Joint Biotechnology Master Program

Palestine Polytechnic University
Deanship of Graduate Studies and
Scientific Research

Bethlehem University
Faculty of Science
Biology Department

Screening for MEIOB Gene Mutations That Cause Premature Ovarian Insufficiency In Five Palestinian Families.

By

Bara'ah Ayman Abu Shamseyah

In Partial Fulfillment of the Requirements for the Degree
Master of Science in Biotechnology

December, 2021

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:

Screening for MEIOB Gene Mutations That Cause Premature Ovarian Insufficiency In Five Palestinian Families.

By

Bara'ah Ayman Abu Shamseyah

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

Graduate Advisory Committee:

Committee Member (Student's Supervisor) — Date: 1/3/2022

Dr. Hashem Shahin, Bethlehem University.

Committee Member (Internal Examiner) — Date: 1/3/2022

Dr. Omar Darissa, Bethlehem University.

External Committee Member (External Examine) — Date: 1/3/2022

Dr. Saad Al-Lahham, An-Najah University.

Approved for the Faculties

Dean of Graduate studies and Scientific Research Palestine Polytechnic University

Dean of Faculty of Science Bethlehem University

1/3/2022

Screening for MEIOB Gene Mutations That Cause Premature Ovarian Insufficiency in Five Palestinian Families.

By: Bara'ah Ayman Abu Shamseyah

Abstract

Infertility is the failure of a couple to achieve clinical pregnancy within 12 months of unprotected regular intercourse according to World Health Organization (WHO)**.** Female factors account for about 50% of all cases. Approximately there is 30% of couples who suffer from unexplained infertility which may be as a result of causes that could have a correlation with endocrinological balance disorders, or maybe an immunology disorder or even by a genetic issues. MEIOB is a single-stranded DNA-binding protein required for repairing double-strand breaks (DSBs) during early steps of meiosis and crossover formation, thus for proper and correct chromosome synapsis. Absence of MEIOB gene in mice led to sterility in both male and female mice due to meiotic arrest. In this study we carried out Sanger sequencing to screen known mutations in the Palestinian population, in premature ovarian insufficiency (POI), specifically mutations in MEIOB gene, in five Palestinian families which have six females who's suffer from POI. No mutation was detected in any of those individuals. After that we implemented linkage exclusion technique to determine if any of our families cannot be excluded for linkage to the MEIOB gene. Two markers was designed before and after MEIOB gene, results showed homozygous data (in affected and un-affected member of chosen families), which mean that this marker is not informative and can't be used for linkage exclusion in this family. Unfortunately, the second marker was not informative for same reasons.

Key words: Infertility , Premature Ovarian Failure (POI) , MEIOB gene.

Screening MEIOB Gene Mutation That Cause Premature Ovarian Insufficiency In Five Palestinian Families .

الملخص

العقم هو فشل الزوجين في تحقيق الحمل في غضون 12 شهرًا من الجماع المنتظم غير المحمي وفقًا لمنظمة الصحة العالمية (WHO). تمثل العوامل الأنثوية حوالي 50 ٪ من جميع الحالات. ما يقرب من 30٪ من الأزواج يعانون من عقم غير معروف سببه والذي قد يكون نتيجة لأسباب قد تكون لها علاقة باضطرابات التوازن في الغدد الصماء ، أو ربما اضطراب في المناعة أو حتى بسبب مشاكل وراثية. MEIOB هو بروتين مرتبط بالحمض النووي أحادي السلسلة مطلوب لإصلاح عملية قطع سلسلتي الحمض النووي أثناء الخطوات المبكرة للانقسام المنصف وتشكيل التقاطع ، وبالتالي من أجل التشابك السليم والصحيح للكروموسوم. أدى فقدان MEIOB في الفئران إلى العقم في كل من الفئران الذكور والإناث بسبب توقف الانقسام المنصف. في هذه الدراسة ، أجرينا فحص باستخدام سانجر لقراءة التسلسل النيوكليوتيدي لفحص الطفرة المسببة في فشل المبايض المبكر (POI) ، وتحديداً الطفرة الجينية MEIOB ، في خمس عائلات فلسطينية لديها ست إناث تم تشخيصهم بفشل المبايض. لم يتم الكشف عن أي طفرة في أي من هؤلاء. بعد ذلك قمنا بتقنية Linkage exclusion للتحقق مما إذا كان الجين مرتبطًا بفشل المبايض المبكر. تم تصميم علامتين قبل وبعد جين MEIOB ، وأظهرت النتائج بيانات متماثلة اللواقح (في الأفراد المتأثرين وغير المتأثرين من العائلات المختارة) ، مما يعني أن هذه العلامة ليست مفيدة ولا يمكن استخدامها لاستبعاد الارتباط في هذه العائلة. لسوء الحظ ، لم تكن العلامة الثانية مفيدة للأسباب نفسها.

DECLARATION

I declare that the Master Thesis entitled "Screening for MEIOB Gene Mutations That Cause Premature Ovarian insufficiency In Five Palestinian Families" 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 taken from the work of others and to the extent that such work has been cited within the text of my work, 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: Bara'ah Ayman Abu Shamseyah

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: Bara'ah Ayman Abu Shamseyah

Date: _____

Dedication

This thesis is faithfully dedicated for my beloved parents, Ayman Abu Shamseyah and Hala Idais, they were my supporters until my research was fully finished and reach this stage, they encouraged me attentively with fullest and truest love and care. To my lovely kids Yamen, Qamar and Nariz, to my siblings Ali, Sara, Salah, Mohammad, Abdullah and Rahaf. To my grandparents Mustafa and Heyam Ideas.

I dedicate this thesis work to my husband Nizar Joulani, who never loses faith in every step I take. This thesis is only the beginning of my journey .

Acknowledgments

Special Thanks, grateful and respect to my supervisor Dr. Hashem Shahin for his assistance at every step of this research project, and his insightful comments and suggestions. It was a real honor to me to be one of your thesis student and work under your supervision, I hope that will be the beginning of my journey in molecular labs.

I would like also to express my special thanks to all my professors during master program (Dr. Muneef Ayyash, , Dr. Ghassan Handal, Dr. Mousa Hindiyeh, Prof. Mazin Qumsiyeh, Dr. Omar Issa, Prof. Moien Kanaan, Dr. Robin Abu Ghazaleh, Dr. Yaqoub Ashhab, Dr. Rami Arafeh, Dr. Fouad Zahdeh, and Dr. Areej Al khatib) for their unwavering support and belief in me.

I would like to thank Dr. Iyad Afaneh and all his team at Palestinian-European Fertilization Clinic in Hebron. Deepest thanks and feeling for patient who help me in this work to get result and information I needed, to whom who struggling to have baby, I want to tell you that life is tough, but so are you .Special thanks to lab colleagues Islam Abu Allan, Lama Juneide, Grace Rabie, Reem Massad, Mariana Kawwas .

Abbreviations

ICMART	International Committee for Monitoring Assisted Reproductive Technology
WHO	World Health Organization
UI	Unexplained infertility
PCOM	polycystic ovarian-morphology
HPG	hypothalamic-pituitary-gonadal
FSH	follicle-stimulating hormone
LH	low luteinizing hormone
PID	pelvic inflammatory disease
MBL	alter Mannose-binding lectin
TS	Turner Syndrome
SHOX	encoding short stature homeobox protein
DNA	Deoxyribonucleic acid
MII	meiosis II
MI	meiosis I
SC	Synaptonemal Complex
AE	axial elements
SYCP2	SC proteins 2
SYCP3	SC proteins 3
TF	transverse filaments
CE	central element
SYCP1	central element proteins 1
SYCE3	Synaptonemal Complex Central Element Protein 3
DSB	double-strand breaks
γH2AX	phosphorylated histone H2AX
RPA	replication protein A
ssDNA	single-stranded DNA

DMC1	DNA Meiotic Recombinase 1
RAD51	recombinase radiation-sensitive 51
D-loop	displacement loop
Dhj	double-Holliday Junction
SPATA22	Spermatogenesis-associated protein 22
CO	Cross over
NCO	non-crossover
SDSA	synthesis-dependent strand-annealing
PGCs	primordial germ cells
MEIOB	Meiosis Specific With OB-Domain
Dbd	DNA binding domains
OB	oligosaccharide/oligonucleotide binding
KEGG SSDB	Kyoto Encyclopedia of Genes and Genomes Sequence Similarity Data Base
Dpc	days post coitum
POI	Primary ovarian insufficiency
SDS	sodium dodecyl sulfate
WBC's	White Blood Cells
RBC's	Red Blood Cells
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase Chain Reaction
Rpm	Rounds per minute
TAE	Tris-acetate-EDTA
AnP	Antarctic Phosphatase
Exo1	Exonuclease I
BDX64	BigDye® Enhancing Buffer
DSBR	DSB repair pathway

List of Figures

List of Tables

Table of Contents

CHAPTER 1

Introduction

2.2.1 Infertility is the failure of a couple to achieve clinical pregnancy within 12 months of unprotected regular intercourse according to International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) (Devroey et al., 2009 ; Oberoi, Khaira and Rai,2017). It is a great life crisis which causes serious Mental, stressful, social, medical and financial effects for infertile individuals. (Direkvand-Moghadam, Delpisheh and Direkvand-Moghadam ,2014). Female factors account for about 50% of all cases (Agarwal et al., 2015). Female Infertility may be caused by many medical conditions that may damage the fallopian tubes, causes hormonal complications, or interferes with ovulation. . Different factors are attributed to infertility, such as ovulation disorders, which considered as the most common cause of infertility in numerous studies (Jacob Farhi,2011). Tubal factor present the second common cause of female infertility (Masoumi et al.,2015). Genetic abnormalities such as chromosomal abnormalities gene mutations (single or multiple) contribute to 5–10% of infertility, often result from meiotic and mitotic errors (Poornimaet al.,2020). There are many chromosomal abnormalities known that lead to infertility, the most common are Turner syndrome, 45,X0/46,XY mosaicism, and Down syndrome. There is also many Chromosomal Structural Anomalies that lead to reproductive impairment in females (Yahaya et al., 2021).Approximately there is about 30% of couples suffer from Unexplained Infertility (UI) which considered idiopathic (Smith, Pfeifer and Collins, 2003).

As a part of studying genetic etiology of female infertility in the Palestinian population, we have applied direct Sanger sequencing to screen for MEIOB gene mutations in 6 infertile Palestinian who suffer from Premature ovarian insufficiency. Most of them having an Azoospermia relatives in their families. We tried to looking for a frame-shift mutation in exon 12 of MEIOB gene (Meiosis Specific with OB-Fold Domain Coding gene) in those six females from five consanguineous families, and two affected females were sisters. MEIOB is a Single-stranded DNA-binding protein required for repairing DSBs during early steps of meiosis and crossover formation, thus for proper and correct synapsis (Guo et al.,2020; Luo et al.,2013). Absence 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). A new MEIOB gene mutation was

discovered in two females who suffer from POI, which was a homozygous variant at the very end of exon 12 on oligonucleotide/oligosaccharide-binding (OB) fold domain 3 of MEIOB. The predicted protein structure result with exon 12 skipping and a premature stop codon. The truncation result from that mutation affects the C-terminal region where SPATA22 supposed to bind, therefor, this will impair the interaction between MEIOB and its partner SPATA22 so there will be no accomplished DSB's repair. Same and different mutations in MEIOB gene were reported in a new three studies, and all of them were found in infertile individuals of Arab origins (Gershoni et al., 2017, 2019; Caburet et al., 2019). Unfortunately, we could not find the same MEIOB gene mutation in any of those females.

CHAPTER 2

Female Infertility

2.1 Infertility Definition and Epidemiology

Infertility is the failure of a couple to achieve clinical pregnancy within 12 months of unprotected regular intercourse according to the International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) (Devroey et al., 2009; Oberoi, Khaira and Rai,2017). It is a great life crisis which causes serious mental, stressful, social, medical and financial effects for infertile women, especially in traditional societies. Many adverse effects of infertile couples, And Thus, there would be a negative impact on the treatment (Direkvand-Moghadam, Delpisheh and Direkvand-Moghadam, 2014). Data collected from population based studies suggest that 10-15 % of couples in reproductive age around the world experience infertility (Sormunen et al.,2018).

However, many regions tend to have much higher rates of infertility, reaching approximately 30% such as South and Central Asia, sub-Saharan Africa, the Middle East and North Africa, Central and Eastern Europe (Inhorn and Patrizio, 2015). Current world population from the WHO states that about , 72.4 million people are infertile (Boivin et al.,2007).

Several large scale studies have shown that female factors account for about 50% of all cases, unlike underreporting of male infertility which accounts for about 20-30%, the high rate evaluation for female factors reflects that female partner is often blamed for infertility, therefore they will undergo fertility evaluation. The rest accounts 20-30% due to a combination of both male and female factors (Agarwal et al., 2015).

Total infertility is divided into primary and secondary infertility. Definitions of primary infertility vary between many studies, but the suitable definition, which is driven by the WHO, "Inability to conceive within 2 years of exposure to pregnancy (*i.e.*- sexually active, non-contracepting, and non-lactating) among women 15 to 49 years old". Secondary infertility refers to the inability to conceive after a previous pregnancy (Adamson et al.,2011). In the past, it has been found that primary infertility was more prevalent than the secondary in many studies.

The proportions vary; many articles came with different results. Muhammad Usman Aziz and his colleagues did a study that confirms the opposite, the ratio number of secondary infertility increased, this could be due to inadequate care during previous pregnancies or previous abortions which resulting in pelvic infections.(Aziz, Anwar and Mahmood,2015)

2.2 Etiology of female Infertility

Etiology of infertility predominance and patterns of causes of infertility in many regions are diverse, that's because there is a difference in the environmental conditions and also in reproductive behaviors, such as age at marriage, changing in life style and diet, environmental pollution and bad habits like smoking and alcohol abuse (Masoumi et al.,2015). Female Infertility may be caused by many medical conditions that may damage the fallopian tubes, causes hormonal complications, or interferes with ovulation. Different factors are attributed to infertility including:

2.2.2 Ovulation disorders

Ovulation disorders are considered as the most common cause of infertility in numerous studies (Jacob Farhi,2011). Those disorders present in more than 25% in infertile females couples, Scenario begin from anovulation, when the ovaries are fail to ovulate duo to dysfunction or any suppression result from drug treatment. Women who do not ovulate also do not menstruate and that called as amenorrhea (Bhattacharya et al.,2010). Amenorrhoea often classified as either primary (absence of menses by 15 years of age in the presence of normal growth and the secondary sexual characteristics) or secondary (absence of menses for more than three cycle intervals or six months in women or girls who had irregular menses) (Kriplani et al .,2017). This disorder spectrum range from amenorrhea go through oligomenorrhea until reaching irregular cycles (Smith, Pfeifer & Collins ,2003).

