

**Arab American University**  
**Faculty of Graduate Studies**  
**Department of Health Sciences**  
**Master Program in Molecular**  
**Genetics and Genetic Toxicology**



**Identification of putative Genes involved in Male infertility in  
selected Palestinian Families using whole-exome sequencing.**

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**This Thesis Was Submitted in Partial Fulfilment of the  
Requirements for the Master Degree in  
Molecular Genetics and Genetic Toxicology**

**Palestine, September /2025**

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**Arab American University**  
**Faculty of Graduate Studies**  
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**Genetics and Genetic Toxicology**



## **Thesis Approval**

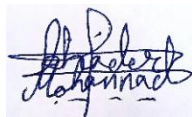
### **Identification of putative Genes involved in Male infertility in selected Palestinian Families using whole-exome sequencing.**

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## **Declaration**

I declare that, except where explicit reference is made to the contribution of others, this thesis is substantially my own work and has not been submitted for any other degree at the Arab American University or any other institution.

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## **Dedication**

I would like to dedicate this thesis to the thousands of individuals silently struggling with infertility who are eager to obtain a reliable resolution and reasoning behind their struggles. Additionally, I would like to raise awareness of this societal taboo to provide compassion and answers to couples who have dealt with these difficulties by using my results from the following thesis. Most importantly, to my family and friends who have encouraged me and made this incredibly gruelling journey possible.

Sumayya Raed Mohammed Abu Raad

## **Acknowledgements**

My journey in obtaining my master's in molecular genetics and toxicology has been difficult yet increasingly rewarding. Moreover, it was possible due to the unshackened efforts from my professor, Hisham Darwish, who has provided me with adequate knowledge that allowed me to thrive in this field, and additionally, thanks to the lab icons Rua Thawabteh and Kholoud Abu Saleh, who have impacted me positively through this challenging journey. Most importantly, I would like to extend my heartfelt appreciation to the Palestinian European Fertility Center, which provided me with the means and samples to conduct this thesis. Furthermore, thanks to Rula Fattouh, my dear friend and head of IVF in the Palestinian European Fertility Centre, as well as my great friend Maysa Thawabteh, who has helped me through this thesis, and also a big thanks to Dr. Diana Alhowari, who helped and stood by me in this thesis.

A major thanks to the unwavering support from my dear father, mother, and two brothers, who have stood by me through these tough times. My supportive uncle Ahmad, who helped me even while my strength was deterred by taking me to and from my university, in order to proceed with my investigation. My loving husband, who supported me and encouraged me to follow my dreams, and inspired me to never give up on my aspirations of gaining my degree in genetic science. And to my future daughter, I extend my greatest appreciation to you, as you provided me with sufficient strength to complete my academic journey.

Sumayya Raed Mohammed Abu Raad

# **Identification of putative Genes involved in Male infertility in selected Palestinian Families using whole-exome sequencing.**

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**Supervision Committee:**

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## **Abstract**

Infertility is defined as the inability to become pregnant after more than 12 months of frequent unprotected intercourse. Male infertility is classified in four categories: spermatogenic quantitative defects, spermatogenic qualitative defects, hypothalamic-pituitary axis disturbances, and ductal obstruction or dysfunction. Genetic factors are responsible for at least 15% of male infertility and significantly contribute to all four aetiological categories of male infertility. Azoospermia is the overall absence of sperm in the ejaculate. Any class other than ductal obstruction or dysfunction is considered non-obstructive azoospermia. In genetic aetiologies, several genes seem to contribute to male infertility, including *MEIOB*, *TEX11*, *STAG3*, *SYCP3*, and *DMC1*. This study aims to identify precise genetic causes of inherited male infertility in Palestinian families with two or more siblings in each family are diagnosed with idiopathic male infertility. Two Palestinian families with several affected members with idiopathic non-obstructive azoospermia were included. one affected member of each family was selected for whole-exome sequencing, and the results of the VCF file were filtered. The variants with the highest phenotype correlation score  $\geq 0.75$ , including two variants from family I and five variants from family II, were tested by Sanger sequencing across all family members. The suspected variants in family 1 were examined and analyzed by in-silico bioinformatics tools, including *CFAP47* and *SYCE1* gene variants. Primarily, the *SYCE1* p.Q241\* variant followed autosomal recessive inheritance and was validated and well segregated in the family. In family II, variants in the *RBBP7*, *CEP112*, *POLG-1*, *POLG-2*, and *HFE* genes were recognized and assessed. The *RBBP7* p.Thr256Pro variant followed X-linked recessive inheritance, which was verified and segregated as expected. In summary, *SYCE1* p.Q241\* and *RBBP7* p.Thr256Pro variants are suggested to be associated with non-obstructive azoospermia in the two affected families in this study. Additional research and functional assays will be required to definitively confirm these results and reveal their distinctive role in the development of male infertility. This data will be helpful for patient screening and evaluation while additionally emphasizing the potential relevance of these genes as therapeutic targets.

**Keywords:** Male Infertility, Azoospermia, RBBP7 Gene, SYCE1 Gene, WES

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## List of Definitions of Abbreviations

|         | Abbreviations                                       | Title |
|---------|-----------------------------------------------------|-------|
| WHO     | World Health Organization                           |       |
| FSH     | Follicle-stimulating hormone                        |       |
| LH      | Luteinizing hormone                                 |       |
| ROS     | Reactive Oxygen Species                             |       |
| SDF     | Sperm DNA Fragmentation                             |       |
| RCTs    | Randomized Controlled Trials                        |       |
| ART     | Assisted Reproductive Technology                    |       |
| GnRH    | Gonadotropin Releasing Hormone                      |       |
| NOA     | Non-Obstructive Azoospermia                         |       |
| CBAVD   | Congenital Bilateral Absence of Vas Deferens        |       |
| CF      | Cystic Fibrosis                                     |       |
| CFTR    | Cystic Fibrosis Transmembrane Conductance Regulator |       |
| SSCs    | Spermatogonial stem cells                           |       |
| AZF     | Azoospermia Factor                                  |       |
| SRY     | Sex-determining Region Y gene                       |       |
| PAR     | Pseudoautosomal Region                              |       |
| TEX11   | Testis Expressed 11                                 |       |
| SPATA16 | Spermatogenesis Associated 16                       |       |
| PICK1   | Protein Interacting With PRKCA 1                    |       |
| DPY19L2 | Dpy-19 Like 2                                       |       |
| ASA     | Anti sperm antibodies                               |       |
| OA      | Obstructive Azoospermia                             |       |
| NGS     | Next Generation Sequencing                          |       |
| IVF     | In Vitro Fertilization                              |       |
| TESE    | Testicular Sperm Extraction                         |       |
| OAT     | Oligoasthenoteratozoospermia                        |       |
| SNP     | Single Neucleotide Polymorphism                     |       |
| GWAS    | Genome Wide Association Studies                     |       |
| SCOS    | Sertoli Cell Only Syndrome                          |       |
| MA      | Maturation Arrest                                   |       |

|          |                                                             |
|----------|-------------------------------------------------------------|
| ADGRG2   | Adhesion G Protein-Coupled Receptor G2                      |
| SLC9A3   | solute carrier family 9 sodium/hydrogen exchanger isoform 3 |
| SGA      | Spermatogonial Arrest                                       |
| ICSI     | Intra Cytoplasmic Sperm Injection                           |
| FANCA    | Fanconi anemia complementation group                        |
| DSB      | Double-Strand Breaks                                        |
| FA       | Fanconi Anemia                                              |
| PLK4     | Polo-Like Kinase 4                                          |
| WNK3     | Lysine Deficient Protein Kinase 3                           |
| MEI1     | Meiotic Double-Stranded Break Formation Protein 1           |
| MEIOB    | Meiosis-Specific with OB Domain-Containing Protein          |
| CGH      | Comparative Genomic Hybridisation                           |
| SYCE1    | Synaptonemal Complex Central Element Protein 1              |
| STAG3    | Cohesin Complex Component                                   |
| SETX     | Senataxin                                                   |
| AOA2     | Oculomotor Apraxia type 2                                   |
| C14orf39 | Chromosome 14 Open Reading Frame 39                         |
| DMC1     | DNA Meiotic Recombinase 1                                   |
| POI      | Primary Ovarian Insufficiency                               |
| SPO11    | Initiator Of Meiotic Double-Strand Breaks                   |
| XRCC2    | X-Ray Repair Cross-Complementing 2                          |
| ZMYND15  | Zinc Finger MYND-Type Containing 15                         |
| RBBP7    | Retinol-Binding Protein 7                                   |
| H4       | Histone H4                                                  |
| BRCA1    | Breast Cancer gene 1                                        |
| ART      | Assisted Reproductive Technology                            |
| USP26    | Ubiquitin Specific Peptidase 26                             |
| PASD1    | PAS domain containing 1                                     |
| SCML1    | Scm Polycomb Group Protein Like 1                           |
| DCAF12L1 | DDB1 And CUL4 Associated Factor 12 Like 1                   |
| GCNA     | Germ Cell Nuclear Acidic Peptidase                          |
| ZFX      | Zinc Finger protein, X-linked                               |
| BCORL1   | BCL6 Corepressor Like 1                                     |

|           |                                          |
|-----------|------------------------------------------|
| FANCM     | FA Complementation Group M               |
| NANOS2    | Nanos C2HC-Type Zinc Finger 2            |
| TEX14     | Testis Expressed 14                      |
| TDRD9     | Tudor Domain Containing 9                |
| ATP       | Adenosine Triphosphate                   |
| SMB3      | Streptavidin Magnetic Beads              |
| EEW       | Enhanced Enrichment Wash                 |
| EE1       | Enrichment Elution Buffer 1              |
| ET2       | Elute Target Buffer 2                    |
| WES       | Whole Exome Sequencing                   |
| CFAP47    | Cilia And Flagella Associated Protein 47 |
| CEP112    | Centrosomal Protein 112                  |
| POLG-1    | DNA Polymerase Gamma, Catalytic Subunit  |
| PRC2      | polycomb repressive complex 2            |
| TNM score | Tumor, Node, and Metastasis score        |

# Chapter One: Introduction

## 1.1 Definition of Infertility

Infertility is defined as the inability to become pregnant after more than 12 months of frequent unprotected intercourse. It is also considered a worldwide health concern, affecting 8-12% of couples. Furthermore, there is no single cause of infertility, with an estimated 30% of infertile couples having no identified cause (idiopathic infertility) (Agarwal et al., 2021; Pereira & Sousa, 2023; Pfeifer et al., 2015). Evidently, between 1950 and 2017, the worldwide fertility rate decreased from 4.7 to 2.4 live births. (Murray et al., 2018). However, between 1981 and 2013, an equivalent decrease in sperm counts (0.70 million/ml/yr) was noted. (Levine et al., 2017).

Male infertility is a complicated, multifaceted disease with variable phenotypic presentations, ranging from a complete lack of spermatozoa in the testes to substantial changes in sperm quality. (Krausz, 2011a; Tournaye et al., 2017). The role of the male partner in infertility has attracted significant attention in recent years, where around half of the cases, a male factor is involved. (Hanson et al., 2020).

The etiology of infertility is extremely diverse, which is not unexpected since the reproductive systems of both males and females must work in a combined and highly coordinated manner to conceive a child. Numerous critical pathways for male infertility have been identified in studies that attempt to clarify the genetic foundation of fertility defects in both humans and other species, such as sexual differentiation, formation of the genitourinary system, and gamete development. (Oud et al., 2019a). Male infertility can arise from either quantitative or qualitative spermatogenic abnormalities. About one in every 100 men is considered to have azoospermia, which is the overall absence of sperms in the ejaculate. Today, roughly 20 percent of azoospermia patients are regarded as idiopathic. (Stephen & Chandra, 2006).

Genetic factors are responsible for at least 15% of male infertility and significantly contribute to all four of the main aetiological categories including spermatogenic quantitative defects, spermatogenic qualitative defects, hypothalamic-pituitary axis disturbances, and ductal obstruction or dysfunction. (Tournaye et al., 2017). Significant studies on the genetics of male infertility have been published since the identification of an extra X chromosome in Klinefelter patients (47, XXY) (A Case of Human

Intersexuality Having a Possible XXY, n.d.). Understanding the particular genetic etiology in each patient improves the management of infertility and related medical problems, which also encourages the development of creative, non-invasive interventions for male infertility. (Kasak & Laan, 2021a).

Spermatogenesis is the key process in forming male germ cells, which is processed in the testis in mammals. It involves a series of steps starting from spermatogonial stem cells division through mitosis, followed by meiosis in which results in dividing the chromosome number in half, producing germ cells that differentiate to form primary and secondary spermatocytes in one and two, respectively, and involves rearrangement of genetic material through DNA cross-over (Zickler & Kleckner, 2025). Crossing over between homologous chromosomes starts especially in the pachytene stage in the first meiotic prophase. Ultimately, in the final step, cells reach the mature sperm cell stage (Nishimura & L'Hernault, 2017). The three indicated main steps in spermatogenesis are shown in Figure (1.1) The mature specialized cells contain the nucleus in the head, mitochondria in the midpiece, and a tail for motility (Galletta et al., 2020). Alteration in the function of any phase, or the change in number, shape, or motility of sperm, may affect the fertility of humans (Yoshida et al., 2008).

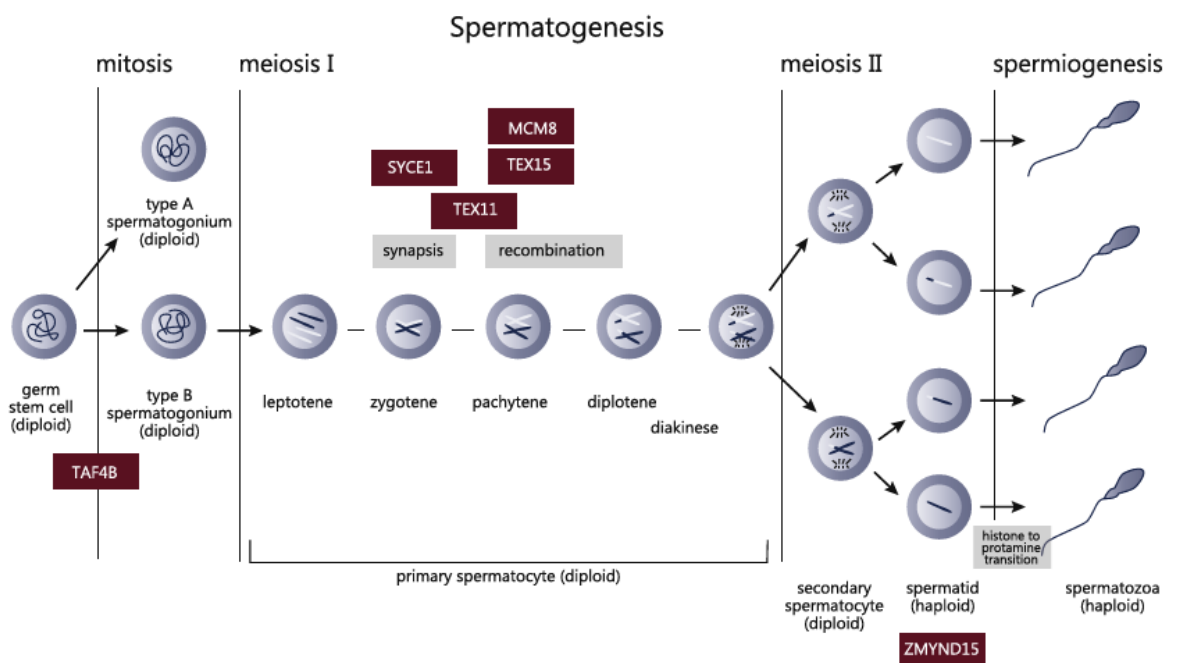


Figure (1.1): Steps of Spermatogenesis (Bracke et al., 2018).

## **1.2 Classification of Infertility and Medical History of Infertility**

Infertility is often defined in two ways: primary (no previous fertility) or secondary (previously fertile but now infertile) (Zegers-Hochschild et al., 2017). Classification of male infertility requires a thorough medical patient history to provide effective therapy.

### **1.2.1 Primary infertility**

Primary infertility is described as "failure to achieve spontaneous clinical pregnancy." (Ghaffari & Arabipoor, 2018).

### **1.2.2 Secondary infertility**

Secondary infertility is described as "failure to produce a spontaneous clinical pregnancy after a prior one." (Ghaffari & Arabipoor, 2018).

### **1.2.3 Medical history of infertility**

Considering the medical history is important to determine whether infertility is primary or secondary. History is classified into many sectors, including medical and surgical history, cancer treatment, spinal cord injury, testicular surgery, and vas deferens surgery. Other factors that may contribute to this condition include drug history, delayed or absent puberty, family history, erectile dysfunction, and finally lifestyle aspects such as the use of certain lubricants, alcohol, exposure to chemicals, smoking, recreational drugs, and obesity are one of the main factors to be taken into consideration. (Pillai & Mceleny, n.d.).

Reviewing the medical history of male infertility provides the opportunity to increase the chance for successful treatment and can help guide treatment options.

## **1.3 Diagnostic Criteria of Infertility**

Precise diagnosis of male infertility is necessary because it is an important step for effective treatment and might be complicated since reproduction requires several organs and demands assessment of the couple. The first approach to assessing infertility is

acquiring a detailed medical history (Krausz, 2011b) and physical examination (Pfeifer et al., 2015). However, according to World Health Organization (WHO) guidelines, the gold standard for the evaluation and diagnosis of infertility is semen analysis, which is classified as one of the key investigations. (WHO Laboratory Manual for the Examination and Processing of Human Semen, Sixth Edition, n.d.-a Year??). The pertinent second-level examinations include hormone assays (Follicle-stimulating hormone (FSH), Luteinizing Hormone (LH), total testosterone), genetic analysis, microbiological evaluation of semen and urine, urethral swab, scrotal and testicular ultrasonography, scrotal color Doppler, and transrectal ultrasound. (Krausz, 2011c). The diagnosis of male infertility includes several steps, explained below in the flowchart as shown in Figure (2).

### **1.3.1 Medical History and Physical Examination**

The medical history of the patient might reveal risk factors and lifestyle behaviors that may significantly affect male infertility. Medical history, as mentioned above, should include an examination of all systems, a family reproductive history, and a thorough social history that covers any previous or present use of anabolic steroids, recreational drugs, alcohol, and tobacco.(Krausz, 2011b). In addition, a complete physical examination is a necessary component of the assessment of infertile males, which includes examining the penis and noting the position of the urethral meatus, palpation, and measurement of the testicles, the existence consistency of both vasa and epididymides, and the existence or absence of a varicocele. Furthermore, secondary sex features must be examined including body habitus, hair distribution, and breast growth. (Krausz, 2011b).

### **1.3.2 Semen Analysis**

Semen analysis is considered the cornerstone of the initial diagnosis of infertility patients. It provides information about the most important characteristics of sperm, including their concentration, morphology, and motility (Pfeifer et al., 2015). The most important information provided by semen analysis is the total spermatozoa count, which indicates testicular sperm production and patency of the post-testicular duct system, also giving information about secretory activity for accessory glands by measuring a total fluid

volume for semen (*WHO Laboratory Manual for the Examination and Processing of Human Semen*, 2010). Table 1.1 shows the most recent criteria developed by the World Health Organization for semen analysis, with the last revision being in 2010. This suggests that there has been a worldwide decline in fertility during the last decade.

Table (1.1): Differences between Semen Analysis Reference Values by WHO in 2010 and 2021 (*WHO Laboratory Manual for the Examination and Processing of Human Semen*, 2010) (*WHO Laboratory Manual for the Examination and Processing of Human Semen* Sixth Edition, n.d.-b).

|                                                          | WHO 2010      | WHO 2021      |
|----------------------------------------------------------|---------------|---------------|
| <b>Semen volume (mL)</b>                                 | 1.5 (1.4–1.7) | 1.4 (1.3–1.5) |
| <b>Total sperm number (10<sup>6</sup> per ejaculate)</b> | 39 (33–46)    | 39 (35–40)    |
| <b>Total motility (%)</b>                                | 40 (38–42)    | 42 (40–43)    |
| <b>Progressive motility (%)</b>                          | 32 (31–34)    | 30 (29–31)    |
| <b>Non progressive motility (%)</b>                      | 1             | 1 (1–1)       |
| <b>Immotile sperm (%)</b>                                | 22            | 20 (19–20)    |
| <b>Vitality (%)</b>                                      | 58 (55–63)    | 54 (50–56)    |
| <b>Normal forms (%)</b>                                  | 4 (3–4)       | 4 (3.9–4)     |

### 1.3.3 Molecular Testing

With the development of science and studies dealing with male infertility in the world, there has been increasing emphasis on developing diagnostic tools to analyze the chemical and molecular activities of sperm, involving assessment of seminal Reactive Oxygen Species (ROS) and Sperm DNA Fragmentation (SDF) (Pasqualotto et al., 2001; Simon et al., 2019).

#### 1.3.3.1 Reactive Oxygen Species (ROS)

Oxidative stress, also known as ROS, has been identified as one of the primary causes of low-quality semen. It is a state characterized by an elevated rate of cell damage caused by oxygen and oxygen-derived oxidants. The uncontrolled formation of ROS that surpasses the antioxidant capacity of the seminal plasma causes oxidative stress (OS), which is damaging to spermatozoa (Bansal & Bilaspuri, 2011). Therefore, oxidative stress is considered one of the causes of male infertility (Minhas et al., 2021a). Furthermore, oxidative stress has been linked to decreased sperm motility, sperm count, and aberrant sperm morphology; for that, low quantities of ROS (physiological levels) are important for spermatozoa maturation, capacitation, hyperactivation, and acrosome reaction. (Agarwal et al., 2014). A delicate balance between ROS and antioxidants is required for

optimum sperm activity, and excess seminal ROS produces oxidative stress, leading to SDF and damage to the sperm plasma membrane (Minhas et al., 2021a). Currently, there is no standardized way of detecting seminal ROS, with a dearth of prospective randomized controlled trials (RCTs) with proper cost-benefit analysis, assessing its efficacy as a predictive biochemical marker for reproductive outcomes.

#### **1.3.3.2 Sperm DNA Fragmentation (SDF)**

Sperm DNA is extremely structured; the damage in sperm DNA can result from errors in chromatin condensation or an increase in reactive oxygen species amount, consequently reducing reproductive potential (Agarwal, Majzoub, et al., 2019). The reasons for aberrant SDF involve varicoceles, smoking, oncological medications (such as chemotherapy), and male genital tract infections (Minhas et al., 2021b). It has been demonstrated that a rise in SDF has a negative impact on the findings of each Assisted Reproductive Technology (ART) and spontaneous conception (Evenson & Wixon, 2006). Furthermore, aberrant SDF is implicated in both recurrent loss of pregnancy and idiopathic infertility (Faduola & Kolade, 2015).

The significant advantage of SDF testing is that it allows the possibility of treating the potentially curable reasons for SDF, incorporating lifestyle adjustments, diagnosing accessory gland infections, and treating a varicocele to enhance spontaneous conception and increase ART outcomes (Kirby et al., 2016).

#### **1.3.4 Hormonal Testing**

Spermatogenesis (Sperm cell differentiation into mature spermatozoa) is regulated by many hormones. (Griswold, 2016; Liu & Handelsman, 2003). Therefore, hormones that are involved in spermatogenesis must be measured. It is considered that primary hormonal abnormalities are the leading cause of male infertility (Esteves et al., 2011), therefore, endocrine abnormalities are crucial for evaluating infertility assessment since up to 70% of males with infertility have associated hormonal impairment (Krausz, 2011b), as shown in Table 1.2. Hypothalamic-pituitary-testicular axis integrity is necessary for the endocrine control of spermatogenesis and testicular function, as shown in Figure (1.2) Pituitary gonadotropin secretion is stimulated by the hypothalamus, Gonadotropin-Releasing Hormone (GnRH). LH primarily stimulates steroidogenesis by

acting on Leydig cells, whereas FSH primarily impacts Sertoli cells. Testosterone controls both gonadotropins by negative feedback. Therefore, the Pituitary gland's release of FSH and LH is necessary for the proper start of spermatogenesis (Liu & Handelsman, 2003).

