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Investigating the Molecular Basis of Immotile Sperms and Azoospermia Associated with Male Infertility in Palestinian Individuals

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Investigating the Molecular Basis of Immotile Sperms and Azoospermia Associated with Male Infertility in Palestinian individuals

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Thesis Approval

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Jerusalem-Palestine

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Dedication

To my family

Declaration

I certify that this thesis submitted for the degree of master, is the result of our research, the content of the thesis is the result of work which has been carried out since the date of approval of research program. All ethics procedures and guidelines have been followed properly while preparing thesis

Signed:  .

John Edward Tawil.

Date: 03/01/2023

Acknowledgment

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Special thanks to the French Consulate for providing a grant for our trip to France to Sorbonne University, it was a very enriching experience that encouraged us, the team in UMR_S933 , INSERM molecular genetics lab. is very inspiring and motivational.

Thanks to my beloved wife and my children for giving me the time to study and search.

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Abbreviations

A: Adenine.

Ala: Alanine.

ATP: Adenosine tri-phosphate.

BTB: Blood testes barrier.

C: Cytosine.

CCDC: Coiled –coil domain.

CFAP: Cilia and flagella associated protein.

CFTR: Cystic fibrosis transmembrane conductance regulator.

CNV: Copy number variant.

Conc.: Concentration.

CP: Central pair.

DA: Dynein arm.

DHDH: Dihydrodiol dehydrogenases.

DMT/d MT: Double microtubule.

DNA: Deoxyribonucleic acid.

DNAAF: Dynein axonemal assembly factor.

DNAH: Dynein axonemal heavy chain.

DNAL: Dynein axonemal light chain.

ESC: Embryonic stem cells.

FSH: Follicle-stimulating hormone.

G: Guanine.

GEO: Gene Expression Omnibus.

GnRH: Gonadotropin-releasing hormone.

gm: Gram.

HG: Human genome.

IGV: Integrative genomics viewer.

IMIGC: International Male Infertility Genomics Consortium.

IVF: In vitro fertilization.

LH: Luteinizing hormone.

MAF: Minor allele frequency.

MDM1: Mouse double minute 1.

min.: Minute.

MMAF: Multiple morphological abnormalities of sperm flagella.

MPS: Micro-physiological Systems.

MSC: Mesenchymal stem cell.

MtDNA: Mitochondrial DNA.

ng.: Nano gram.

Nm: Nanometer.

NOA: Non obstructive azoospermia.

nTPM: normalized protein-coding transcripts per million

OA: Obstructive azoospermia.

Pro: Proline.

PCD: Primary ciliary dyskinesia.

PCR: Polymerase chain reaction.

PGD: Preimplantation genetic diagnosis.

RSPH: Radial spoke head protein.

sMT: singlet microtubules,

T: Thymine.

Thr: Threonine.

TOC: Testis on a chip.

TSH: Thyroid stimulating hormone.

WES: Whole exome sequence.

wt: Wild type.

WHO: World health organization.

µl: Microliter.

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Figure 20. Multiple Sequence Alignment Viewer 1.22. we can tell that the site in which our mutation is very conserved among multiple systems and in multiple variants of *MDM1*.

Abstract

Title: Investigating the Molecular Basis of Immotile Sperms and Azoospermia Associated with Male Infertility in Palestinian Individuals

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Infertility in men seems to be very obscure and lacks appropriate explanations in terms of genetic causes, though as a definition infertility, in general, is the inability of couples to conceive within a year of trying to achieve pregnancy without using contraceptives. Considerations in the definition means that the male is incapable of producing healthy sperms and/or sometimes no sperms at all that can fertilize an egg.

The quality and the quantity of the produced sperms is very important, and a sperm should owe to specific criteria in order to be fertile, it needs a good tail or flagellum that can provide capacity to move, a good head that provide capacity to penetrate the egg in a quick manner and enough mitochondria in the neck, all with an integrated morphological feature and avide quantity. In term of Quality for instance, Multiple morphological abnormalities of the sperm flagellum (MMAF) is a disorder that results in the inability of sperm to move effectively. It encompasses a range of flagellar disorders. The genetic basis of MMAF is complex and could be owed to multiple genes. And, in terms of quantity oligospermia and azoospermia refer to decreased sperm quantity, which plays a critical role in increasing the chances of conception. Advances in genetic and molecular research are helping to address this issue and improve reproductive outcomes.

In Palestinian community investigation is worthy because we have many consanguineous marriages which is set at a rate of ~ 40% this is according to the central bureau of Statistics in Palestine, which may help in finding variations in genes associated to MMAF that is not related to environmental factors.

In this study, we utilized whole exome sequencing (WES) to analyze two independent individuals with different forms of infertility: asthenospermia and non-obstructive azoospermia. Our WES analysis led to the identification of several candidate genetic variations that may contribute to these phenotypes. In the asthenospermia case, we identified

variants in four candidate genes (*MROH8*, *MUC4*, *FADS6*, *TAS2R43*) that may explain the MMAF phenotype. In the azoospermia case, we identified a homozygous missense variation in the *MDM1* gene (NM_017440.4: c.1981G>A: p.Ala661Thr), which may explain the observed spermatogenesis failure. Segregation analysis revealed that the affected brother of the proband is also homozygous for the same *MDM1*, further supporting its potential role in the azoospermia observed in this family. Additionally, we identified variations in other four candidate genes (*MAGEC1*, *OSBP2*, *NAT10*, *CD248*) in the azoospermia case that may also play a role in infertility. We believe that there are still many unknown genes that causes azoospermia and asthenospermia. Trying to find a signature that exist in our Palestinian community would make innovative solutions in the future in the world of genetics practical.

ملخص

العنوان: تقصي الأساس الجزيئي للحيوانات المنوية غير متحركة و فقد النطاف المرتبط بالعقم عند الذكور لدى أفراد فلسطينيين.

إعداد: جون طويل.

أشرف على الرسالة: د. فواز عواد.

العقم عند الرجال يميل الى كونه موضوع غامض للغاية, و يفتقد الى تفسير بالمعنى و المستوى الجيني بشكل لائق, بالرغم من ذلك, كتعريف العقم بشكل عام هو عدم قدرة الأزواج على الإنجاب في غضون عام من محاولة الإنجاب دون استخدام وسائل منع الحمل. الاعتبارات التي يحملها التعريف تعني أن الذكر غير قادر على إنتاج حيوانات منوية صحية (متكاملة من حيث البناء و الوظيفة) و / أو أحيانًا عدم انتاج حيوانات منوية على الإطلاق و بالتالي عدم القدرة على تخصيب البويضة.

تعتبر جودة و كمية الحيوانات المنوية المنتجة مهمة جدًا ويجب أن يكون الحيوان المنوي حائد لمعايير محددة من أجل أن يكون خصبًا, فهو يحتاج إلى ذيل جيد أو سوط يمكنه توفير القدرة على الحركة ، ورأس جيد يوفر القدرة على اختراق البيضة بطريقة سريعة وميتوكوندريا كافية في الرقبة ، وكل ذلك بخاصية مورفولوجية متكاملة و وفرة بالكمية. يُطلق على الاضطراب الذي يفسر عدم قدرة الحيوانات المنوية على الحركة اسم التشوهات المورفولوجية المتعددة في السوط (MMAF) التي تصف كوكبة من اضطرابات الأسواط. وراثيًا ، تشارك العديد من الجينات في MMAF . فيما يتعلق بقلة النطاف oligospermia وانعدام النطاف azoospermia هي مصطلحات تتعامل مع الحيوانات المنوية من حيث الكمية ، لأن عدد الحيوانات المنوية مهم ، في زيادة احتمالات تحقيق الحمل , يساعد التقدم في البحث الجيني والجزيئي على معالجة هذه المشكلة وتحسين النتائج الإنجابية.في المجتمع الفلسطيني يعتبر الاستقصاء الجيني أمرًا جديرًا لأن لدينا العديد من زيجات الأقارب التي قدرت بمعدل 40٪ تقريبًا وهذا وفقًا للمكتب المركزي للإحصاء في فلسطين مما قد يساعد في إيجاد متغيرات في الجينات المرتبطة بـ MMAF غير مرتبطة بالعوامل البيئية.