The most common cause of ovulation disorders is Poly Cystic Ovarian Syndrome (PCOS). The diagnostic criteria that have been used include : hyperandrogenism, oligo- or anovulation , that criteria give it a nomenclature to Poly Cystic Ovarian Morphology (PCOM) , after examination , PCOM defined as the number of follicle 12 or more at least in one ovary in addition to the ovarian volume of more than 10 mm (Anagnostis,Tarlatzis, & Kauffman,2018 ; Murphy et al.,2003).

2.2.3 Hypothalamic-pituitary-axis Disturbances .

For an appropriate female sexual development and function, hormonal systems should work together to regulate the gonadal role. This is achieved by hypothalamic-pituitary-gonadal (HPG) axis, which is composed of hypothalamic gland, pituitary gland, ovaries and uterus as end organs, if there is any alteration in their function, the normal feedback of this axis are troubled. The coordinator in this axis called gonadotropic releasing hormone (GnRH), It has to be regularly provided in the right concentrations, to assure oocyte maturation and well ovulation.(Luciano, Lanzone and Goverde ,2013 ; Acevedo-Rodriguez et al.,2018)

GnRH stimulates the secretion of many gonadotrophins like low follicle-stimulating hormone (FSH) and low luteinizing hormone (LH), that will encourage the secretion of gonadal sex steroids such as E_2. If the secretion altered, anovulation will take place either by hypergonadotropic or hypogonadotropic hypogonadal (Acevedo-Rodriguez et al.,2018).

2.2.4 Tubal damage

Tubal factor present the second common cause of female infertility (Masoumi et al.,2015) define as the inability to become pregnant because there is a blockage in one or both fallopian tubes. It's usually caused by infection in the pelvic, such as Pelvic Inflammatory Disease (PID) . It also could be endometriosis when uterus and fallopian tubes are stuck to each other, this occlusion prevent the egg transfer to the tube and thus produce infertility (Deshpande and Gupta ,2019).

There is many genes regulating the immune response in the body, any alteration in those bring an unstable protein thus reduced antimicrobial activity, for example , substitution of

guanine for adenine in MBL2 gene, therefor alter Mannose-binding lectin (MBL) and make it ineffective in preventing reproduction tract microbes such as C. trachomatis (Vinagre et al.,2019)

2.2.5 Chromosomal anomalies

For anonymous reason, gametes of females are more susceptible to errors during meiotic recombination and nondisjunction. It could occur at chromosome segregation or pairing (Hultén, Smith and Delhanty, 2010). Genetic abnormalities such as chromosomal abnormalities gene mutations (single or multi) contribute to 5–10% of infertility (Poornimaet al.,2020). For example, Turner syndrome (45,X or mosaic with 45,X/46,XX ; 45,X/47,XXX) is a neurogenic disorder known by partial or complete loss of X chromosome (Gravholt et al., 2019) . It's a rare condition that found in approximately 1 in 2,000 baby girl (Ouarezki et al, 2018). Girl with TS express only one normal X chromosome resulting in 45,X karyotype, but it could express also another types of karyotyping, anyway, in all types the x chromosomal material still missed. There are many features that exist in TS female, the most common are delayed in puberty, ovarian failure and in addition to hypergonadotropic hypogonadism and infertility (Yahaya et al.,2021).

The only gene which thought to be responsible for those features is SHOX (encoding short stature homeobox protein), which exist in pseudoautosomal region of Y and X chromosome. The growth defect correlated with the under-expression of SHOX because it's escape from X-inactivation (Gravholt et al.,2019).

There is also Chromosomal Structural Anomalies that lead to reproductive impairment in females. It occurs when part of a chromosome is inverted, joined with another chromosome, adding a new segment or when a deletion in chromosome segments happened. However, infertility could arise as a result of balanced rearrangement which produce un-balanced gametes (Yahaya et al.,2021).

2.2.6 Idiopathic female infertility.

Idiopathic or unexplained female infertility refer to females whose try to get pregnant but failed even though all of tests are normal, such as ovulation or tubal patency (Ray et al., 2012). Approximately there is about 30% of couples experience UI (Smith, Pfeifer and Collins, 2003; Farren and DiBenedetto, 2021). The causes of UI could

have a correlation with endocrinological balance disorders, or maybe an immunology disorder or even by a genetic issues. (Ray et al., 2012).

2.2.7 Epigenetic Factors

Epigenetic refers to any modification at molecular level in chromatin that alters gene expression and stability without any alteration in DNA sequence. Mainly correlated with phenotype changes (Das et al.,2017). Many of the epigenetic changes such as DNA methylation or histone modification occur in sperm and egg cells of parents, and could escape from the reprogramming. This could lead to an acquired event of epigenetic, which passed to next generation (Li, 2002).

Epigenetic is very essential during oogenesis, post implantation and placentation, this leads to long and short term of adverse effect for female or even for the fetus(Pisarska et al.,2019). Many environmental changes and lifestyle factors lead to epigenetic alterations, such as smoking, the exposure to chemical detergents, or even stress (Das et al.,2017).

CHAPTER 3

Meiosis

3.1 Meiosis and Meiotic Recombination

Meiosis is the process by which haploid gametes (egg and sperm) are generated from their diploid precursor cells. It produces variation through the involvement of two rounds; one round of DNA replication followed by two rounds of chromosome segregation, called meiosis I (MI) and meiosis II (MII) in order. The first reductional division MI comprises homologous chromosomes segregation, while the second equational division is MII, where the sister chromatids segregate (Hunt and Hassold,2008). Meiosis I start with a very complicated phase called prophase, where the genetic information of homologous chromosomes is exchanged. (Hunt and Hassold,2008 ; Fragouli et al.,2011).This stage is divided into five sub-groups, including leptotene and zygotene where the pairing is accomplished between each chromosome and its homologue, through the formation of chiasmata along the length of that pair (Yatsenko and Rajkovic,2019). Once this happens, a tri-partite protein structure called Synaptonemal Complex (SC) will mediate that assembly by being a key component that maintains synapsis at the pachytene stage (Schramm et al.,2011). The major components of the SC are the axial elements (AE), which have SC proteins 2 and 3 (SYCP2, SYCP3). The other components are central element (CE) and transverse filaments (TF) which are mainly composed of central element proteins 1 (SYCP1) and (SYCE3) (Roeder, 1997; Costa and Cooke, 2007).

Meiotic crossover recombination accomplished between the aligned homologs. It ensures that the segregation of homologs at MI is appropriate and correct, thus it will produce a genetic diversity in turn (Allers and Lichten, 2001). The introduction of the crossover recombination is the formation of double-strand breaks (DSB), if the double-strand DNA breaks do not go correctly, it could be deleterious to the cell or even introduce a cell death (Marcon and Moens,2005). Many large sites around the DNA break are marked by phosphorylated histone H2AX (γH2AX) (Rogakou et al.,1998).

DSB formation needs many mitotic recombination genes, such as *SPO11* and *RAD50* (Cao et al., 1990), any disruption or mutation in genes that have a role in that formation, a blockage will happen, this will result in a chromosome –nondisjunction at meiosis I (Keeney, Giroux and Kleckner,1997).

SPO11 is involved as a catalytic protein that acts directly on DSB's sites through topoisomerase-like reaction, which is a cleaving activity that cut DNA (Baudat et al., 2000; Keeney, Giroux and Kleckner,1997).

There are many biochemical stages included after DSB formation, initiate with resection the DNA breaks by helicases and nucleases to produce an extended region of over hanged 3' single-stranded DNA (ssDNA), which will then bounded by a replication protein A (RPA) (Heyer, Ehmsen and Liu,2010; Kowalczykowski,2015). RPA coats ssDNA and protect it from degradation by inducting many DNA repairs and replication factors(Yates et al.,2018), and taking off many inhibitory secondary structures (Wang and Haber, 2004). during that, RPA will rapidly be displaced after facilitating the binding of recombination mediators, DNA Meiotic Recombinase 1 (DMC1) and recombinase radiation-sensitive 51 (RAD51), the result will come here as a nucleoprotein filament which boosts single end strand invasion and homology search (Symington,2016; Anand at al.,2017). Here, 3' strand extended and also invades the existing homologous duplex, displacement loop (D-loop) and heteroduplex generation accomplished (Lisby and Rothstein,2015). Two possible consequences come after that, first known as the DSB repair pathway (DSBR). The D loop here grab the second 3' end, DNA synthesis fills the gaps formed by the resection, in accordance with that, end ligation occurs and a double-Holliday Junction (dHJ) formed (de Massy,2003; Bishop and Zickler,2004).

Here we have reached the point that we describes the role of the MEIOB gene; all stages that begin with DNA synthesis until dHJ formation depends on MEIOB protein with help from its cofactor named Spermatogenesis-associated protein 22 (SPATA22); specifically after the invasion of the strand; MEIOB linked with ssDNA and react with RPA proteins which immediately coats the formed D loop until the step of dHJ figuration (Luo et al.,2013). The dHJ take one of two scenarios, it could be either resolved through endonucleolytic cleavage and then a ligation to produce two types of recombination products, cross over (CO) often referred to as reciprocal exchanges. The alternative model is non-crossover (NCO), its often denomination as gene conversions (de Massy, 2003; Bishop and Zickler,2004).

On the other hand, dHJ may not be created, instead it take other model, the synthesis-dependent strand-annealing (SDSA). The D-loop disjointed by the displacement of the strand which newly created, repair of the break also done by DNA synthesis and ligation,

gene conversion generated without crossover (de Massy, 2003). For more eludication follow (Figure 3.1) below.

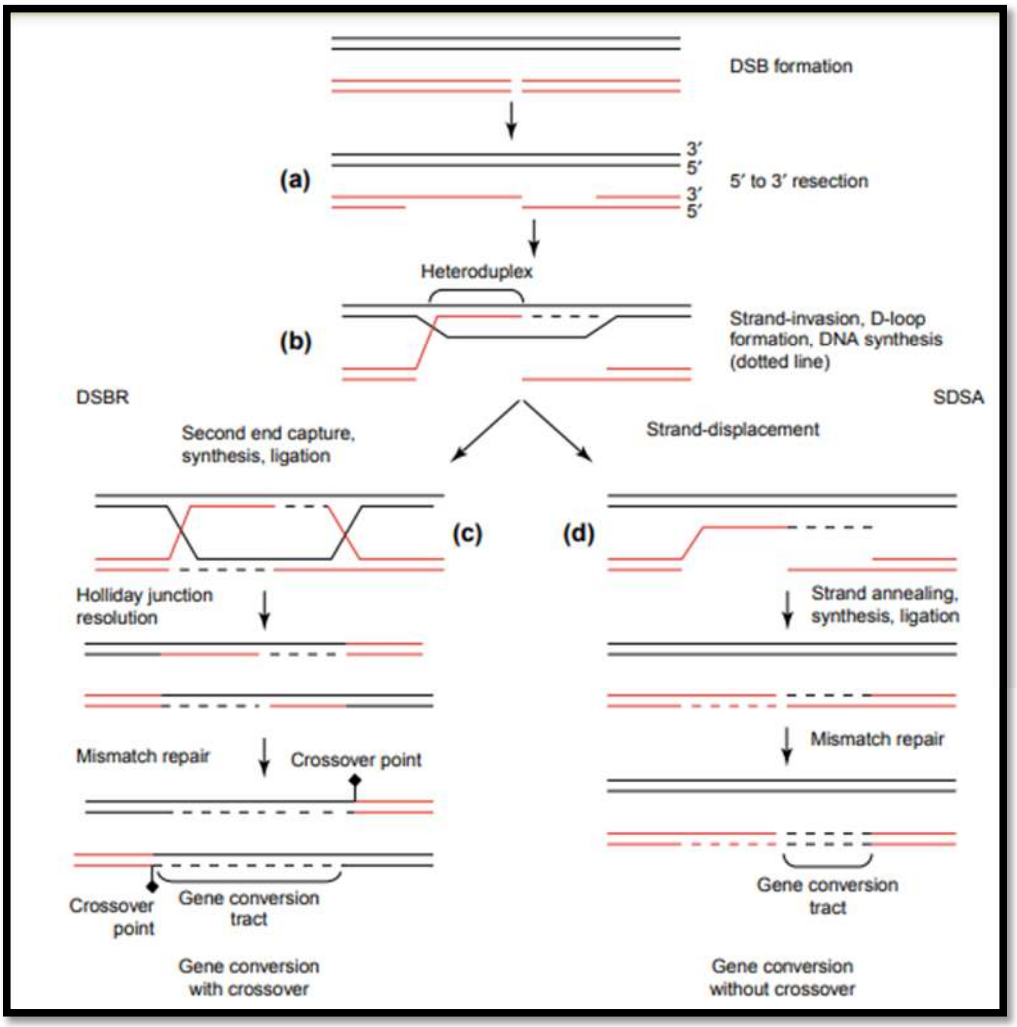


Figure 3.1: Represents the formation of double-Holliday Junctions (dHJs) by Double-strand break repair (DSBR) and synthesis-dependent strand-annealing (SDSA) pathways. After DSB formation and resection of their 5' ends, 3' single strand invades the homologous duplex, heteroduplex and D loop formed, dHJ generated. This recombination intermediate may be resolved to generate crossover products. Or taking the other pathway to generate a non-crossover product (de Massy, 2003).

3.2 Oogenesis

Oogenesis is an process that begins from the embryonic phase lasting until reaching menopause (Shao et al.,2018). Primary cells that used in oogenesis are primordial germ cells (PGCs),which will differentiate later through mitosis to generate oogonia, at that point, primary oocyte will arrested in prophase I (Nikolic et al., 2016 ; Alam and Miyano, 2020). However, prophase stage include synapsis and cohesion to get then program of mitotic recombination (discussed previously in details), which together are fundamental to obtain (DSB) repair and succeeded MI chromosome segregation (Biswas et al.,2021). As mentioned before, SPO11 topoisomerase begins chromosome DSB repair mechanism (Baudat et al., 2000). An extended region of ssDNA produced and then coated by RPA complex to protect it from degradation (Liu,2010) (Yates et al.,2018). There are many markers and transcription factors included such as SYCP3, SYCP2, SYCE1, TRIP13 and STAG3 whom indicated in (figure 3.2) (Biswas et al.,2021).