Table (1.2): Table of Basal Hormone Levels. The Table Illustrates the Normal and Abnormal Levels of Hormones According to the Diagnosis of Infertility (Pfeifer et al., 2015).

| Basal hormone levels in various clinical states.           |             |            |            |        |
|------------------------------------------------------------|-------------|------------|------------|--------|
| Clinical condition                                         | FSH         | LH         | T          | PRL    |
| Normal spermatogenesis                                     | Normal      | Normal     | Normal     | Normal |
| Hypogonadotropic hypogonadism                              | Low         | Low        | Low        | Normal |
| Abnormal spermatogenesis <sup>a</sup>                      | High/normal | Normal     | Normal     | Normal |
| Complete testicular failure/hypergonadotropic hypogonadism | High        | High       | Normal/low | Normal |
| PRL-secreting pituitary tumor                              | Normal/low  | Normal/low | Low        | High   |

<sup>a</sup> Many men with abnormal spermatogenesis have a normal serum FSH, but a marked elevation of serum FSH is clearly indicative of an abnormality in spermatogenesis.

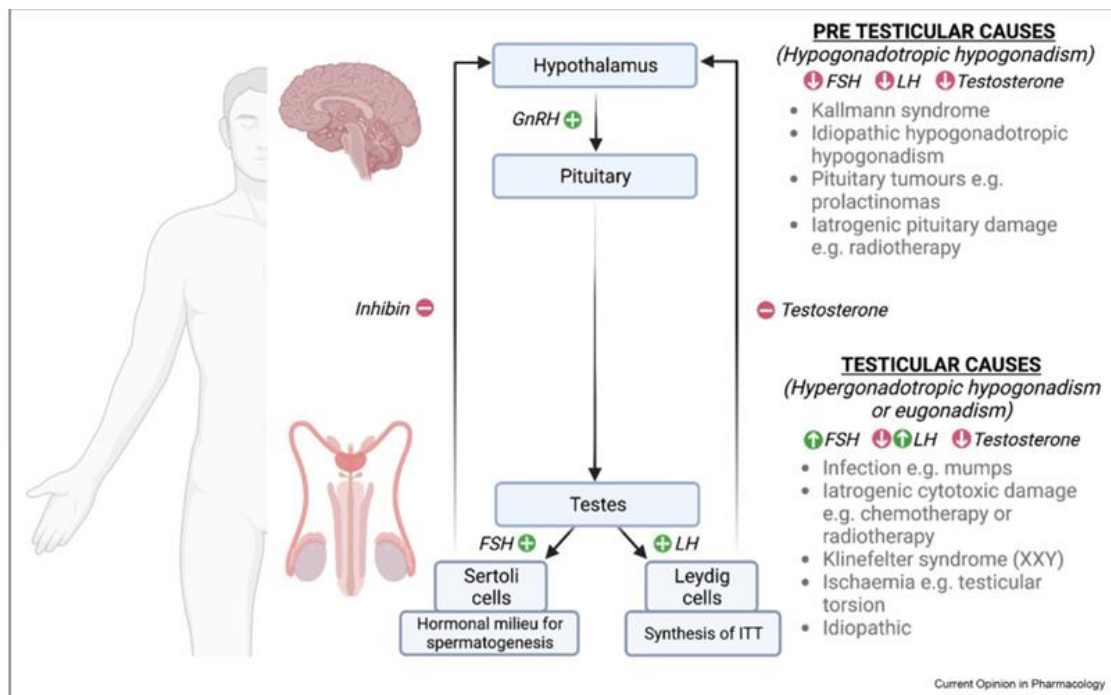


Figure (1.2 ): A schematic Diagram Demonstrating the Hypothalamic-Pituitary-Gonadal Axis and the Hormonal Signaling Networks Influencing the Manufacture of Testosterone and Spermatogenesis (Foran et al., 2023).

### 1.3.5 Genetic Testing

Determining whether a patient needs a genetic test or not is essential because genetic testing is becoming increasingly significant in identifying individuals who are likely to benefit from surgical sperm retrieval techniques and counseling of infertile males. Also,

to ascertain hereditary genetic disorders that may be transmitted to the progeny (Hwang et al., 2018). However, there are specific criteria for genetic testing for male infertility with severe oligospermia or azoospermia (Jarow et al., 2010). A comprehensive medical history, like semen analysis and hormone tests, assists in determining the patient's diagnostic category (Barratt et al., 2017).

The production of normal spermatogenesis results from the interactions of as many as 2300 genes working together (Hotaling & Carrell, 2014). Therefore, genetics testing is concentrated mainly on karyotype analysis, Y microdeletions, and cystic fibrosis mutation assessment. Karyotype testing must be conducted on all males with severe oligozoospermia ( $<5 \times 10^6/\text{ml}$ ) or non-obstructive azoospermia before any therapeutic intervention (Dul et al., 2012). On the other hand, Y chromosome microdeletion testing (YCMDs) is recommended for all males with severe oligozoospermia or non-obstructive azoospermia before any treatment intervention (Rozen et al., 2012). In addition, patients with congenital bilateral absence of the vas deferens (CBAVD) or cystic fibrosis (CF) should be provided with suitable CFTR mutation analysis (Barratt et al., 2017). Collectively, genetic counseling for infertility cases comes at advanced stages after basic tests such as semen analysis, hormone levels, and physical examination were performed, as described in Figure (1.3).

### **1.3.6 Imaging**

Ultrasound imaging helps to effectively examine the whole male genital system, thus providing a valuable tool for diagnosing anomalies that may impact fertility (Pfeifer et al., 2015). Scrotal ultrasonography has been utilized to show the existence or absence of the vas deferens, seminal vesicles, and epididymal abnormalities (Lotti & Maggi, 2015). Transrectal ultrasonography might be able to detect ejaculatory duct cysts or blockage and dilatation, and atrophy or hypoplasia of the seminal vesicles in males with azoospermia and poor ejaculate volume (Lotti & Maggi, 2015) while colour Doppler ultrasound was used to confirm the diagnosis of varicoceles (Bertolotto et al., 2020).

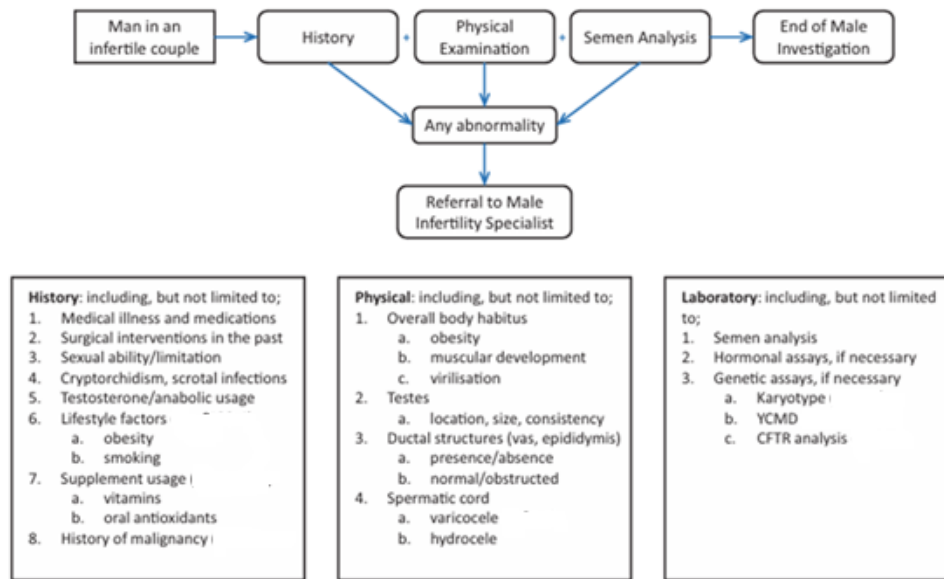


Figure (1.3): Flowchart Illustrating the Algorithm for Diagnostic Criteria of Male Infertility. The Primary Phase of Investigations Should Include Physical Examination, Medical History, and Semen Analysis (Barratt et al., 2017)

#### 1.4 Causes of Infertility

Male infertility becomes more frequent due to a wide variety of reasons and risk factors, which can be classified as acquired, congenital, and idiopathic (Minhas et al., 2021b). Some of the most prevalent acquired factors include varicocele, testicular trauma, acquired hypogonadism, recurring urogenital infections (prostatitis), exogenous factors (chemotherapy, medications, radiation, heat), and Anti-sperm antibodies (Agarwal et al., 2021), whereas the most common genetic reasons for male infertility are congenital bilateral vas deferens absence, which is involved in cystic fibrosis gene mutations (CFTR) (Stuhrmann & Dörk, 2000), chromosomal or genetic abnormalities (P. Zhan et al., 2022), Such as Klinefelter syndrome and its variations, Kallmann syndrome, Robertsonian translocation, Mild androgen insensitivity syndrome, which inhibits testicular function, and Y chromosome microdeletions that cause spermatogenic abnormalities (Vogt, 2004). In addition, idiopathic male infertility occurs in 30-50% of instances, with no obvious etiology or attributed to female infertility. Idiopathic male infertility is likely attributable to genetic and environmental causes. It is anticipated that more than 1000 genes are implicated in spermatogenesis. A confluence of genetic predisposition and environmental variables (e.g., pollution and reactive oxygen species) may lead to testicular dysfunction

and compromised sperm production (Jungwirth et al., 2012). Furthermore, male oxidative stress infertility is caused by altered sperm properties and oxidative stress, affecting roughly 37 million males with idiopathic male infertility. Moreover, toxic chemical exposure at work or in the environment, as well as various lifestyle factors (for example, smoking, alcohol consumption, recreational drug use, obesity, and psychological stress), are all possible causes of male infertility. (Agarwal, Parekh, et al., 2019; Qutub et al., 2022). Consequently, the etiology of compromised sperm production and function may be attributed to several congenital or acquired factors operating at pre-testicular, post-testicular, or directly at the testicular level (Krausz, 2011c).

### **1.5 Main Aetiological Categories of Male Infertility**

Male infertility is categorized into four primary etiological groups: Spermatogenic Quantitative Defects, Spermatogenic Qualitative Defects, Hypothalamic-pituitary axis Disturbances, and Ductal Obstruction or Dysfunction. There is a growing identification of genetic foundations for male reproductive problems, prompting discussions over their etiology concerning genetic, non-genetic, and environmental variables hereafter (Tournaye et al., 2017).

#### **1.5.1 Spermatogenic Quantitative Defects**

Disrupted spermatogenic mechanisms adversely impact sperm production and quantity. Spermatogenesis is a stem-cell-mediated pathway encompassing mitotic and meiotic phases, wherein spermatogonial stem cells SSCs undergo self-renewal and differentiation into viable male gametes (Caires et al., 2010). Spermatogenesis is a complex process regulated by endocrine, paracrine, and autocrine aspects (Varner, 2015).

Following continual mitotic self-renewal from puberty onwards, spermatogonial stem cells (SSCs) initiate meiosis soon after differentiating into primary spermatocytes.

The initial meiotic division generates two secondary spermatocytes, whereas the subsequent division results in four haploid spermatids. During spermiogenesis, spherical spermatids lengthen, form an acrosomal cap, and differentiate into flagellated spermatozoa, which are subsequently discharged by Sertoli cells into the luminal fluid (Muciaccia et al., 2013). Therefore, any defect in the spermatogenic mechanism will

impact on quantity of sperm production, and the defect can be genetic, non-genetic, or could be environmental, as shown in Figure (1.4).

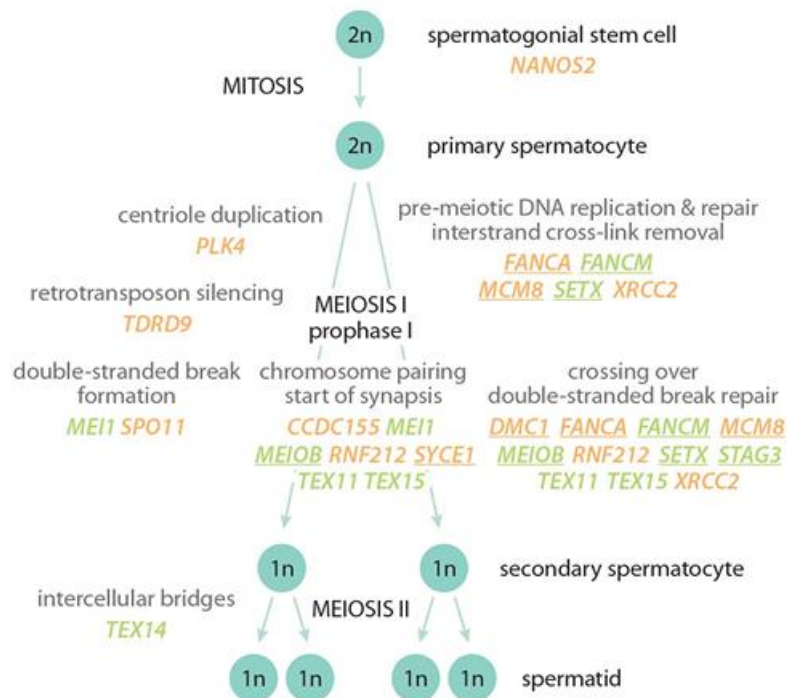


Figure (1.4): Genes Involved in Spermatogenesis and Mitosis with Meiosis (These Genes Will be Explained in Detail in the Second Chapter) (Kasak & Laan, 2021a).

### 1.5.1.1 Genetic Causes of Spermatogenic Quantitative Defects

Spermatogenesis may be affected by significant chromosomal abnormalities, detectable by karyotype analysis, as well as by genetic variation or submicroscopic deletions. The degree of spermatogenic impairment often escalates with the increased incidence of genetic conditions (Tournaye et al., 2017).

#### 1.5.1.1.1 Y- Chromosomal Microdeletion

Y-chromosomal microdeletions are the most prevalent identified molecular genetic factors contributing to significant spermatogenesis dysfunction, constituting approximately 10% of non-obstructive azoospermia cases and 3–5% of severe oligozoospermia instances (Krausz et al., 2014). This is due to the presence of several genes essential for the beginning and maintenance of spermatogenesis located in the Azoospermia Factor (AZF) region of the long arm of the Y chromosome (Skaletsky et al., 2003). The three subregions, AZFa, AZFb, and AZFc, are typically recognized in

clinical practice because they contain highly homologous repeated sequences that are susceptible to deletions (Krausz et al., 2014).

#### **1.5.1.1.2 Klinefelter Syndrome**

Klinefelter syndrome (47, XXY) is a chromosomal anomaly that impacts embryonic and fetal development, live birth, and health during childhood and adulthood. It is the main reason for male reproductive failure in 15% of azoospermic males, occurring in one out of every 600 men (Ghorbel et al., 2012). The additional X chromosome in Klinefelter patients causes the loss of spermatogonial stem cells (SSCs) by inhibiting self-renewal and obstructing the initiation of the meiotic cascade post-puberty, resulting in SSC death. Consequently, in the testes of adulthood, the tubules mostly include Sertoli cells, accompanied by extensive fibrosis (Davis et al., 2015; Oates, 2012).

#### **1.5.1.1.3 46XX Syndrome**

Known as de la Chapelle syndrome, the frequency of 46XX male syndrome is one in 20,000 male newborns (Vorona et al., 2007). The embryonic gonad differentiates into a testis or an ovary by a regulatory cascade of genes. One of the genes is the sex-determining region Y gene (*SRY*), situated on the distal short arm of the Y chromosome, adjacent to the pseudoautosomal region (PAR). Both ends of the Y chromosome possess pseudoautosomal regions that align with similar areas on the X chromosome during replication processes, including spermatogenesis. A translocation transpires within the Y and X chromosomes, resulting in the *SRY* gene being located on the X chromosome. When sperm with an altered X chromosome fertilizes an egg, the embryo will be genetically female but will have a masculine phenotype due to functioning *SRY*. Nonetheless, the extended arm of the Y chromosome, encompassing all the AZF subregions, will be absent, thereby inhibiting spermatogenesis. Infrequently, *SRY* is not responsible, and gonadal morphogenesis is dictated by one of the several other genes participating in the cascade (Q. Y. Wu et al., 2014).

#### **1.5.1.1.4 Isodicentric Y Chromosome**

Structural alterations that may modify the natural makeup and integrity of the Y chromosome include isodicentricism, truncation, or ring formation. The presence of spermatogenesis is contingent upon the ratio of cells exhibiting the aberrant Y chromosome and the integrity of the AZF subregions (Lange et al., 2009).

#### **1.5.1.1.5 Chromosomal Structure Anomalies**

Structural anomalies of the autosomes, including Robertsonian translocations, inversions, and reciprocal translocations, may impede spermatogenesis. These anomalies are more frequently observed in oligozoospermic males than in normozoospermic men, occurring at a rate of 4–8%, which is tenfold higher than in the overall population (*Chromosomal Alterations and Male Infertility*, n.d.).

#### **1.5.1.1.6 X-linked Genetic Anomalies**

Alterations in the androgen-receptor gene on the X chromosome can result in many phenotypic manifestations, including reduced sperm production in males with mild androgen insensitivity syndrome (O’Hara & Smith, 2015). Also, frequent deletions and genetic variation on the X chromosome, such as in *TEX11*, have clinical significance for male infertility, due to a genetic variation in *TEX11* leading to maturation arrest at spermatogenesis, resulting in NOA (Lo Giacco et al., 2014; Yatsenko et al., 2015; Sha et al., 2018a)

#### **1.5.1.1.7 Cryptorchidism**

Testicular descent in males is the most prevalent congenital anomaly in children, with a frequency of 2–9% at birth and 1–2% at three months of age. It may occur independently or with other congenital defects, known as syndromic cryptorchidism (Hutson & Thorup, 2015). Sperm production is diminished in males with cryptorchidism relative to that in normal people.

The cause of cryptorchidism is related to genetic and environmental variables, mostly endocrine disruptors, which have been suggested to operate alone or synergistically to

induce cryptorchidism (Hauser et al., 2015). This disease may occur in males with Klinefelter syndrome, Kallmann syndrome, or in boys with partial androgen insensitivity syndrome (Krausz, 2011c).

#### **1.5.1.1.8 Idiopathic Oligozoospermia and Azoospermia**

Idiopathic infertility from azoospermia and oligozoospermia likely results from singular genetic alterations, small nucleotide polymorphisms, or environmental influences that interact cumulatively with a predisposed genetic framework. Numerous single-nucleotide polymorphisms of more than 1500 genes have been associated with different forms of spermatogenic impairment (Krausz et al., 2015).

#### **1.5.1.2 Non-Genetic Causes of Spermatogenic Quantitative Defect**

Numerous non-genetic variables adversely impact male fertility. Such as a varicocele. According to the WHO, varicoceles are associated with semen anomalies, reduced testicular volume, and diminished Leydig-cell activity, hence contributing to poor spermatogenesis (The Influence of Varicocele on Parameters of Fertility in a Large Group of Men Presenting to Infertility Clinics\*, 1992). Also, fertility can be affected by mumps, viral orchitis, testicular torsion, gonadal cancer, and severe scrotal trauma. Common drugs may negatively impact male reproductive potential; in addition, chemotherapy significantly adversely impacts spermatogenesis (Samplaski & Nangia, 2015).

#### **1.5.2 Spermatogenic Qualitative Defects**

Despite the production of sufficient spermatozoa, their activities, including motility, fertilization, and genetic competence, may be inadequate. Genomic competence is essential for both early and late embryonic development. Here, delineate a few of the most clinically significant illnesses that impact sperm quality.

### **1.5.2.1 Genetic Causes of Spermatogenic Qualitative Defects**

Numerous disorders possess established genetic foundations that influence sperm morphology and functionality. Globozoospermia (round-headed sperm) and macrozoospermia (macrocephalic sperm head syndrome) are two variants of severe teratozoospermia that, in addition to prominent morphological deformities, are marked by atypical chromosomal compositions in the nuclei. Defects or alterations in *SPATA16*, *PICK1*, and *DPY19L2* can result in globozoospermia (Coutton et al., 2014). *DPY19L2* genetic variation is among the most prevalent, impacting 20–80% of individuals, contingent upon the degree of globozoospermia (Chianese et al., 2015). Four pathogenic variations in *AURKC* have been linked to macrozoospermia (macrocephaly, occurring in 50–80% of selected cases) (Chianese et al., 2015; Coutton et al., 2014).

### **1.5.2.2 Non-Genetic Causes of Spermatogenic Qualitative Defects**

Low levels of reactive oxygen species are important for signal transduction pathways linked to capacitation. So the excessive generation of reactive oxygen species by activated leukocytes, due to inflammation or infection of the urogenital tract or aberrant spermatozoa, may compromise the functional competence of spermatozoa (Gharagozloo & Aitken, 2011).

### **1.5.2.3 Environmental Causes of Spermatogenic Qualitative Defects**

One of the environmental causes of spermatogenic qualitative defects is Anti sperm antibodies (ASA). An autoimmune response targeting sperm cells occurs due to abundant antibodies against sperm, because of prior infections or inflammatory conditions of the testis or epididymis that compromise the blood-testis barrier (Yasin et al., 2016).

### **1.5.3 Hypothalamic-Pituitary Axis Disturbances**

Hypogonadotropic hypogonadism is defined as a deficiency in LH and FSH secretion. It results from acquired or congenital causes, is associated with a primary pituitary lesion in the pituitary gland, and is attributable to inadequate hypothalamic GnRH production or function (Bouvattier et al., 2012).

#### **1.5.3.1 Genetic Causes for Hypothalamic-Pituitary Axis Disturbances**

Congenital hypogonadotropic hypogonadism has genetic heterogeneity, many genes play a role in hypogonadotropic hypogonadism such as *FGFR1*, *FGF8*, *HS6ST1*, *SOX10*, which has a function in embryonic differentiation, *KAL1*, *PROK2*, *PROKR2*, *WDR11*, *SEMA3A*, *FEZF1*, which has a function in migration of gonadotropin-releasing hormone neurons, *GNRH1* which has a role in GnRH synthesis, and *GNRHR* has a role in GnRH activation. Although, the discovery of several novel candidate genes have been discovered, the etiology remains undetermined in around 60% of cases (Boehm et al., 2015).

#### **1.5.3.2 Non-Genetic Causes for Hypothalamic-Pituitary Axis Disturbances**

Acquired hypogonadotropic hypogonadism in adulthood exhibits few symptoms. decreased semen volume and facial hair development, diminished libido, and fatigue. The diagnosis of acquired hypogonadotropic hypogonadism is crucial for infertility and its possible implications for overall health, including life-threatening disorders like craniopharyngioma or meningioma. The condition may occur due to ablative therapies, such as surgery or radiation (Crowne et al., 2015).

#### **1.5.4 Ductal Obstruction or Dysfunction**

Congenital bilateral absence of the vas deferens (CBAVD) is the most prevalent genetically determined disorder that impedes sperm transport. The majority of cases arise from inherited genetic variations in both maternal and paternal copies of *CFTR*, and thus, are classified as genital manifestations of cystic fibrosis (Yu et al., 2012). Another cause of ductal obstruction is any deliberate or unexplained lesion to the ductal system, which may lead to obstructive azoospermia. Also, Vasectomy, epididymal blockage, or prior inguinal hernia repair resulting in unintentional disruption of the vasa may impact sperm transmission. Bilateral occlusion of the ejaculatory ducts can result in decreased semen volume, acidic pH, and azoospermia (Tournaye et al., 2017).

## **1.6 Etiology of Azoospermia**

Azoospermia, one of the most severe types of male infertility, is an overall loss of sperm in the ejaculate. However, it is a serious phenotype; the predicted incidence of azoospermia worldwide is extremely high, impacting one in every 100 men (Stephen & Chandra, 2006). While the diagnosis of azoospermia by semen analysis and hormonal testing is essential, there is a variety of heterogeneity of medical subphenotypes that complicate the determination of the etiology in each male suffering from azoospermia.

Azoospermia may result from hypothalamic-pituitary dysfunction, main quantitative spermatogenic disorders, or urogenital duct blockage (Tournaye et al., 2017). Based on several studies that examined the etiology of azoospermia, obstructive azoospermia represents 30% of cases, and 70% of cases represent non-obstructive azoospermia (NOA). The large percentage of NOA patients manifest primary testicular failure leading to failure of spermatogenesis, and also the lowest percentage of NOA patients suffer from endocrine abnormalities (Punab et al., 2017).

Statistics indicate that 2.4% of patients with azoospermia are due to congenital secondary hypogonadism, 14.2% of azoospermia cases are due to congenital anomalies in the urogenital tract, 25.8% of azoospermia because of acquired seminal tract obstruction, 2.6% of OA due to CFTR mutations, 6.4% of NOA cases are result from Y chromosome microdeletions, 16.2% of azoospermia cases due to sex chromosome abnormalities and 17.1% of azoospermia cases because of idiopathic reasons (Punab et al., 2017).