في هذه الدراسة ، استخدمنا whole exome sequencing (WES) لتحليل شخصين مستقلين مصابين بأشكال مختلفة من العقم: وهن النطاف وفقدان النطاف غير الانسدادي. أدى تحليل WES الخاص بنا إلى تحديد العديد من الاختلافات الجينية المرشحة التي قد تساهم في هذه الأنماط الظاهرية. في حالة وهن النطاف ، حددنا المتغيرات في أربعة جينات مرشحة (MROH8، MUC4، FADS6، TAS2R43) التي قد تفسر النمط الظاهري لـ MMAF. في حالة فقد النطاف ، حددنا تباينًا متماثلًا في الخطأ في جين MDM1 (NM_017440.4: c.1981G> A: p.Ala661Thr) ، مما قد يفسر فشل تكوين الحيوانات المنوية الملحوظ. كشف ال Segregation analysis أن الأخ المصاب للproband هو أيضًا متماثل الزيجوت homozygous للMDM1 ، مما يدعم دوره المحتمل في فقد النطاف الذي لوحظ في هذه العائلة. بالإضافة إلى ذلك ، حددنا أختلافات في أربعة جينات اخرى (MAGEC1، OSBP2، NAT10، CD248) و هي مرشحة لحالة فقد النطاف التي قد تلعب أيضًا دورًا في العقم. نعتقد أنه لا يزال هناك العديد من الجينات المجهولة التي تسبب فقد النطاف و وهن النطاف. إن محاولة العثور على توقيع موجود في مجتمعنا الفلسطيني من شأنه أن يجعل الحلول المبتكرة في المستقبل في عالم علم الوراثة عملية.

Chapter 1

Introduction

1.1 Male reproductive system.

Reproduction in humans depends highly on the integrity of both the male and the female reproductive system [1]. In respect to the male reproductive system, it is very crucial that this system would be able to produce good sperm quality, and also be able to deliver them through ejaculation into the vagina in order to make conception possible [2]. The male reproductive machinery is an integrated system, that has been classified into primary and secondary reproductive organs along with three accessory sex glands. The primary male reproductive organ is the testis, which is housed and protected by the scrotum, as seen in figure (1), whereas the secondary male reproductive organs are the epididymis, vas deferens, the penis, and the three sex glands which are the seminal gland (vesicle), prostate gland, and bulbourethral bulb (Cowper's gland) [3].

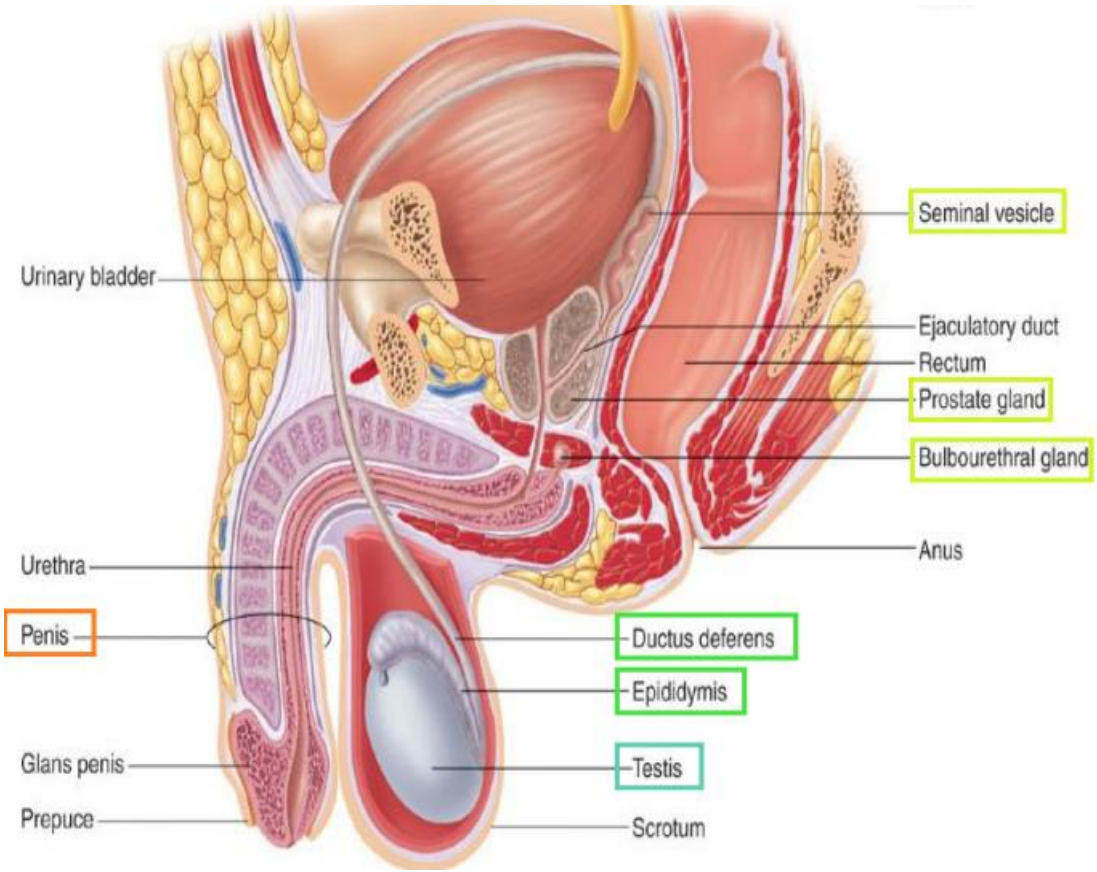


Figure (1): Male pelvis, showing multi-organs that make up part of the male urinary tract along with the male reproductive system [4].

1.1.1 Testicular structure and cellular organization

One important and interesting fact about the testis is that they are located outside the body cavity in the scrotum, which grants the capacity to make an isolated system in terms of temperature, putting and incubating the testis at typically 2.5-3 Celsius degrees below body temperature [5]. As a structure, the testis is a bicompartmental organ that is divided as follows:

1. The seminiferous tubules: forms ~90% of the testis, which are the long, tiny, coiled tubes that are well demonstrated in figure (2) where the sperm is produced and mature. These tubules are lined with germinal epithelium which is composed of epithelia cells, spermatogonia, and Sertoli cells in the basal lumina. In the body of the testis, there are hundreds of seminiferous tubules that combine together forming dozens that exit the testis from the efferent ducts where they meet together leading to the epididymis [6].

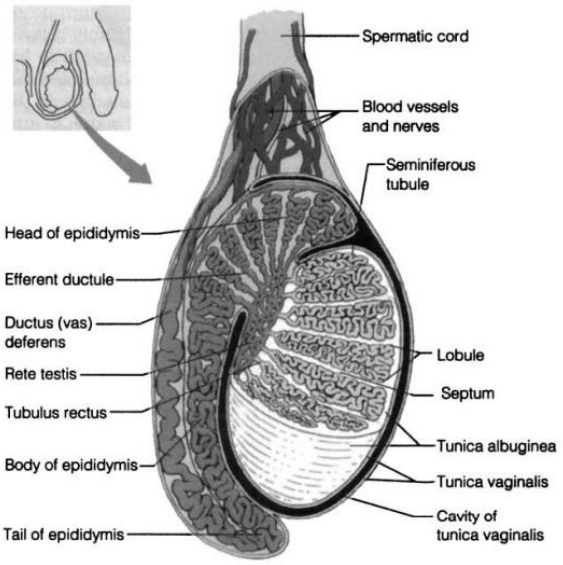


Figure (2): The testis structure, a schematic photo showing the overall structure of the testis: epididymis structure, seminiferous tubules [4].

2. Interstitial space between seminiferous tubules: forms ~10% of the testis, which contain a variety of specialized cells and most importantly the blood vascular element that are crucial in providing functionality in terms of fertility and cues for sperm maturation and development [6,7]. Referring to figure (3) we can see how components come together in the interstitial space.