Posteriorly, RPA will be displaced after facilitating the binding of many recombination mediators including BRCA1,BRCA2,HFM1,MEIOB, SPATA 22, RAD51, DMC1, MSH4,MSH5, MLH3 and other indicate in (figure 3.2) (Symington,2016) (Biswas et al.,2021). Any defect in prophase I steps cause many infertility defects such as POI, because that damaged oocyte will picked out from ovaries reserve.

Each oocyte will be surrounded by pre-granulosa cells to become a primary follicles, and this pool of follicles will represent the exact female ovarian reserve and reproduction ability after birth, but it should be at dormant arrest until puberty, oocyte continue growing in size inner the follicles (Nikolic et al.,2015; Chen et al.,2020).

Resumption of meiosis is initiated by a hormonal stimulation at puberty, causing oocyte to become mature in size and ready to get out from the ovary and move on until reaching the oviduct to start ovulation, once it starts, maternal RNA translation occurs. (Biswas et al.,2021). Resuming begins with spindle formation at metaphase I, which is fundamental for correct chromosome segregation, any defect in genes responsible in that, infertility could outcome. Following asymmetric cytokinesis, the oocyte will arrest again at metaphase II until it is fertilized. (Overviewed in the figure 3.2) (Nikolic et al.,2015).

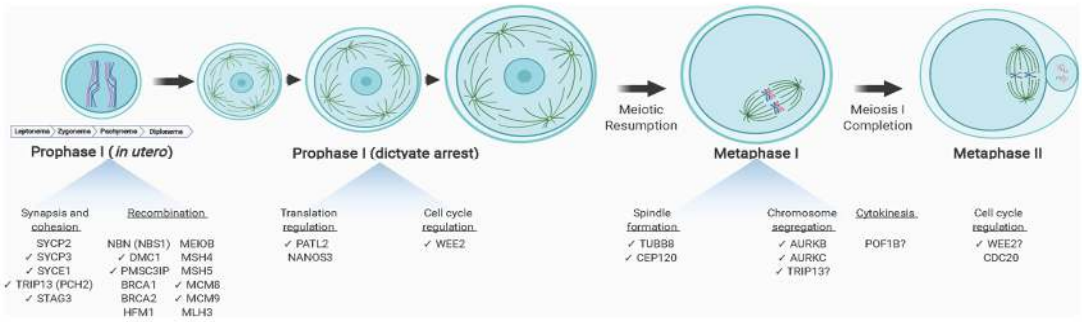


Figure 3.2: illustration of oocyte meiotic maturation steps, including prophase I and oocyte arresting, meiotic resumption, meiosis I completion include complete chromosome segregation and finally meiotic cell cycle regulation, with involvement of molecular factors in each phase. (✔) indicates genes only involved in that review (Nikolic et al.,2015).

CHAPTER 4

MEIOB Gene

4.1 Human MEIOB gene representation and its chromosomal location.

MEIOB is abbreviation for Meiosis Specific with oligonucleotide/oligosaccharide-binding OB-Fold Domain Coding gene. It is a Single-stranded DNA-binding protein required for repairing DSBs during early steps of meiosis and crossover formation, proper and correct synapsis, maintain an efficient number of some recombinases such as RAD51 and DMC1 foci after the stage of zygotene. Preserving the stabilization of many recombinases required for successful meiotic recombination. Furthermore it shows Single-stranded DNA 3'-5' exonuclease activity in vitro (Guo et al.,2020; Luo et al.,2013) .Those functions will be discussed minutely in later sections. By using UCSC genome browser, the MEIOB gene is localized on chromosome 16p13.3 (chr16:1,883,984-1,922,179) in humans with length of 38,196 bp, its composed of 14 exons. The (figure 4.3) represent the human MEIOB gene from UCSC genome browser and its chromosomal location.

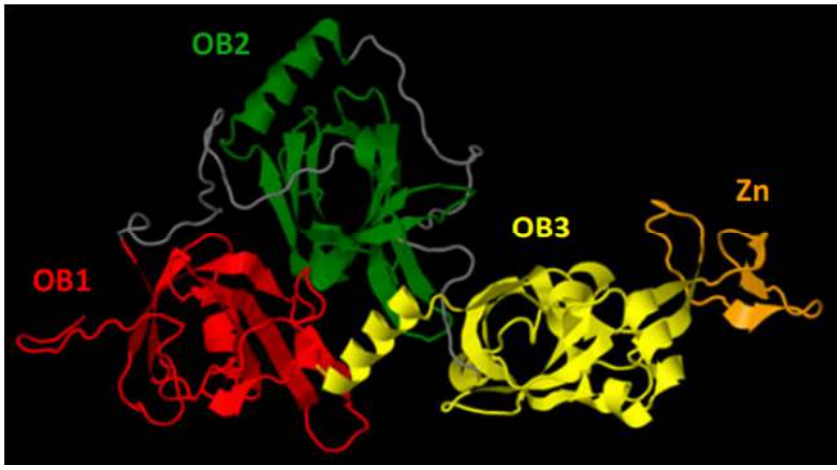
Figure 4.3 : UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly

4.2 MEIOB protein structure

MEIOB protein structure have been detected by Souquet and his colleagues by using an InterProScan Search. They identify a 3 DNA binding domains (dbd), called oligosaccharide/oligonucleotide binding (OB) folds, which allow a binding activity on ssDNA. Those OB folds are same as those of RPA1, both are homologue (Souquet et al., 2013). If we take a look on the similarities between MEIOB and RPA1, we note that both have 2 OB fold domains, and another third long C-terminal OB domain (figure 4.4). Ribeiro and his colleagues used the Sequence Similarity DataBase of the Kyoto Encyclopedia of Genes and Genomes (KEGG SSDB), they discovered that there is a 30-amino acid inserted, which contain a putative zinc ion-binding domain in Homo-sapiens. This motif take a shape as double zinc ribbon in both MEIOB and RPA1, but they different in the number of amino

acid residues subsist between cysteines. However, there is N-terminal domain exists in RPA1 which is missing in MEIOB (Souquet et al., 2013; Ribeiro et al., 2016). This absent domain create another specific interactions for MEIOB, RPA1 still cannot recompense the missing of MEIOB, that showed in mice which mutated and become MEIOB$^{-/-}$, every one of them has its own interactions, but both are remarkable in meiosis (Souquet et al., 2013).

a



b

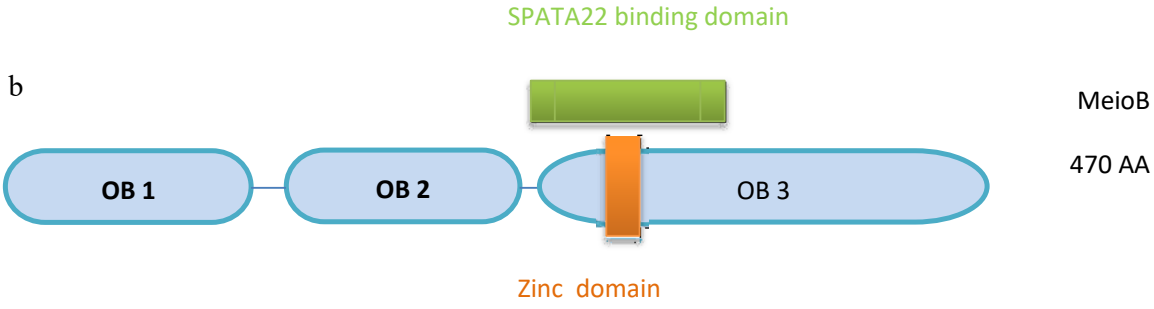


Figure 4.4: (a) Stereo ribbon presentation for MEIOB domains structure. Yellow, green and red ribbons reflect the OB fold domains 1,2 and 3. Orange ribbon represent zinc ion-binding domain, the gray shows linkers that are unfolded (Ribeiro et al., 2016). (b) modest illustration for MEIOB domains structures, blue boxes represent MEIOB OB fold domains, orange block represents zinc ion-binding domain

4.3 MEIOB and evolution

Decades ago, RPA4, was the only RPA paralog in mammals, its belonging to RPA2 family (Ribeiro et al., 2016). But recently, a new work discover MEIOB protein as a novel paralog for Rpa1 in metazoans. That work was done by Luo's and Souquet's squad in 2013. Souquet's squad try to find out MEIOB homologs by using tBlastn searching in the full length sequence or even in the short conserved motifs, they found that there is homologs in the genomes of almost all metazoan but not in Nematoda. They also did multiple alignment for the amino acid sequence in full length, to indicate the high degree of conservation of the different MEIOB homologs in vertebrates, for example, the identity between mouse and human was 85% and similarity count 90%. However, invertebrates showed extremely lower conservation such as in mouse and fly, identity was only 23% . Furthermore, no MEIOB orthologs was indicated in the genomes of fungi and plants (Souquet et al., 2013). Interestingly, souquet's squad also found out an ortholog in a uni-cellular organism named as Capsaspora owczarzaki, this bring a strong clue that MEIOB evolved from RPA1 before 600 million years ago, by suggesting that a duplication event execute prior the metazoans manifestation (Souquet et al., 2013). Ribeiro's group recently puzzled out MEIOB sequence similarity between some fungal genome and metazoan. That’s mean, MEIOB evolved from ancient time (Ribeiro et al., 2016). A phylogenetic tree placed below for MEIOB orthologs (figure 4.5)

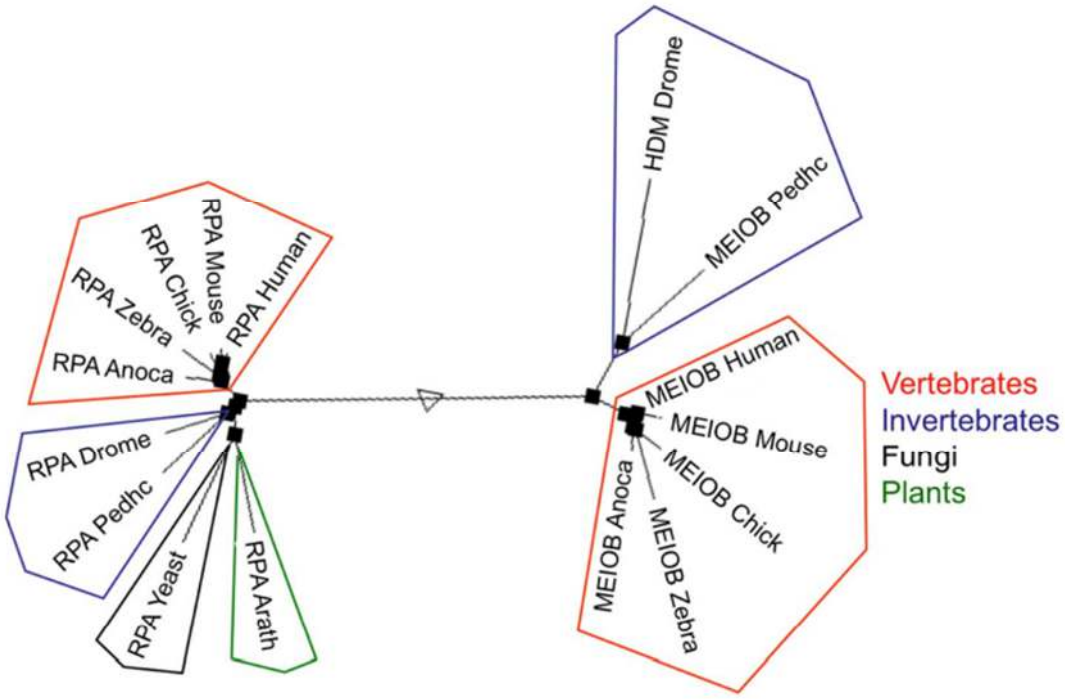


figure 4.5: Phylogenetic tree for MEIOB orthlogs in vertebrates and invertebrates, the missing of that orthlogs in plants and fungi. (Souquet et al., 2013).

4.4 Putative roles of MEIOB

Souquet's squad investigate the role of MEIOB protein in-vivo by introducing a MEIOB$^{-/-}$ mutant male and female mice. They investigate the MEIOB is one of the core genes that essential in meiosis, mutation result in sterility because there was arresting in prophase 1 stage. Impairment in chromosome synapsis and DSB's repair was clear in MEIOB$^{-/-}$ meiocytes (Souquet et al., 2013). Same result in Luo's group were indicated, they proceed systematical proteomics screening for novel meiotic chromatin-associated proteins in mice (Luo et al.,2013). Consequences of the deleted MEIOB indicated as destabilization of DMC1 and RAD51. Suggestions tells that after strand invasion the meiotic recombination failed, or there would be instability in DMC1 and RAD51 filaments. . Immunolocalizations was applied with antibodies recognition, staining indicate a huge decrease in DMC1 and RAD51 at zygotene and pachytene-like stages. That's mean, MEIOB is essential for

maintain a proper number of recombinases, on the other hand, MEIOB not vital in the initial induction of those recombinases (Souquet et al., 2013).

Another study performed next by a different group, they discovered that spermatocytes in rats which is missing SPATA22 have been arrested in zygotene-like stage of meiosis. SPATA22 known as a partner for MEIOB protein. Although, RPA1 foci was in a normal range as usual, but RAD51 foci was not in regular situation. At first, the number is normal, but then, it start to descend from the leptotene-like stage. All of those results suggest that SPATA22 deficiency impair the stabilization of RAD51, but not the localization of it during meiosis (Ishishita, Matsuda and Kitada, 2014).

Examination of truncated MEIOB binding activity to o ssDNA and dsDNA (double stranded DNA) was done by Luo's group. MEIOB demonstrate 3'-5' exonuclease activity specifically for ssDNA, and depend on Mg^{2+}. Binding affinity was droopy in the shorter oligonucleotides of 5-15 nt in vitro, but firmly binds to those greater than 18 nt, this is a hint predict that the enzymatic activity of MEIOB expand together with DNA binding (Luo et al., 2013).

4.5 RPA–MEIOB–SPATA22 complex model.

During early meiotic recombination steps, exactly in DSB formation, there is no role for MEIOB gene, until completing strand invasion. After that, MEIOB and its partner SPATA22 react with RPA after binding ssDNA.

