics*. Springer, 138(2), pp. 125–140.

Casals, T. *et al.* (2000) 'Heterogeneity for mutations in the CFTR gene and clinical correlations in patients with congenital absence of the vas deferens', *Human Reproduction*. Oxford University Press, 15(7), pp. 1476–1483.

Corradi, P. F., Corradi, R. B. and Greene, L. W. (2016) 'Physiology of the hypothalamic pituitary gonadal axis in the male', *Urologic Clinics*. Elsevier, 43(2), pp. 151–162.

Costa, Y. and Cooke, H. J. (2007) 'Dissecting the mammalian synaptonemal complex using targeted mutations', *Chromosome Research*. Springer, 15(5), pp. 579–589.

Coutton, C. *et al.* (2015) 'Teratozoospermia: spotlight on the main genetic actors in the human', *Human reproduction update*. Oxford University Press, 21(4), pp. 455–485.

Dieterich, K. *et al.* (2007) 'Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility', *Nature genetics*. Nature Publishing Group, 39(5), pp. 661–665.

Doerksen, T. and Trasler, J. M. (1996) 'Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats', *Biology of reproduction*. Oxford University Press, 55(5), pp. 1155–1162.

Eggler, A. L., Inman, R. B. and Cox, M. M. (2002) 'The Rad51-dependent pairing of long DNA substrates is stabilized by replication protein A', *Journal of Biological Chemistry*. ASBMB, 277(42), pp. 39280–39288.

Estivill, X., Bancells, C. and Ramos, C. (1997) 'Geographic distribution and regional origin of 272 cystic fibrosis mutations in European populations', *Human mutation*. Wiley Online Library, 10(2), pp. 135–154.

Ferlin, A., Dipresa, S. and Foresta, C. (2019) 'Genetic Testing in Male Infertility', in *Human Reproductive and Prenatal Genetics*. Elsevier, pp. 383–398.

Gershoni, M. *et al.* (2017) 'A familial study of azoospermic men identifies three novel causative mutations in three new human azoospermia genes', *Genetics in Medicine*. Nature Publishing Group, 19(9), pp. 998–1006.

Gershoni, M. *et al.* (2019) 'A new MEIOB mutation is a recurrent cause for azoospermia and testicular meiotic arrest', *Human Reproduction*. Oxford University Press, 34(4), pp. 666–671.

Gottlieb, B. *et al.* (2012) 'The androgen receptor gene mutations database: 2012 update', *Human mutation*. Wiley Online Library, 33(5), pp. 887–894.

Greenbaum, M. P. *et al.* (2006) 'TEX14 is essential for intercellular bridges and fertility in male mice', *Proceedings of the National Academy of Sciences*. National Acad Sciences, 103(13), pp. 4982–4987.

Greil, A. L., Slauson-Blevins, K. and McQuillan, J. (2010) 'The experience of infertility: a review of recent literature', *Sociology of health & illness*. Wiley Online Library, 32(1), pp. 140–162.

Grimes, D. A. and Lopez, L. M. (2007) '"Oligozoospermia,""azoospermia," and other semen-analysis terminology: the need for better science'. Elsevier.

Harewood, L. and Fraser, P. (2014) 'The impact of chromosomal rearrangements on regulation of gene expression', *Human molecular genetics*. Oxford University Press, 23(R1), pp. R76--R82.

Hochstenbach, R. and Hackstein, J. H. P. (2000) 'The comparative genetics of human spermatogenesis: clues from flies and other model organisms', in *The genetic basis of male infertility*. Springer, pp. 271–298.

Hughes, I. A. *et al.* (2012) 'Androgen insensitivity syndrome', in *Seminars in reproductive medicine*, pp. 432–442.

Inhorn, M. C. and Patrizio, P. (2015) 'Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century', *Human reproduction update*. Oxford University Press, 21(4), pp. 411–426.