Even so, the determination of monogenic etiologies of NOA remains restricted (Jarvi et al., 2015). Also, one of the causes of azoospermia is related to epigenetics.

## **1.7 Epigenetics of Infertility**

Besides genetic screens for idiopathic infertility, the examination of epigenetics represents a potential domain for investigating idiopathic infertility. The epigenetic changes, generated by genetic or environmental influences, can serve as biomarkers for idiopathic male infertility. Epigenetics can be described as the investigation of modifications in gene function that are inheritable during mitosis and/or meiosis without requiring changes in the DNA sequence (Dupont et al., 2009).

The main types of epigenetic modifications that occur in sperm cells involve: the exchange of histones with protamines, the preservation of certain histones at critical regulatory genomic sites, and the hypomethylation of certain specific segments of the genome. Therefore, Epigenetic deregulation has been hypothesized as an element in the pathogenesis of idiopathic male infertility (Carrell, 2012).

### **1.8 Prevalence and Epidemiology of Infertility**

Infertility afflicts millions globally, which frequently results in severe consequences. Consequently, between 1950 and 2017, the worldwide fertility rate decreased from 4.7 to 2.4 live births (Murray et al., 2018). According to a statistical analysis by the WHO from 1990 to 2021, the 2022 global prevalence predictions indicate that one in six men has experienced infertility at some point in their lives (Infertility Prevalence Estimates, n.d.). Infertility prevalence differs by region due to a lack of data, giving proper prevalence statistics. WHO data indicates the predicted lifetime prevalence of infertility is greatest in the Western Pacific Region at 23.2%, whereas it is the least in the Eastern Mediterranean Region at 10.7% (Infertility Prevalence Estimates, n.d.). About one in every 100 men is considered to have azoospermia, which is the overall absence of sperm in the ejaculate. Today, roughly 20 percent of azoospermia patients are regarded as idiopathic (Stephen & Chandra, 2006).

### **1.9 Significance of the study**

This study is designed to identify precise genetic causes of inherited male infertility in Palestinian families with two or more siblings in the same family diagnosed with idiopathic male infertility, using Whole Exome Sequencing (WES), to understand the molecular genetic basis of male infertility in these families. Identifying the molecular cause of infertility will allow the establishment of a strategy in cooperation with fertility and clinical genetics specialists to enhance and manage treatments to improve the IVF success rate, and the identified genes will be very valuable for screening and identifying males in families with infertility and carriers. Furthermore, the findings of this study will be useful both for genetic and reproductive counseling, helping to develop a diagnostic gene panel, and for strengthening the understanding of familial male infertility.

### **1.10 Problem of the study**

There is a notable rise in instances of male infertility globally and especially in Palestine. Also, there is a significant rise in the number of families requiring assistance in reproductive technology programs, such as in vitro fertilization (IVF), particularly couples experiencing severe male infertility who necessitate specialized procedures and techniques, such as testicular sperm extraction (TESE), to obtain viable sperm for fertilization and to achieve a healthy pregnancy. In addition, male infertility may indicate poor general health, since men with aberrant sperm parameters have an elevated risk of malignancy, with the likelihood of testicular cancer increasing by up to 20 times. The projected variation of the putative genetic factors in different men with idiopathic infertility makes it difficult to make a precise diagnosis before the development of the various complications. So, it is essential to precisely identify the genetic loci and individual genetic variations directly associated with inherited male infertility and their role in infertility prognosis.

### **1.11 Objectives of the Study**

The objective of the study is to identify candidate gene(s) implicated in the etiology of male infertility across several unrelated Palestinian families, each having two or more siblings diagnosed with idiopathic male infertility. The phenotypes of infertile males are azoospermia. This will include, first, identifying the susceptible loci and causative genetic variations responsible for male infertility in these families, then validating the identified genetic variations in the family members and their relatives through direct segregation analysis. Therefore, it will be very useful information for family gene counseling to minimize the birth of more affected individuals, develop broad diagnostic gene panels, and provide direct assistance to familial male infertility in order to improve the IVF success rate.

### **1.12 Hypothesis of the Study**

This study hypothesizes that the identification of several families with more than one affected male with infertility, including twins, provides strong evidence for the

presence of specific genetic factors involved in the development of infertility in these families. Therefore, it is expected to identify the putative gene(s) directly linked to inherited infertility in affected males. The identification of the different genes is anticipated in these families due to their unrelated family backgrounds and variation in the clinical symptoms expressed in infertile males. The identified genetic variants will eventually be confirmed with broad segregation analysis, including parents and other closely related family members

### **1.13 Limitations of the study**

The limitation of the study is the absence of data about inherited male infertility, with no references for specific genes associated with inherited male infertility, with OAT and Azoospermia in Palestine. Furthermore, a limited number of samples can be analyzed due to the high cost of WES. Moreover, some families may withdraw from the study due to social complications. Along with the potential involvement of epigenetic factors in the process, which need additional specific tools for their detection and identification.

## **Chapter Two: Literature Review**

Numerous studies in European and American populations have shown that the prevalence of infertility varies between 10% and 15% globally (Rapino et al., 2014), with male infertility accounting for 40–50% of all instances. Over fifty percent of cases are idiopathic, whereas 10–20% of the affected men are azoospermic (Pashaei et al., 2020a). The most serious type of identified male infertility is the lack of spermatozoa in ejaculation, known as nonobstructive azoospermia (NOA) (Chiba et al., 2016). While evidence is deficient about the precise diagnosis and etiology of azoospermia, particularly due to genetic factors, multiple studies have identified several single-nucleotide polymorphisms (SNPs) that significantly contribute to azoospermia in males. 38 potential genes for idiopathic nonobstructive azoospermia have been identified according to analogous reproductive phenotypes in mice or familial segregation. Currently, 17 of them have attained a "moderate" or superior level of clinical evidence, per the categorization standards based on previous literature (Oud et al., 2019b).

The accelerated advancement of bioinformatics and its massive expansion have facilitated the enhancement of genetic information and the establishment of extensive databases, assisting researchers in examining gene variations, frequencies of alleles, and genotype-phenotype correlations at familial, social, and global scales. Several genetic loci linked to male infertility phenotypes have been found using next-generation sequencing (NGS) and genome-wide association studies (GWAS) from various populations. Distinctive results have been obtained from research performed on populations from different regions. The most common genetic susceptibilities associated with azoospermia will be discussed based on prior studies.

### **2.1 Genetic Susceptibility of Azoospermia**

Recent studies using NGS, particularly in family cases of NOA, have significantly improved the identification of novel NOA candidate genes. This chapter will investigate the genetic susceptibility of azoospermia by obstructive azoospermia and idiopathic non-obstructive azoospermia concerning multiple associated testicular histologies, which include Sertoli cell only syndrome (SCOS), maturation arrest (MA), and various kinds of testis phenotype.

### 2.1.1 Congenital Bilateral Absence of Vas Deferens (CBAVD)

CBAVD is a congenital developmental disorder occurring in 1 in 1000 males, defined by the absence of both vas deferens, where clinical characteristics are contingent on whether the disease is associated with another abnormality in the urogenital tract, specifically affecting seminal vessels (50% of cases) and renal agenesis (5–10% of CBAVD patients) (Bieth et al., 2021). The incidence of CBAVD in azoospermic males is reported to be 4–17%, also up to 25% in cases of obstructive azoospermia (Tournaye et al., 2017). The genetic basis of Congenital Bilateral Absence of the Vas Deferens without renal agenesis is restricted to two genes, the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene, involved in 75–80% of cases, and the Adhesion G Protein-Coupled Receptor G2 (*ADGRG2*), engaged in 5–10% of cases (Bieth et al., 2021; Patat et al., 2016).

#### 2.1.1.1 Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*)

The *CFTR* gene is located on the long arm of chromosome 7(7q31.2), as shown in Figure (2.1), and contains 27 exons. It encodes a functional protein of 1480 amino acids that controls many ion transporters, such as proton exchangers, sodium channels, water channels, and chloride/bicarbonate exchangers. It is responsible for the cystic fibrosis phenotype. Almost 2000 genetic variations have been identified in this gene. Genetic variations in *CFTR* could affect not just coding regions but also the promoter and deep intronic areas (Bergougnoux et al., 2019; J. Feng et al., 2019).

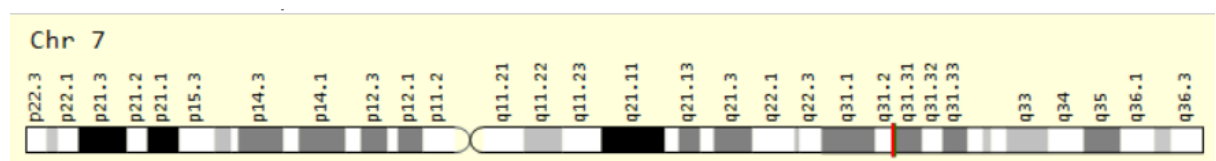


Figure (2.1): Location of the *CFTR* gene on the human genome.

**Role in Azoospermia:** Defects in the *CFTR* gene affect chloride channel function, blocking them from managing water and ion flow through cell membranes. Thus, male genital tract cells develop thick, sticky mucus. This mucus blocked the vas deferens throughout formation, causing it to degrade before delivery (Cuppens & Cassiman, 2004).

CFTR disease is an autosomal recessive inherited condition. There are basically two types of causative alleles: severe mutations, which arise from homozygous mutation, and mild mutations, which arise from heterozygous mutation and do not cause the disease. The combination of two mild mutations or a single severe allele together with a single mild allele is the reason for CBAVD. More than 33% of CBAVD patients in the European population have a compound heterozygous mutation F508del with an intronic mutation IVS8-5T allele (de Souza et al., 2018).

### 2.1.1.2 Adhesion G Protein-Coupled Receptor G2 (*ADGRG2*)

The *ADGRG2* gene is an X-linked gene, as shown in Figure (2.2), that encodes an adhesion-class G protein-coupled receptor, mostly expressed in the efferent ducts. It is associated with CBAVD disease. Various findings were documented from research conducted among populations from several regions. For example, Patat and colleagues in 2016, found three hemizygous truncating genetic variations of the *ADGRG2* gene in four of the 26 determined patients who had the congenital bilateral absence of the vas deferens, all having the wild-type *CFTR* gene with normal kidneys (Patat et al., 2016). However, other studies have discovered five relatively rare variants of *ADGRG2* in six congenital bilateral absence of the vas deferens patients of Asian descent, none of whom have mutant *CFTR* (Khan et al., 2018; B. Yang et al., 2017; Yuan et al., 2019). That indicates the limited percentage of CBAVD cases with healthy kidneys that don't have a genetic diagnosis may be related to abnormalities in other genes. For example, the solute carrier family 9 sodium/hydrogen exchanger isoform 3 (*SLC9A3*) gene was recently indicated to play a role in some cases of idiopathic CBAVD by causing obstructive azoospermia and damaged epithelial morphology in the reproductive tract, in Taiwanese patients (Y. N. Wu et al., 2019).

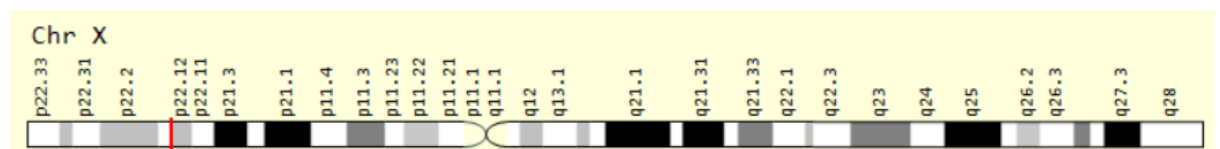


Figure (2.2): Location of the *ADGRG2* Gene on the Human Genome.

### **2.1.2 Primary Testicular Failure**

Non-obstructive azoospermia (NOA) constitutes 70% of azoospermia in infertile patients, with the majority leading to primary testicular failure in spermatogenesis anomalies. Furthermore, the monogenic causes of NOA associated with primary testicular failure are unclear; also, none of the available clinical recommendations utilize mutational analysis for the NOA genes (Kasak & Laan, 2021a). Nonetheless, the spermatogenic process is complicated and involves more than 2000 genes (Chalmel et al., 2012). NOA may present with several testicular histologies, including Sertoli-Cell-Only Syndrome (SCOS), Maturation Arrest (MA) at many stages, such as Spermatocyte Arrest (SCA) and Spermatogonial Arrest (SGA), and hypospermatogenesis (Soraggi et al., 2021).

#### **2.1.2.1 Genes for the Sertoli-Cell-Only Syndrome (SCOS) Phenotype**

SCOS and full MA can be distinguished by the lack of haploid cells in the testes, making the TESE technique for spermatozoa recovery ineffective for subsequent ICSI. Some tubules may contain spherical or advanced spermatids in situations of incomplete MA. So, creating a pre-TESE diagnostic gene panel would significantly assist clinical practice by avoiding surgery among individuals with pure SCOS and MA (Soraggi et al., 2021).

##### **2.1.2.1.1 Fanconi Anemia Complementation Group (*FANCA*)**

The *FANCA* gene is located on the long arm of chromosome 16(16q24.3), as shown in Figure (2.3). It encodes the protein for complementation group A, it's a DNA repair protein for post-replication or cell cycle checkpoints, and it is involved in interstrand crosslink repair and DNA double-strand breaks (DSB) repair. It is associated with the Fanconi Anemia (FA) pathway. It plays a significant role in adult-onset syndromic non-obstructive azoospermia cases (Nepal et al., 2017), through several mechanisms: DNA damage during the spermatogenesis process, testicular atrophy through impaired sperm production because of the reduced size of testes in Fanconi Anemia (FA) patients, and hormonal imbalance, such as testosterone, LH, and FSH. A study by Krausz and colleagues detected homozygous *FANCA* genetic variations in three out of 29 idiopathic NOA patients showing SCOS. (Krausz et al., 2019).

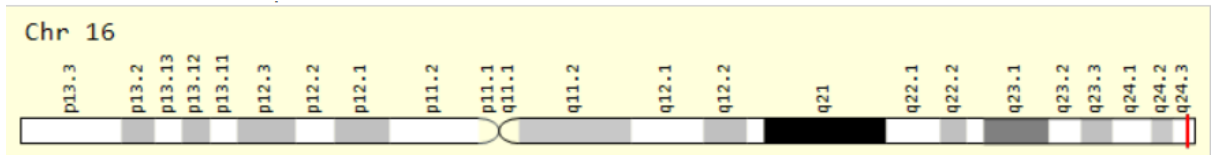


Figure (2.3): Location of the *FANCA* Gene on the Human Genome.

#### 2.1.2.1.2 Polo-Like Kinase 4 (*PLK4*)

The *PLK4* is located on the long arm of chromosome 4(4q28.1), as shown in Figure (2.4). It encodes a serine-threonine protein kinase that functions in centriole duplication, an essential process that must be achieved before primary spermatocytes initiate meiosis, which is mainly expressed in the testis and other actively proliferative tissues (Habedanck et al., 2005). In a study by Miyamoto in 2016, one patient suffering from idiopathic NOA caused by SCOS was recently identified as a carrier 13 bp deletion in the Ser/Thr kinase domain, heterozygous for *PLK4* (Miyamoto et al., 2016). Also, a study conducted on mice proves that the heterozygous mutation in *PLK4* led to patchy germ cell depletion in testes, comparable to humans, emphasizing the potential significance of *PLK4* as a main contributor to SCOS, and so to NOA (Harris et al., 2011).

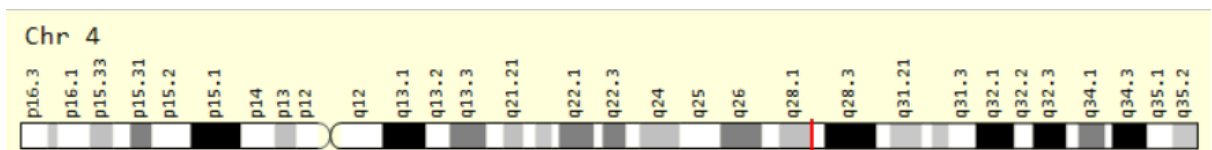


Figure (2.4): Location of the *PLK4* Gene on the Human Genome.

#### 2.1.2.1.3 Lysine Deficient Protein Kinase 3 (*WNK3*)

The *WNK3* is located on the short arm of chromosome X (Xp11.22), as shown in Figure (2.5). It encodes a protein related to the 'with no lysine' subclass of serine-threonine protein kinases, which control cation-chloride exchangers across many tissues (Rodan & Jenny, 2017), which is highly expressed in the testes. It also codes an activator of *NKCC1*. In a study conducted on mice, knockdown of the *WNK3*-activated *NKCC1* gene in mice, caused spermatogenic anomalies, concluding in an overall lack of spermatocytes (Pace et al., 2000). Also, another study conducted in Oman has shown that a rare deleterious recessive variant in *WNK3* leads to NOA in two brothers in the same family (Fakhro et al., 2018).

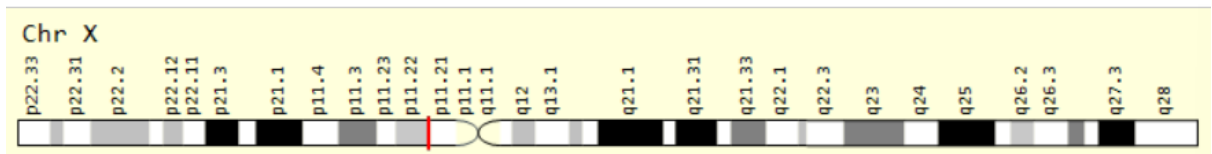


Figure (2.5): Location of the *WNK3* Gene on the Human Genome.

#### 2.1.2.2 Genes for the Maturation Arrest (MA) Phenotype

Maturation arrest can be defined when histological inspection of the testicular biopsy shows significant impairment of spermatogenesis throughout all tubules at a particular point of germ cell development, encompassing spermatogonia, spermatocytes, or spermatids.

##### 2.1.2.2.1 Meiotic Double-Stranded Break Formation Protein 1 (*MEI1*)

The *MEI1* gene is located on the long arm of chromosome 22 (22q13.2), as shown in Figure (2.6). The molecular function of the *MEI1* gene is essential for appropriate meiotic chromosome synapsis, and it is also associated with spermatocyte meiotic double-strand breaks (DSBs) (Kasak & Laan, 2021b). A case report study conducted in a consanguineous Tunisian family on infertile brothers diagnosed with non-obstructive azoospermia showed that a homozygous missense genetic variation in the gene *MEI1* (*MEI1*; c C3307T p. R1103W) was significantly associated with meiotic arrest in spermatocytes, and the mode of inheritance was autosomal recessive (Ben Khelifa et al., 2018). This finding confirms the study conducted on mice, wherein the knockout of the *MEI1* gene causes spermatocyte arrest at the zygotene stage, due to the homologous chromosomes being unable to undergo synapsis (Libby et al., 2002).

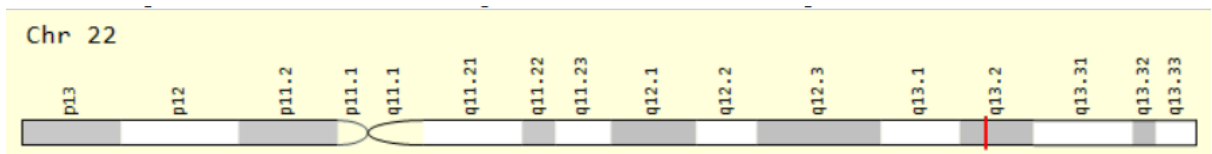


Figure (2.6): Location of the *MEI1* Gene on the Human Genome.

##### 2.1.2.2.2 Meiosis-Specific with OB Domain-Containing Protein (*MEIOB*)

The *MEIOB* gene is located on the short arm of chromosome 16 (16p13.3), as shown in Figure (2.7), which is highly expressed in testes. The molecular function of the *MEIOB* gene is important for the repair of double-strand breaks (DSBs), crossover development,

and the promotion of complete synapsis in meiosis (Luo et al., 2013). Nevertheless, there has been a paucity of published literature on *MEIOB* and how it causes NOA by maturation arrest. One of these studies was conducted on consanguineous families with familial NOA and testicular spermatocyte maturation arrest by WES and revealed a distinctive putative NOA-causative genetic variation in the *MEIOB* gene; the mode of inheritance is autosomal recessive (Gershoni et al., 2017a). This finding confirms that the study was conducted on mice, wherein the knockout of the *MEIOB* gene in mice was unable to develop crossover in germ cells, leading to infertility in both male and female *MEIOB* knockout mice because of meiotic arrest (Luo et al., 2013).

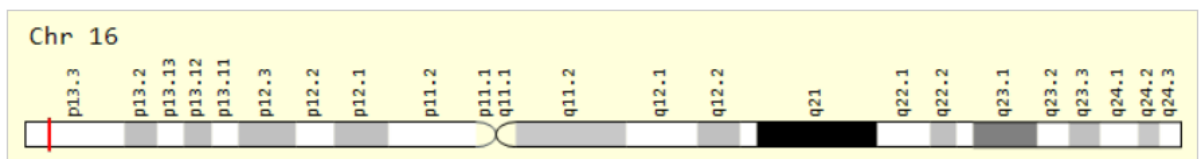


Figure (2.7): Location of the *MEIOB* Gene on the Human Genome.

#### 2.1.2.2.3 Testis Expressed 11 (*TEX11*)

*TEX11* is located on the long arm of chromosome X (Xq13.1), as shown in Figure (2.8). It is highly expressed in the testis. *TEX11* molecular function regulates meiotic crossing over, which is also associated with the initiation and continuation of chromosome synapsis. Many studies have considered that it is linked to the NOA because of MA (Sha et al., 2018b; F. Yang et al., 2015). By applying a high-resolution array of Comparative Genomic Hybridization (CGH) to investigate males with NOA, the result was a repetitive deletion of three exons of *TEX11* was found in two patients with azoospermia, with the mode of inheritance X-linked recessive. Moreover, several studies have revealed the genetic basis of azoospermia by *TEX11*, up to 1% of NOA conditions (Yatsenko et al., 2015).



Figure (2.8): Location of the *TEX11* Gene on the Human Genome.

#### 2.1.2.2.4 Synaptonemal Complex Central Element Protein 1 (*SYCE1*)

*SYCE1* is located on the long arm of chromosome 10 (10q26.3), as shown in Figure (2.9). It is highly expressed in the reproductive tissues and stem cells. The molecular function of *SYCE1* is a major component of the synaptonemal complex, which integrates homologous chromosomes during meiotic prophase. Mutant *SYCE1* leads to spermatogenic failure and premature ovarian failure, as the meiotic study conducted on testis tissue for a patient carrier of a homozygous mutation in *SYCE1* identified a pachytene arrest. This result is consistent with a study conducted on a mouse model, which proved that mutant *SYCE1* leads to unrepaired meiotic double-strand DNA breaks in spermatocytes (Krausz et al., 2020). The first study conducted on consanguineous parents in 2014 identified a homozygous point mutation in a 13-member family where two sisters have primary ovarian insufficiency, the NGS results were: two sisters were carried a nonsense homozygous mutation (c.613C>T), the parents and three brothers were heterozygous for mutant *SYCE1*, while a healthy sister does not carry the mutant variant of *SYCE1*, this research revealed the important role of *SYCE1* in ovarian function (De Vries et al., 2014). In another study conducted in 2015 to recognize the genetic causes of NOA in two infertile patients from consanguineous parents, the result was that the two patients carried a homozygous mutation at a splice site in *SYCE1*, causing a premature stop codon. In addition, the result of the histological study of the testis tissue was lacking in mature spermatocytes; this research confirms that the mutant in *SYCE* leads to maturation arrest in spermatogenesis (Maor-Sagie et al., 2015).

In a study conducted in 2020 by whole-exome sequencing in infertile patients suffering from azoospermia, the result provided further support for the involvement of *SYCE1* in NOA, because the patient carried a genetic variation in a splice site in *SYCE1* c.375-2A> G. This result confirms that the genetic variation in *SYCE1* causes azoospermia (Pashaei et al., 2020b). According to a study conducted in China in 2021, on a NOA patient, to understand the genetic cause of his case. First, using pathological examination, the study found that testicular spermatogenesis was blocked. Second, using target area capture combined with a high-throughput sequencing strategy, to search for mutant genes related to male infertility, the result revealed for the first time a frameshift genetic variation (c.689\_690del; p. F230fs) in the exon region of *SYCE1* in an NOA patient (K. Feng et al., 2022). All the previous studies illustrate the role of the *SYCE1* gene in both male and female fertility.