That tri-complex coats the ssDNA of the second end and also the D-loop as well.it interposes second end-capture. The second end capture is precondition for dHJ formation. After strand invasion First-end initiate the DNA synthesis to expand the D-loop across to the second end, ligation is needed here for the second end and the first end. Another DSB's repair scenario could occur through SDSA pathway (explained in details previously). MEIOB exonuclease activity removes the 3' flaps of the ssDNA from the first-end DNA synthesis in both scenarios.(figure 4.6) (Luo et al., 2013)

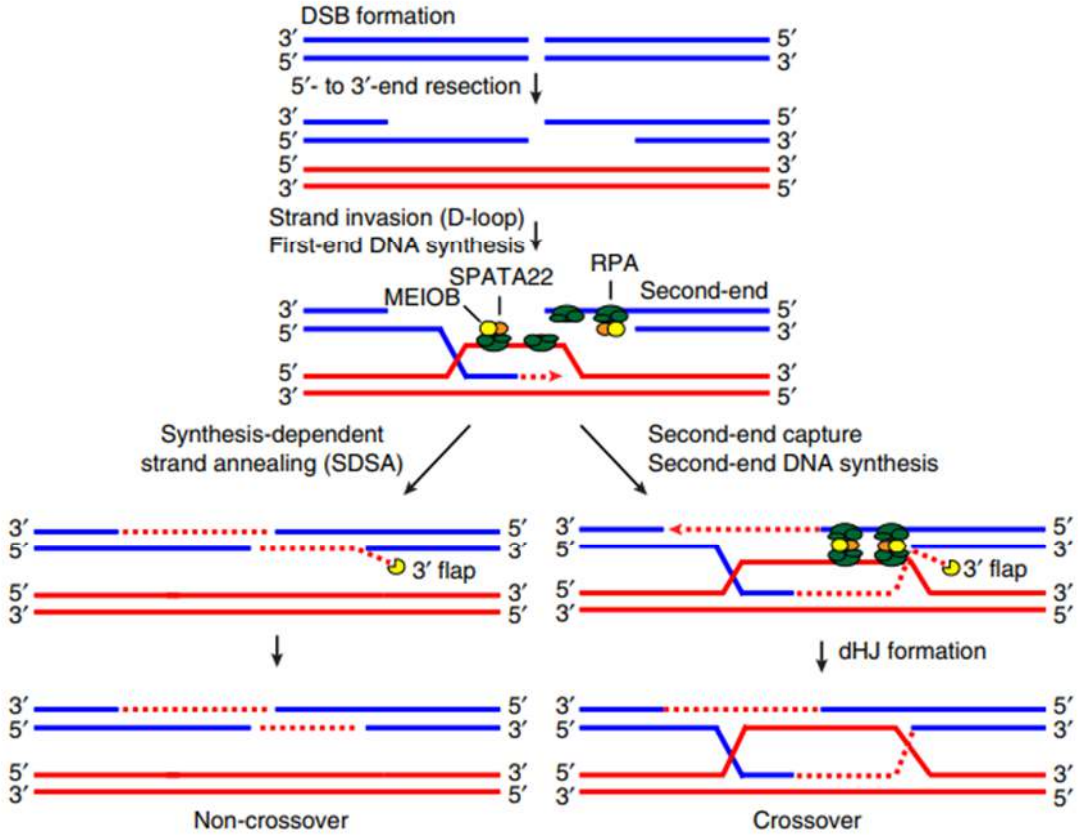
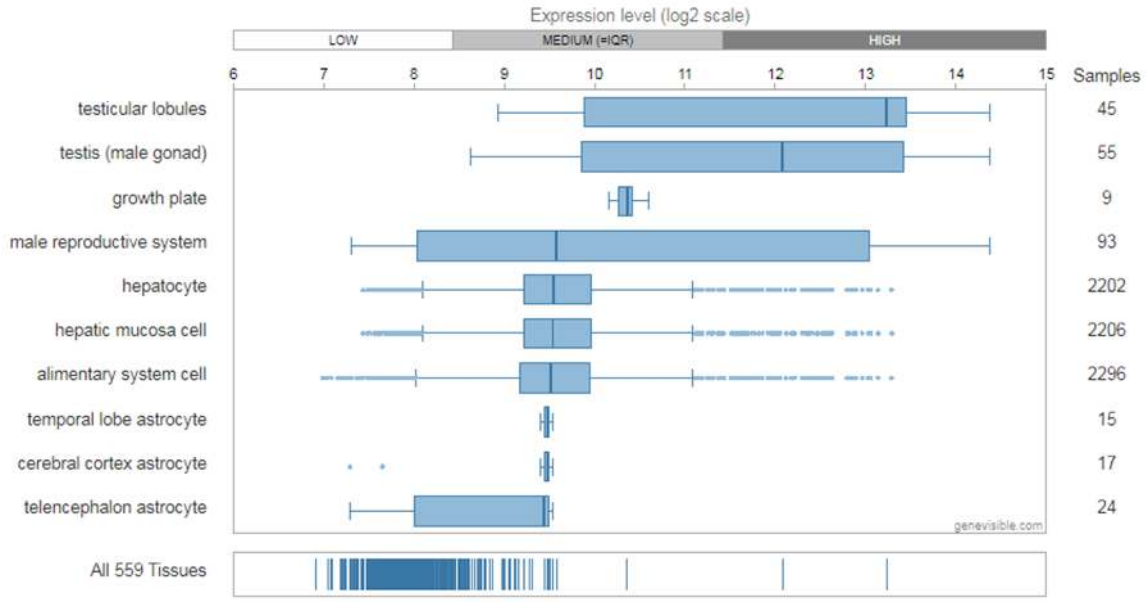


Figure 4.6: MEIOB-SPATA22-RPA complex and their key function during meiotic recombination.

4.6 MEIOB expression

MEIOB reverse transcription was used in mice tissue (adult and fetal). It was expressed in fetal ovaries, liver and in postnatal testis also. MEIOB expression in the mouse ovary begins at 12.5 days post coitum (dpc) and the crest was at 15.5 dpc, but in post-natal life it becomes un reveal. In the fetal gonads of human, MEIOB gene expression only found in the ovary, which begin at the 14th weeks post fertilization. MEIOB protein was also detected through early meiosis prophase I, 4n fraction containing leptotene, zygotene and few pachytenes spermatocytes, but its absent in the following meiosis stages (Souquet et al., 2013).

If you see the (figure 4.7) below, you can view the expression of MEIOB gene in the top 10 tissues across 559 tissue tested by GENEVESTIGATOR



Organism: Homo sapiens

Gene: MEIOB

Platform: Affymetrix Human Genome U133 Plus 2.0 Array

Figure 4.7: the expression of MEIOB gene in the top 10 tissues across 559 tissue tested by GENEVESTIGATOR

4.7 MEIOB and female Infertility

Lately, many motivating studies have been done to detect the exact role of MEIOB gene in female infertility. In 2019, Caburet and his collogues discovered a new MEIOB gene mutation in two females who suffer from POI. a homozygous variant was detected at the very end of exon 12 on the third OB fold domain of MEIOB. The predicted protein structure result with exon 12 skipping and a premature stop codon. This study showed that the truncation result from that mutation affects the C-terminal region where SPATA22 supposed to bind, therefor, this will impair the interaction between MEIOB and its partner SPATA22 so there will be no accomplished DSB's repair. This may stimulate the depletion in the follicular stock of the ovaries and thus meiotic defection. Those results came compatible with phenotype of MEIOB$^{-/-}$mouse as previous studies of Luo's and Souquet's (Caburet et al., 2019).

CHAPTER 5

Premature ovarian Insufficiency (POI)

5.1 Background for premature ovarian insufficiency

Premature ovarian insufficiency is the major known cause of infertility in females around the world (Persani, Rossetti and Cacciatore, 2010). This heterogeneous disease characterized by early truncation of ovarian physiology with depletion in follicles in addition to high level of follicle stimulating hormone (FSH) before the age of 40 (Veitia, 2020). About 7 million of primordial follicles exist in ovaries during embryogenesis, most of them will lost by apoptosis, only 400-500 of them will continue dividing until enter ovulation cycle before menopause. POI occur when those follicles lost and estrogen production decreased (Panay et al., 2020). 70% of POI cases still idiopathic, the majority percent of it thought to be genetic origin (Persani, Rossetti and Cacciatore, 2010; Heddar, 2020). About 10 to 15% of cases are positive in family history, those who have affected relative at first degree (Nelson, 2009). First indicator of POI is the menstrual irregularities until amenorrhea occur (primary or even secondary), in addition to hypergonadotrophic hypogonadism. Hormone profile may be fluctuated which make the exact clinical diagnosis become harder in many POI females (Torrealday, Kodaman and Pal, 2017). Elevated FSH levels (higher than 25 IU/L) in two tests at least one month apart, and low estradiol (E2) levels (lower than 50 pg/mL) presented too with 4 months of amenorrhea at least, collectively help in right diagnosis. A pelvic ultrasound give further investigation in POI by doing a vaginal ultrasound to check up the ovaries situation. POI females have a thin endometrial echo (<4 mm) with small ovarian volumes (Torrealday, Kodaman and Pal, 2017).

5.3 Etiology of POI

5.2.1 Genetic factors

Many studies showed that there is a role of genetic causes in the idiopathic POI (Persani, Rossetti and Cacciatore, 2010). Advanced genetic screening technologies such as whole-genome sequencing help in investigation of genetic causes in POI about 20-25% of cases (Torrealday, Kodaman and Pal, 2017). Those identified genes believed to affect X chromosome at first step, or even the autosomal variation (in rare conditions), because they

have a vital function in DSP repair, hormonal, meiosis and metabolic pathways (Panay et al., 2020). Partial loss of X chromosome occur in Turner syndrome, this partial loss by a deletion, translocation or inversion lead to the loss of X-linked genes, and this is included as syndromic POI (Panay et al., 2020). There are many syndromes that included as a reason factor for POI.

MEIOB gene which have ssDNA binding activity similar in those of RPA1. During DSB repair, MEIOB and its partner SPATA22 bind to ssDNA. Caburet and his colleagues found a homozygous variant that lead to exon 12 skipping in two sister affected with POI in consanguineous family (Veitia, 2020). This result same as in previous studies, in a MEIOB mutant mice which have a depletion in follicular pool (Souquet et al., 2013) (Luo et al., 2013).

5.2.2 Other factors

There are many causes of POI such as toxic factors, include the exposure to polycyclic aromatic hydrocarbon in cigarette or other air polluters. autoimmune causes also play a role in POI such as inflammatory bowel disease and many others. Iatrogenic causes include surgical therapies, chemotherapy and radiation can lead to POI (Panay et al., 2020).

CHAPTER 6

Materials and Methods

6.1 Materials (Consumables and Equipment)

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

Table 6.1: List of required buffers, solutions, and gels including their compositions.

Gels and Buffers	Composition
1X White Blood Cells (WBCs)/ DNA lysis buffer	50 mM Tris HCL (pH = 7.5) 100 mM NaCl 1 mM EDTA (pH=8.0)
1X Red Blood Cells (RBCs) lysis buffer	155 mM NH4Cl 100 M NH4HCO3 0.1 mM EDTA (pH=7.4)
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

6.1.2 List of Reagents and Kits Table

6.2: List of required reagents and kits including their specifications

Reagent	Manufacturer/ Company	Product Specifications
Proteinase K (5 mg/ mL)	**aMReSCO®**	**Cat# : E195**
20% Sodium Dodecyl Sulfate (SDS)	**aMReSCO®**	**Cat# : 083754-500ml**
Agarose	**aMReSCO®**	**CAS# : 9012-36-6**
10X polymerase buffer	**Eisenberg Bros**	**Cat# : JMR-420**
Q-Solution	**QIAGEN**	
0.02% Sodium Azide	**Sigma Aldrich**	**Cat# : S2002**
Nucleotides dNTPs (2.5 mM)	**TAMAR**	**Cat# : R0181,4X0.25mM**
DNA Oligonucleotide primers (other details in tables below)	**Hylabs**	
Super Therm Taq DNA polymerase	**Eisenberg Bros**	**Cat# : JMR-801**
100bp DNA ladder H3 RTU (size: 54 µg/ 500 µL)	**GeneDireX®**	**Cat# : DM003-R500**
GoTaq® Green Master Mix (100 reactions)	**Promega**	**Cat# : M7122**
Ultra-Pure Water (DNase and RNase-free)	**Biological Industries (BI)**	**Cat# : 01-866-1A**
Exonuclease I (20,000	**BioLabs**	**Cat# : M0293L**

units/ml)		
Antarctic Phosphatase AnP (5,000 units/mL)	**BioLabs**	**Cat# : M0289L**
BDX64 (BigDye® Enhancing Buffer) (2 x1.25 ml)	**MCLAB**	**Cat# : BDX-100**
BigDye Terminator 5X Sequencing Buffer	**MCLAB**	**Cat# : SBUF-100**
BigDyeTM Terminators v1.1 Cycle Sequencing Reaction Kit	**Applied Biosystems**	**Cat# : 4337451-100**
HiDi ™ Formamide	**Applied biosystems**	**Lot # : 2102754**
GS 400 HD ROX SIZE STD	**Applied biosystems**	**Lot# : 1201394**

6.1.3 List of Equipment

Table 6.3: 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# : 1704401
PowerPac™ Basic Power Supply/ Gel electrophoresis	BioRad	Power PAC 300 Cat# : 1645050

power supply		
Molecular Imager, Gel DOC™ XR+ Imaging System/ Gel documentation system	BioRad	Cat# : 1708195
ABI 3130XL Genetic Analyzer/ Sanger sequencing machine	Applied Biosystems	ABI 3130XL Genetic Analyzer

6.2 Methods

6.2.1 Samples Recruitment

In collaboration with Palestine European Fertility Center, 6 females from different parts of West Bank mainly from Yatta and Hebron diagnosed with POI, after doing a hormonal test (FSH,E2 and LH) with addition of transvaginal ultrasound to check the ovaries size and reserve, according to their physician diagnosis they were recruited to this study. Three of the patients have no history of infertility in their family, but two POI females were sisters with a family history of infertility, and one affected female also has two brothers who had non-obstructive azoospermia. All of these females were checked at Palestine European Fertility Center, and asked to sign an informed consent form provided by Bethlehem University Institutional Review Board. We met each female family independently at their home to explain the goals of our research and to get agreement for blood sample collection from each person in the family. Family pedigree was drawn for each one and about 10-mL blood was withdrawn and collected in EDTA vacutainer blood tube. Samples were also taken from all relevant relatives in and family members who agreed to participate in our study.