Ishishita, S., Matsuda, Y. and Kitada, K. (2014) 'Genetic evidence suggests that Spata22 is required for the maintenance of Rad51 foci in mammalian meiosis', *Scientific reports*. Nature Publishing Group, 4, p. 6148.

Jenkins, T. and Carrell, D. T. (2012) 'Dynamic alterations in the paternal epigenetic landscape

following fertilization', *Frontiers in genetics*. Frontiers, 3, p. 143.

Joyce, E. F., Tanneti, S. N. and McKim, K. S. (2009) 'Drosophila hold'em is required for a subset of meiotic crossovers and interacts with the dna repair endonuclease complex subunits MEI-9 and ERCC1', *Genetics*. Genetics Soc America, 181(1), pp. 335–340.

Jungwirth, A. *et al.* (2012) 'European Association of Urology guidelines on Male Infertility: the 2012 update', *European urology*. Elsevier, 62(2), pp. 324–332.

Juul, A. *et al.* (2014) 'Possible fetal determinants of male infertility', *Nature Reviews Endocrinology*. Nature Publishing Group, 10(9), p. 553.

Kelly, T. L. J., Li, E. N. and Trasler, J. M. (2003) '5-Aza-2′-deoxycytidine induces alterations in murine spermatogenesis and pregnancy outcome', *Journal of andrology*. Wiley Online Library, 24(6), pp. 822–830.

Khelifa, M. Ben *et al.* (2014) 'Mutations in DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple morphological abnormalities of the sperm flagella', *The American Journal of Human Genetics*. Elsevier, 94(1), pp. 95–104.

Kim, D.-K., Stigger, E. and Lee, S.-H. (1996) 'Role of the 70-kDa Subunit of Human Replication Protein A (I) SINGLE-STRANDED DNA BINDING ACTIVITY, BUT NOT POLYMERASE STIMULATORY ACTIVITY, IS REQUIRED FOR DNA REPLICATION', *Journal of Biological Chemistry*. ASBMB, 271(25), pp. 15124–15129.

Knowles, M. R., Zariwala, M. and Leigh, M. (2016) 'Primary ciliary dyskinesia', *Clinics in chest medicine*. Elsevier, 37(3), pp. 449–461.

Krausz, C. (2011) 'Male infertility: pathogenesis and clinical diagnosis', *Best practice & research Clinical endocrinology & metabolism*. Elsevier, 25(2), pp. 271–285.

Krausz, C. *et al.* (2014) 'EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013', *Andrology*. Wiley Online Library, 2(1), pp. 5–19.

Krausz, C. and Casamonti, E. (2017) 'Spermatogenic failure and the Y chromosome', *Human*

genetics. Springer, 136(5), pp. 637–655.

Krausz, C. and Consultant, S. (2011) 'Endocrinology & Metabolism Male infertility : Pathogenesis and clinical diagnosis', *Best Practice & Research Clinical Endocrinology & Metabolism*. Elsevier Ltd, 25(2), pp. 271–285. doi: 10.1016/j.beem.2010.08.006.

Krausz, C. and Riera-escamilla, A. (2018) 'Genetics of male infertility', *Nature Reviews Urology*. Springer US, 15(June). doi: 10.1038/s41585-018-0003-3.

Krausz, C. and Riera-Escamilla, A. (2018) 'Genetics of male infertility', *Nature Reviews Urology*. Nature Publishing Group, 15(6), pp. 369–384.

Lawo, S. *et al.* (2009) 'HAUS, the 8-subunit human Augmin complex, regulates centrosome and spindle integrity', *Current Biology*. Elsevier, 19(10), pp. 816–826.

Luo, M. *et al.* (2013) 'MEIOB exhibits single-stranded DNA-binding and exonuclease activities and is essential for meiotic recombination', *Nature communications*. Nature Publishing Group, 4(1), pp. 1–12.

Martini, E. *et al.* (2006) 'Crossover homeostasis in yeast meiosis', *Cell*. Elsevier, 126(2), pp. 285–295.