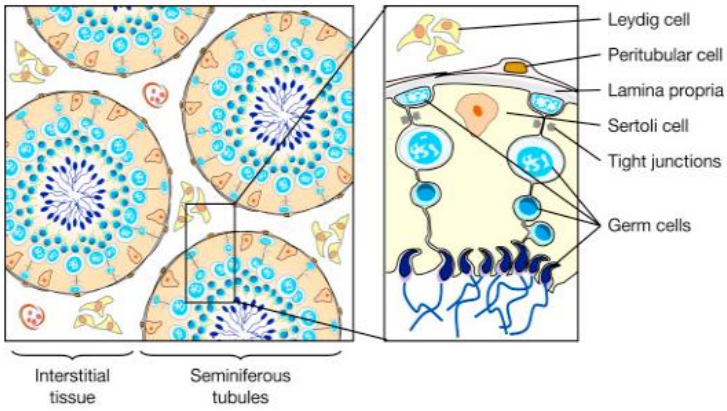


Figure (3): A schematic Cross section showing the lumen of the seminiferous tubules with the interstitial tissue, also cells involved in the niche and structure [14].

The testes contain three specialized cells: Leydig cells, Sertoli cells, and spermatogonium. Leydig cells are responsible for producing testosterone and insulin growth factor 3. Sertoli cells provide nutrition and support for the developing sperm cells, known as spermatids. These cells also play a role in the elimination of degenerating germ cells and excess cytoplasm through phagocytosis. Spermatogonium are the stem or precursor cells of sperm. [8,9,10,11,12]. Adhesion and tight junction proteins provide the basis for connection between Sertoli cells; these junctions prevent any possible diffusion from the interstitial space to the tubules, thus creating a specialized milieu capable of providing a niche for germ cell development. Consequently, the passage of substances from the circulation is prevented from entering the inner part of the seminiferous tubules. Amazingly, immature germ cells are located nearby the basement membrane of the seminiferous tubules, which provide those cells with access to signals and cues from the interstitial space [13] (figure 3).

Meanwhile, germ cells passing through meiosis I&II and haploid cell differentiation leading to spermatogenesis are placed within the blood-testis-barrier. Which provide and protect the space where division takes place happen, and thus cells there are being catered by BTB to be subjected to a unique Sertoli cell microenvironment [15]. Interestingly, the BTB creates an immune-privileged environment out of the seminiferous tubules [16].

1.1.2 Inducers of sperm production.

Sperm production is an orchestrated and highly regulated process, where each cell type in the male reproductive system plays a central role. As mentioned before, Sertoli cells are

responsible for nurturing the sperm cell and assist in releasing mature spermatids from the seminiferous tubules into the tubular lumen. As this step is very crucial, it is being facilitated by the production of two main hormones, inhibin and activin, which control the feedback of the anterior pituitary gland that secretes follicle-stimulating hormone (FSH). This system is in equilibrium and is quantitatively determined by means of the ability of a single Sertoli cell to provide cues to a certain amount of surrounding germ cells.

The Sertoli cell niche equilibrium, which is controlled [17] by factors such as species type, density of Sertoli cells, optimal Sertoli cell development before puberty, levels of germ cell apoptosis, and the endocrine environment, determines the effectiveness of germ cell development.

It is of paramount importance to know that Sertoli cells become mitotically inactive upon puberty, this takes place when the first germ cell goes into mitotic division along with the presence of a tight junction between Sertoli cells (a readily built BTB) [18].

Notably, the process of spermatogenesis begins in the fetal testis, when the Sertoli cell population is specified in the embryonic testis under the influence of male sex determining factors, such as SRY and SOX9. Destined and differentiated Sertoli cells are responsible for the seminiferous cord structures and dictate primordial germ cells to commit to the male pathway of gene expression. On the other hand, Leydig cells are the source of testosterone production. As the fetal Leydig cells are apparent after gonadal sex differentiation (gestational weeks 7-17 in humans) and, under the stimulation of placental human chorionic gonadotropin (hCG), results in the production of testosterone during gestation [19].

In the testis, testosterone is indispensable for sperm production. This takes place upon secretion of gonadotropin-releasing hormone (GnRH) which induces luteinizing hormone (LH) secretion, which in turn travels to the testis and induces Leydig cells to produce testosterone. The testes, in turn, feedback on the hypothalamus and the pituitary via testosterone and inhibin secretion, in a negative feedback loop to limit GnRH and gonadotropin production [20]. Good sperm production initially requires the dual action of testosterone and FSH [21].

1.1.3 Spermatogenesis.

As the name implies, spermatogenesis means sperm production or creation, where sperm are produced through a collaboration of systems, starting from spermatogonia and ending with a sperm capable of excelling in conception. This is ideally what we expect in a spermatogenesis. The main region where this process takes place, as mentioned before, is in the seminiferous tubules. This process passes through multiple steps and usually takes ~64 days to be accomplished [22].

Germ cells pass through multiple successive stages in order to form a sperm [23]. These steps are presented in figure (4) in terms of differentiation stage and its position in the testis.

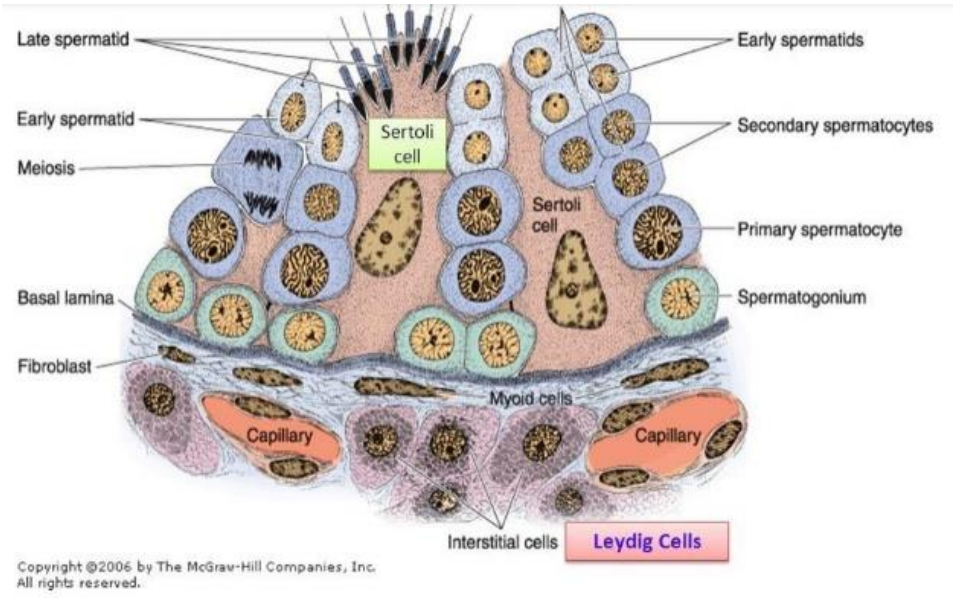


Figure (4): A cellular cross section about an eighth of the seminiferous epithelium with germ lines and interstitial tissue. From this scheme we can see the pre-meiotic cells (spermatogonia), located in the basal region of the seminiferous tubules, whereas the meiotic (spermatocytes) and the post-meiotic early and late spermatid cells are found organized in differential state order and/or maturation heading to the lumen of the seminiferous tubule [26].

Differentiation of spermatogenesis consists of several stages, including first spermatogonia, followed by primary spermatocytes, then secondary spermatocytes, making spermatids, and finally spermatozoa. During the spermatogonia stage, the cells have a large nucleus with dense heterochromatin and Golgi apparatus with small dense granular material in the

cytoplasm. In the primary spermatocyte stage, synaptonemal complexes form in the nucleus, and few mitochondria are present in the cytoplasm. In the secondary spermatocyte stage, there is very dense heterochromatin in the nucleus and small mitochondria, endoplasmic reticulum, and vacuoles in the cytoplasm. During spermiogenesis, the acrosomal vesicle moves towards the tip of the nucleus and becomes an elongated acrosome, the nucleus becomes elongated, and the head of the spermatozoon becomes elongated. In the final stage, spermatozoa, the centrioles form the axial filament of the flagellum and the axoneme of the tail flagellum is (9+2) structure, nine outer doubles with 2 two central singlets, regarding 9+2 structure it will be discussed further in coming chapters.