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

About 5-10 ml of the blood was collected from each participant in a 10-mL sterile EDTA vacutainer tube. The blood sample was transferred to 50 ml conical tube. Then the tube was filled with Red Blood Cells (RBCs) lysis buffer, after that, the tube will kept on ice for about 20-30 minutes, with gently being shaken by hand from time to time, until blood becomes transparent. Centrifugation was done at 2000 round per minute (rpm) for 12 min at 4C. The

supernatant was discarded carefully after centrifugation, while the pellet was maintained at the bottom. Then, this pellet was re-suspended by adding again a 15 ml of RBCs lysis buffer and centrifuge it again with the same parameter that used in previous step. Then supernatant discarded again and the pellet was suspended in a mix of 3 ml White Blood Cells (WBCs) lysis buffer, 100-µL of 5 mg/ mL proteinase K and 20% sodium dodecyl sulfate (SDS). After that, the tube was incubated 37°C with continuous shaking overnight in shaking incubator. After digestion was complete, 3ml of 6M NaCl was added to the homogeneous solution without a clear pellet with vigorously shaken until the solution appeared foamy and then centrifuged at 3000 rpm for 20 minutes at 25 °C. The supernatant containing the DNA was transferred to a new other clean 15ml falcon tube. 100% cold ethanol was added to the supernatant (twice the volume of the supernatant; approximately 8 mL). With a gently inversion for the tube, DNA strands were became visible as white thin fibers, then the DNA network fibers was fished from the solution by using a sterile glass Pasteur pipette, and washed in 70% ethanol. Next step include air drying for 2 minutes on Pasteur pipette. DNA was then transferred into a screw cap tube that contained 200-600µl of 0.02% sodium azide depending on the amount of DNA that was fished.

6.2.3 Genotyping the Palestinian MEIOB mutation

6.2.3.1 MEIOB (NM_001163560; Chr16: 1889376 del G MEIOB, exon 12 – S366fs*) Primers

Table 6.4: MEIOB Gene Primers

MEIOB exon Number	Oligo Sequence	Annealing Temp. (°C)	Length (bases)	Product size (bp)
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	

6.2.3.2 MEIOB Polymerase Chain Reaction

According to previous studies the mutation of MEIOB gene was detected in exon number 12. So for this amplification reaction we add a recipe that shown in table below which contain a reverse and forward oligonucleotide primers, 2.5 mM dNTPs, Super Therm Taq DNA polymerase, DNase RNase free H_2O, X polymerase buffer and the DNA template. Those ratio in the table was done for one reaction, and could multiplied correlate with the number of reactions that we need.

Table 6.5: MEIOB PCR reaction recipe.

Material	**For 1 X (µL)**
Q-Solution	**5.00**
X polymerase buffer	**2.50**
F – primer	**0.50**
R – primer	**0.50**
2.5 mM dNTPs	**2.00**
Super Therm Taq DNA polymerase	**0.25**
DNase RNase free H2O	**13.25**
DNA template	**1.00**

Touch down 60 °C was used, Refer to the Table 6.6 for PCR program illustrations.

Table 6.6: Touch down 60° C PCR pragramme for Exon 12.

Phase	Temperature	Time (min)	# of 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	872.0 °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	∞	

6.2.3.3 Agarose Gel electrophoresis for Amplified MEIOB PCR Products

After amplification of MEIOB samples, 1.5 % agarose gel was prepared by adding 1.50 gr of agarose powder to 100ml of 1X TAE. For samples loading, 4-µL of 5X loading dye was mixed with 4-µL of PCR product and then loaded into the gel. Running of Electrophoresis was at 120V for about 30 minutes in 1X TAE buffer, was visualized using gel imaging documentation system.

6.2.3.4 Enzymatic Cleanup of MEIOB PCR Products

After making sure that the amplification of all MEIOB exons was successful and we can see single bands on the gel, we carried out an enzymatic clean-up procedure to clean excess primers or remove excess unincorporated dNTPs. Antarctic Phosphatase (AnP) and Exonuclease I (Exo I) were added to this procedure for cleaning purpose, also PCR product will added and the volume will continue to reach 7-µL by adding DNase RNase free H2O

Table 6.7: Enzymatic cleanup reaction of MEIOB PCR products.

Reagent	**For 1X (µL)**
Exonuclease I (Exo I)	0.25
Antarctic Phosphatase (AnP)	0.25
DNase RNase free H2O	1.50
PCR product	5.00

Thermal cycler machine were used to run the reaction with the program that showed below in Table 6.8

Table 6.8: MEIOB PCR cleanup program

Phase	**Temperature**	**Time of incubation (mins)**
Enzymes activation	37 °C	30:00
Enzymes in-activation	80 °C	20:00
Hold in cold	4 °C	∞

6.2.3.5 Direct sequencing of purified PCR Products

Direct Sanger sequencing method was applied for sequencing using BigDyeTM Terminators V1.1 Cycle Sequencing Reaction Kit. Recipe include BDX64 (BigDye® Enhancing Buffer) which enhance the activity of the polymerase by reducing the extension time from 4 minute to 1 minute, forward or reverse primer, PCR clean product, BigDye® Terminator 5X

Sequencing Buffer, and BigDye terminator. Adding DNase RNase free H2O until volume reach 16-µL for I reaction (1X). Volumes of reagent are shown in table 5.14

Table 6.9: Direct sequencing reaction of purified MEIOB PCR products

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

Thermal cycler machine was carried out the reaction, by using a program shown below in Table 6.10

Table 6.10 : Cycle-sequencing of MEIOB program

Phase	Temperature	Time (min)	# of 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	4 °C	∞	

The cycle-sequenced products will then have an extra step of purifying to prepare it for ABI 3130XL Genetic Analyzer.

6.2.3.6 cleaning of the cycle-sequenced products using EDTA/Ethanol Precipitation method and applying Capillary Electrophoresis

EDTA/Ethanol precipitation method was used to purify and clean Cycle-Sequenced products from primers, excess dyes or unincorporated dNTPs. For each 16µl of the cycle-sequenced

products 5µl of 125mM EDTA and 100µl of 99.8% cold Ethanol were added and mixed very well. The mixture was incubated in -20°C freezer for 30 min. samples the centrifuged at 3800 rpm for 30 minutes at 4 °C. we discard the supernatant and 60-µL of 70% Ethanol was added for washing and centrifuged again at 3800 RPM for 20 min at 4C°. 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. the pellet was dried by putting the opened strips on a 95 °C heated hotplate for 5 minutes to make sure that Ethanol is completely gone. . Finally the samples were re-suspended in 10µl Hi Di Formamide with enough vortex. Samples then put on 95C° hot plate for 5 minutes for the denaturation of DNA strands.

Followed immediately by a 5 minutes incubation on ice. Samples were then loaded on the 96 –well Optical Reaction Plate from Applied Biosystems and run on sequencing machine (3130XL Genetic Analyzer from Applied Biosystems).

6.2.3.7 Analysis of Sequencing Data

Opening the DNA chromatogram files was achieved by using FinchTV program. DNA sequence displays then in a graph (electropherogram) for easy analyzing.

6.4 Linkage exclusion analysis

After we obtained the pure DNA, we prepare the product for linkage exclusion, but before that step, Two microsatellite markers [Marker1 (M1) and Marker2 (M2)] were chosen; one before the MEIOB gene and the other after this gene. (figure 6.8)

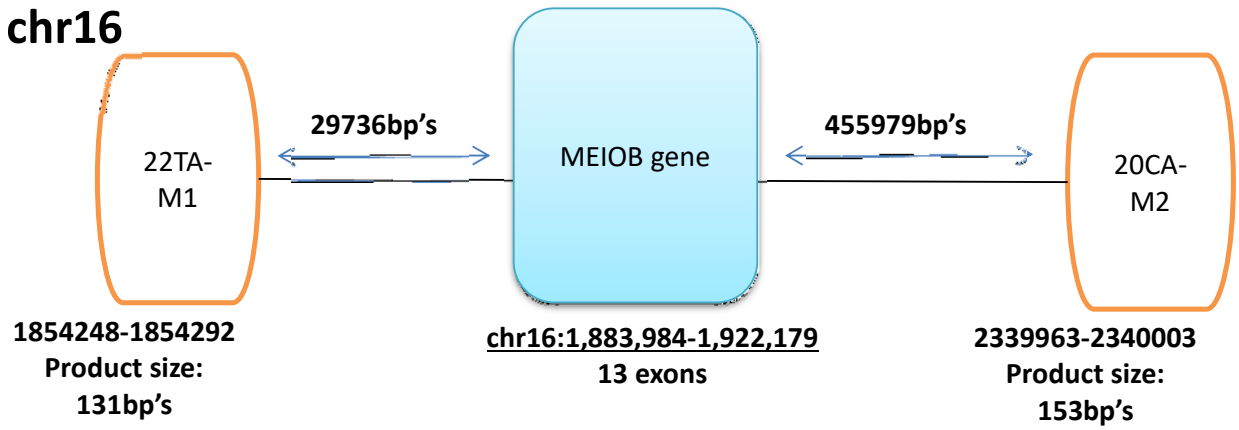


Figure 6.8**:** M1, M2 and MEIOB gene position on chromosome 16 and the distance between this gene and these two markers.

Primer 3 online software was used to design 4 primers to amplify this region around the markers for the MEIOB gene, two primers for M1; Forward (F) and Reverse (R) primers, and the other two primers for M2; Forward (F) and Reverse (R). (table 6.11) below discuss primer sequences with touch down temperature for each one.

Table 6.11: Microsatellite markers used to study linkage for POI.

Microsatellite	**FAM-F-primer**	**R-primer**	**T.D**
22xTA marker	gtgcagagcatcatgc GGATCCTCGTGCTATGAACAG	TCCCTCTAAAGG CAGGAACA	55 °C
20xCA marker	gtgcagagcatcatgc TTACAGGCATGAACCACCAA	GCTGCAGCCTCA GTTGTGT	55°C

F-primers for M1 and M2 fluorescently labeled with FAM (blue color).

6.4.1 Genotyping PCR program

The sequences of the DNA from two families (OD-E AND OD-D) were amplified for genotyping by PCR with M1 and M2 primers. F-primers for M1 and M2 were labeled with FAM florescent (blue color). Recipe of the reaction showed in Table 6.12 below

Table 6.12: Genotyping PCR reaction mix per 25µl of total volume

Reagents	**For 1X (µL)**
10X Polymerase Buffer	**2.50**
Polymerase enzyme (super therm polymerase)	**0.25**
Q-Solution	**2**
dNTPs (2.5Mm)	**2**
MgCl2	**1**
Forward primer with FAM tail	**0.10**
Reverse primer	**0.50**

Uni FAM	**0.50**
Nuclease free H2O	**15.15**
100ng/µl DNA Template	**1**

Table 6.13: Touch down 55 °C PCR program for linkage.

Phase	Temperature	Time (min)	# of 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	∞	

6.4.1.1 Genotyping

For genotyping, a mixture 0.25 of 400 µl Rox with 10.75 µl Hi Di and 1 µl of PCR product were put together. This step was done for each PCR product then the mix was incubated for 5 minutes at 95 °C followed by an immediate incubation for five minutes on ice. The

Samples were run on 3130XL Genetic Analyzer machine on special optical Reaction Plate the results were analyzed using the genemapper 4.0. These results were represented in the form of peaks. The number of peaks and their sizes are proportional to the length of repeats and the number of repeats in the PCR product.

Table 6.14 : Reaction mix for genotyping per 12 µl of total volume

Reagents	Volume in µl
GS-400HD ROX	**0.25 µl**
Hi Di	**10.75 µl**
PCR product	**1 µl**

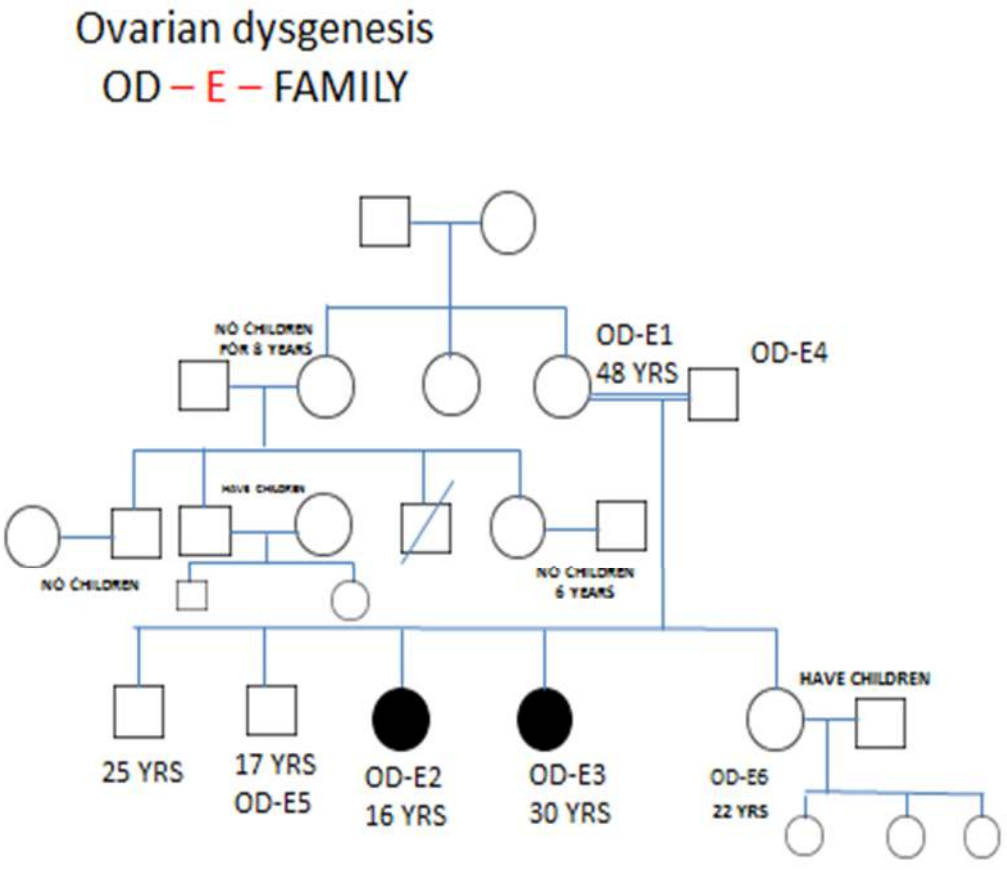
CHAPTER 7

Results

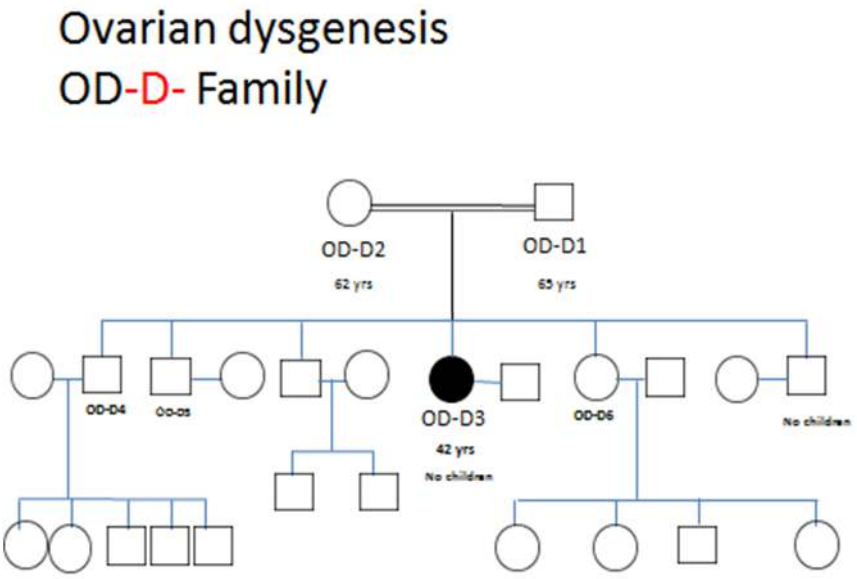
7.1 Family Pedigrees of Affected Individuals