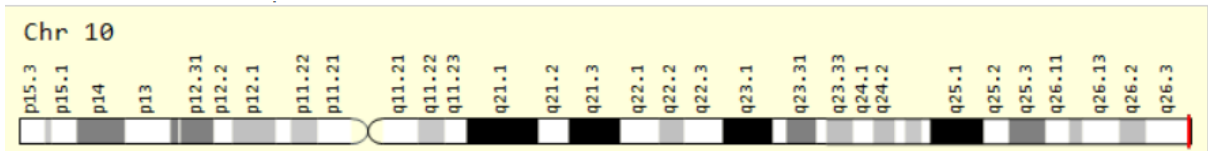


Figure (2.9): Location of the *SYCE1* GENE on the Human Genome.

#### 2.1.2.2.5 Cohesin Complex Component (*STAG3*)

*STAG3* is located on the long arm of chromosome 7 (7q22.1), as shown in Figure (2.10). It is highly expressed in the stem cells. *STAG3* molecular function, development of the chromosomal axis, and cohesion of sister chromatids are consequences of DNA replication. A study conducted by Riera-Escamilla and colleagues showed that biallelic loss-of-function mutations in the *STAG3* gene produce the persistence of meiotic double-strand breaks and the inability to complete chromosome pairing (Riera-Escamilla et al., 2019). In addition, there was a study conducted on a consanguineous family involving a primary ovarian insufficiency patient and an NOA patient to understand the genetic cause of infertility in the family. The result was a homozygous missense genetic variation in *STAG3* in the family that is responsible for infertility (Jaillard et al., n.d.).

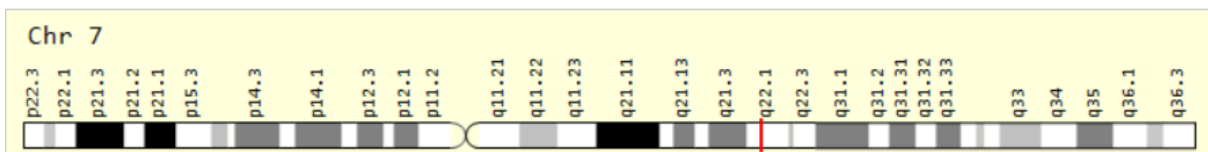


Figure (2.10): Location of the *STAG3* Gene on the Human Genome.

#### 2.1.2.2.6 Senataxin (*SETX*)

The *SETX* gene is located on the long arm of chromosome 9 (9q34.13), as shown in Figure (2.11), which is highly expressed in stem cells. The molecular function of the *SETX* gene is important for transcription regulation by regulating RNA Polymerase II, attaching to chromatin, and interacting with transcriptional proteins. Mutant *SETX* leads to impairment of function, causing syndromic disease, for example, Ataxia with Oculomotor Apraxia type 2 (AOA2), which is an autosomal recessive cerebellar ataxia (Suraweera et al., 2009). In Australia, a study was conducted on patients suffering from AOA2 to investigate the genetic causes of azoospermia. The study found impaired spermatogenesis in AOA2 patients because of the mutant *SETX*. This gene illustrates a “phenocopy” gene related to a syndromic disorder, during which male primary gonadal

failure can appear as one of the clinical signs of the disorder. Therefore, it is critical to focus on the clinical assessment of azoospermic individuals who carry recessive *SETX* mutations (Becherel et al., 2019)

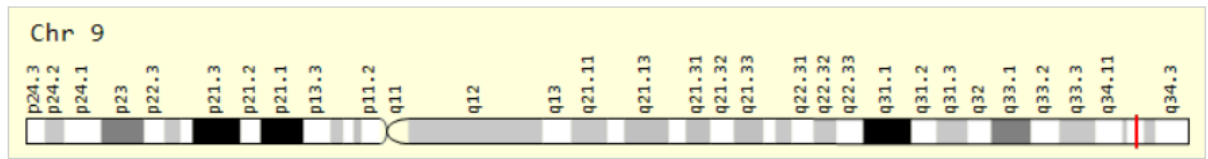


Figure (2.11): Location of the *SETX* Gene on the Human Genome.

#### 2.1.2.2.7 Chromosome 14 Open Reading Frame 39 (*C14orf39*)

The *C14orf39* gene is located on the long arm of chromosome 14 (14q23.1), as shown in Figure (2.12). It is considered a meiotic protein, is localized to the central element of the synaptonemal complex. Also, the molecular function of the *C14orf39* gene is important for chromosome synapsis during meiotic recombination; it works with *SYCE1* via its alpha-helical domain (Gómez-H et al., 2016). In a study conducted on two brothers suffering from NOA, to understand the genetic causes of infertility, a homozygous frameshift variant of the *C14orf39* gene was discovered in the two brothers with NOA and their sister, who is additionally affected by Primary Ovarian Insufficiency. In addition, testicular tissue examination to analyze meiotic stage showed no XY body development and significant synaptic abnormalities, confirming that meiosis was discontinued at the pachytene-like stage. This study demonstrated that infertile males with *C14orf39* frameshift mutations had complete asynapsis of homologous chromosomes during meiosis (Fan et al., 2021).



Figure (2.12): Location of the *C14orf39* Gene on the Human Genome.

#### 2.1.2.2.8 DNA Meiotic Recombinase 1 (*DMC1*)

The *DMC1* gene is located on the long arm of chromosome 22 (22q13.1), as shown in Figure (2.13). The *DMC1* gene is important for meiotic homologous recombination, also in double-strand break repair, which is considered a Meiosis-specific recombinase that works with different DNA repair proteins during the Fanconi anemia pathway

(Meiotic Prophase Arrest with Failure of Chromosome Synapsis in Mice Deficient for *Dmc1*, a Germline-Specific RecA Homolog, n.d.). A recent study conducted on consanguineous Chinese family, using NGS to understand the genetic basis of infertility in family, the study recognized a homozygous missense genetic variation in the *DMC1* gene as the genetic basis of both MA at the spermatocyte stage and POI in two brothers and a sister from family, this study demonstrated that, an absence of this protein produces an obstruction throughout the leptotene or zygotene stage of meiotic prophase I, leading to the incapacity to form synaptonemal complexes (He et al., 2018).

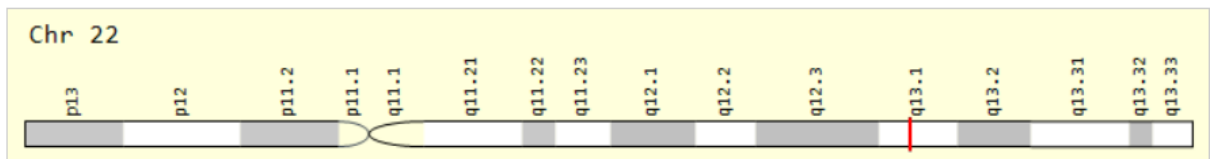


Figure (2.13): Location of the *DMC1* Gene on the Human Genome.

#### 2.1.2.2.9 Initiator of Meiotic Double-Strand Breaks (*SPO11*)

The *SPO11* gene is located on the long arm of chromosome 20 (20q13.31), as shown in Figure (2.14). The molecular function of the *SPO11* gene is important for beginning meiotic recombination and the development of the synaptonemal complex among homologous chromosomes (Tock & Henderson, 2018). So far, a homozygous missense variant in *SPO11* has been found in two brothers diagnosed with maturation arrest, which means the mutant in *SPO11* leads to failure in spermatogenesis (Fakhro et al., 2018).

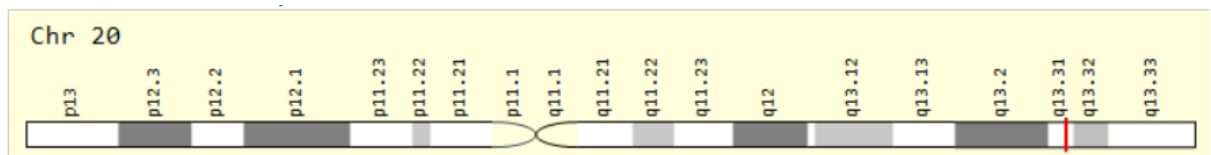


Figure (2.14): Location of the *SPO11* Gene on the Human Genome.

#### 2.1.2.2.10 X-Ray Repair Cross-Complementing 2 (*XRCC2*)

The *XRCC2* gene is located on the long arm of chromosome 7 (7q36.1), as shown in Figure (2.15). The molecular function of the *XRCC2* gene is important for DNA double-strand break repair and is involved in interstrand crosslink repair in DNA. Through a study conducted on a consanguineous Chinese family to investigate the genetic basis of infertility, the result was a meiosis-specific mutation (p.Leu14Pro) in the *XRCC2*, causing maturation arrest in males and POI in females. Further study conducted on a mouse model

revealed this result by the male mouse model carrying the mutant *XRCC2* exhibited MA phenotype. This result concluded that a mutant *XRCC2* gene may result in infertility by meiotic arrest and azoospermia (Y. Yang et al., 2018).

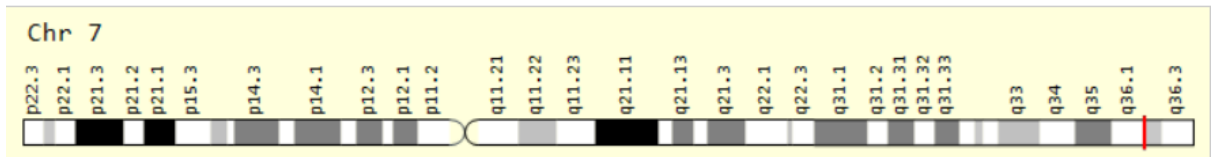


Figure (2.15): Location of the *XRCC2* Gene on the Human Genome.

#### 2.1.2.2.11 Zinc Finger MYND-Type Containing 15 (*ZMYND15*)

The *ZMYND15* gene is located on the short arm of chromosome 17 (17p13.2), as shown in Figure (2.16). The molecular function of the *ZMYND15* gene is important for spermiogenesis because it performs as a transcriptional repressor by binding to histone deacetylases, so it regulates haploid genes expression during spermiogenesis (W. Yan et al., 2010). In Turkey, a study demonstrated mutant *ZMYND15* in two consanguineous families, which causes recessive azoospermia in two families, indicating that NOA can be due to a genetic variation in *ZMYND15* by post-meiotic defect (Ayhan et al., 2014).

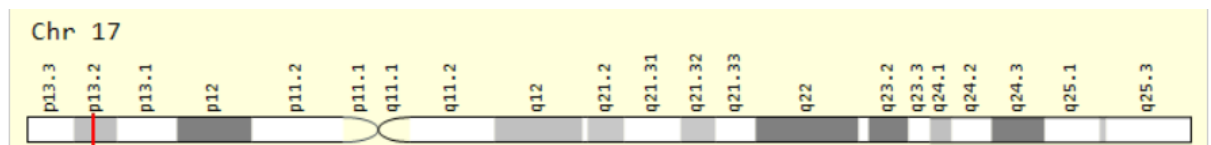


Figure (2.16): Location of the *ZMYND15* gene on the human genome.

#### 2.1.2.2.12 Retinol-Binding Protein 7 (*RBBP7*)

The *RBBP7* gene is located on the short arm of the X chromosome (Xp22.2), as shown in Figure (2.18). The main molecular function of *RBBP7* is vital to chromatin remodeling after DNA replication, transcription repression, differentiation, proliferation, and apoptosis of germ cells. It is highly expressed in the testis, particularly in the germ cells and Sertoli cells (J. Li et al., 2023; Riera-Escamilla et al., 2022). In a study conducted on two Asian brothers by Jingping Li et al, in 2023, to detect the genetic basis of the early maturation arrest in this family, by whole exome sequencing, the result was a hemizygous X-linked *RBBP7* genetic variation in two brothers (NM\_002893.4, c.1201ins1, p.W401M fs\*4), this genetic variation lead to premature ending of protein translation, disrupting the WD40 domain of *RBBP7*, thus this affected on the binding of protein with H4 and

*BRCA1*, as shown in the Figure (2.17), which the figure illustrates the structure of *RBBP7* protein (J. Li et al., 2023).

To study the effect of the mutant *RBBP7* gene on male fertility, a mouse model of two cell lines was generated: spermatogonia and spermatocyte cell lines, and *Drosophila* models. The knockout of *RBBP7* from both models caused a Lack of germ cells and male gametes and contributed to testicular atrophy. In addition, artificial transgenic for the wild and mutant types of human *RBBP7* was done, replacing the mutant variant showed no effect on fertility, while the ectopic expression of wild-type human *RBBP7* recovered it. This study suggests the importance of this gene in all species, as the genetic variation leads to a defect in spermatogenesis, contributing to non-obstructive azoospermia and increasing the predisposition to testicular malignancy, as infertility is a known risk factor of testicular oncogenicity (J. Li et al., 2023). In another study conducted on 2354 infertile men by Riera-Escamilla et al, in 2022, to access the repetitively impaired x-linked genes in idiopathic male infertility, data was analyzed for 2354 infertile men who participated in four cohort studies from the European Union, United Kingdom and the United States of America, they compared the results of x chromosome sequencing centering on protein-coding gene mutations with the wild-type x-linked genes from controls and genetic database to assess the role of each gene in the spermatogenesis process, they confirmed the association of seventeen x-linked genes mutation known to affect male spermatogenesis as *AR*, *TEX11*, and *USP26* and newly seven x-linked genes were added in the offending causes of idiopathic male infertility from this research including, *RBBP7*, *PASD1*, *SCML1*, *DCAF12L1*, *GCNA*, *ZFX*, and *BCORL1* (Riera-Escamilla et al., 2022).

The *RBBP7* gene was recurrently affected in the cohort studies, nine patients carried mutated variants that are not classified in male infertility in the genome aggregation database yet, from these, three of them were loss of function mutation, knock out of *RBBP7* on *drosophila* models were done, results showed the small testicular size and complete sterility of male germ cells that supports the importance of *RBBP7* in germline stem cells preservation (Riera-Escamilla et al., 2022).

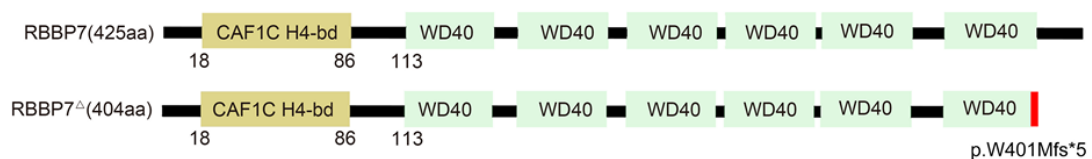


Figure (2.17): Structure of Wild-Type *RBBP7* Protein, Which Contains 425 Amino Acids, and the Mutant Structure of *RBBP7* Protein in the Sixth WD40 Domain(J. Li et al., 2023) .



Figure (2.18): Location of the *RBPP7* gene on the human genome.

### 2.1.3 Genes Associated with Different Types of Testicular Phenotypes

#### 2.1.3.1 FA Complementation Group M (*FANCM*)

The *FANCM* gene is located on the long arm of chromosome 14 (14q21.2), as shown in Figure (2.19). The molecular function of the *FANCM* gene is important for cellular function, especially DNA replication, and has been associated with the FA pathway, in addition anti crossover process, which keeps genomic stability (Z. Yan et al., 2010). In a study conducted on a consanguineous Pakistani family, in which three brothers were diagnosed with oligoasthenozoospermia and azoospermia, to investigate the genetic basis of infertility in the family, the result identified a homozygous loss-of-function mutation in *FANCM*. Furthermore, the mouse model knock-out of *FANCM* exhibited spermatogenic failure (Yin et al., n.d.).

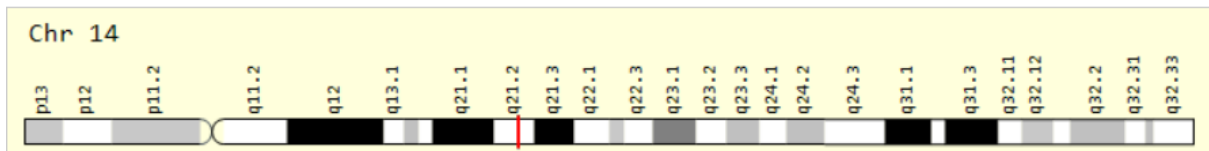


Figure (2.19): Location of the *FANCM* Gene on the Human Genome.

#### 2.1.3.2 Nanos C2HC-Type Zinc Finger 2 (*NANOS2*)

The *NANOS2* gene is located on the long arm of chromosome 19 (19q13.32), as shown in Figure (2.20). The molecular function of the *NANOS2* gene is important for encoding an RNA-binding protein that assists in the continued survival of the spermatogonial stem cell and prevents the stimulation of meiotic entry (Saba et al., 2014). In a study on a mouse model to understand the role of *NANOS2* in infertility, the male mouse exhibited a total loss of germ cells with the knockout of *NANOS2* (Tsuda et al., n.d.). A homozygous mutation in this gene has just been found to cosegregate with SCOS

in two siblings from a consanguineous family. However, another sporadic patient with a homozygous loss-of-function mutation in *NANOS2* had MA (Fakhro et al., 2018).

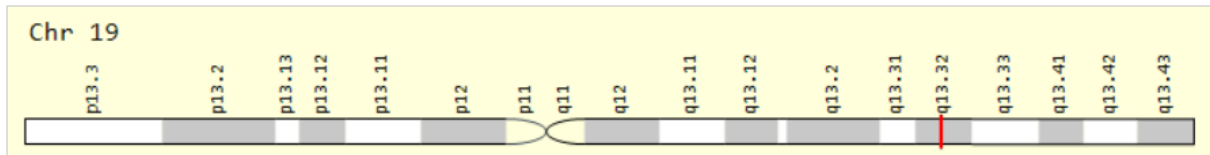


Figure (2.20): Location of the *NANOS2* Gene on the Human Genome.

### 2.1.3.3 Testis Expressed 14 (*TEX14*)

The *TEX14* gene is located on the long arm of chromosome 17 (17q13.32), as shown in Figure (2.21). It is highly expressed in the testis. The molecular function of the *TEX14* gene is essential for the building and preservation of intercellular bridges in vertebrate germ cells, which are necessary for meiosis during spermatogenesis. Massive spermatocyte reduction has been detected in *TEX14* knockout mice (Greenbaum et al., 2011). In a study conducted to investigate the genetic basis of NOA by whole-exome sequencing, the result was recessive genetic variations in *TEX14* in humans constitute significant candidates for non-obstructive azoospermia, demonstrating testis histology examination, which fluctuates from Sertoli cell-only syndrome, to early maturation arrest phenotype, alongside negative testicular sperm extraction results (Gershoni et al., 2017b).

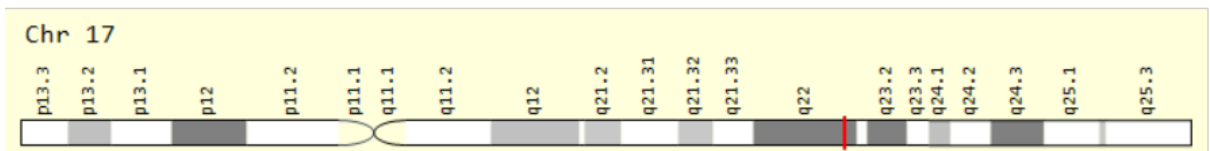


Figure (2.21): Location of the *TEX14* Gene on the Human Genome.

### 2.1.3.4 Tudor Domain Containing 9 (*TDRD9*)

The *TDRD9* gene is located on the long arm of chromosome 14 (14q32.33), as shown in Figure (2.22). The molecular function of the *TDRD9* gene is necessary for spermatogenesis in ATP-binding RNA helicase (Arafat et al., 2017). Five infertile men from a consanguineous Bedouin family have been identified with a homozygous frameshift genetic variation in the *TDRD9* gene. They have been detected with azoospermia corresponding with incomplete MA. This study estimates that the genetic variation is responsible for the spermatogenic failure, similar to that shown in *TDRD9* knockout mice, but it is not related to female infertility (Arafat et al., 2017).



Figure (2.22): Location of the *TDRD9* Gene on the Human Genome.

## Chapter Three: Methodology

### 3.1 Study Subjects

This study was conducted on two Palestinian families affected by idiopathic azoospermia infertility. They were confirmed to have idiopathic azoospermia infertility in the Palestinian European Fertility Center based on specific parameters illustrated in Table (3.1). Family I is composed of fertile consanguineous parents, 2 infertile twin offspring, 4 fertile offspring, and one of the grandchildren in family 1 who was diagnosed with idiopathic azoospermia infertility. Family II comprises fertile parents, 3 idiopathic azoospermia infertile offspring, two fertile offspring, and one fertile daughter. Figure (3.1) shows the pedigree of both families. Written informed consent was obtained from all subjects to participate in the study. (Enclosed in Appendix)

Table (3.1): Diagnosed Characteristics of Idiopathic Azoospermia Infertility Patients in Family I and II.

| Diagonestic Parameter                                                               | Family I<br>(Proband)                  | Family II<br>(Proband)                                              | Reference Range                                                                                                                                       |
|-------------------------------------------------------------------------------------|----------------------------------------|---------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|
| Semen Analysis<br>- Volume (ml)<br>-Appearance<br>-Viscosity<br>-Total sperm number | 1.5 - 2.0<br>Normal<br>Liquid<br>0     | 1.5 - 2.0<br>Normal<br>Liquid<br>0                                  | Normal $\geq 1.5$ mL<br><br>Liquified within 60 mm<br>Azoospermia = not a<br>single sperm was seen,<br>even after concentration<br>and centrifugation |
| Karyotype                                                                           | 46, XY<br>Normal                       | 46, XY<br>Normal                                                    | 46, XY<br>Normal                                                                                                                                      |
| Y chromosome Microdeletion                                                          | Not detected                           | Not detected                                                        | AZF a/b/c<br>regions tested                                                                                                                           |
| Hormonal Profile<br>-FSH<br>-TSH<br>-LH<br>-Testosterone<br>-Prolactin              | 7.8 mIU/mL<br>-<br>-<br>404 ng/dL<br>- | 28.0 mIU/mL<br>2.85 mIU/mL<br>9.9 mIU/mL<br>415 ng/dL<br>25.7 ng/mL | Men: 1-11<br>Adult: 0.35-5.5<br>Men: 1.79-7.68<br>Men: 245-1600<br>Men: 3.0-25                                                                        |
| Obstructive Causes                                                                  | Not detected                           | Not detected                                                        |                                                                                                                                                       |
| Surgical Causes                                                                     | Not detected                           | Not detected                                                        |                                                                                                                                                       |
| Infections                                                                          | Not detected                           | Not detected                                                        |                                                                                                                                                       |
| Malignancy                                                                          | Not detected                           | Not detected                                                        |                                                                                                                                                       |
| Drug exposure                                                                       | Not detected                           | Not detected                                                        |                                                                                                                                                       |

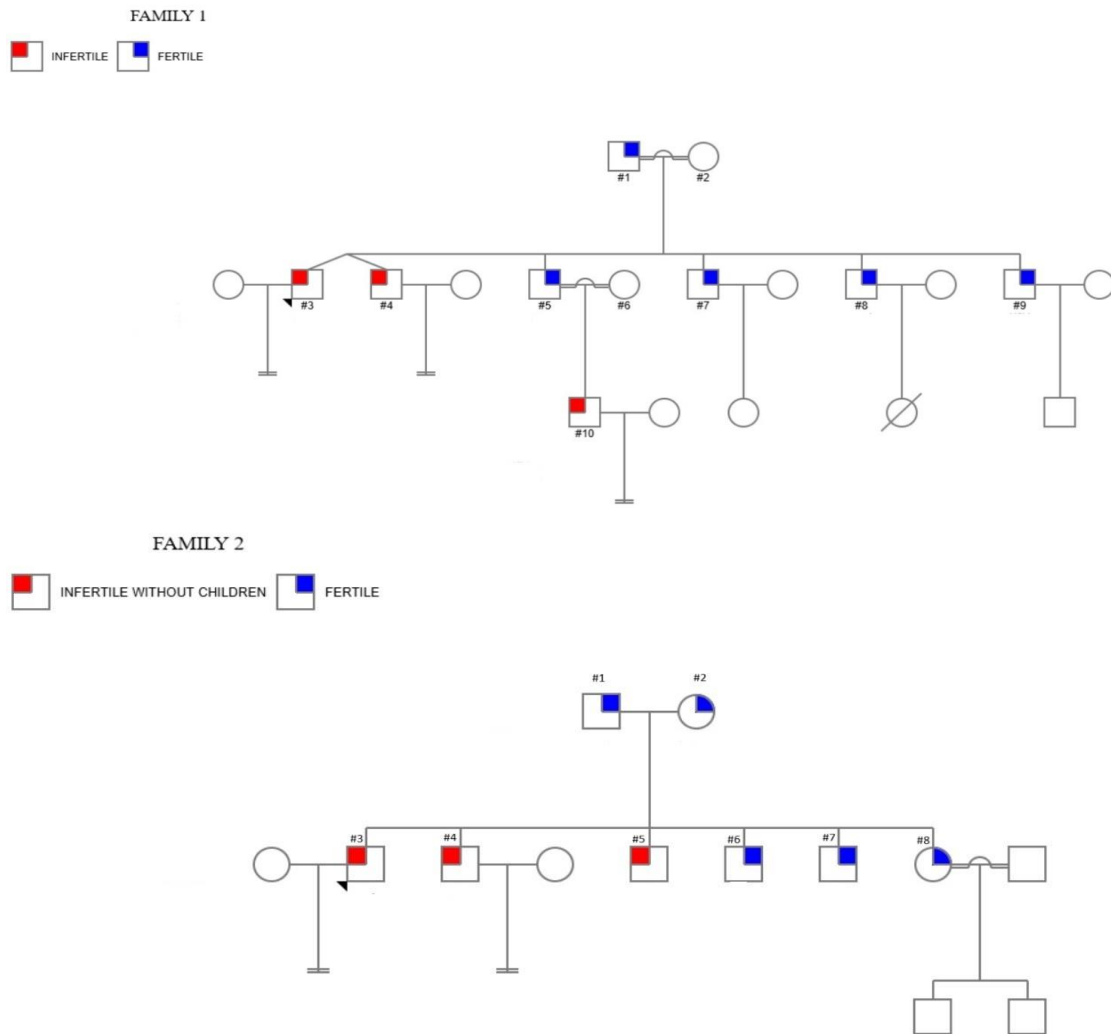


Figure (3.1): Families' Pedigree. Family I is composed of fertile parents, 2 idiopathic azoospermia infertile offspring, and one of the grandchildren is diagnosed with idiopathic azoospermia infertility. Family II comprises fertile parents, 3 idiopathic azoospermia infertile offspring, two fertile offspring, and one fertile daughter.