Epithelial cells in the seminal vesicles undergo morphological changes that are correlated with the accumulation of spermatozoa in the lumen. These changes can be divided into four phases: S-I (resting), S-II (early accumulating), S-III (active accumulating), and S-IV (spent). These phases are part of a periodic cycle of changes in the seminiferous epithelium, the morphological changes that occur in the seminal vesicle epithelium during spermatogenesis, are thought to play a role in the transport and storage of spermatozoa catering the process. [24].

A very interesting fact is that while this cycle of the seminiferous epithelium is taking place during spermatogenesis, the tight junctions in the BTB are disassembled and rebuilt, the mechanism underlying this process is still unclear [25].

Spermatogenesis is an important stage in human reproduction. Investigating and studying genes that appear to be associated in this process can help to identify genes involved in fertility [27].

1.2 Male infertility.

Infertility represents a steep health burden worldwide [28]. As a health concern, male infertility is heterogenous and represents a constellation of phenotypes. It is not a one term for one disease, but rather ranges from the first moment of spermatogenesis till the moment of sperm trying to penetrate the zona pellucida in the ova [29,30]. In each step of spermatogenesis, there are specific requirements that a sperm must meet in order to be considered fertile, such as proper tail production in terms of flagellar architecture, proper head shape, and enough mitochondria [31]. Proper signaling between the cells, especially

Sertoli cells, is necessary to create an appropriate microenvironment [32] and an appropriate hypothalamic pituitary testicular hormonal axis signaling capacity [33].

To start an investigation in male infertility, many medical institutions agree that after a year of failure to conceive while practicing unprotected intercourse, patients should come to the clinic and semen analysis is ordered as a first step in the investigation [34,35].

Semen analysis provides valuable information about the presence of sperm defects. The normal semen volume, sperm count, sperm vitality, sperm morphology, and presence of other cells in the ejaculate are important factors to consider when evaluating sperm quantity and quality.

Semen analysis is best done at a minimum of 2 days – and a maximum of 7 days of sexual abstinences. Parameters and recommendations are taken into considerations accordingly with the semen examination and processing taken from (WHO Laboratory Manual, 2010). Semen analysis provide us with phenotypes describing male fecundity [37], in terms of infertility these phenotypes should be followed by clinical, molecular and cellular investigations, However on the genetic and molecular scale around 2000 proteins are involved in spermatogenesis making the investigation in male infertility avide for the appropriate tool in the cellular and molecular lab investigations [38]. Since 15% of male infertility cases could directly be owed to genetical causes[39], efforts have been made in this respect such as the International Male Infertility Genomics Consortium (IMIGC), which provide overview on gene disease relationship through genomic analysis, statistical, bioinformatics data analysis along with functional studies and the goal is to accelerate genomic findings sharing, and introducing new genotype/s associated with certain phenotypes to databases, in which the ultimate goal is to facilitate patient care, As we can see in figure(5) efforts have been made shedding light on variety of genes that if mutated could be a cause for male infertility. Therefore, the identification of new genetic factors associated with idiopathic cases will significantly enhance our understanding of personalized patient care and provide capacity for proper genetic counseling, and, probably be able to avoid unwanted phenotypes in future generations through offering preimplantation genetic diagnosis (PGD) for cases with familial

male infertility history [40].

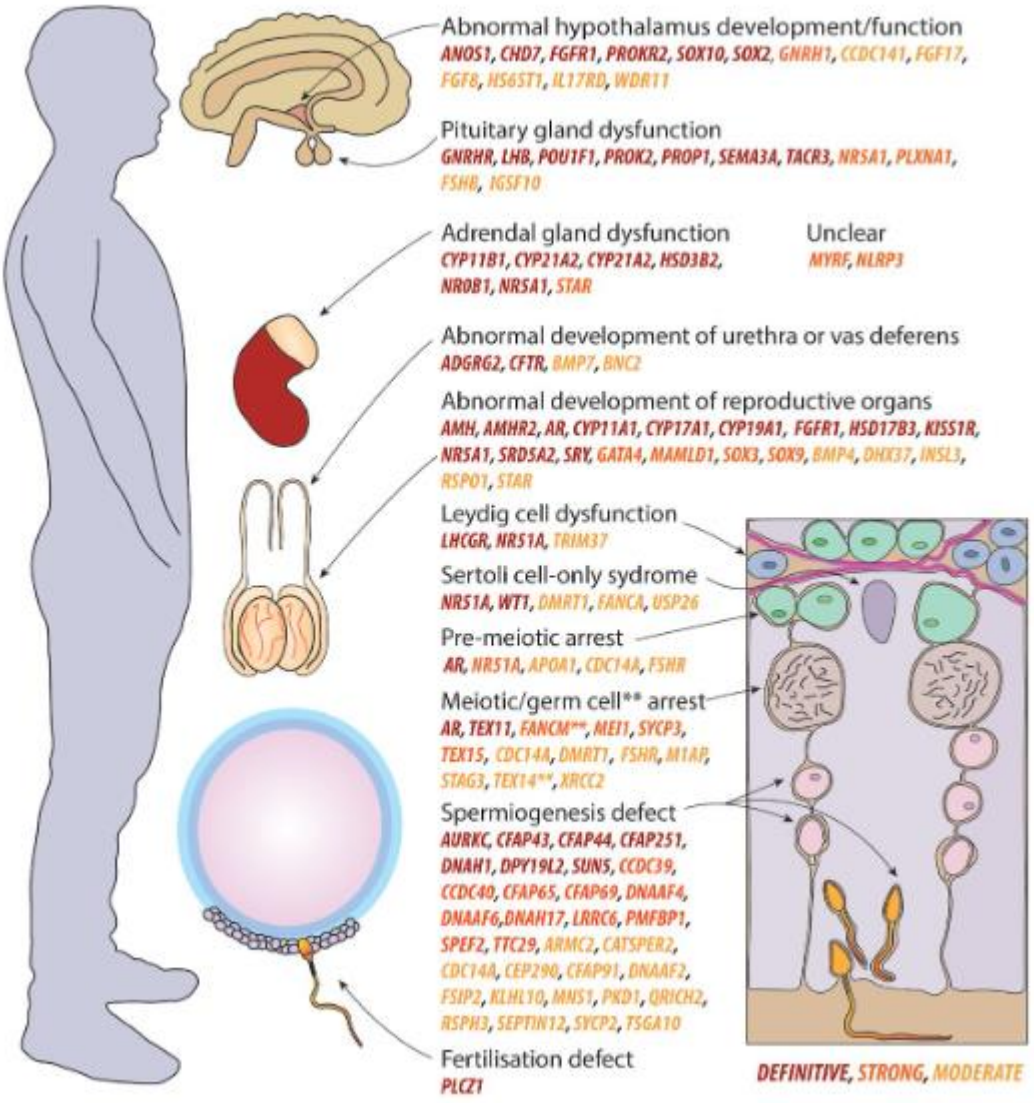


Figure (5): Overview of most commonly known monogenic possible mutations related to male infertility, with association of genes to affected organ or cell [41**].**

1.2.1 Multiple morphological abnormalities of the sperm flagella (MMAF) and Primary ciliary dyskinesia (PCD).

Cellular capacity to move is associated with functionality of the cell [42]. Although different in name, ciliary and the flagellar machinery of motility share similar structural unit, which is

called the axoneme.