Five families were recruited for this study which contain females who have POI as in physical examination. Figure 7.9 showed the 5 families pedigree's

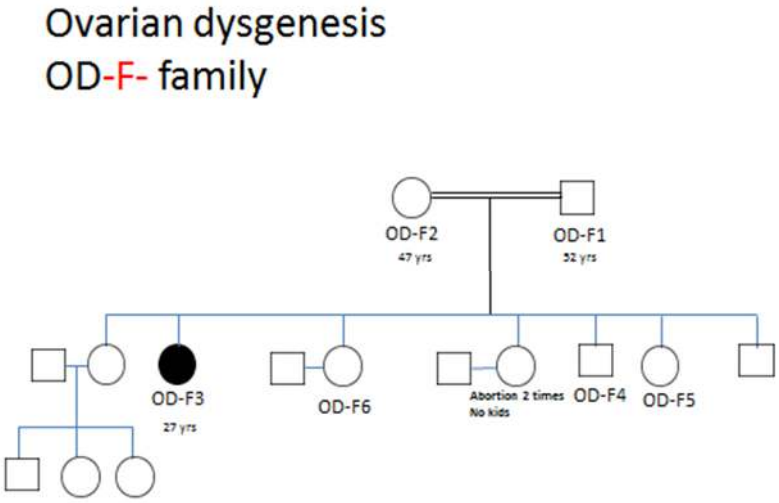
(a)



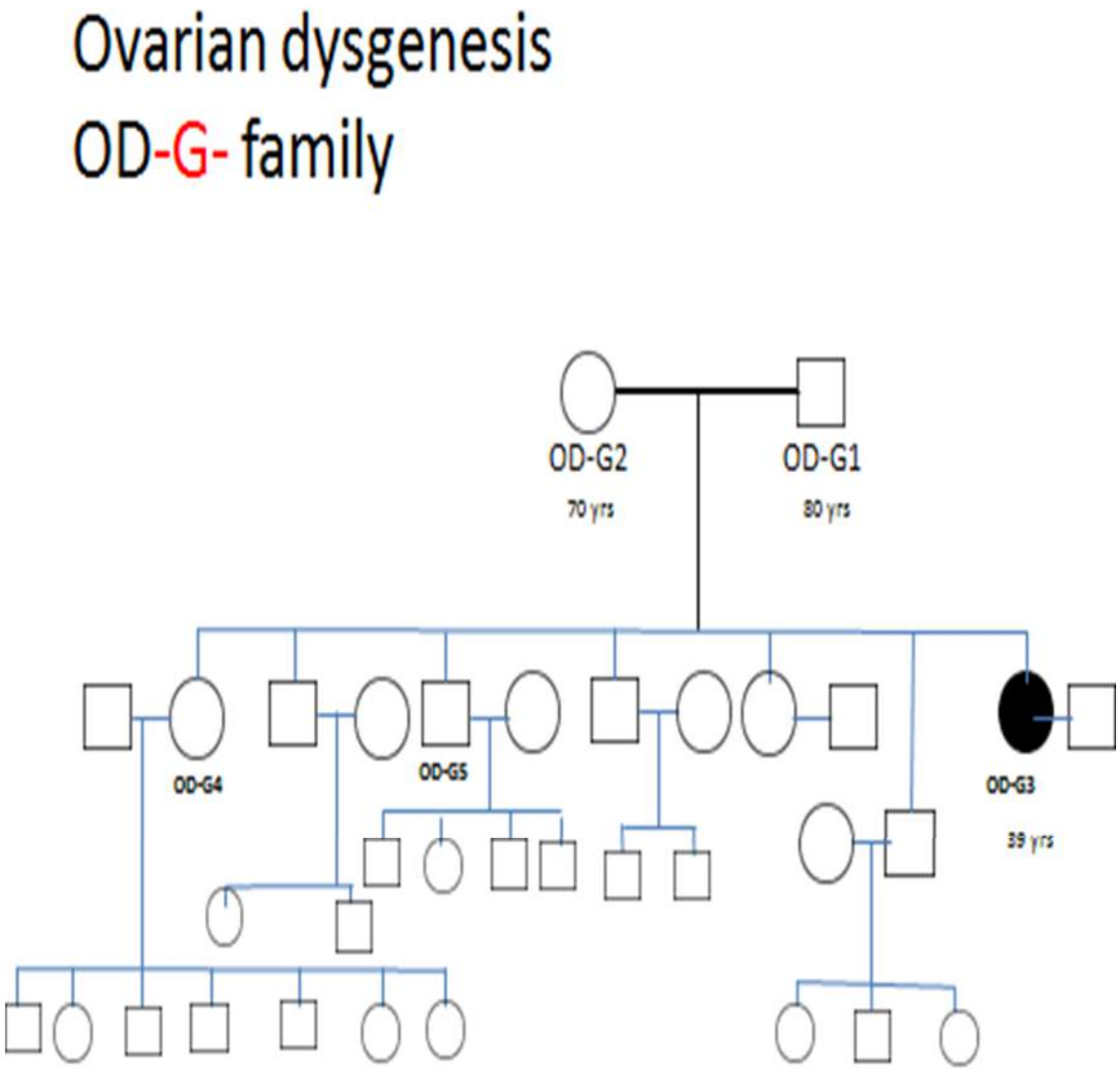
(b)


Ovarian dysgenesis
OD-D- Family
OD-D2
62 yrs
OD-D1
65 yrs
OD-D4
OD-D5
OD-D3
42 yrs
No children
OD-D6
No children

(c)


Ovarian dysgenesis
OD-F- family
OD-F2
47 yrs
OD-F1
52 yrs
OD-F3
27 yrs
OD-F6
Abortion 2 times
No kids
OD-F4
OD-F5

(d)


Ovarian dysgenesis
OD-G- family
OD-G2
70 yrs
OD-G1
80 yrs
OD-G4
OD-G5
OD-G3
39 yrs

(e)

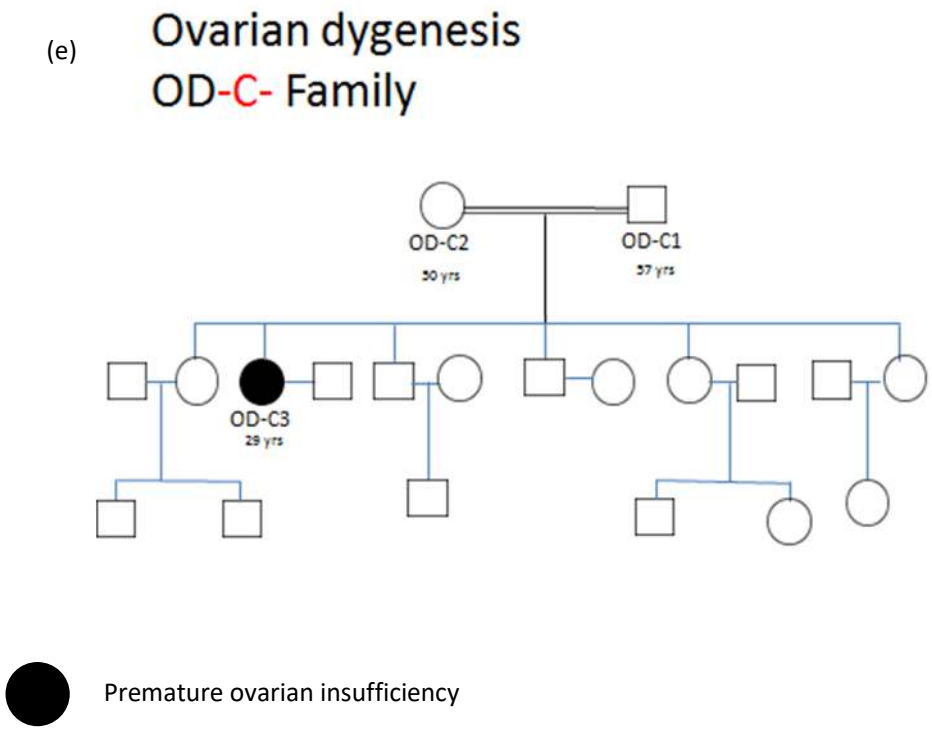


Figure 7.9 : a,b,c,d and e are family pedigrees of affected individuals. Coded individuals were sampled and sequenced for the mutation.

7.2 Ultrasound images for ovaries

Patients were examined by Dr. Iyad Afaneh and diagnosed with POI. The diagnosis was confirmed by a trans-vaginal ultrasound images that showed ovaries size and reserve. All of results showed that all of them are POI, size of ovaries was very small as showed in (figure 7.10) (figure 7.11) (figure 7.12)

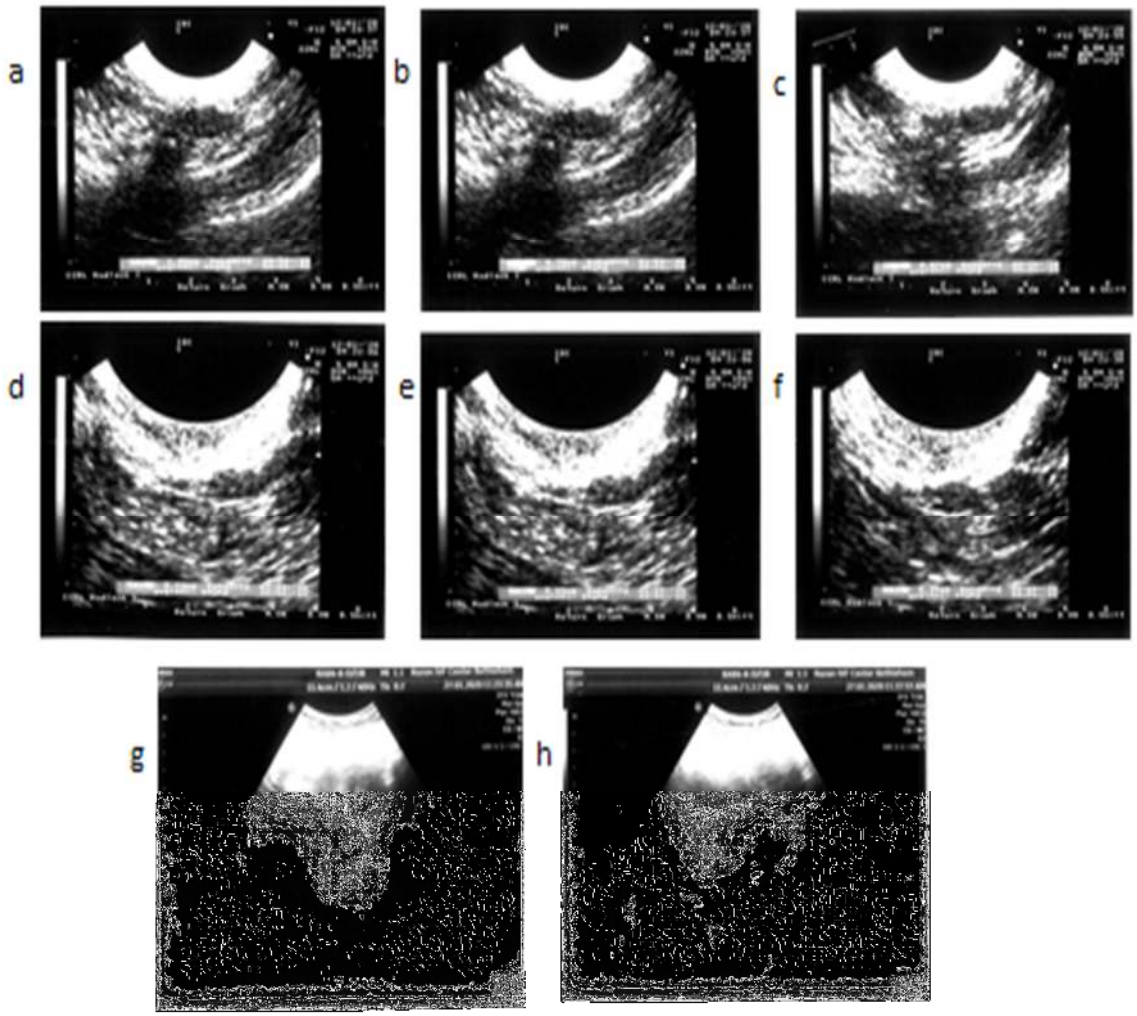


Figure 7.10 : Ultrasound images for OD-G and OD-E3. a,b,c,d,e and f showed small uterus 4.3X1.5X1.2cm, also both ovaries were seen and measuring about 0.39X 0.45cm and 1.05X0.75cm in diameter, no clear follicles were seen on them. g and h for OD-E3 illustrated an absence of adult form of uterus. Both ovaries could not be reliably visualized with probable existence of rudimentary uterus.