## 3.2 Genomic DNA Preparation

### 3.2.1 DNA Extraction

Whole EDTA blood samples (3 mL) were collected from one infertile patient from each family and from non-infertile family members and centrifuged for 15 minutes at 2000 rpm by the Centrifuge, model Universal 320, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany. The buffy coat was utilized for DNA extraction using the protocol of a commercially available kit (Master Pure™ Genomic DNA Purification Kit,

Epicenter Technology Co., Cat. No MG71100). Complying with the manufacturer's guidelines with some adjustments.

Initially, 150 µl of the buffy coat was mixed with 600 µl of Cell Lysing Solution in a sterile microcentrifuge tube. The tube was inverted multiple times and incubated for 5 minutes at room temperature. This was repeated once. The samples were centrifuged at high speed (14,000 rpm) for 25 seconds. The supernatant was eliminated, leaving 50 µl of lysate, and the residual white pellet was instantly vortexed and resuspended in 300 µl of lysis buffer 2. After adding 250 µl of a precipitation solution, the mixture was vortexed for approximately forty-five to sixty seconds, and the sample was centrifuged at 14,000 rpm for 10 minutes. The supernatant was moved to a new Eppendorf tube, followed by the addition of 700 µl of ice-cold isopropanol. Following, the tube was inverted thirty to forty times until a stringy DNA precipitate was obvious. Upon centrifuging, the DNA was pelleted after 10 minutes at 14,000 rpm, the supernatant was thrown away. The DNA pellet had two rinses with 200 µl of 75% cold ethanol to wash residual isopropanol. Ethanol was eventually aspirated, and the tube was inverted for drying in the air. In the meantime, 200 µl of TE buffer had been added. The DNA pellet was dissolved, and the samples were stored at -30°C until required.

### **3.2.2 DNA Quantification**

The purity and concentration of extracted DNA were measured utilizing a Nanodrop spectrophotometer. Absorption measurements have been performed at 260 nm for nucleic acids, 230 nm for salts, and 280 nm for proteins. The concentration of DNA can be measured in µg/mL by multiplying the absorbance at 260 nm by the dilution factor and 50 µg/mL, with reference that 1 OD indicates 50 µg/mL dsDNA.

### **3.2.3 DNA Qualification**

DNA was examined using Agarose Gel Electrophoresis to determine its size and integrity. 0.8 grams of agarose had been added to TAE buffer, warmed to a boil, and finally added to ethidium bromide.

### **3.3 Next Generation Sequencing**

A single participant who has idiopathic azoospermia infertility was selected from each family. The samples undergone WES employing the Illumina 5500, which follows the manufacturer's guidelines (Illumina, 2021). Outlined as follows (these steps were performed in the research molecular genetics lab of AAUP by the lab staff):

#### **1. Genomic DNA Tagmentation:**

For fragmenting and tagging the DNA with adaptor sequences, 30  $\mu\text{L}$  (with a total of 500 ng) of each sample was transferred to each of the wells in a 96-well PCR plate. A tagmentation master mix, which includes enzymatic Bead-Linked Transposome (eBLT), 10  $\mu\text{L}$ , and Tagmentation Buffer (TB1), 10  $\mu\text{L}$ , had been added to each well. The plate was tightly sealed with Microseal B and installed on the thermal cycler. Preheat the lid to 100°C, establish the reaction volume to 50  $\mu\text{L}$ , and remain incubated at 55°C for 5 minutes, afterward hold at 10°C.

#### **2. Post-Tagmentation cleanup**

To clean the adaptor-tagged DNA, 10  $\mu\text{L}$  of Stop Tagment Buffer 2 (ST2) was added to the tagmentation reaction, mixed-centrifuged at 1600 rpm for 1 minute, and subsequently placed on a magnetic stand. Samples were eventually washed with 100  $\mu\text{L}$  of Tagment Wash buffer (TWB) added to the beads and installed on the magnetic stand for two minutes. The supernatant was thereafter discarded. The steps were repeated a second time.

#### **3. Tagmented DNA Amplification**

To increase the amplified form of the tagmented DNA, 40  $\mu\text{L}$  of PCR master mix (EPM) was added to each well. Index 1 and index 2 adaptors, pre-paired in 10 base pairs, have been placed from the index adaptor plate through each well. The tagged DNA went through amplification using the eBLT PCR Program on the thermal cycler. This procedure involved heating the lid to 100 °C in a reaction volume of 50  $\mu\text{L}$ . The cycling procedure conditions included 72 °C for 3 minutes, followed by 98 °C for 3 minutes, and 9 cycles which included 98 °C for 20 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute. The final extension was performed at 72 °C for 3 minutes, with a hold at 10 °C.

#### **4. Library Cleanup**

To purify the amplified libraries, 45  $\mu$ L of supernatant from each well was cautiously moved to the corresponding well of a new MIDI plate. 77  $\mu$ L of nuclease-free water and 88  $\mu$ L of AMPure XP beads had been added to the mixture. Before the addition of 17  $\mu$ L RSB, 200  $\mu$ L of fresh 80% ethanol was used for washing.

#### **5. Pre-Enriched Libraries Qualification**

The quality of each library was evaluated by applying the Agilent Technology 2100 Bioanalyzer via the DNA 1000 kit.

#### **6. Pre-Enriched Libraries Pooling**

DNA libraries have been combined with different indexes for each library into an individual pool. For a final volume of 30  $\mu$ L (150 ng DNA), the precise volume from each sample was estimated through on its concentration and afterward mixed in the PCR tube.

#### **7. Probes Hybridization**

Certain areas of DNA have been attached to capture probes. 50  $\mu$ L of Nextera Hybridization Buffer 2 (NHB2), 10  $\mu$ L of the enrichment probe panel, and 10  $\mu$ L of EHB2 were put together with 30  $\mu$ L of the sample in a new tube, and thereafter placed in the thermal cycler using the NF-HYB program as described: Preheat lid option at 100°C, reaction volume of 50  $\mu$ L, incubation at 95°C for 5 minutes, then perform 16 cycles of 1 minute each, beginning at 94°C, then decreasing by 2°C during each cycle. Sustain at 62°C for 24 hours.

#### **8. Hybridized Probes Capture**

The sample was put through to centrifugation at 280g for 30 seconds. 250  $\mu$ L of Streptavidin Magnetic Beads (SMB3) were put into 100  $\mu$ L of the sample in a new tube to capture probes hybridized to the target regions. Afterwards, 200  $\mu$ L of Enhanced Enrichment Wash (EEW) was utilized for the washing step. To prepare the elution mix, 342  $\mu$ L of Enrichment Elution Buffer 1 (EE1) was added to 18  $\mu$ L of 2N NaOH (HP3). 23  $\mu$ L of this solution was added to the sample, incubated at room temperature for 2 minutes, centrifuged at 280g for 30 seconds, and then positioned on the magnetic stand

for 2 minutes. 21  $\mu$ L of the supernatant had been mixed with 4  $\mu$ L of Elute Target Buffer 2 (ET2) in a new tube.

### **9. Enriched Library Amplification**

To amplify the enriched library, 5  $\mu$ L from the PCR primer cocktail (PPC) and 20  $\mu$ L from the enhanced PCR mix (EPM) were added. The sample was centrifuged and afterward placed in the thermal cycler for processing based on the following program: Preheat lid to 100°C, adjust reaction volume to 50  $\mu$ L, incubated at 98°C for 60 seconds, then perform by 10 cycles of: (98°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 seconds), then incubation at 72°C for 5 minutes, and at last hold at 10°C.

### **10. Amplified Enriched Library Cleanup**

To clean the library, 45  $\mu$ L of AMPure XP Beads were inserted into the sample tube. Following that, the sample went through two washing steps, including 200  $\mu$ L of 80% EtOH, followed by a final addition of 32  $\mu$ L of RSB.

### **11. Purification and Quality Control**

The library concentration was measured using the Qubit dsDNA HS assay kit (#Q23850). The average fragment size was measured using the High Sensitivity DNA kit (catalog #5067).

## **3.4 Data Analysis**

FastQ paired-end reads were assigned to the reference human genome version GRCh38 through the BWA-MEM software application, which produced mapped reads in BAM format. The mapped reads were filtered based on two criteria: At first, we maintained only paired reads for which both the forward and reverse reads were properly mapped to the reference using Samtools. Next, PCR duplicates have been removed with the RmDup tool. Filtered mapped reads were then utilized to call variants, applying the FreeBayes variant detector to identify SNPs (single-nucleotide polymorphisms) and indels (insertions and deletions). The list of variants produced in a VCF format has been filtered and prioritized for their potential relevance for Azoospermia infertility. The SnpEff tool, which annotates variants with their anticipated effects on existing genomic characteristics, has been employed to generate an annotated VCF file that includes variant

effect annotations. The VCF file covers chromosome number, position, reference and variant nucleotides, gene name, genomic context (which suggest whether the variant resides in an exon, intron, splice site, or other regions), synonymy (whether the variant is synonymous or not), amino acid modification, clinical significance of the variant (pathogenic or benign), phenotypes corresponding with the variant based on HPO and OMIM, average variant frequency (population occurrence rate), quality by depth (QD), RMS mapping quality (MQ), predicted pathogenicity scores via tools such as SIFT and Polyphen2, and the phenotype correlation score from Exomiser.

In conclusion, we eliminated variants that are highly unlikely to carry a pathogenic impact, among them those located in UTRs, upstream or downstream of the gene, and deep intronic variants, whereas keeping variants that impact splice sites. In addition, variants displaying low QD (below 3) or low phenotype correlation scores were excluded from assessment. A small number of variants were eventually identified. We investigated them in OMIM to prioritize an opportunity for causative variants informed by the genotype-phenotype relationship. Because of the absence of WES for the parents' samples, we were not able to discover a single candidate variant. As a result, we decided on the variants with the highest correlation score across each family for the family segregation study. Figure (3.2) provides a brief description of the methods of analysis performed.

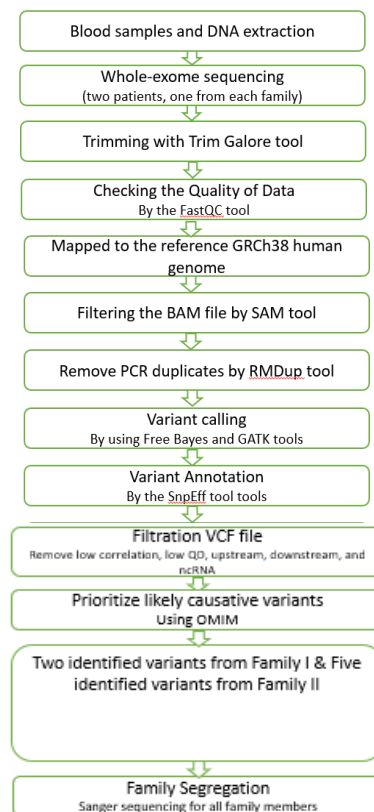


Figure (3.2): Flowchart of Analysis Steps

### 3.5 Family Segregation

Two suspected gene variants were identified in the first family, including the *CFAP47* and *SYCE1* genes. In the second family, five suspected variants were identified in the *RBBP7*, *CEP112*, *POLG-1*, *POLG-2*, and *HFE* genes. Diverse primers were designed for each variant. For PCR amplification of specific DNA fragments encompassing the indicated variants, followed by Sanger sequencing.

#### 3.5.1 Primer Design

Primer 3 web (<https://primer3.ut.ee/>) was employed for designing the indicated list of primers described in Table (3.2).

Table (3.2): The Sequences and Properties of the Primers for the Genetic Variations.

| Gene name     | Sequence of primer               | DNA Fragment | GC% content | T <sub>m</sub> °C |
|---------------|----------------------------------|--------------|-------------|-------------------|
| <i>CFAP47</i> | Forward: GTGGTTATCGGAATTTTGTTGCA | 139          | 39%         | 60                |
|               | Reverse: ACTCATGTTTCGGGTATTGCC   | 139          | 48%         | 60                |
| <i>SYCE1</i>  | Forward: CCTCCACACGCACCTCTG      | 253          | 67%         | 60                |
|               | Reverse: CCCCTCCACCCTTGATGAG     | 253          | 63%         | 60                |
| <i>RBBP7</i>  | Forward: AGGCACAAGAGAACTGGGTT    | 249          | 50.0%       | 60                |
|               | Reverse: CAGTGATGTGAGGCCGAATG    | 249          | 55.0%       | 60                |
| <i>CEP112</i> | Forward: CCCCTAAATCCTTGAAAATGCC  | 401          | 48%         | 62.3              |
|               | Reverse: TCACAGGGTCAGACTGGGTA    | 401          | 55%         | 62.3              |
| <i>POLG-1</i> | Forward: GACACGTGCCAGGAAAGGAG    | 401          | 60%         | 64.9              |
|               | Reverse: CAGCTGCCAATGCACCATC     | 401          | 58%         | 64.9              |
| <i>POLG-2</i> | Forward: TTACCTGTAGGGGCAGACCA    | 327          | 55%         | 62.3              |
|               | Reverse: GGGTGGGGACATTGTGAGAG    | 327          | 60%         | 62.3              |
| <i>HEF</i>    | Forward: TGATGGTATGAGTTGATGCAGGT | 401          | 43%         | 62.3              |
|               | Reverse: GAAAAGCTCTGACAACCTCAGG  | 401          | 50%         | 62.3              |

### 3.5.2 PCR Amplifications

All family members of the first family were tested for the indicated variants in the *CFAP47* and *SYCE1* genes. Similarly, all individuals of the second family were tested for the indicated variants in the *RBBP7*, *CEP112*, *POLG-1*, *POLG-2*, and *HFE* genes. The PCR reactions were prepared as described in Table (3.3).

Table (3.3): Reagents and Volumes Used for PCR Reaction.

| Reagent                        | Volume |
|--------------------------------|--------|
| PCR master mix (1X)            | 10 µl  |
| Forward primer (10 picomole)   | 0.5 µl |
| Reverse primer (10 picomole)   | 0.5 µl |
| DNA sample (100 ng)            | 1.0 µl |
| Nuclease-free H <sub>2</sub> O | 8.0 µl |
| Total volume                   | 20 µl  |

All PCR mixes were run in the thermocycler, Thermal Cycler, FlexCycler<sup>2</sup>, Biometra GmbH, Göttingen, Germany. using the amplification program described in Table (3.4).

Table (3.4): PCR Guidelines Utilized for the amplification of DNA fragments with the indicated Variants.

| Temperature °C | Time        | Cycle numbers |
|----------------|-------------|---------------|
| 99 °C          | Preheat lid | -             |
| 95 °C          | 5 min       | 1 cycle       |
| 95 °C          | 30 seconds  | 35 cycles     |
| 56 °C          | 30 seconds  |               |
| 72 °C          | 30 seconds  |               |
| 72 °C          | 5 min       | 1 cycle       |
| 4 °C           | Hold        | ∞             |

The relevant PCR amplified DNA fragments were visualized on a 2% agarose gel, which prepare by 2g of agarose dissolved in 100 mL of TAE buffer. NTC and a 50 bp ladder of DNA were included in the gel.

### 3.5.3 Sanger Sequencing

Samples were sequenced utilizing the BigDye™ Terminator Cycle Sequencing Kit and Applied Biosystems Genetic Analyzer in keeping with the manufacturer's procedures (Fisher Scientific, n.d.). For PCR purification, 1 µL of EPPIC-FAST reagent (Catalog #1021- 100F A&A Biotechnology), which is including of a mixture of thermolabile nucleotide hydrolase and recombinant exonuclease I with increased efficiency, was added to 5 µL of PCR product. Products were positioned on a thermocycler at 37°C for 10 minutes, followed by 1 minute at 80°C. Afterwards, cycle sequencing was started by adding 18 µL of Big Dye Terminator (BDRR) mix with 2 µL of cleaned PCR and then placed on a thermocycler according to the following conditions in the table (3.5). Table (3.6) shows the reagents needed to make the BDRR mix. Sequenced sample was then followed by EDTA Ethanol precipitation; At first, the sample was mixed with 60 µl of 100% cold ethanol and 5 µl EDTA (12.5 mM). Secondly, cold centrifugation for 30 minutes at 2200g was done. Third, the supernatant was discarded, and 80 µl of 80% ethanol was added to the pellet. Fourth, 15 minutes of centrifugation at 1600g was completed. Fifth, the supernatant was also dropped, and the pellet was air dried. Sixth, High dye was added, and then the sample was put on a hot plate at 95°C for 5 minutes, and then directly cooled by ice incubation for another 5 minutes.

Table (3.5): BDRR PCR Program Used.

| Temperature °C | Time       | Cycle numbers |
|----------------|------------|---------------|
| 96 °C          | 20 seconds | 1 cycle       |
| 96 °C          | 10 seconds | 25 cycles     |
| 50 °C          | 5 seconds  |               |
| 60 °C          | 4 minutes  |               |
| 4 °C           | Hold       | ∞             |

Table (3.6): Reagents and Volumes Used to Prepare BDRR Mix.

| Reagent                | Volume  |
|------------------------|---------|
| Sequencing buffer (5x) | 3.5 µl  |
| H2O                    | 11.5 µl |
| Sequencing primer      | 2.0 µl  |
| BDRR                   | 1.0 µl  |
| Total volume           | 18 µl   |

### 3.5.4 In Silico Analysis

The validated and segregated variants, *SYCE1* p. Gln241\* from Family 1 and *RBBP7* p. Thr256Pro from Family II, were investigated using several bioinformatics prediction approaches, including:

- Mutation Taster <https://www.mutationtaster.org/>
- Clinvar <https://www.ncbi.nlm.nih.gov/clinvar/>
- SIFT (Sorting Intolerant from Tolerant). <https://sift.bii.a-star.edu.sg/>
- PolyPhen-2 (Polymorphism Phenotyping version2)  
<http://genetics.bwh.harvard.edu/pph2/>
- GVGd (Grantham Variation, Grantham Difference).  
<http://agvgd.hci.utah.edu/index.php>
- LIST-S2 <https://precomputed.list-s2.msl.ubc.ca/>
- PROVEAN (Protein Variation Effect Analyzer) accessible at  
<http://provean.jcvi.org/index.php>
- The COBALT Alignment tool has been used to determine the conservation of the variant locus. <https://www.ncbi.nlm.nih.gov/tools/cobalt>
- FATHMM (Functional Analysis via Hidden Markov Models (version 2.3)  
<http://fathmm.biocompute.org.uk/>

These investigations were used to predict the consequences of amino acid substitutions or insertions and deletions on protein structure and function. In addition, the AlphaFold tool program was used to predict the structural implications of the *SYCE1* mutant variant and the *RBBP7* mutant variant. The DUET server was used to predict the stability of the mutant protein of the *RBBP7* mutant variant

# Chapter Four: Results

To identify candidate gene variants associated with azoospermic infertility, two Palestinian families afflicted with idiopathic azoospermic infertility were included in this study. Each patient exhibited regular physical examinations, hormonal profiles (FSH, LH, testosterone, prolactin, and TSH), karyotypes (46,XY), and no Y-chromosome microdeletions. The scrotal ultrasound confirmed the absence of varicocele and obstruction. Semen analysis showed a complete absence of spermatozoa in the ejaculate, with no identifiable etiology.

One infertile patient from each family (the proband member number 3 from family one, and proband member number 3 from family 2) was selected for next-generation sequencing (NGS) by performing whole-exome sequencing (WES). After NGS data analysis and variant filtration, two variants in the first family and five variants in the second family were identified and subjected to direct confirmation by family segregation.

## 4.1 Family I

The first family is composed of fertile consanguineous parents, 2 infertile identical twin offspring, 4 fertile offspring, and one of the grandchildren in family 1 who was diagnosed with idiopathic azoospermia infertility. Two variants have been identified, and segregation information for each variant is presented in Figure (4.1) and Table (4.1). Family segregation can be observed in the pedigree for every variant below.

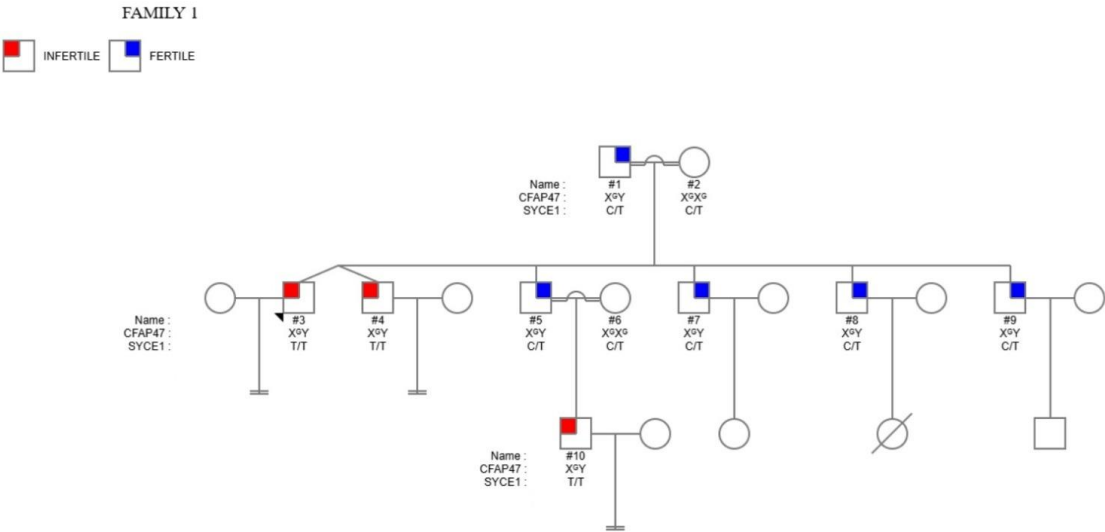


Figure (4.1): Family I Pedigree, illustrating the Genotype Results of the indicated variants among all Members in the Family.