Nistal, M., Paniagua, R. and Herruzo, A. (1978) 'Multi-tailed spermatozoa in a case with asthenospermia and teratospermia', *Virchows Archiv B*. Springer, 26(1), pp. 111–118.

O'Donnell, L. (2014) 'Mechanisms of spermiogenesis and spermiation and how they are disturbed', *Spermatogenesis*. Taylor & Francis, 4(2), p. e979623.

Oakes, C. C. *et al.* (2007) 'Adverse effects of 5-aza-2′-deoxycytidine on spermatogenesis include reduced sperm function and selective inhibition of de novo DNA methylation', *Journal of Pharmacology and Experimental Therapeutics*. ASPET, 322(3), pp. 1171–1180.

Pawlowski, W. P. and Cande, W. Z. (2005) 'Coordinating the events of the meiotic prophase', *Trends in cell biology*. Elsevier, 15(12), pp. 674–681.

Potter, S. J. and DeFalco, T. (2017) 'Role of the testis interstitial compartment in spermatogonial stem cell function', *Reproduction*. Society for Reproduction and Fertility, 153(4), pp. R151--

R162.

Punab, M. *et al.* (2017) 'Causes of male infertility: a 9-year prospective monocentre study on 1737 patients with reduced total sperm counts', *Human reproduction*. Oxford University Press, 32(1), pp. 18–31.

Rao, L. *et al.* (2008) 'Y chromosome microchimerism in female peripheral blood', *Reproductive biomedicine online*. Elsevier, 17(4), pp. 575–578.

Ray, P. F. *et al.* (2017) 'Genetic abnormalities leading to qualitative defects of sperm morphology or function', *Clinical Genetics*. Wiley Online Library, 91(2), pp. 217–232.

Repping, S. *et al.* (2003) 'Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection', *Nature genetics*. Nature Publishing Group, 35(3), pp. 247–251.

Ribeiro, J. *et al.* (2016) 'RPA homologs and ssDNA processing during meiotic recombination', *Chromosoma*. Springer, 125(2), pp. 265–276.

Robert, T. *et al.* (2016) 'The TopoVIB-Like protein family is required for meiotic DNA double-strand break formation', *Science*. American Association for the Advancement of Science, 351(6276), pp. 943–949.

Roeder, G. S. (1997) 'Meiotic chromosomes: it takes two to tango', *Genes & development*. Cold Spring Harbor Lab, 11(20), pp. 2600–2621.

Rutstein, S. O. and Shah, I. H. (2004) *Infecundity, infertility, and childlessness in developing countries*. ORC Macro, MEASURE DHS.

Sharlip, I. D. *et al.* (2002) 'Best practice policies for male infertility', *Fertility and sterility*. Elsevier, 77(5), pp. 873–882.

Skaletsky, H. *et al.* (2003) 'The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes', *Nature*. Nature Publishing Group, 423(6942), pp. 825–837.

Souquet, B. *et al.* (2013) 'MEIOB targets single-strand DNA and is necessary for meiotic recombination', *PLoS genetics*. Public Library of Science, 9(9).

de Souza, D. A. S. *et al.* (2018) 'Congenital bilateral absence of the vas deferens as an atypical form of cystic fibrosis: reproductive implications and genetic counseling', *Andrology*. Wiley Online Library, 6(1), pp. 127–135.

Sun, F. *et al.* (2007) 'Abnormal progression through meiosis in men with nonobstructive azoospermia', *Fertility and sterility*. Elsevier, 87(3), pp. 565–571.

Sun, H., Treco, D. and Szostak, J. W. (1991) 'Extensive 3′-overhanging, single-stranded DNA associated with the meiosis-specific double-strand breaks at the ARG4 recombination initiation site', *Cell*. Cell Press, 64(6), pp. 1155–1161.

Tiepolo, L. and Zuffardi, O. (1976) 'Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm', *Human genetics*. Springer, 34(2), pp. 119–124.