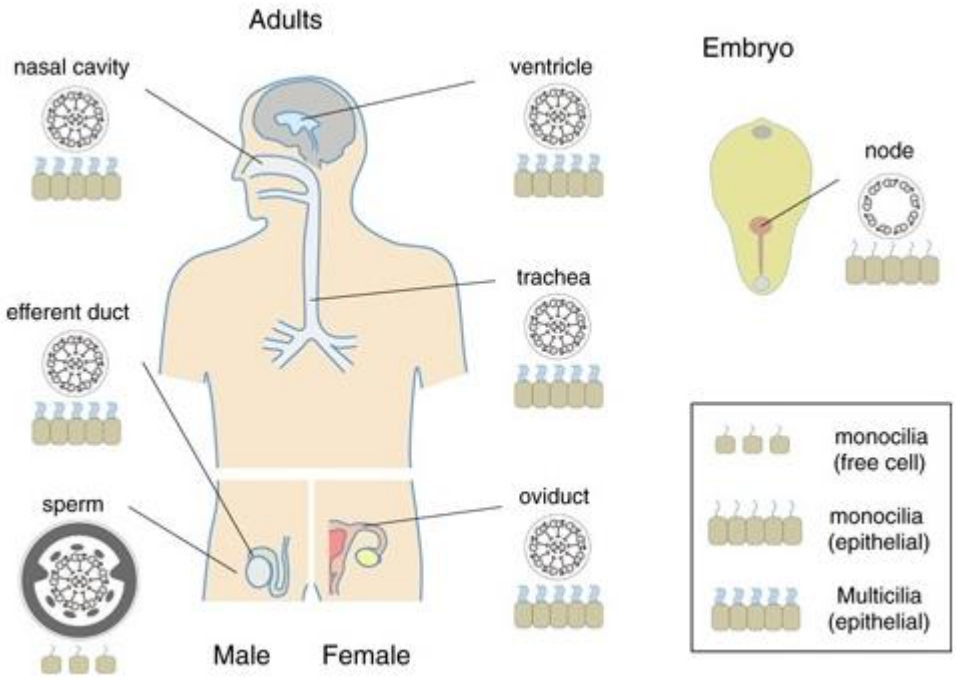


Figure (6): Possible occurrence of motile cilia and its type classified in the figure as mono-cilia like sperm flagellum or multi-cilia like epithelium of airway cilia. Also, the figure shows major similarities in the structure (9+2) axoneme though out different regions **[**43**]**.

Any disturbance in the protein content (genetic mutations) causes phenotypic off margins, which may lead to disease. Many diseases could be attributed to defective cilia or flagella, of which MMAF and PCD are examples. Their appearance is dependent on defective axonemal structure, but both are distinctive. In order to go through our visualization of MMAF vs PCD, we have to introduce two main definitions.

MMAF is an autosomal recessive disorder, responsible for defective formation of the sperm flagellum, leading to defective movement of the sperm, hence lack of appropriate capacity of performing functionality, which at the ultimate view is granting conception [44]. MMAF as a term has been highlighted and proposed in 2014 by Ben Khelifa, Mariem et al. and is considered one of the types of Astheno-teratozoospermia that is associated with mosaic flagellar defect, including (absent, bent, coiled, irregular, short) Sperm flagella [45].

PCD is usually an autosomal recessive genetic disorder, associated in a lot of cases with genetic heterogeneity and a wide spectrum of phenotypes, mainly caused by defective

formation of cilia and flagella, associated with multisystemic defects. Male infertility due to Asthenozoospermia is common among PCD patients [46].

Interestingly, a good question to ask is: since the cilia and flagella share an evolutionary conserved structure in humans, which could be deducted from figure (6), and these structures are called the axoneme, then why do we have variation in the phenotypes between MMAF and PCD, or would MMAF be a variation of PCD?

This question, as many suggest, remains elusive and needs further investigation. The spermatozoon of infertile patient with PCD resembles that of MMAF patient to some extent, however, this pathophysiological aspect of PCD require more research to offer better understanding. Indeed, the etiology of PCD patients may overlap with MMAF patients. However, sometimes PCD patients are not infertile, so it has been suggested that MMAF is independent from PCD. This suggestion is backed by the fact that there are phenotypic discordances between flagella and cilia, and that so far around 40 genes have been found to cause 70% of PCD cases, but few causes sperm flagellar defect. According to (Lores et al.), some proteins have different structural properties depending on the cell type, or presuming that there is something such as sperm-specific quality control, for example, ubiquitylation pathways to degrade a certain mutated protein for example [47], making MMAF appealing in certain mutations rather than PCD, and vice versa. Some proteins might not be crucial function wise in cilia as in flagella, and vice versa, and the site of expression plays a role, meaning some genes might be higher in expression in testis, whereas lower in expression in lung or trachea, such as (Dynein Axonemal Heavy Chain 1) DNAH1, which eliminate PCD symptoms in some instances [48]. Regarding phenotypes, PCD is different from MMAF in that it affects multiple organs. This is due to defective ciliary movement, which can cause respiratory distress in term infants, bronchiectasis, chronic rhinosinusitis, and conductive hearing impairment. The progression of PCD varies among patients, creating a spectrum of symptoms. These symptoms have been attributed to different mutations, with some mutations being more aggressive than others. Additionally, Situs inversus (Kartagener syndrome) can be detected in 50% of PCD patients. In contrast, MMAF only affects Sperm Flagella function and patients with MMAF are typically characterized by male infertility.

1.2.2 Cilia and flagella architecture.

Cellular movement in general is facilitated by many means, e.g. (amoeboid movement, ciliary, gliding motility, flagella, mechanotaxis, chemotaxis etc.). Notably, ciliary and flagellar means share the same molecular structure, but are different in nomenclature and distribution. Years before, they were studied, and a flagellum used to be the name of a beating one-eight flagella, whereas ciliated cells tend to have larger numbers (hundreds) of short cilia. Several studies, which were pioneered by Fawcett and Porter in 1954, which followed Manton et al 1952, showed that cilia and flagella share the same building block, which is termed as axoneme, showing 9+2 uniformity [49,50]. This was followed by the discovery, identification, and characterization of the dynein family proteins, which appeared to be a conserved and a multi-functional molecular motor protein. This work was accomplished by Ian Gibbons and colleagues [51], also describing the mechanochemical cycle corresponding to the sliding coiled-coil mechanism between the microtubules and the dyneins arms [52].

As shown in figure (7), an axoneme is composed of a fibrillar bundle, where a central pair of singlet microtubules is surrounded by nine outer doublet microtubules. In a transverse section, this structure is continuous longitudinally in the axoneme, and is referred to as (9+2) arrangement, and is contained within the plasma membrane of the cell.