Table (4.1): Data and Information on the Identified Variants in Family I.

|                           |                |              |
|---------------------------|----------------|--------------|
| Gene                      | <i>CFAP47</i>  | <i>SYCE1</i> |
| Chromosome                | Chr. X         | Chr. 10      |
| Type of genetic variation | Missense       | Nonsense     |
| Nucleotide change         | G6103A         | C721T        |
| Protein change            | Asp2035Asn     | Gln241*      |
| Sanger sequencing         | Validated      | Validated    |
| Segregation in the family | No Segregation | Confirmed    |

#### 4.1.1 *CFAP47* Gene

The *CFAP47* gene is located on chromosome X. The identified variant G6103A is a missense genetic variation, where Guanine is replaced by Adenine at position 6103, resulting in Aspartic acid being replaced by Asparagine at this location. This variant was tested and confirmed by Sanger sequencing. However, it failed to segregate in the family as expected. Confirmed Direct sequencing all male family members have X<sup>G</sup>Y for males and X<sup>G</sup>X<sup>G</sup> for females, indicating the absence of positive segregation of this genetic variation among all family members. In addition, NGS results predicted an uncertain significance of the mutant variant change (c.6103G>A) in the gene, as shown in Table (4.1) and Figure (4.2) below. Therefore, it is concluded that this gene is not primarily involved in the observed phenotype of the affected males in this family; however, it may play a role in the severity of the case among selected family members with this mutant variant which needs further testing and evaluation.

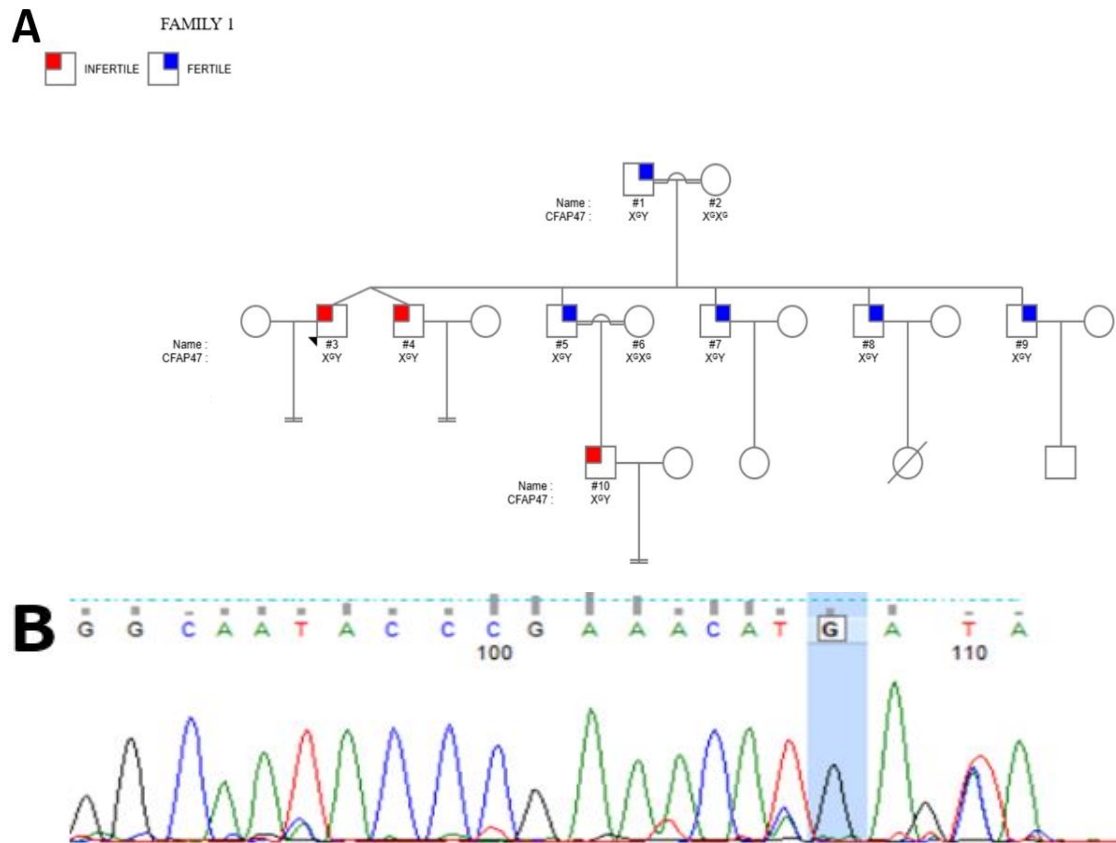


Figure (4.2): Family Segregation of the *CFAP47* Variant. (A) Genotype Results of Each Family Member are Shown in the Family Pedigree. (B) Sanger Sequencing Results of the Detected Variant (for members number 1,2,3,4,5,7,8, and 9) in Family I.

#### 4.1.2 *SYCE1* Gene

The *SYCE1* is located on chromosome 10. The identified variant C721T is a nonsense genetic variation where Cytosine is replaced by Thymidine at position 721, leading to replacement of Glutamine with a premature stop codon, resulting in a truncated protein at codon number 241. This variant was confirmed by Sanger sequencing and segregated in the family by genotype matching the phenotype. The *SYCE1* gene encodes synaptonemal complex central element protein 1 (*SYCP1*), a tetrameric protein structure that plays a major role in synapsis during meiosis (De Vries et al., 2014; Hamer et al., 2008).

In family I, both twin cases showed a homozygous genotype with this gene (T/T) while their four brothers showed a heterozygous genotype (C/T). In addition, one brother with a heterozygous genotype (C/T) is married to a wife from the same family, predicted with a heterozygous genotype with this gene (C/T), gave birth to a child, who was

confirmed as an Azoospermia case and infertile, and is predicted to have a homozygous genotype (T/T). So, based on family information, we predict that the genotype of member number 10 is (T/T), and his mother, member number 6, is (C/T), which needs direct confirmation. These results show the indicated genetic variation segregates well in the family, and strongly support direct involvement of this gene in the pathogenesis of this family, as described in Figure (4.3).

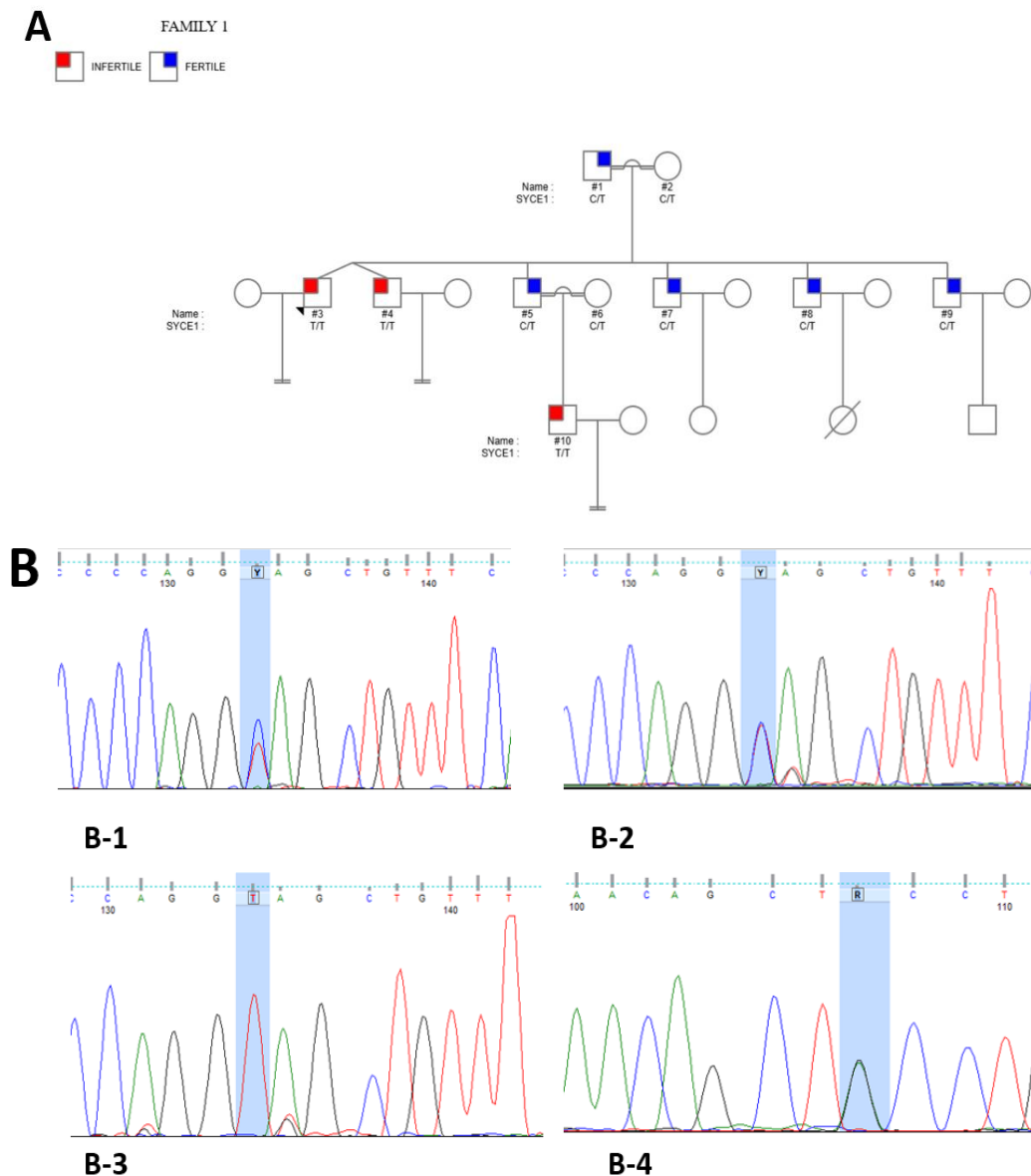


Figure (4.3): Family Segregation of the *SYCE1* Variant. (A) Genotype Results of Each Family Member are Shown in the Family Pedigree. (B) Sanger Sequencing Results of the Detected Variant in Family I, B-1: Illustrate the Sequence of the Father (member number 1) with the (CT) Genotype, B-2: Illustrate the Mother (member number 2) Sequence with the (CT) Genotype, B-3: Illustrate the Sequence of the Proband (member number 3) with the (TT) Genotype, and B-4: Illustrate the fertile brother Sequence (members number 5,7,8 and 9) with the (CT) Genotype.

## 4.2 Family II

The second family is composed of two fertile parents, 3 idiopathic azoospermia infertile offspring, two fertile offspring, and one fertile daughter. In this family, five variants were identified, and information and segregation about each variant are described in Figure (4.4) and Table (4.2).

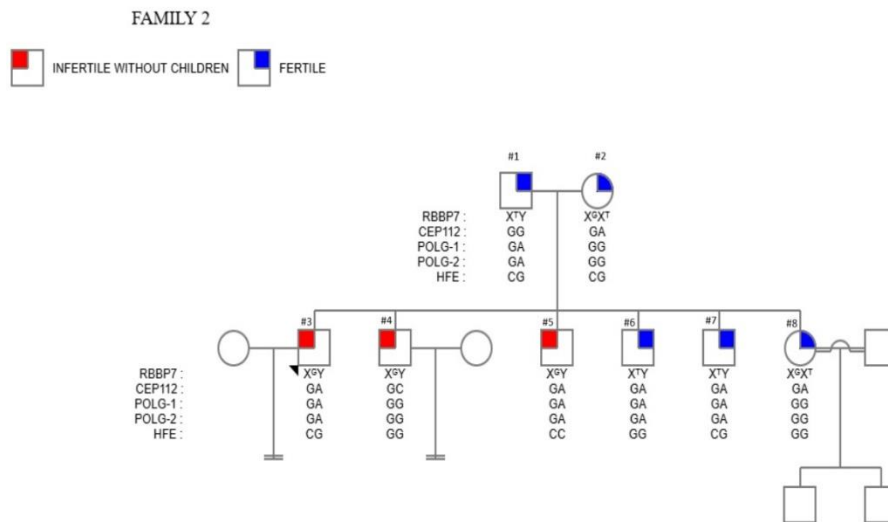


Figure (4.4): Family II Pedigree, Which Illustrates the Genotype Results of Each Member in the Family.

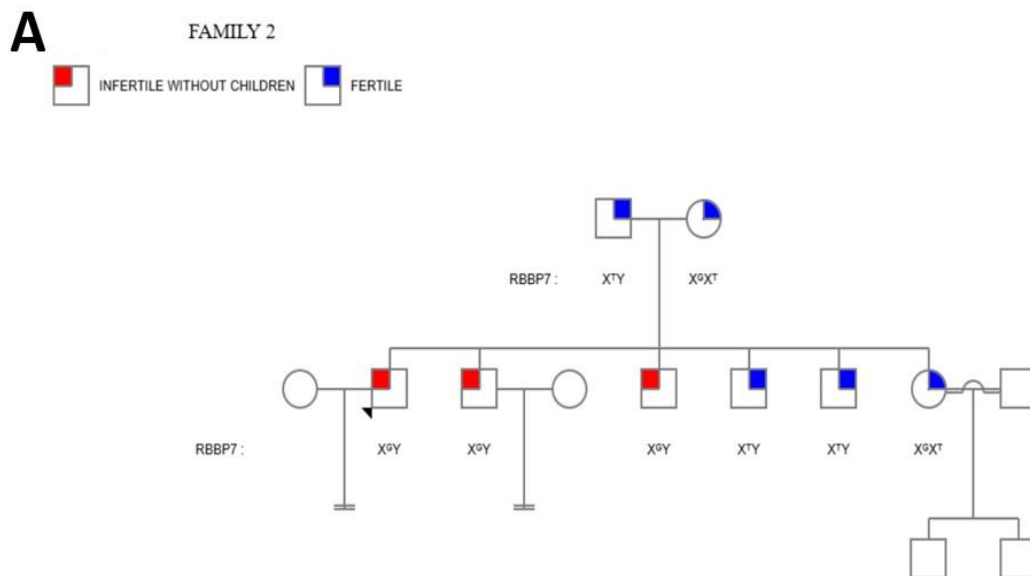
Table (4.2): Information and Details About the Identified Variants in the Second Family.

| Gene                      | <i>RBBP7</i> | <i>CEP112</i>  | <i>POLG-1</i>  | <i>POLG-2</i>  | <i>HFE</i>     |
|---------------------------|--------------|----------------|----------------|----------------|----------------|
| Chromosome                | Chr. X       | Chr. 17        | Chr. 15        | Chr. 15        | Chr. 6         |
| Type of genetic variation | Missense     | Nonsense       | Missense       | Missense       | Missense       |
| Nucleotide change         | A766C        | C2710T         | C752T          | C1760T         | C187G          |
| Protein change            | Thr256Pro    | Arg904*        | Thr251Ile      | Pro587Ileu     | His63Asp       |
| Sanger sequencing         | Validated    | Validated      | Validated      | Validated      | Validated      |
| Segregation in the family | Segregated   | No Segregation | No Segregation | No Segregation | No Segregation |

#### 4.2.1 *RBBP7* Gene

The *RBBP7* is located on chromosome X. The identified variant A766C is a missense genetic variation, where Adenine is replaced by Cytosine at position 766, leading to a Proline amino acid instead of Threonine at codon 256. The *RBBP7* gene encodes a histone-binding protein that is essential for chromatin remodeling and regulation of gene expression (Rutherford & Daggett, 2010).

This variant was validated by Sanger sequencing. It segregated as expected in Family II since the three infertile brothers with the proband showed a similar genotype ( $X^G Y$ ). In contrast, their two fertile brothers showed the wild-type variant genotype ( $X^T Y$ ), the fertile sister showed a heterozygous genotype ( $X^G X^T$ ), and the fertile mother had the same heterozygous genotype ( $X^G X^T$ ), while the fertile father had the wild-type genotype ( $X^T Y$ ). This result strongly supports direct involvement of this gene in the pathogenesis of this family, as shown in Figure (4.5).



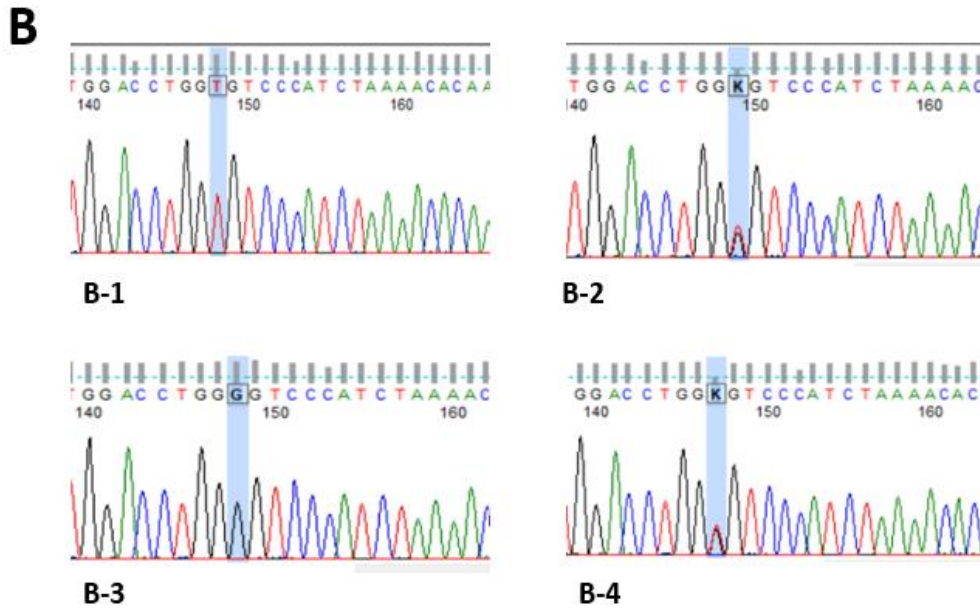


Figure (4.5): Family Segregation of the *RBBP7* Variant. (A) The Genotype Results of Each Family Member in the Pedigree. (B) Sanger Sequencing Results of the *RBBP7* Variant in Family II, B-1: Illustrate the Sequence of the Father, B-2: Illustrate the Mother Sequence, B-3: Illustrate the Sequence of the Proband, and B-4: Illustrate the Sister Sequence.

#### 4.2.2 *CEP112* Gene

*CEP112* is located on chromosome X. The identified variant C2710T is a nonsense genetic variation, in which Cytosine is replaced by Thymine at position 2710, leading to a truncated protein stop codon instead of Arginine at codon 904. It was verified and validated by Sanger sequencing. Even so, its segregation did not correlate with the confirmed manifestation of the condition among family members; all family members have a heterozygous GA genotype, and member number 1 has a GG genotype. As a result, this gene is not involved in the observed phenotype of the affected males in family II, as illustrated in Figure (4.6), and Figure (4.7) below.

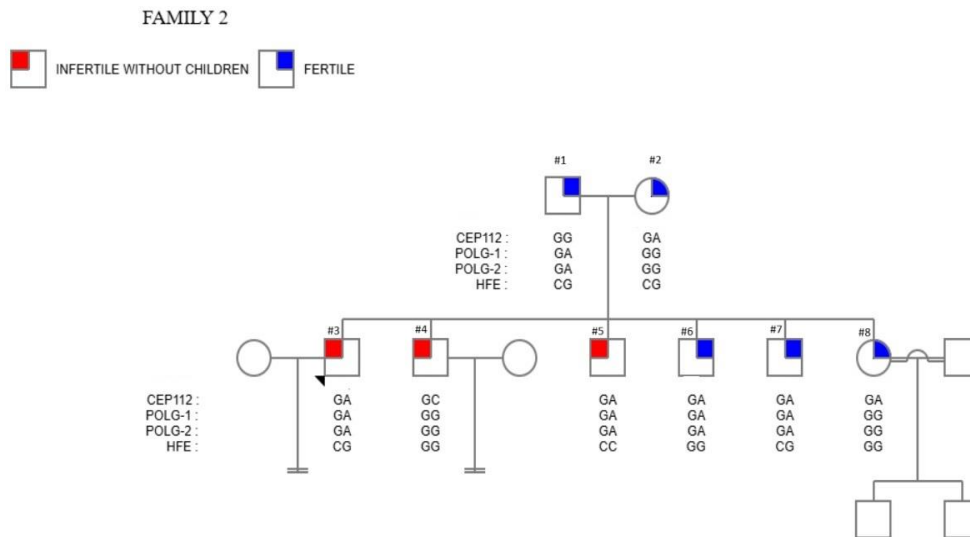


Figure (4.6): Family II Pedigree, Which Illustrates the Genotype Results of Each Member for the *CEP112*, *POLG-1*, *POLG-2*, and *HFE* Variants.

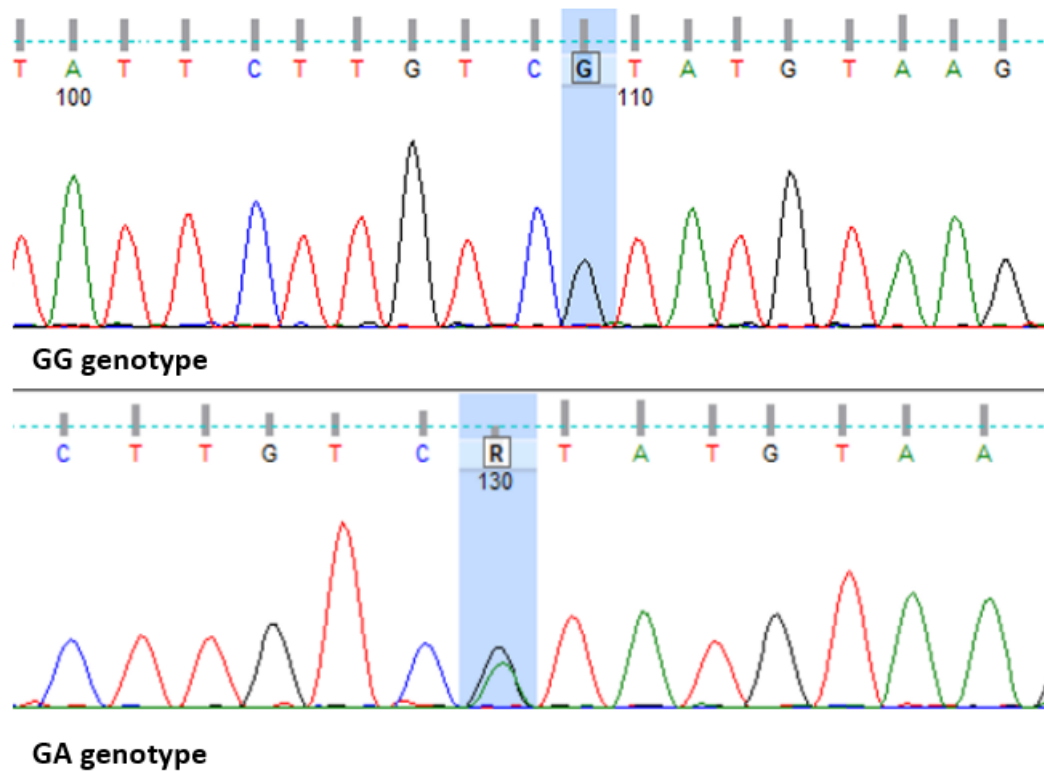


Figure (4.7): Sanger Sequencing Results of the *CEP112* Variant in Family II ( GG genotype for the member #1, GA genotype for the members #2,3,5,6,7, and 8).

#### 4.2.3 *POLG-1* Gene

*POLG-1* is located on chromosome 15. The identified variant C752T is a missense genetic variation in which Cytosine is replaced by Thymine at position 752, leading to the production of Isoleucine instead of Threonine at codon 752. It was verified and validated by Sanger sequencing. Even so, its segregation did not correlate with the confirmed manifestation of the condition among family members; all family members have a heterozygous GA and wild-type GG genotype. As a result, this gene is not involved in the observed phenotype of the affected males in family II, as illustrated in Figure (4.6) above and in Figure (4.8) below.