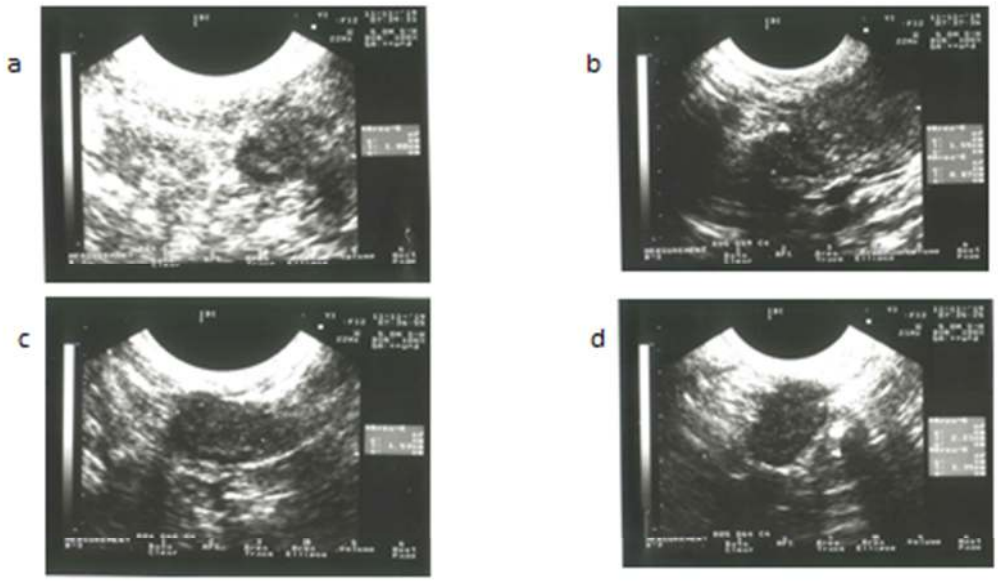


Figure 7.11: Ultrasound images for OD-D female. a and b showed right ovary with measurement about 1X0.87 cm. b and d for the left ovary, which is 2.21X1.35cm .

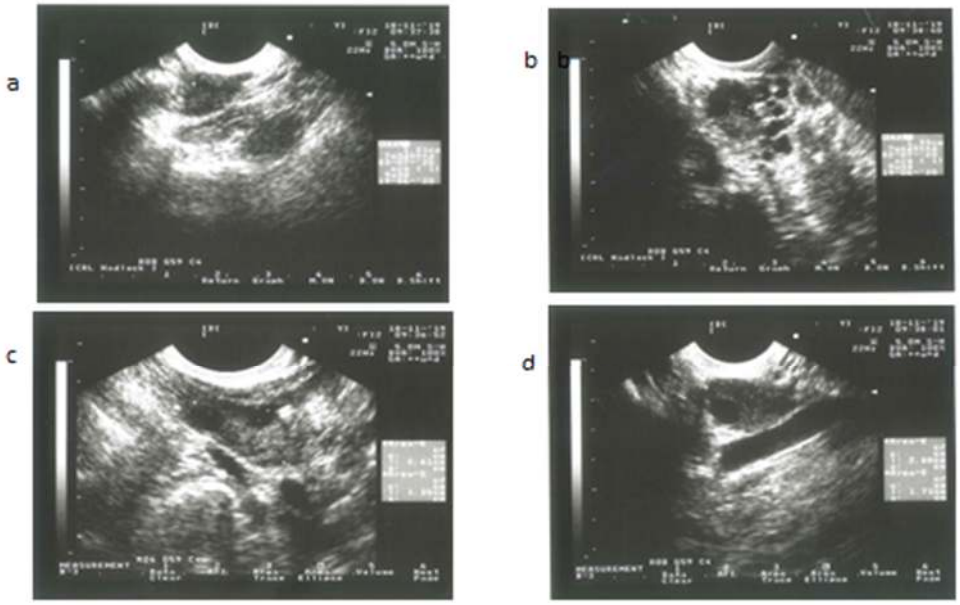


Figure 7.12 : ultrasound images for OD-C female. a and b showed right ovary with measurement about 2.61X1.35 cm. b and d for the left ovary, which is 1.35X2.66cm .

7.3 Hormones Level.

Table 7.15: Hormonal Level For Affected POI.

Females Codes	FSH	LH
OD-D	95.03mIU/ml	19mIU/ml
OD-E3	130.3 mIU/ml	30.5 IU/L
OD-G	46.2 mIU/ml	21.8 mIU/ml
OD-C	44.59 mIU/ml	1.42 mIU/ml
OD-F	87.31 mIU/ml	28.16 mIU/ml
OD-E2	86.42 mIU/ml	34.9 IU/L

7.4 Sanger Sequencing Results of the Identified MEIOB Mutation

The novel mutation which was detected in MEIOB gene in POI females is the deletion of G on the position on 1889376 of chromosome 16 at exon 12 of the gene. Which leads to a frameshift result in changing the amino acid serine to a stop codon resulting in a premature stop, thus, a truncation in the conserved C'-terminal DNA binding domain of MEIOB protein will occur. Exon 12 of MEIOB gene was sequenced in all females samples. No mutation detected in that exon in all POI females. Look to (Figure 7.13) to see Sanger sequencing results (electropherograms) of exon 12.

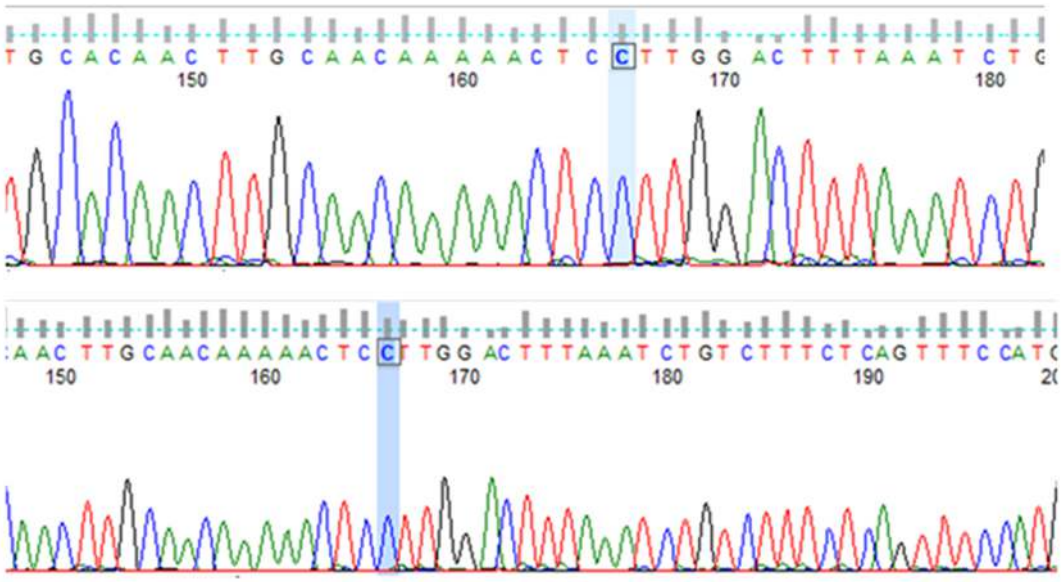


Figure 7.13 : Sanger sequencing electropherograms of exon 12 of MEIOB gene.

Both showed a normal sequence which has no mutation in MEIOB gene area.

7.5 Linkage Exclusion Analysis Results

After testing for the candidate 1889376 del G, exon 12 – S366fs* MEIOB gene mutation, the plan was to sequence the remaining exons, but before doing that, we preferred to do linkage analysis first to check if our gene of interest is linked to POI. We chose OD-E Family since we have two affected females within the same family. Two markers before and after MEIOB gene were used, M1 is 22TA, M2 is 20CA.

22TA marker was Homozygous for all individuals in OD-E family (affected and un-affected members). Fragment analysis of TA marker of unaffected parents and two affected sisters and one unaffected individual, from OD-E family are shown in (figure 7.14)

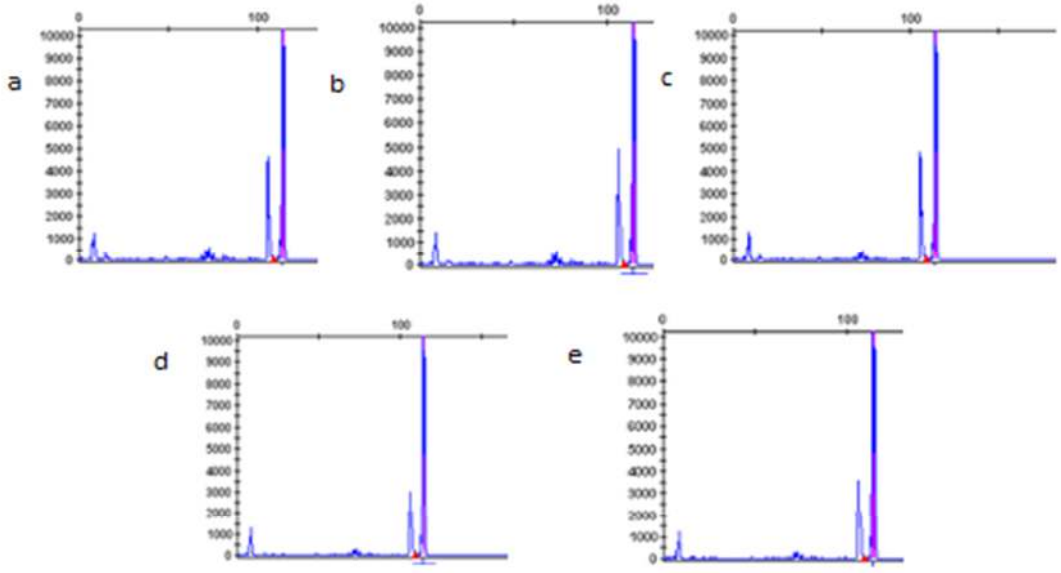


Figure 7.14 : Fragment analysis of M1- 22TA from OD-E family. (a) for E1 un-affected mother, (b) for E2 affected female,(c) For E3 affected female, (d) E4 for unaffected father and (e) E5 for unaffected brother.

The other marker CA20, was not informative in results, which showed no peaks in interested area around the MEIOB gene. Pedigree of the family used for linkage analysis with Haplotypes showed in (figure 7.15)

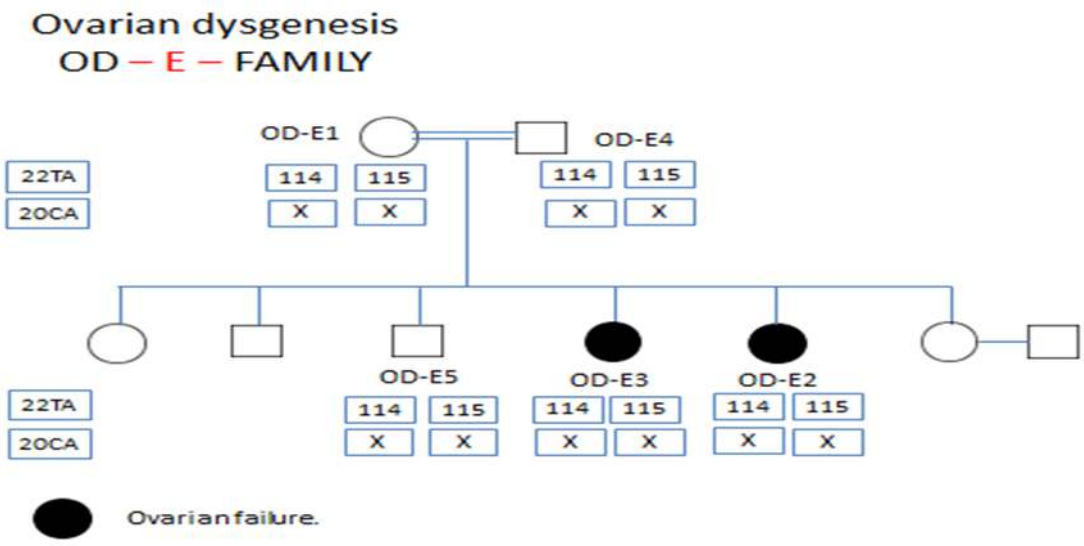


Figure 7.15: Pedigree of OD-E family which used for linkage test, blue boxes showed the Haplotypes of 22TA-M1 and 20CA-M2 around MEIOB gene.

CHAPTER 8

Discussion

In our study, we screened for the presence of MEIOB gene mutations in five Palestinian families who have six females diagnosed with POI according to their physician. Two of them were sisters with relatives diagnosed with Azoospermia. Lately, there was a study that showed a mutation in the MEIOB gene with a truncating affects the C-terminal region where SPATA22 supposed to bind, therefor, this will impair the interaction between MEIOB and its partner SPATA22 so there will be no accomplished DSB's repair. This may stimulate the depletion in the follicular stock of the ovaries and thus meiotic defection. Those results came compatible with phenotype of MEIOB$^{-/-}$mouse as previous studies of Luo's and Souquet's (Caburet et al., 2019). All the affected individuals were of Arab ethnicity in previous studies, this raises a big question whether this mutation is an Arab founder mutation or not. When we used Sanger sequencing to sequence MEIOB gene at exon 12, no mutation were found in that gene in our samples. Which indicates that they must harbor a different mutation in the MEIOB gene or any, other yet unknown fertility gene,

The next step after excluded the candidate mutation of MEIOB gene as a reason of POI, we try in this study to check if our gene of interest might be involved in causing this phenotype and linked to POI. We designed two markers before and after MEIOB gene. We chose the family which had at least two affected females and one unaffected female, OD-E family were the best to be chosen.

22TA marker was homozygous for all OD-E family members (affected and un-affected), which mean that this marker is not informative and can't be used for linkage exclusion in this family. Unfortunately, the second marker (20CA) was not informative for same reasons.

CHAPTER 9

Conclusion

In our study, we performed direct Sanger sequencing to screen for a previously identified MEIOB gene mutation in six infertile Arab-Palestinian females, at exon 12 only because of time and cost. All the patients were wild type for the 1889376delG mutation in exon 12 of MEIOB. Linkage exclusion test made to check if MEIOB gene correlated with phenotype of POI, both markers we tested were uninformative; because all family members had the same number of repeats for each marker. We could look for and design more markers and repeat the experiment, but we could also sequence all of the coding exons of MEIOB gene in only the proband from each family. This might be a bit expensive, but would give us more details on other variants such as SNPs that can be used for genotyping purposed as well. We also need to recruit more families with patients who suffer from POI as this could be of great importance once we discover a novel variant.