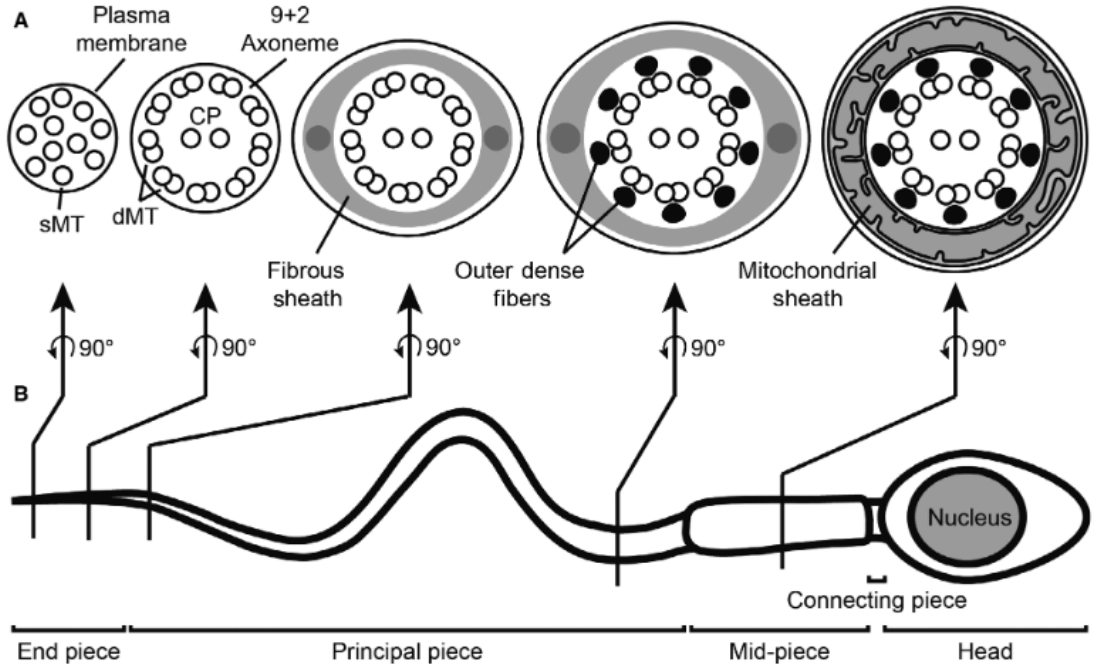
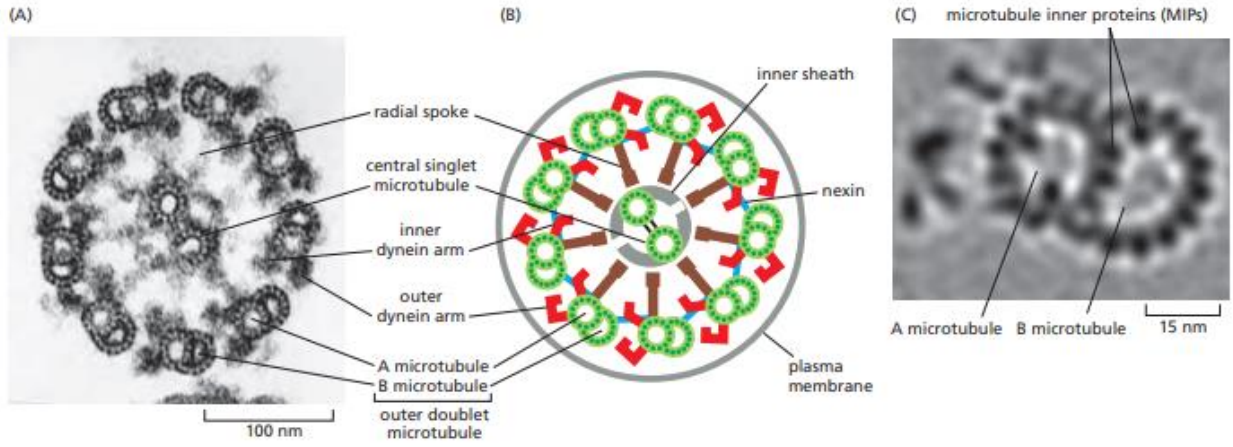


Figure (7): A cross section along the sperm flagellum showing internal ultrastructure [53].



Figure(8): Axonemal Architectural structure of cilia and sperm flagella [54**].**

This composition is found virtually in all eukaryotic cilia and flagella, and is about 5- to 10-μm in length, and around ~300-nm in diameter [55].These specialized tubulin polymers are arranged transversely, where Microtubular doublet A and B account for each doublet microtubule, and are arranged as in figure (8) were the A tubule appears as a complete microtubule with 13 protofilaments, while the B tubule, which doesn't appear as a full tubule, has 10 protofilaments [56].

From the inner side and the outer side of the A tubule of each doublet, is attached dynein arms all along the axoneme, forming a row of this attachment, with these dyneins reaching to the adjacent B tubule, as explained in the figure above.

Tektins, which represent a very interesting set of microtubule-associated proteins, have been sought as possible contributors to the structural property of the axoneme. They are present in the axoneme in the place where the B tubule attaches to the A tubule and near the binding sites of the radial spokes' inner dynein arms and the nexin links. Notably, they are stable filaments that remain after the extraction of doublet microtubules [57].

An axonemal structure holds itself mainly through three sets of protein cross-link system and are as follow.

Periodic bridges and the inner sheath connect central singlet microtubules, forming a fibrous structure that surrounds central singlets. Nexin protein joins adjacent outer doublet microtubules and is suggested to be a part of the dynein regulatory complex, which regulates motility and axonemal dynein [58]. Radial spokes bind only to the A tubule of the outer

doublets and are a crucial part in regulating flagellar motility and forming the third linkage system [59].

Interestingly ciliary and flagellar axoneme is a closed system meaning if we isolated an integrated axonemal structure from cellular body, it would still be able to generate bending motion when ATP is available. The movement or the beating of the cilia or the flagella could be described as a progression of curves, starting from the bottom of the pattern spreading toward the tip. This waveform (beat) or bending pattern can be evaluated using high-speed strobe microscopy [60].

1.2.3 Molecular aspects of MMAF.

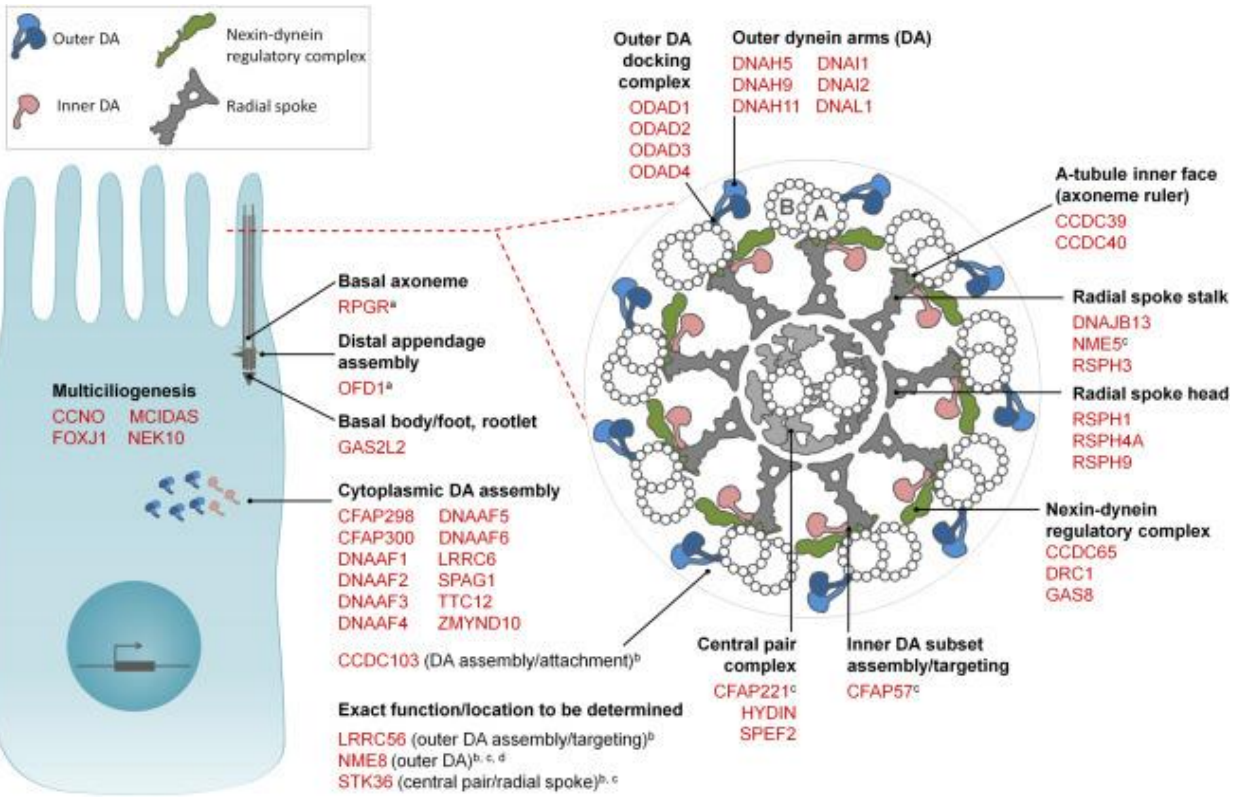


Figure (9): Functionality possible loss associated with possible mutations in the genes shown, showing Known Genes associated with Ciliopathies [61**].**

As we can see from Figure 9, ciliopathies genetic causes vary and come along with architectural defects of the axoneme. This gives rise to multiple different phenotypes. Amazingly, in MMAF infertility is the only obvious phenotype and could be highly and with confidence attributed to specific proteins. As Table 1 shows, proved genes in terms of functionality that are associated and reported with MMAF.