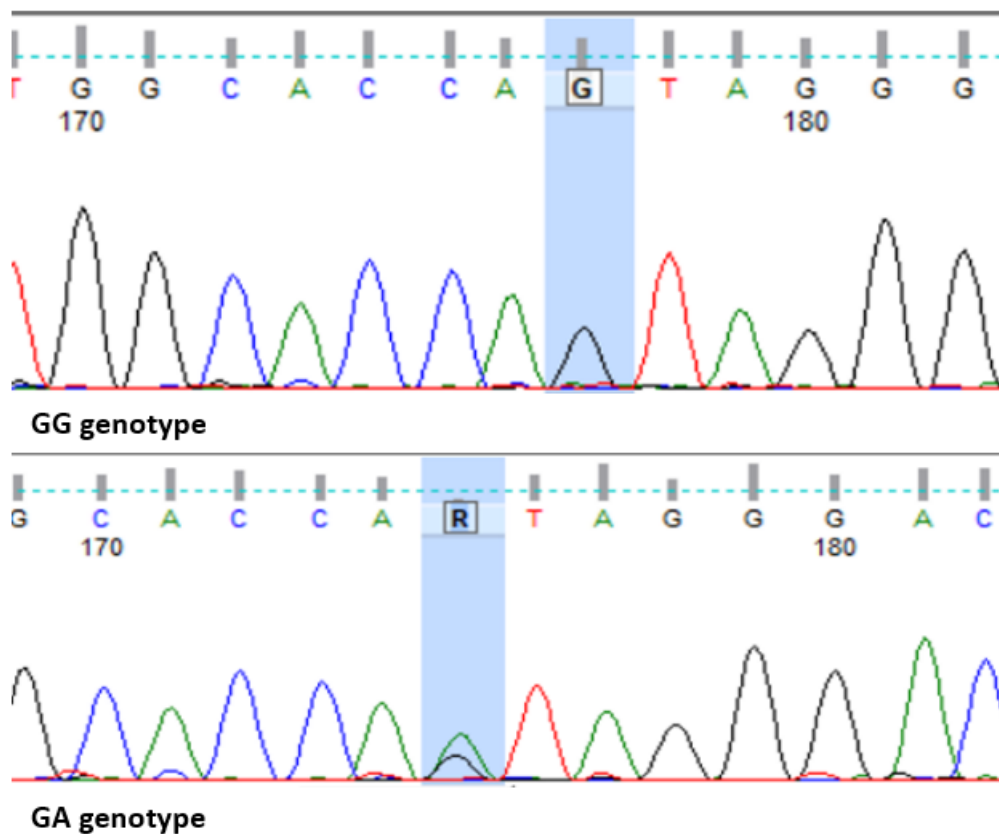


Figure (4.8): Sanger Sequencing Results of the *POLG-1* Variant in Family II.

#### 4.2.4 *POLG-2* Gene

*POLG-2* is located on chromosome 15. The identified variant C1760T is a missense genetic variation, in which Cytosine is replaced by Thymine at position 1760, producing Leucine instead of Proline at codon 587. It was verified and validated by Sanger sequencing. Even so, its segregation did not correlate with the confirmed manifestation of

the condition among family members; all family members have a heterozygous GA and wild-type GG genotype. As a result, this gene is not involved in the observed phenotype of the affected males in family II, as illustrated in Figure (4.6) above and in Figure (4.9) below.

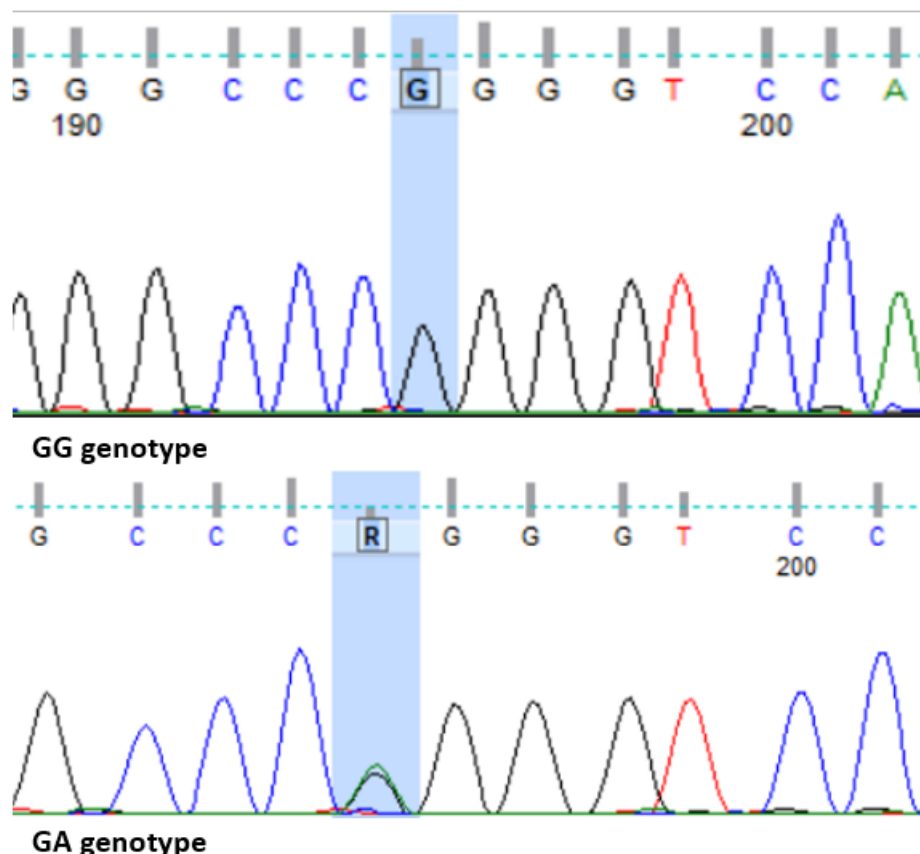


Figure (4.9): Sanger Sequencing Results of the *POLG-2* Variant in Family II.

#### 4.2.5 *HFE* Gene

*HEF* is located on chromosome 6. The identified variant C187G is a missense genetic variation, in which Cytosine is replaced by Guanine at position 187, producing Aspartic acid instead of Histidine at codon 63. It was verified and validated by Sanger sequencing. Even so, its segregation did not correlate with the confirmed manifestation of the condition among family members; all family members have a heterozygous CG, homozygous GG, and wild-type CC genotype. As a result, this gene is not involved in the observed phenotype of the affected males in family II, as illustrated in Figure (4.6) above and in Figure (4.10) below.

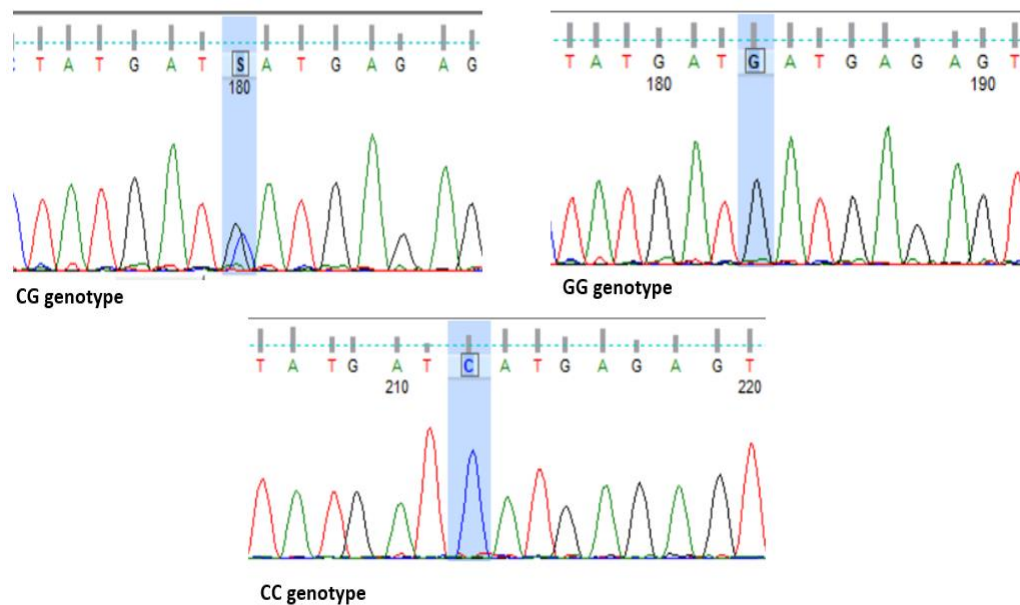


Figure (4.10): Sanger Sequencing results of the *HEF* variant in Family II.

### 4.3 In-silico Analysis for indicated *SYCE1* and *RBBP7* genes variants.

To evaluate the consequences of the amino acid substitution on the protein's structure and function for the confirmed and segregated variants, several bioinformatics methods were utilized.

#### 4.3.1 In Silico Analysis for *SYCE1* p.Gln241\* Variant

##### 4.3.1.1 COBAL T Alignment Software

The COBAL T alignment software was utilized to assess the conservation of the *SYCE1* p.Gln241\* locus. The finding shows that the *SYCE1* p.Q241\* variant is highly conserved, as shown in Figure (4.11).

|                            |                |     |                          |                                                    |     |
|----------------------------|----------------|-----|--------------------------|----------------------------------------------------|-----|
| Homo sapiens               | NP_001137236.1 | 238 | ATVQLFQEEHRKAEELAAAAQRH  | QKCCQCCQKQRLKEELEKHGMQVPAQAQSTQEEAGPGDVASPKPLKGERP | 317 |
| Pan troglodytes            | NP_001267863.1 | 202 | ATVQLFQEEHRKAEELAAAAQRH  | QKCCQCCQKQRLKEELEKHGMQVPAQAQSTQEEAGPGDVASPKPLKGERP | 281 |
| Equus asinus               | XP_070376298.1 | 177 | AAVHLFEEENRNAQRLLEAAAQRH | QKCCQCCQKQRLKEELEKGVQVPAQAQSTQEEAGPGDVASPKPLKGERP  | 251 |
| Mus musculus               | NP_001137237.1 | 240 | AAMQMFKDEHKAEFLAAAAQRH   | QKCCQCCQKQRLKEELEKHGMQVPAQAQSTQEEAGPGDVASPKPLKGERP | 317 |
| Scorilla scorilla scorilla | XP_018889876.1 | 239 | ATVQLFQEEHRKAEELAAAAQRH  | QKCCQCCQKQRLKEELEKHGMQVPAQAQSTQEEAGPGDVASPKPLKGERP | 318 |
| Symphalangus syndactylus   | XP_055100580.2 | 238 | ATVQLFQEEHRKAEELAAAAQRH  | QKCCQCCQKQRLKEELEKHGMQVPAQAQSTQEEAGPGDVASPKPLKGERP | 317 |
| Normascus leucodermis      | XP_003275546.1 | 238 | ATVQLFQEEHRKAEELAAAAQRH  | QKCCQCCQKQRLKEELEKHGMQVPAQAQSTQEEAGPGDVASPKPLKGERP | 313 |

Figure (4.11): Conservation Analysis of *SYCE1* p.Q241\* variant Using the COBAL T Alignment Tool.

#### 4.3.1.2 Prediction Tools

Multiple prediction tools were used to investigate whether the *SYCE1* p.Q241\* variant has an impact on the biological function and structure of the protein. The variant is predicted to be deleterious according to PROVEAN and SIFT tools. Based on the FATHMM prediction tool, *SYCE1* p.Q241\* will be pathogenic. However, by the ClinVar and Mutation tester tools, it is predicted to be pathogenic and disease-causing. Therefore, the *SYCE1* p.Q241\* variant seems strongly pathogenic and disease-causing.

#### 4.3.1.3 Structural Prediction Tools for *SYCE1*

##### Structural Modeling of the *SYCE1* p.Gln241 Truncating Variant\*

In silico analysis was performed to evaluate the structural impact of the predicted pathogenic *p.Gln241\** nonsense variant. This variant is located in exon 10 of the *SYCE1* gene, which is not in the end of the exon. Therefore, introduces a premature stop codon at amino acid position 241, resulting in a truncated protein lacking the C-terminal region. Figure (4.12) shows the superposition of the wild-type (yellow) and truncated (blue) models. Figure (4.12) shows the superposition of the wild-type protein (yellow) and the truncated protein (purple). In the wild-type model, the C-terminal segment contains multiple  $\alpha$ -helices that help maintain the protein's overall structure. In contrast, the truncated model lacks this entire region. The overlay clearly illustrates the loss of several helical segments in the mutant protein. Also, in silico analyses suggest that this variant leads to a loss of function of the *SYCE1* identified variant.

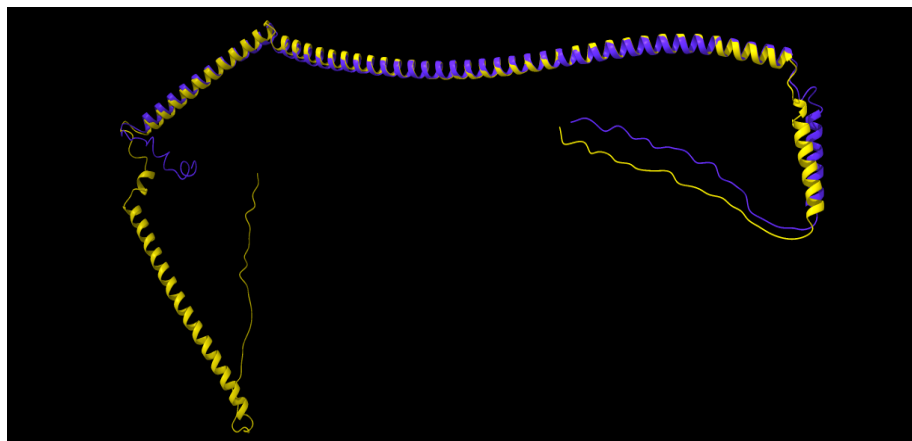


Figure (4.12): Structural superposition of wild-type (Yellow) and mutant (Blue) *SYCE1* models.

### 4.3.2 In silico Analysis for *RBBP7* p.Thr256Pro

#### 4.3.2.1 COBALT Alignment Software

The COBALT alignment software was utilized to assess the conservation of the *RBBP7* p.Thr256Pro locus. The finding shows that *this variant is highly conserved*, as shown in Figure (4.13).

|                         |                                |     |                                                                                |     |
|-------------------------|--------------------------------|-----|--------------------------------------------------------------------------------|-----|
| Homo sapiens            | <a href="#">NP_002884.1</a>    | 238 | HESLFGSVADDQKLMIVTSNNTSKPSHLVDAHTAEVNCLSFNIPYSEFILATGSADKTVALWDLRNLKLLHTFESHKD | 317 |
| Mus musculus            | <a href="#">NP_033057.3</a>    | 238 | HESLFGSVADDQKLMIVTSNNTSKPSHLVDAHTAEVNCLSFNIPYSEFILATGSADKTVALWDLRNLKLLHTFESHKD | 317 |
| Danio rerio             | <a href="#">XP_068080210.1</a> | 238 | HESLFGSVADDQKLMIVTSNNTSKPSHSDAHTAEVNCLSFNIPYSEFILATGSADKTVALWDLRNLKLLHSFESHKD  | 317 |
| Rattus norvegicus       | <a href="#">NP_001101382.1</a> | 239 | HESLFGSVADDQKLMIVTSNNTSKPSHSDAHTAEVNCLSFNIPYSEFILATGSADKTVALWDLRNLKLLHSFESHKD  | 318 |
| Drosophila melanogaster | <a href="#">NP_524354.1</a>    | 243 | HESLFGSVADDQKLMIVTSNNTSKPSHTVDAHTAEVNCLSFNIPYSEFILATGSADKTVALWDLRNLKLLHSFESHKD | 322 |
| Onychomys torridus      | <a href="#">XP_036030125.1</a> | 238 | HESLFGSVADDQKLMIVTSNNTSKPSHLVDAHTAEVNCLSFNIPYSEFILATGSADKTVALWDLRNLKLLHTFESHKD | 317 |

Figure (4.13): Conservation Analysis of *RBBP7* p.Thr256Pro Locus Using the COBALT Alignment Tool.

#### 4.3.2.2 Prediction Tools

Multiple prediction tools were used to investigate whether the *RBBP7* p.Thr256Pro variant has an impact on the biological function and structure of the protein. The variant is predicted to be deleterious according to PROVEAN and LIST-S2 tools. Based on the Mutation Taster prediction tool, *RBBP7* p.Thr256Pro will be disease-causing. However, by the ClinVar, the mutant variant of *RBBP7* p.Thr256Pro is not reported, and according to GVG D is predicted to be likely pathogenic. Also, the variant is predicted to be tolerated according to FATHMM, but not tolerated according to the SIFT tool. In addition, it is expected to be benign by the PolyPhen-2 tool.

#### 4.3.2.3 Structural prediction Tools for the *RBBP7* variant.

##### Structural Modeling of the *RBBP7* p.Thr256Pro Variant

To investigate the potential structural impact of the novel *RBBP7* variant c.766A>C (p.Thr256Pro), comparative in silico models were generated of the wild-type and mutant proteins. Figure (4.14) in section A, illustrates the wild-type structure, highlighting the threonine residue at position 256, which is located within the WD40 domain, a  $\beta$ -sheet-rich region forming part of the conserved  $\beta$ -propeller architecture. WD stands for tryptophan-aspartic acid (W–D) dipeptide motif, often found near the C-terminus of each

repeat. “40” refers to ~40 amino acids per repeat, and multiple repeats (usually 7 in *RBBP7*) fold together into a  $\beta$ -propeller structure (Rutherford & Daggett, 2010).

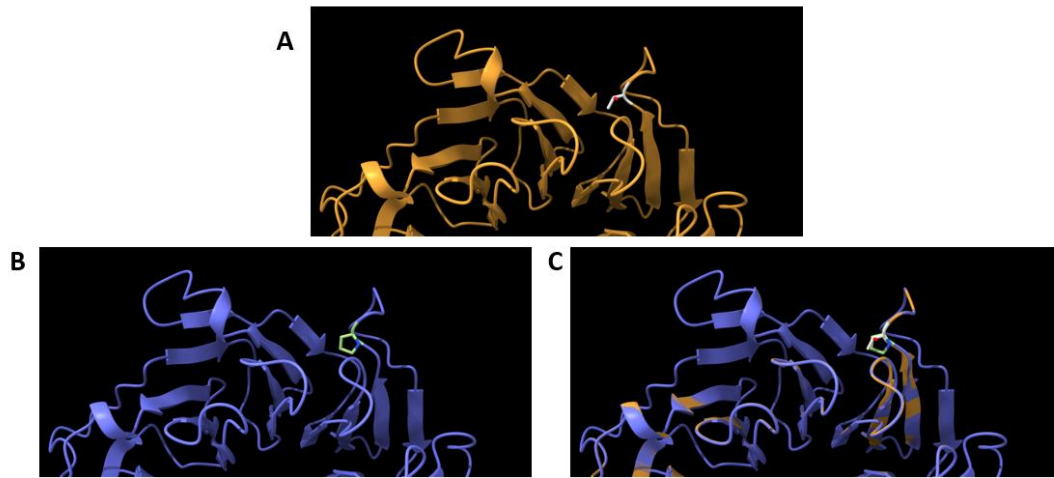


Figure (4.14): (A) Wild-type *RBBP7* Structural Model Highlighting Thr256. (B) Mutant *RBBP7* Structural Model Carrying the p.Thr256Pro Substitution. (C) Structural Superposition of Wild-Type and Mutant *RBBP7* Models

Substitution of threonine by proline introduced a marked local conformational alteration. The proline residue, due to its rigid cyclic structure, disrupted the adjacent  $\beta$ -strand and caused visible distortion of the backbone orientation relative to the wild-type configuration. When superimposed, the mutant model displayed local displacement of neighboring  $\beta$ -sheets and loops near residue 256, suggesting a perturbation of the secondary structure, Figure (4.14) in section B. No gross unfolding of the global tertiary fold was observed, but localized distortion in the vicinity of the substitution was evident. Also, *in silico* analyses suggest that this variant leads to a loss of function of the *RBBP7* identified variant.

In addition to structural modeling, the predicted effect of this substitution on protein stability was assessed using the DUET server, which combines mCSM and SDM approaches. All three predictions indicated that the p.Thr256Pro substitution is destabilizing, with estimated free energy changes ( $\Delta\Delta G$ ) as follows:

- **mCSM:**  $-0.762$  kcal/mol
- **SDM:**  $-0.99$  kcal/mol
- **DUET consensus prediction:**  $-0.848$  kcal/mol

These values suggest a modest but consistent destabilizing effect on the protein fold.

The table (4.3) presents a summary of the in-Silico analysis for the identified variants of *SYCE1* and *RBBP7*.

Table (4.3): Summary of in-Silico Analysis for *SYCE1* p.Gln241\* and *RBBP7* p.Thr256Pro Variants.

| Variant / Tool                    | Conservation | PROVEN      | FATHMM     | SIFT          | Mutation Taster | ClinVar      | LIST - S2   | Polyphen - 2 | GVGD              |
|-----------------------------------|--------------|-------------|------------|---------------|-----------------|--------------|-------------|--------------|-------------------|
| <b>SYCE1</b><br><b>P.Gln 241*</b> | Conserved    | Deleterious | Pathogenic | Deleterious   | Causing Disease | Pathogenic   | N/A         | N/A          | N/A               |
| <b>RBBP7</b><br><b>Thr256 Pro</b> | Conserved    | Deleterious | Tolerated  | Not Tolerated | Causing Disease | Not Reported | Deleterious | Benign       | Likely Pathogenic |

## Chapter Five: Discussion and Conclusion

Despite various studies associated with non-obstructive azoospermia, the molecular basis of the azoospermia remains unclear. More efforts are needed to understand the genetic causes of male infertility, which will produce guidance for assessing expected monogenic human azoospermia causes and provide personalized infertility treatment. Very limited data on the molecular genetics on azoospermia is available among the Arab population. Therefore, this project was carried out as an attempt to identify precise genetic factors involved in the development of inherited male infertility among afflicted Palestinian families. The generated data is projected to help control birth of new affected individuals, provide insights to new therapies and expand our understanding of the molecular genetics of this abnormality at the global level.

Two Palestinian families with two or more affected siblings diagnosed with idiopathic non-obstructive azoospermia were included in this study. One infertile patient from these families was chosen for whole-exome sequencing. After analysing data by bioinformatics techniques, putative genetic variants were selected, and these variants were examined using direct DNA sequencing to confirm proper family segregation and evaluate the pathogenicity of the identified mutant variants. In the first family, the results showed an X-linked mutation in the *CFAP47* gene that was confirmed by Sanger sequencing; however, it did not segregate within the family as expected. All family members have  $X^G Y$  for males and  $X^G X^G$  for females. However, next-generation sequencing (NGS) results detect an uncertain significance of the hemizygous variant change (c.6103G>A) in the gene. Direct Sanger sequencing showed all family members with the wild-type GG genotype. Therefore, this gene does not seem to be involved in the observed phenotype of the affected males in this family. Another autosomal mutation in the *SYCE1* gene was identified in this family. The identified variant, C721T, is a nonsense genetic variation where Cytosine is replaced by Thymidine at position 721, leading to the replacement of Glutamine with a premature stop codon, resulting in a truncated protein at codon number 241. Also, in silico analyses suggest that this variant leads to a loss of function of the *SYCE1* identified variant. This genetic variation leads to an error in synapses in meiosis, leading to primary ovarian insufficiency and a non-obstructive form of male infertility (De Vries et al., 2014; Pashaei et al., 2020a). The *SYCE1* gene is located on chromosome 10. and the indicated variant segregated as expected in the family by

genotype and phenotype matching. This result strongly suggests that this variant may play a crucial role in the main etiology of infertility in this family. This variant in the *SYCE1* gene (rs875989885), was associated with primary ovarian insufficiency, follicular dysfunction, and female infertility before the age of forty (*Primary Ovarian Insufficiency*, n.d.), in a single study among two Israeli Arab sisters of a consanguineous family, which was the first study that demonstrated this nonsense homozygous mutation as a cause of autosomal recessive POI in humans (De Vries et al., 2014), which indicated the important role of SYCE1 in the synaptonemal complex and meiosis in ovarian function. In the present study, this variant seems to be involved in non-obstructive azoospermia. Several *in-silico* prediction tools were applied, including FATHMM, PROVEN, ClinVar, SIFT, and Mutation Taster, to evaluate the variant damaging and disease-causing effect. The results collectively predicted the indicated p.Q241\* variant to be deleterious. This variant generated a premature stop codon that results in a truncated protein missing a significant part of its structure. The lost C-terminal region contains several  $\alpha$ -helical segments that are likely important for the protein's stability and function. Such truncating mutations often lead to loss of function, either because the shortened protein cannot fold correctly or because the mRNA is degraded by nonsense-mediated decay, preventing any protein from being made. This variant was not found in the gnomAD database, suggesting it is very rare. Combined with the predicted severe structural loss shown by modeling, this supports the interpretation that the *SYCE1* p.Gln241\* variant is highly pathogenic.

The *SYCE1* gene encodes synaptonemal complex central element protein 1 (SYCP1), a tetrameric protein structure that plays a major role in synapsis during meiosis (De Vries et al., 2014; Hamer et al., 2008). SYCP1 links the axial components of each homologous chromosome molecule to the main part of the complex, which involves two proteins, SYCE1 and SYCE2 (Bolcun-Filas et al., 2009). The three proteins (SYCP1, SYCE1, and SYCE2) are important for the development of the synaptonemal complex. The vertebrate synaptonemal complex is composed of a zipper-like configuration of SYCP1 subunits that interbind homologous chromosomes by self-assembly at the center, directly stabilized by the central element. The SC central element includes five proteins, SYCE1, SYCE3, SIX6OS1, SYCE2, and TEX12, that promote the formation of SYCP1. The structural core of SYCE1 consists of amino acids from 25-179, situated in the N-terminal part of the protein, that promotes the dimerization of SYCE1. The *SYCE1* functions as a structure that is important in the stability of the synaptonemal complex, as illustrated in Figure (5.1) (Dunne & Davies, 2019).