CHAPTER 9

Bibliography

Acevedo-Rodriguez, A., Kauffman, A. S., Cherrington, B. D., Borges, C. S., Roepke, T. A., & Laconi, M. (2018). Emerging insights into hypothalamic-pituitary-gonadal axis regulation and interaction with stress signalling. *Journal of neuroendocrinology*, *30*(10), e12590.

Adamson, P. C., Krupp, K., Freeman, A. H., Klausner, J. D., Reingold, A. L., & Madhivanan, P. (2011). Prevalence & correlates of primary infertility among young women in Mysore, India. *The Indian journal of medical research*, *134*(4), 440.

Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the globe. *Reproductive biology and endocrinology*, *13*(1), 1-9.

Alam, M. H., & Miyano, T. (2020). Interaction between growing oocytes and granulosa cells in vitro. *Reproductive medicine and biology*, *19*(1), 13-23.

Allers, T., & Lichten, M. (2001). Differential timing and control of noncrossover and crossover recombination during meiosis. *Cell*, *106*(1), 47-57.

Anagnostis, P., Tarlatzis, B. C., & Kauffman, R. P. (2018). Polycystic ovarian syndrome (PCOS): Long-term metabolic consequences. *Metabolism*, *86*, 33-43.

Anand, R., Beach, A., Li, K., & Haber, J. (2017). Rad51-mediated double-strand break repair and mismatch correction of divergent substrates. *Nature*, *544*(7650), 377-380.

Aziz, M. U., Anwar, S., & Mahmood, S. (2015). Hysterosalpingographic evaluation of primary and secondary infertility. *Pakistan journal of medical sciences*, *31*(5), 1188.

Baudat, F., Manova, K., Yuen, J. P., Jasin, M., & Keeney, S. (2000). Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. *Molecular cell*, *6*(5), 989-998.

Bhattacharya, S., Johnson, N., Tijani, H. A., Hart, R., Pandey, S., & Gibreel, A. F. (2010). Female infertility. *BMJ clinical evidence*, *2010*, 0819.

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

Biswas, L., Tyc, K., El Yakoubi, W., Morgan, K., Xing, J., & Schindler, K. (2021). Meiosis interrupted: the genetics of female infertility via meiotic failure. *Reproduction*, *161*(2), R13-R35.

Boivin, J., Bunting, L., Collins, J. A., & Nygren, K. G. (2007). International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Human reproduction*, *22*(6), 1506-1512.

Caburet, S., Todeschini, A. L., Petrillo, C., Martini, E., Farran, N. D., Legois, B., ... & Veitia, R. A. (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*, *42*, 524-531.

Cao, L., Alani, E., & Kleckner, N. (1990). A pathway for generation and processing of double-strand breaks during meiotic recombination in S. cerevisiae. *Cell*, *61*(6), 1089-1101.

Chen, Y., Yang, W., Shi, X., Zhang, C., Song, G., & Huang, D. (2020). The factors and pathways regulating the activation of mammalian primordial follicles in vivo. *Frontiers in Cell and Developmental Biology*, *8*, 1018.

Costa, Y., & Cooke, H. J. (2007). Dissecting the mammalian synaptonemal complex using targeted mutations. *Chromosome Research*, *15*(5), 579-589.

Das, L., Parbin, S., Pradhan, N., Kausar, C., & Patra, S. K. (2017). Epigenetics of reproductive infertility. *Front Biosci (Schol Ed)*, *9*, 509-535.

de Massy, B. (2003). Distribution of meiotic recombination sites. *TRENDS in Genetics*, *19*(9), 514-522.

Deshpande, P. S., & Gupta, A. S. (2019). Causes and Prevalence of Factors Causing Infertility in a Public Health Facility. *Journal of human reproductive sciences*, *12*(4), 287–293. https://doi.org/10.4103/jhrs.JHRS_140_18

Devroey, P., Fauser, B. C. J. M., Diedrich, K., & Evian Annual Reproduction (EVAR) Workshop Group 2008. (2009). Approaches to improve the diagnosis and management of infertility. *Human Reproduction Update*, *15*(4), 391-408.

Direkvand-Moghadam, A., Delpisheh, A., & Direkvand-Moghadam, A. (2014). Effect of infertility on the quality of life, a cross-sectional study. *Journal of clinical and diagnostic research: JCDR*, *8*(10), OC13.

Farren, A. T., & DiBenedetto, A. (2021). One couple's experience with infertility: Nursing theory-based practice case study. *International Journal of Nursing Knowledge*.

Fragouli, E., Alfarawati, S., Goodall, N. N., Sánchez-García, J. F., Colls, P., & Wells, D. (2011). The cytogenetics of polar bodies: insights into female meiosis and the diagnosis of aneuploidy. *MHR: Basic science of reproductive medicine*, *17*(5), 286-295.

Gravholt, C. H., Viuff, M. H., Brun, S., Stochholm, K., & Andersen, N. H. (2019). Turner syndrome: mechanisms and management. *Nature Reviews Endocrinology*, *15*(10), 601-614.

Guo, R., Xu, Y., Leu, N. A., Zhang, L., Fuchs, S. Y., Ye, L., & Wang, P. J. (2020). The ssDNA-binding protein MEIOB acts as a dosage-sensitive regulator of meiotic recombination. *Nucleic acids research*, *48*(21), 12219-12233.

Luo, M., Yang, F., Leu, N. A., Landaiche, J., Handel, M. A., Benavente, R., ... & Wang, P. J. (2013). MEIOB exhibits single-stranded DNA-binding and exonuclease activities and is essential for meiotic recombination. *Nature communications*, *4*(1), 1-12.

Heddar, A., Beckers, D., Fouquet, B., Roland, D., & Misrahi, M. (2020). A novel phenotype combining primary ovarian insufficiency growth retardation and pilomatricomas with MCM8 mutation. *The Journal of Clinical Endocrinology & Metabolism*, *105*(6), 1973-1982.

Heyer, W. D., Ehmsen, K. T., & Liu, J. (2010). Regulation of homologous recombination in eukaryotes. *Annual review of genetics*, *44*, 113-139.

Hultén, M., Smith, E., & Delhanty, J. (2010). Errors in chromosome segregation during oogenesis and early embryogenesis. In *Reproductive Endocrinology and Infertility* (pp. 325-342). Springer, New York, NY.

Hunt, P. A., & Hassold, T. J. (2008). Human female meiosis: what makes a good egg go bad?. *Trends in Genetics*, *24*(2), 86-93.

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

Ishishita, S., Matsuda, Y., & Kitada, K. (2014). Genetic evidence suggests that Spata22 is required for the maintenance of Rad51 foci in mammalian meiosis. *Scientific reports*, *4*(1), 1-8.

Jacob Farhi, M. D. (2011). Distribution of causes of infertility in patients attending primary fertility clinics in Israel. *IMAJ Jan*, *13*, 51-4.

Keeney, S., Giroux, C. N., & Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell*, *88*(3), 375-384.

Kowalczykowski, S. C. (2015). An overview of the molecular mechanisms of recombinational DNA repair. *Cold Spring Harbor Perspectives in Biology*, *7*(11), a016410.

Kriplani, A., Goyal, M., Kachhawa, G., Mahey, R., & Kulshrestha, V. (2017). Etiology and management of primary amenorrhoea: A study of 102 cases at tertiary centre. *Taiwanese Journal of Obstetrics and Gynecology*, *56*(6), 761-764.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews Genetics*, *3*(9), 662-673.

Lisby, M., & Rothstein, R. (2015). Cell biology of mitotic recombination. *Cold Spring Harbor perspectives in biology*, *7*(3), a016535.

Lisby, M., & Rothstein, R. (2015). Cell biology of mitotic recombination. *Cold Spring Harbor perspectives in biology*, *7*(3), a016535.

Luciano, A. A., Lanzone, A., & Goverde, A. J. (2013). Management of female infertility from hormonal causes. *International Journal of Gynecology & Obstetrics*, *123*, S9-S17.

Luo, M., Yang, F., Leu, N. A., Landaiche, J., Handel, M. A., Benavente, R., ... & Wang, P. J. (2013). MEIOB exhibits single-stranded DNA-binding and exonuclease activities and is essential for meiotic recombination. *Nature communications*, *4*(1), 1-12.

Marcon, E., & Moens, P. B. (2005). The evolution of meiosis: recruitment and modification of somatic DNA-repair proteins. *Bioessays*, *27*(8), 795-808.

Masoumi, S. Z., Parsa, P., Darvish, N., Mokhtari, S., Yavangi, M., & Roshanaei, G. (2015). An epidemiologic survey on the causes of infertility in patients referred to infertility center in Fatemieh Hospital in Hamadan. *Iranian journal of reproductive medicine*, *13*(8), 513.

Murphy, M. K., Hall, J. E., Adams, J. M., Lee, H., & Welt, C. K. (2006). Polycystic ovarian morphology in normal women does not predict the development of polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism*, *91*(10), 3878-3884.

Nelson, L. M. (2009). Primary ovarian insufficiency. *New England Journal of Medicine*, *360*(6), 606-614.

Nikolic, A., Volarevic, V., Armstrong, L., Lako, M., & Stojkovic, M. (2016). Primordial germ cells: current knowledge and perspectives. *Stem cells international*, *2016*.

Oberoi, S., Khaira, R., & Rai, S. K. (2017). Aetiology of Infertility: An Epidemiological Study. *Ntl J Community Med*, *8*(1), 17-21.

Ouarezki, Y., Cizmecioglu, F. M., Mansour, C., Jones, J. H., Gault, E. J., Mason, A., & Donaldson, M. D. (2018). Measured parental height in Turner syndrome—a valuable but underused diagnostic tool. *European journal of pediatrics*, *177*(2), 171-179.

Panay, N., Anderson, R. A., Nappi, R. E., Vincent, A. J., Vujovic, S., Webber, L., & Wolfman, W. (2020). Premature ovarian insufficiency: an international menopause society white paper. *Climacteric*, *23*(5), 426-446.

Persani, L., Rossetti, R., & Cacciatore, C. (2010). Genes involved in human premature ovarian failure. *Journal of molecular endocrinology*, *45*(5), 257.

Pisarska, M. D., Chan, J. L., Lawrenson, K., Gonzalez, T. L., & Wang, E. T. (2019). Genetics and epigenetics of infertility and treatments on outcomes. *The Journal of Clinical Endocrinology & Metabolism*, *104*(6), 1871-1886.

Poornima, S., Daram, S., Devaki, R. K., & Qurratulain, H. (2020). Chromosomal abnormalities in couples with primary and secondary infertility: Genetic counseling for assisted reproductive techniques (ART). *Journal of Reproduction & Infertility*, *21*(4), 269.

Ray, A., Shah, A., Gudi, A., & Homburg, R. (2012). Unexplained infertility: an update and review of practice. *Reproductive biomedicine online*, *24*(6), 591-602.

Ribeiro, J., Abby, E., Livera, G., & Martini, E. (2016). RPA homologs and ssDNA processing during meiotic recombination. *Chromosoma*, *125*(2), 265-276.

Roeder, G. S. (1997). Meiotic chromosomes: it takes two to tango. *Genes & development*, *11*(20), 2600-2621.

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., & Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *Journal of biological chemistry*, *273*(10), 5858-5868.

Schramm, S., Fraune, J., Naumann, R., Hernandez-Hernandez, A., Höög, C., Cooke, H. J., ... & Benavente, R. (2011). A novel mouse synaptonemal complex protein is essential for loading of central element proteins, recombination, and fertility. *PLoS genetics*, *7*(5), e1002088.

Shao, R., Zheng, H., Jia, S., Jiang, Y., Yang, Q., & Kang, G. (2018). Nitric oxide enhancing resistance to PEG-induced water deficiency is associated with the primary photosynthesis reaction in Triticum aestivum L. *International journal of molecular sciences*, *19*(9), 2819.

Smith, S., Pfeifer, S. M., & Collins, J. A. (2003). Diagnosis and management of female infertility. *Jama*, *290*(13), 1767-1770.

Sormunen, T., Aanesen, A., Fossum, B., Karlgren, K., & Westerbotn, M. (2018). Infertility-related communication and coping strategies among women affected by primary or secondary infertility. *Journal of clinical nursing*, *27*(1-2), e335-e344.

Souquet, B., Abby, E., Hervé, R., Finsterbusch, F., Tourpin, S., Le Bouffant, R., ... & Livera, G. (2013). MEIOB targets single-strand DNA and is necessary for meiotic recombination. *PLoS genetics*, *9*(9), e1003784.

Symington, L. S. (2016). Mechanism and regulation of DNA end resection in eukaryotes. *Critical reviews in biochemistry and molecular biology*, *51*(3), 195-212.

Torrealday, S., Kodaman, P., & Pal, L. (2017). Premature ovarian insufficiency-an update on recent advances in understanding and management. *F1000Research*, *6*.

Veitia, R. A. (2020). Primary ovarian insufficiency, meiosis and DNA repair. *biomedical journal*, *43*(2), 115-123.

Vinagre, J. G., Witkin, S. S., Ribeiro, S. C., Robial, R., Fukazawa, E. I., Ortolani, C. C., ... & Linhares, I. M. (2019). Influence of a mannose-binding lectin gene polymorphism and exposure to Chlamydia trachomatis on fallopian tube obstruction in Brazilian woman. *Archives of gynecology and obstetrics*, *300*(3), 641-645.

Wang, X., Haber, J. E., & West, S. (2004). Role of Saccharomyces single-stranded DNA-binding protein RPA in the strand invasion step of double-strand break repair. *PLoS biology*, *2*(1), e21.

Yahaya, T. O., Oladele, E. O., Anyebe, D., Obi, C., Bunza, M. D. A., Sulaiman, R., & Liman, U. U. (2021). Chromosomal abnormalities predisposing to infertility, testing, and management: a narrative review. *Bulletin of the National Research Centre*, *45*(1), 1-15.

Yates, L. A., Aramayo, R. J., Pokhrel, N., Caldwell, C. C., Kaplan, J. A., Perera, R. L., ... & Zhang, X. (2018). A structural and dynamic model for the assembly of Replication Protein A on single-stranded DNA. *Nature communications*, *9*(1), 1-14.

Yatsenko, S. A., & Rajkovic, A. (2019). Genetics of human female infertility. *Biology of reproduction*, *101*(3), 549-566.