Table (1): Genes associated with MMAF, data about gene chromosomal position and accession number along with structural-mutation affect in the table have been adapted from (NCBI website - gene category) [62,61].

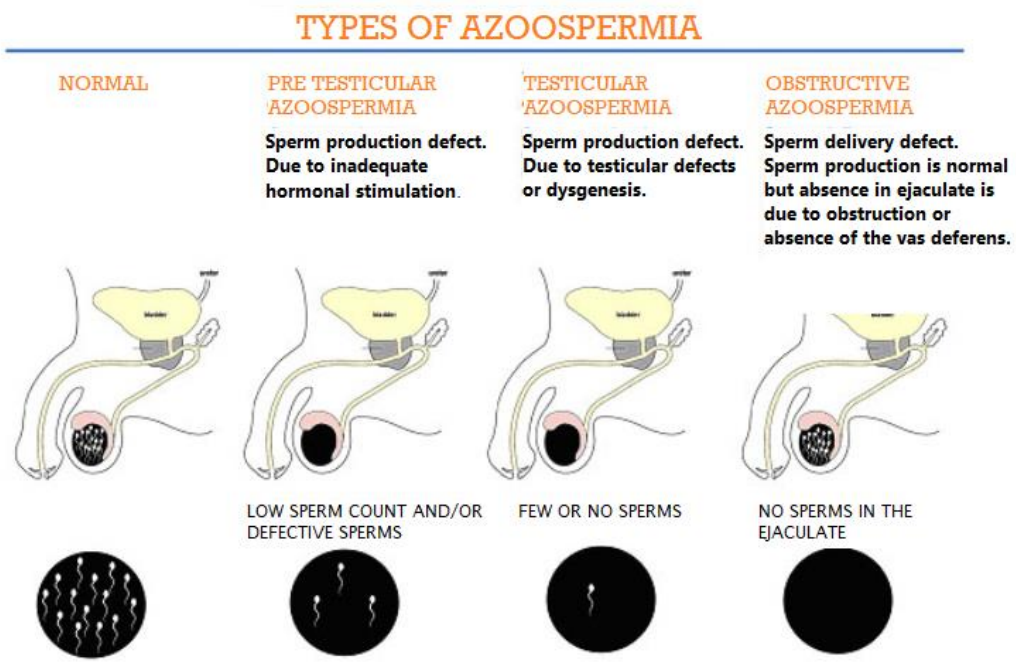
Gene Symbol according to HGNC	phenotype OMIM Number	Gene/Locus OMIM Number/NM accession number	Chromosomal location	Mode of inheritance	Mutation position in the structural entity of the sperm	Observed phenotype under electron microscopy
AK	617965	615364 / (NM_152327)	14q32.2	Autosomal recessive	mutation result in (adenylate kinase-7) defect	Severe disorganization of the 9+2 axonemal structure due to Absence of CP and IDA
AKAP4		(NM_003886)				
ARMC2	618433	618424 / (NM_032131)	6q21	Autosomal recessive	Mutation result in (armadillo repeat-containing protein-2) defect	Absence of the CP producing (9 + 0) structure lead to Severe axonemal disorganization
CFAP43	617592	617558 / (NM_025145)	10q25.1	Autosomal recessive	Mutation result in (cilia- and flagella-associated protein-43) defect	Disorganization of IDA, Absence of CP leading to (9+0).
CFAP44	617593	617559 / (NM_001164496)	3q13.2	Autosomal recessive	Mutation result in (cilia- and flagella-associated protein-44) defect	Absence of CP, leading to (9+0).
CFAP65	618664	614270 / (NM_194302)	2q35	Autosomal recessive	Mutation result in (cilia- and flagella-associated protein-65) defect	Absence of CP, disorganized or absent doublet microtubules
CFAP69	617959	617949/ (NM_001039706)	7q21.13	Autosomal recessive	Mutation results in (cilia- and flagella-associated protein-69) defect	Required for flagellum assembly and stability63
CFAP251	618152	618146 / (NM_144668)	12q24.31	Autosomal recessive	Mutation results in (WD repeat-containing protein-66)	Disorganization in the axonemal and periaxonemal

					defect	structure.
DNAH1	617576	603332/ (NM_015512)	3p21.1	Autosomal recessive	Mutation results in (axonemal dynein heavy chain-1) defect	Disorganization of inner dynein arms, absent CP.
DNAH6	603336	NM_001370	2p11.2			dynein axonemal heavy chain 6
DNAH17	618643	610063 / NM_173628)	17q25.3	Autosomal recessive	mutation result in (axonemal dynein heavy chain-17) defect	Absence of central pairs and outer dynein arms
FSIP2	618153	615796 / (NM_173651)	2q32.1	Autosomal recessive	Mutation results in (fibrous sheath-interacting protein-2)defect	Associated with Abnormal conformation of axoneme
MNS1	618948	610766/ (NM_018365)	15q21.3	Autosomal recessive	meiosis-specific nuclear structural protein-1	
ORICH2	---	(NM_032134)				
SPAG17	---	(NM_206996)				
TTC21A	618429	611430/ (NM_145755)	3p22.2	Autosomal recessive	Mutation results in (tetratricopeptide repeat domain-containing protein-21A) defect	Misplaced or absent central-pair and/or peripheral microtubules
TTC29	618745	618735/ (NM_031956)	4q31.22	Autosomal recessive	Mutation results in (tetratricopeptide repeat domain-containing protein-29) defect	

As we can see from Table 1, several genes have been identified as being associated with abnormalities in the flagellum, or tail, of a sperm. These abnormalities may range from defects in the structure or shape of the flagellum to defects in sperm motility. These genes are involved in the development and function of cilia and flagella, as Figure 9 suggests, which help sperms to move. Abnormalities in these genes can lead to a variety of problems with the structure and function of the

flagellum, which can ultimately affect fertility. Still, along with this effort, 50% of cases showing asthenospermia are remained unsolved [64]. As for the mode of inheritance, most are found in the autosomal recessive pattern, though that does not mean it cannot be found in a compound heterozygotic form [65].

1.2.4 Azoospermia approach and definition.



Figure(10):A simple representation and an explanatory figure for multiple types of azoospermia [66].

As a definition azoospermia is a condition in which there are no sperm in the semen when a man ejaculates [67],which is well presented in figure (10).

Azoospermia, characterized by the absence of sperm in semen, is classified into two main categories - non-obstructive azoospermia (NOA) and obstructive azoospermia (OA). NOA results from primary or secondary testicular failure in spermatogenesis while OA is caused by an obstruction in the male reproductive tract[68].

Reasons behind non-obstructive azoospermia could be complicated and need deep investigation on multiple levels such as karyotyping looking for chromosomal anomalies, Y-chromosome microdeletions, hormonal investigation, monogenic defects such as loss of specific gene signaling involved in spermatogenesis leading to maturation arrest, TFNA.

The OA differs in that its diagnosis depends on imaging, genetic testing such as mutations leading to obstruction such as cystic fibrosis transmembrane conductance regulator (CFTR)

mutations which may cause congenital unilateral or bilateral obstructive vas deferens [69], physical examination, and testicular fine needle aspiration or testicular biopsy.

1.2.5 Molecular and cellular aspects of non-obstructive azoospermia.

Testicular azoospermia or non-obstructive azoospermia, more broadly refers to the absence of germ cells which happen to be due to spermatogenic failure, even though proper functioning of the hypothalamus and the pituitary glands is present [70]. Importantly, germ cells in testicular azoospermia may be totally absent. This case is known as Sertoli cell only syndrome (SCO) or it may be a maturation arrest in spermatogenesis [71].

Factors that promote testicular azoospermia are multifactorial and phenotypes are in many cases heterogenous. Though, below we mention the most common reasons for an investigator to search in, when the concern is studying azoospermia [72,73,74]:

1. **Chromosomal abnormalities (Aneuploidy).**
    A. Klinefelter syndrome, karyotype (47XXY), is the most common syndrome associated with azoospermia [75]. Klinefelter syndrome accounts for around 14% of azoospermia cases [76]. It happens to occur due to the presence of an extra X-chromosome, in which evidence suggest that it causes degeneration of early germ cells epithelium [77].