Tournaye, H., Krausz, C. and Oates, R. D. (2017) 'Novel concepts in the aetiology of male reproductive impairment', *The Lancet Diabetes & Endocrinology*. Elsevier, 5(7), pp. 544–553.

Tüttelmann, F. *et al.* (2007) 'Gene polymorphisms and male infertility--a meta-analysis and literature review', *Reproductive biomedicine online*. Elsevier, 15(6), pp. 643–658.

Vog, P. H. *et al.* (1996) 'Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11', *Human molecular genetics*. Oxford University Press, 5(7), pp. 933–943.

Vorona, E. *et al.* (2007) 'Clinical, endocrinological, and epigenetic features of the 46, XX male syndrome, compared with 47, XXY Klinefelter patients', *The Journal of Clinical Endocrinology & Metabolism*. Oxford University Press, 92(9), pp. 3458–3465.

Wang, X. and Haber, J. E. (2004) 'Role of Saccharomyces single-stranded DNA-binding protein RPA in the strand invasion step of double-strand break repair', *PLoS biology*. Public Library of Science, 2(1).

Weiske, W.-H. *et al.* (2000) 'Clinical findings in congenital absence of the vasa deferentia', *Andrologia*. Wiley Online Library, 32(1), pp. 13–18.

Wyatt, H. D. M. and West, S. C. (2014) 'Holliday junction resolvases', *Cold Spring Harbor*

perspectives in biology. Cold Spring Harbor Lab, 6(9), p. a023192.

Xu, Y. *et al.* (2017) 'Meiosis-specific proteins MEIOB and SPATA22 cooperatively associate with the single-stranded DNA-binding replication protein A complex and DNA double-strand breaks', *Biology of reproduction*. Oxford University Press, 96(5), pp. 1096–1104.

Yang, Y. *et al.* (2010) 'Differential effect of specific gr/gr deletion subtypes on spermatogenesis in the Chinese Han population', *International journal of andrology*. Wiley Online Library, 33(5), pp. 745–754.

Yoshida, S. *et al.* (2006) 'The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage', *Development*. Oxford University Press for The Company of Biologists Limited, 133(8), pp. 1495–1505.

Zegers-Hochschild, F. *et al.* (2009) 'The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology, 2009', *Human reproduction*. Oxford University Press, 24(11), pp. 2683–2687.

CHAPTER 10

Appendix I

Patient's Questionnaire

BETHLEHEM UNIVERSITY

جامعة بيت لحم

الاسم الرباعي للزّوج : ..... تاريخ الميلاد : ..... / ..... / .....

الاسم الرباعي للزّوجة: ..... تاريخ الميلاد : ..... / ..... / .....

مكان السكن : ..... الحي: .....

رقم النقال : ..... رقم الهاتف: .....

العمل: .....

يرجى الإجابة على الأسئلة التالية :

	نعم	لا	
هل كان العدد أكثر من 20 مليون؟	☐	☐	
هل تعرضت إلى حادث من قبل ؟	☐	☐	
هل أجريت عملية جراحية؟	☐	☐	أذكرها .....
هل أصبت بمرض أبو دغيم في حياتك؟	☐	☐	
هل أصبت بدوالي؟	☐	☐	
هل لديك أمراض مزمنة ؟	☐	☐	
هل كان عندك ارتفاع في الخصيتين ؟	☐	☐	
هل قمت بفحص كروموسومات من قبل؟	☐	☐	
هل قمت بعمل خزعة (TFNA) ؟	☐	☐	النتيجة؟ .....

مدة الزواج ؟ .....

هل يوجد قرابة بين الزوجين ؟ ..... ما هي ؟ .....

P.O. Box 9- Bethlehem-Palestine
Tel: + 972-2-2741241
Fax:+ 972-2-2744440

ص.ب 9 – بيت لحم – فلسطين
هاتف: 2741241-2-972+
فاكس: 2744440-2-972+