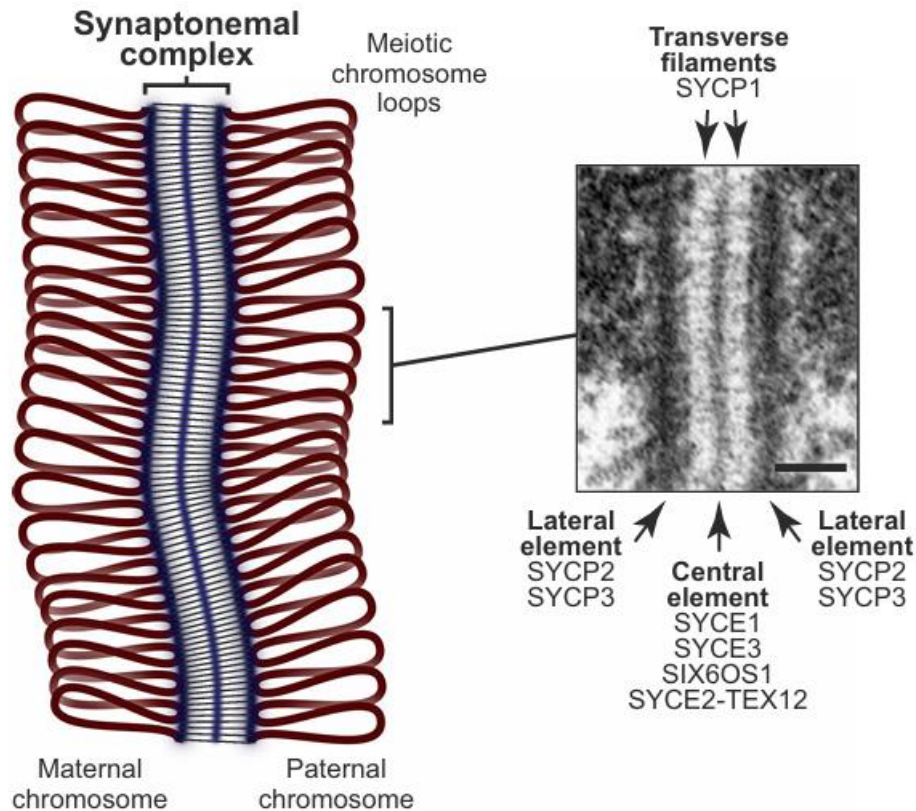


Figure (5.1): Schematic illustration of the synaptonemal complex, integrating a pair of homologous chromosomes throughout meiosis, illustrated with an insert electron micrograph of the SC (Dunne & Davies, 2019).

The *SYCE1* is highly expressed in the primary spermatocytes of the testis, and to a lesser extent in the meiotic prophase 1 in the oocytes in the ovaries, with much less in other tissues (Wang et al., 2018). In a previous study on mice, it was demonstrated that the *SYCE1* gene is expressed in both mouse genders, and when mutated, it results in infertile female mice and smaller testicular size than average in mouse's testicular size (Bolcun-Filas et al., 2009; Costa et al., 2005). Knockout of the *SYCE1* gene in mice led to the arrest of prophase I in meiosis, marked by the absence of synaptonemal complexes, because *SYCE1* has an important role in the stability of the homologous interactions controlled by the synaptonemal complex (De Vries et al., 2014). These observations indicate the major role of the *SYCE1* gene in sperm development, which is central for controlling infertility. Other previous studies in mice showed that the mutant *SYCE1* leads to failure in spermatogenesis, as it leads to pairing failure of meiotic DNA double-strand breaks in spermatocytes. Other studies on a non-obstructive azoospermia patient affected by a homozygous genetic variation in *SYCE1* demonstrated the arrest of the pachytene stage during meiosis in testicular histopathology (Krausz et al., 2020). In 2014, a study

was conducted on thirteen family members who had parents of consanguineous marriage, with two **sisters diagnosed with primary ovarian insufficiency** carrying a nonsense homozygous genetic variation (c.613C>T). The parents and three asymptomatic brothers were heterozygous for the genetic variation, while other healthy siblings did not carry the genetic variation (De Vries et al., 2014). In addition, a homozygous genetic variation at a splice site in *SYCE1* leads to a premature stop codon, which results in a lack of mature spermatocytes based on histological examination that can undergo fertilization. **This confirms maturation arrest in spermatogenesis directly caused by SYCE1 gene mutation** (Maor-Sagie et al., 2015) that was previously supported among infertile patients (Pashaei et al., 2020a). Another study confirmed a frameshift mutation (c.689\_690del; p. F230fs) in the *SYCE1* gene in an NOA patient (K. Feng et al., 2022). These studies strongly support the major impact of the *SYCE1* gene on male and female fertility. Interestingly, there seems to be a link between *SYCE1* genetic variation and the recurrence of prostate cancer after radical prostatectomy (X. Wu et al., 2020), which further supports the impact of this gene on prostate cell proliferation, which is not detected in patients in the family included in this study.

In the second family, several gene mutant variants were identified in *RBBP7*, *CEP112*, *POLG-1*, *POLG-2*, and *HFE* genes. The variants did not show the expected matched segregation except for the *RBBP7* gene A766C mutant variant. It follows hemizygous X-linked recessive inheritance, where in male offspring, a single mutated gene leads to pathogenicity, while in females, two mutated X-linked gene copies are required for the offspring to be affected. The *RBBP7* gene mutation is suggested to predispose to **maturation arrest in spermatogenesis**, leading to non-obstructive azoospermia ((J. Li et al., 2023).

The *RBBP7* gene is located on the X chromosome. The identified variant A766C is a missense genetic variation, where Adenine is replaced by Cytosine at position 766, leading to a Proline amino acid instead of Threonine at codon 256, resulting in instability and damage of the protein structure, causing a loss of function in the *RBBP7* protein in the identified variant. This variant segregated well in the family by genotype, matching the phenotype. In this family, three infertile males had the mutant *RBBP7* variant ( $X^G Y$ ), whereas two fertile males carried the wild-type *RBBP7* variant genotype ( $X^T Y$ ). The single female who is fertile showed a heterozygous genotype ( $X^G X^T$ ), the mother had the same heterozygous genotype ( $X^G X^T$ ) while the father had the wild type of *RBBP7* variant genotype ( $X^T Y$ ). These results strongly suggest direct involvement of this genetic

variation in the non-obstructive azoospermia in this family.. In previous study, two Asian brothers who exhibited maturation arrest of germ cells, showed X-linked *RBBP7* genetic variation, NM\_002893.4, c.1201ins1, p.W401M fs\*4) located on chromosome Xp22.2. Mouse and drosophila models were generated to assess the effect of knockout of *RBBP7* which led to the Lack of germ cells and male sterility and resulted into testicular atrophy, which supported the importance of *RBBP7* in all species spermatogenesis (J. Li et al., 2023). In another report that analysed data from 2354 infertile men included in four cohort studies, seven X-linked genes were newly discovered to affect male idiopathic infertility, including the *RBBP7* genetic variations detected in nine infertile patients mutated *RBBP7* gene that were not classified in male infertility affection in the genome aggregation database. Following knock out of the *RBBP7* gene in drosophila, it showed a decrease in testicular size and complete lack of male germ cells, demonstrating the importance of *RBBP7* gene in spermatogenesis (Riera-Escamilla et al., 2022). Several *in-silico* prediction tools were applied to evaluate the currently identified *RBBP7* variant including, FATHMM, PROVEN, ClinVar, SIFT, Mutation Taster, LIST-S2, PolyPhen-2, and GVGDD to test the impact of this mutation on the protein function. Evidently, the variant is predicted to be deleterious with pathogenic and disease-causing effect.

The *RBBP7* gene encodes a histone-binding protein that is essential for chromatin remodelling and regulation of gene expression. This protein contains a highly conserved WD40 domain, which folds into a seven-bladed  $\beta$ -propeller structure. This fold creates large surfaces that mediate interactions with histones and other proteins involved in chromatin-modifying complexes (Rutherford & Daggett, 2010). The p.Thr256Pro substitution identified in this study has not been previously reported in disease databases or population cohorts. Structural modelling showed that replacing threonine with proline at position 256 causes a localized distortion of the  $\beta$ -sheet architecture within the WD40 domain, since proline introduces rigidity and disrupts  $\beta$ -strand formation and this substitution is predicted to affect the geometry of the  $\beta$ -propeller blade where it is located. Computational stability predictions using DUET indicated that the variant has a destabilizing effect, with  $\Delta\Delta G$  values between  $-0.762$  and  $-0.99$  kcal/mol. Although these values suggest only moderate destabilization, they may still be relevant, as the WD40 domain relies on precise  $\beta$ -sheet packing to maintain structural integrity and binding capacity. Even subtle structural changes can impair the protein's ability to interact with histones and assemble into chromatin complexes.

The *RBBP7* is a member of the WD40 protein group and it is a major part of multiple protein complexes including nucleosome remodelling and deacetylase (NuRD) complex which include HDAC1/2, MTA1/2/3, MBD2/3 CHD3/4, GATAD2A/B, and RBBP7. It participates in histone deacetylation, transcriptional repression, condense chromatin that leads to gene silencing, as shown in Figure (5.2). Any defect in its mechanism of action may lead to disturbance in gene expression regulation. It is worth mentioning that RBBP7 contributes to DNA repair with NURD complex participation since both recruit BRCA1 to enable DNA damage repair, and when RBBP7 is mutated, the process fails in the regulation of repair, leading to the accumulation of defective cells and oncogenesis during cell cycle (Y. Zhan et al., 2024). During the cell cycle process, the *RBBP7* gene is necessary for DNA repair and appropriate cell division in mitosis. It contributes to the insertion of centromere protein A on centromeres, playing an important role in chromosomal segregation. A mutant *RBBP7* causes an arrest of the cell cycle, particularly at the G2/M phase (Y. Zhan et al., 2024). *RBBP7* is also a part of the polycomb repressive complex 2 (PRC2) complex composed of EZH1/2, EED, SUZ12, and RBBP4/7 that contribute to histone methylation at the core of EZH1/2 that results in methylating histone 3 at lysine 27, forming H3K27me3 that condense chromatin and resulting in gene transcription repression. The participation of *RBBP7* gene in these complexes indicates its importance in epigenetic regulation, DNA repair, the cell cycle process, and the remodelling of chromatin, as shown in Figure (5.3) (Y. Zhan et al., 2024).

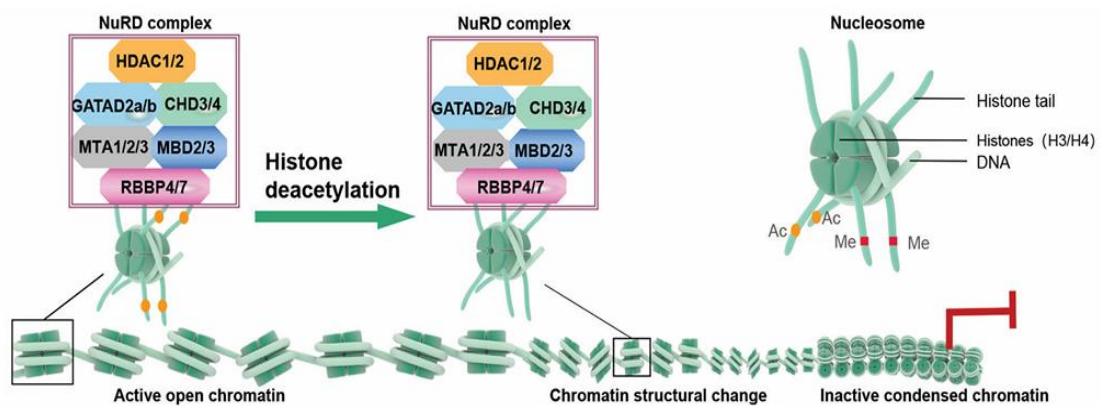


Figure (5.2): Illustrates the Components and the Mechanism of Action of the NURD Complex (Y. Zhan et al., 2024).

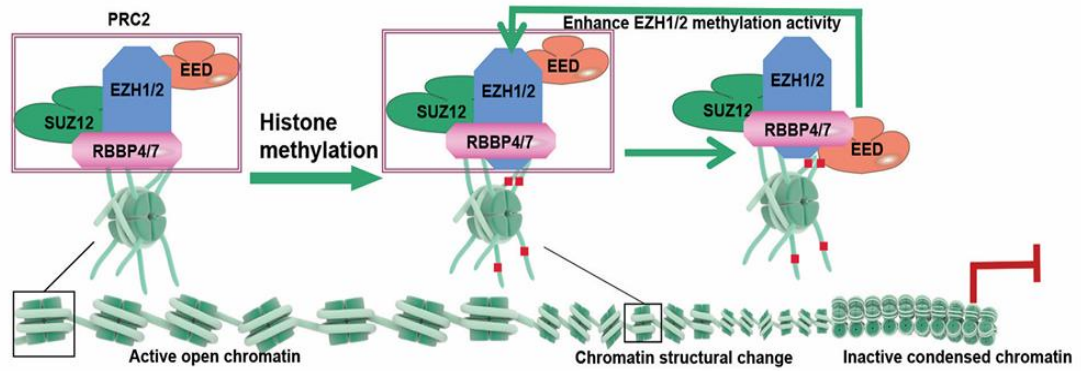


Figure (5.3): Illustrates the Components and the Mechanism of Action of the PRC2 Complex.

In spermatogenesis, *RBBP7* contributes to histone interaction, chromatin modification, regulation of cell cycle and arrest, and interaction with *BRCA1* in DNA damage repair as we mentioned above. It shares in mitotic and meiotic processes of spermatocytes and spermatogonia formation, although, the specific role of the *RBBP7* gene that alters male fertility remains under exploration and research (J. Li et al., 2023; Y. Zhan et al., 2024). The *RBBP7* gene is expressed in the majority of organ systems, including respiratory, digestive, endocrine, nervous, immune, and urogenital systems, and previous studies linked *RBBP7* genetic variation to many disease processes. Overexpression of the gene indicates poor prognosis, distant metastasis, and recurrence of non-small cell lung cancer (Y. Zhan et al., 2024). It also increases the chance of acute and chronic myeloid leukemia occurrence (Y. Zhan et al., 2024). In addition, its overexpression surges the tumour size, lymph node involvement, and metastasis (TNM) score in oesophageal squamous cell carcinoma (Y. Zhan et al., 2024). In breast cancer, several studies showed a link between *RBBP7* and breast malignancy development, where some studies linked the down-regulation of this gene with breast malignancy (G. C. Li et al., 2003; Y. Zhan et al., 2024), while others, especially those focused on ductal carcinoma in situ and the oestrogen-hormone state of cancer, supported that upregulation of this gene is the cause of pathogenicity (Ebata et al., 2012). In summary, disturbances in the *RBBP7* gene activity status are linked with oncogenicity in many tissues (Y. Zhan et al., 2024).

Since it is highly expressed in testicular germ cells and Sertoli cells, modification of this gene is predicted to contribute to non-obstructive azoospermia. Previous studies revealed that mutated *RBBP7* gene is associated with defects in male reproductivity, spermatogenesis, and germ cell maturation (J. Li et al., 2023; Riera-Escamilla et al., 2022). Based on the significant role of this gene in major cellular functions and the

deleterious effect caused by distortion in its structure and expression, the currently identified variant in this study (A766C) in the *RBBP7* gene is strongly implicated in male infertility observed in affected patients in the studied family. Definitely, further work is needed to confirm the direct functional impact of the detected genetic variation on sperm cell development in cellular and animal models. This study recommends extended efforts in investigation to assess the causal relationship between *SYCE1* p.Q241\* and *RBBP7* p.Thr256Pro genetic variations in non-obstructive azoospermia. One approach is in vitro modeling experiments with embryonic stem cells transfected with the genetic variation generated by the CRISPR-CAS9 system, followed by their differentiation into spermatogenic cells, and functional investigations can be done in mouse models. Additionally, Semen samples could be obtained from patients to quantify mutated genes at both RNA and protein levels.

## Conclusion

The data presented in this project indicate the probable involvement of two novel candidate genes in the pathological process of non-obstructive azoospermia, the first variant, *SYCE1* p.Q241\*, which follows autosomal recessive inheritance. Previously, a homozygous mutation in this gene led to male infertility by the formation of a premature stop codon followed by spermatogenic failure. The second variant, *RBBP7* p.Thr256Pro which follows X-linked recessive inheritance. the gene product is part of many protein complexes and plays an important role during the cell cycle, where it contributes to chromatin remodelling and DNA damage repair, functions in transcription repression, differentiation, proliferation, and apoptosis of germ cells. A mutant variant of this gene may lead to maturation arrest in the spermatogenesis process. Cell and mouse models are needed to confirm the results and assess the causative association between the *SYCE1* p.Q241\* and *RBBP7* p.Thr256Pro in non-obstructive azoospermia

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## Appendices

### Appendix (1): Consent Form

#### نموذج الموافقة على المشاركة في البحث العلمي

##### عنوان البحث العلمي:

تحديد الجينات المحتملة المرتبطة بالعقم الذكوري في عائلات فلسطينية مختارة باستخدام تسلسل الإكسوم الكامل.

اسم الباحث الرئيسي: سميرة رائد محمد ابورعد

اسم المشرف على البحث: البروفيسور هشام درويش

##### ملخص البحث:

يُعرَّف العقم بأنه عدم القدرة على الحمل بعد أكثر من 12 شهرًا من الجماع المتكرر دون وقاية. ويُصنَّف العقم إلى أربع فئات: عيوب كمية في تكوين الحيوانات المنوية، وعيوب نوعية في تكوين الحيوانات المنوية، واضطرابات محور الوطاء-النخامية، وانسداد أو خلل في القناة التناسلية. تُعزى ١٥٪ على الأقل من حالات العقم عند الرجال إلى العوامل الوراثية، وتسهم بشكل كبير في جميع الفئات الأربع الرئيسية المسببة للعقم عند الرجال. انعدام الحيوانات المنوية هو غياب الحيوانات المنوية تمامًا في السائل المنوي. ويُعتبر انعدام الحيوانات المنوية غير الانسدادي أي فئة أخرى غير انسداد القناة التناسلية أو خللها. وفي المسببات الوراثية، تسهم عدة جينات في عقم الرجال. تهدف هذه الدراسة إلى تحديد الأسباب الجينية الدقيقة للعقم الذكوري الوراثي في العائلات الفلسطينية .

##### المطلوب للمشاركة في البحث:

سحب عينات دم من المشارك لفحص الحمض النووي.

##### الفائدة المرجوة من البحث:

البحث العلمي سيساعد في المجال الطبي في إيجاد السبل العلاجية الملائمة للمرضى الذين يعانون من انعدام الحيوانات المنوية وبالتالي إيجاد حلول للعقم الذكوري المنتشر في المجتمع الفلسطيني.

##### المخاطر المتوقعة من البحث:

لا يوجد أي مخاطر تذكر على المشاركين خلال هذا البحث العلمي.

### الخصوصية:

حرصا على خصوصية المشاركين في البحث العلمي سيتم التعامل مع العينات بأرقام تحدد هوية المشارك بدون ذكر الاسماء.

### حقوق المشاركين في البحث:

يحق للمشاركين التوقف عن المشاركة في هذا البحث لأي سبب شخصي.  
يحق للمشاركين بمعرفة النتائج النهائية للبحث العلمي عن طريق التواصل المباشر مع الباحث.  
في حال وجود اي استفسارات عن البحث يستطيع المشاركون التواصل المباشر مع الباحث عبر البريد الالكتروني:

[Suma\\_aburaed@hotmail.com](mailto:Suma_aburaed@hotmail.com)

في حال عدم الشعور بالراحة او الرضى عن كيفية اجراء الدراسة يمكن للمشاركين التواصل مع:

Ethical Review Committee

Deanship of Scientific Research

Arab American University–Palestine (AAUP)

Email: [src@aaup.edu](mailto:src@aaup.edu)

### الموافقة على المشاركة:

بعد قراءة كل المعلومات المذكورة اعلاه وفهم جميع البنود انا الموقع ادناه اوافق على المشاركة في هذا البحث العلمي مع احتفاظي بجميع الحقوق المذكورة.

اسم المشارك:

التاريخ:

التوقيع:

تحديد العوامل الوراثية (الجينات) التي تساهم في تطور العقم عند الذكور لدى بعض العائلات الفلسطينية المنتقاه (لديها أكثر من فرد مصاب بالعقم) باستخدام تقنية تسلسل النيوكليدات في كافة الاكسونات في الجينوم.

سمية رائد محمد أبو رعد

أسماء لجنة الإشراف:

الأستاذ الدكتور هشام محمد أحمد درويش

الدكتور مهند محمود أحمد خضر

الأستاذ المساعد فواز عوض

## ملخص

يُعرّف العقم بأنه عدم القدرة على الحمل بعد أكثر من 12 شهراً من الجماع المتكرر دون وقاية. ويُصنّف العقم إلى أربع فئات: عيوب كمية في تكوين الحيوانات المنوية، وعيوب نوعية في تكوين الحيوانات المنوية، واضطرابات محور الوطاء-النخامية، وانسداد أو خلل في القناة التناسلية. تُعزى ١٥٪ على الأقل من حالات العقم عند الرجال إلى العوامل الوراثية، وتُساهم بشكل كبير في جميع الفئات الأربع الرئيسية المسببة للعقم عند الرجال. انعدام الحيوانات المنوية هو غياب الحيوانات المنوية تماماً في السائل المنوي. ويُعتبر انعدام الحيوانات المنوية غير الانسدادي أي فئة أخرى غير انسداد القناة التناسلية أو خللها. وفي المسببات الوراثية، تُساهم عدة جينات في عقم الرجال، بما في ذلك جينات MEIOB وTEX11 وSTAG3 وSYCP3 وDMC1. تهدف هذه الدراسة إلى تحديد الأسباب الجينية الدقيقة للعقم الذكوري الوراثي في العائلات الفلسطينية (تم تضمين اثنين أو أكثر من الأشقاء في نفس العائلة الذين تم تشخيصهم بالعقم الذكوري مجهول السبب في هذه الدراسة).

دُرست عائلتان فلسطينيتان، تضمان عدة أفراد مصابين بانعدام الحيوانات المنوية مجهول السبب غير الانسدادي؛ واختير فرد مصاب واحد من كل عائلة لإجراء تسلسل الإكسوم الكامل، ثم فُلترت نتائج ملف VCF. واختُبرت المتغيرات ذات أعلى درجة ارتباط بالنمط الظاهري، وهي متغيران من العائلة الأولى وخمسة متغيرات من العائلة الثانية، باستخدام تسلسل سانجر لجميع أفراد العائلة.

وفُحصت المتغيرات المؤكدة باستخدام أدوات المعلوماتية الحيوية الحاسوبية، وُحددت متغيرات العائلة الأولى في جينات CFAP47 و SYCE1 وحُللت. في المقام الأول، اتبع متغير SYCE1 p.Q241\* الوراثة الجسدية المتنحية، وتم التحقق من صحته وفصله في العائلة. أما في العائلة الثانية، فقد تم التعرف على المتغيرات في جينات RBBP7 و CEP112 و POLG-1 و POLG-2 و HFE وتقييمها. تم التحقق من وجود متحور RBBP7 p.Thr256Pro، الذي يتبع الوراثة المتنحية المرتبطة بالكروموسوم X، وعزله. باختصار، يُقترح ارتباط متحوري SYCE1 p.Q241\* و RBBP7 p.Thr256Pro بانعدام الحيوانات المنوية غير الانسدادي لدى العائلتين المتأثرتين في هذه الدراسة. سيلزم إجراء المزيد من الأبحاث والاختبارات الوظيفية لتأكيد هذه النتائج بشكل قاطع والكشف عن دورهما المميز في تطور العقم عند الذكور. ستكون هذه البيانات مفيدة لفحص المرضى وتقييمهم، مع التأكيد على الأهمية المحتملة لهذه الجينات كأهداف علاجية.

الكلمات المفتاحية: عقم الذكور، انعدام الحيوانات المنوية، جين RBBP7، جين SYCE1، تسلسل الاكسوم الكامل