    B. De la Chapelle syndrome or (46, XX) male syndrome, is a rare aneuploidy that occurs at a population frequency of 1 in 20,000 live births [78]. 46, XX male syndrome occur due to the translocation of the SRY from the Y-chromosome to the X-chromosome or an autosome, 46, XX males are azoospermic because of lack of AZF region which takes place on the Y-chromosome.

    C. (45, XO/46, XY) is produced due to mitotic loss of Y-chromosome during embryo development producing a mosaic karyotype. (45, XO/46, XY) cases have a normal SRY gene, though, mosaicism in SRY affects testis–determining factor, which along with other factors plausibly may play a role in gonadal failure, leading to azoospermia or oligozoospermia in most cases.

    D. Chromosomal translocations associated with (46, XY).

Robertsonian translocations: The frequency of Robertsonian translocations is 1 in 1000 live births and is considered the most common reason for male infertility [79].this type of chromosomal anomaly take place between two acrocentric chromosomes it occurs as a fusion

between long arms near the centromere and short arms which contain heterochromatin so short arms are lost but with no phenotypic implication, in humans we have six acrocentric chromosomes Chr. Y, Chr.13, Chr.14, Chr.15, Chr.21 and Chr.22 [80].with rob[13:14] as being responsible for 50%-100% of infertile men cases, phenotype regarding this translocation varied between azoospermia and oligozoospermia [81].

Reciprocal translocation: represent a very important aspect when investigating azoospermia and are 9 folds more frequent in infertile males than in fertile males. Reciprocal translocation may Affect testis-specific genes, along with other genes that may regulate testicular function.

Chromosomal inversions: inversions in autosomes 1, 3, 4, 6, 9, 10, and 21 appeared to be more reluctant in infertile men, notably Inversions on chromosome 9 showed association with azoospermia eight folds more common in infertile men.

2. Y-chromosome microdeletion.

Y-chromosome carries a lot of important genes and contain critical regions that define male phenotypes and those regions involve multiple gene families important for germ cell development and differentiation , microdeletions in these regions affect Y-chromosome microdeletions are very common cause explaining azoospermia and is found to be responsible for (5%- 15%) of azoospermia cases this region is called the (Azoospermia factor) AZF region and its frequency is represented in the table 2.

Table (2) : Showing most prevalent microdeletions on the Y chromosome [82].

Azoospermia Factor family	**Frequency in cases**
AZFa	7.7 %
AZFb	0%
AZFc	76.9 %
AZFa+AZFb	7.7%
AZFa+AZFc	0%
AZFb+AZFc	7.7%

Cases that are presented with azoospermia due to AZF deletions are liable for Y chromosome loss. Y-chromosome microdeletions are considered the most common cause of spermatogenic failure after the Klinefelter syndrome.

3. Monogenic factors:

Common genetic factors that cause azoospermia are summarized in Table 3.

Table (3) :List of most common monogenic factors associated with non-obstructive azoospermia [83].

ble 2 Established genes implicated in monogenic causes of non-obstructive azoospermia (NOA)

ene	Function	NOA phenotype [MIM]	Inher. mode	mRNA expression[a]	Reported families/ patients (n)[b]	Reported variant type	Mouse model[c]	Other phenotype [MIM]	Core references link to NOA
olated NOA									
ANCM	DNA repair, inter-strand cross-link removal	SCOS, oligoas-thenospermia [618086]	AR	Testis enhanced	1/3 cons., 3/4 non-cons	LoF	Yes	POI [618096]; breast, ovar-ian and prostate cancer	Kasak et al. (201 and Yin et al. (2019)
EI1	Chromosome syn-apsis in meiosis	MA	AR	Testis enhanced	1/2 cons., 1/1 non-cons	Missense, LoF	Yes	Hydatidiform mole [618431]	Ben Khelifa et a (2018), Nguye et al. (2018)
EIOB	DNA DSB repair, crossover forma-tion and promo-tion to complete synapsis	MA [617706]	AR	Testis enriched	2/6 cons., 1/1 non-cons	LoF, missense	Yes	POI	Gershoni et al. (and Gershoni ((2017)
TAG3	Cohesion of sister chromatids, DNA DSB repair	MA	AR	Testis enriched	2/2 non-cons	LoF, missense	Yes	POI [615723]	Riera-Escamilla (2019) and var Bijl et al. (201
EX11	Chromosome syn-apsis and forma-tion of crossovers	MA, mixed tes-ticular atrophy [309120]	XLR	Pancreas, testis enriched	multiple	LoF, missense	Yes	Not reported	Nakamura et al. (2017), Sha et (2018), Yang e (2015), and Ya senko et al. 20
EX14	Formation of mei-otic intercellular bridges	MA, SCOS [617707]	AR	Testis enriched	2/4 cons., 2/2 non-cons	LoF, missense	Yes	Not reported	Fakhro et al. 201 Gershoni et al. (2017)
EX15	Chromosome, syn-apsis, DNA DSB repair	MA [617960]	AR	Endometrium, smooth muscle, testis	1/3 cons., 1/2 non-cons	LoF	Yes	Not reported	Colombo et al. (2017) and Ok man et al. (201
ndromic NOA									
R5A1	transcription factor (sex determina-tion)	SCOS, MA [613957]	AD incompl	Adrenal gland, ovary, spleen enriched	Multiple	Missense, LoF	Yes	46,XY and 46,XX sex reversal [617480, 612965]; adrenocortical insufficiency [612964]; POI [612964]	Bashamboo et al (2010), Ferlin (2015) and Za Abdollahi et al (2015)
STX	DNA and RNA processing	MA	AR	All tissues	2/2 non-cons	LoF, missense	Yes	Amyotrophic lateral sclerosis [602433], ataxia with oculomotor apraxia type 2 [606002]; POI	Becherel et al. (and Catford et (2019)
T1	Transcription factor	SCOS, MA	AD	Endometrium, fallopian tube, smooth muscle enhanced	Multiple	Missense	Yes	Wilms tumor [194070]; nephrotic syn. [256370]; mesothe-lioma [156240]; Meacham syn. [608978]; Frasier syn.[136680]; Denys-Drash syn. [194080]; POI	Seabra et al. (20 Wang et al. (2 and Xu et al. (

4. **Cryptorchidism.**

As a definition cryptorchidism is the absence of testes from the scrotum, it is considered as one of the most frequent causes responsible for testicular failure and/or non-obstructive azoospermia. Notable in cryptozoospermic men (intermittent detection of spermatozoa in ejaculate) due to isolated foci of active spermatogenesis [84],which allow spermatozoa retrieval in some cryptorchidism cases .

5. **Copy number variants (CNV).**

WES data could bear valuable information regarding CNV explaining some cases in NOA [85]. As multiple studies have reported that infertile men carry more CNVs compared to normal fertile males (controls) which may be leading to instability in the genome making CNVs related to NOA in many cases [86].

1.2.6 Environmental factors affecting sperm motility and spermatogenesis

Environmental factors affecting spermatogenesis vary between reversible (could be transient if the source is avoided) and irreversible (if it was subjected to in certain developing age) and sometimes could be hard to be identified and trace. Several environmental factors affect male infertility, such as Smoking, Drugs, Diet, Psychological stress and Depression, stress, Obesity, Chemicals in the environment such as pesticides, air-pollution, Heat. [87].

Since semen quality is a measure for male fertility [88], it has been found that these adverse environmental factors affect mainly sperm quantity and quality, causing abnormal sperm morphology, abnormalities in the building blocks of the sperm, including deformed head, loss of tail, and also affecting motility. This makes the environment a risk factor for the asthenospermia phenotype. Additionally, sperm viability and thrive can be affected by the environment, leading to the production of dead sperm and sperm with high levels of fragmented DNA. Sometimes, these environmental factors can also block signals of spermatogenesis, resulting in decreased sperm count, as summarized in figure (11) below.

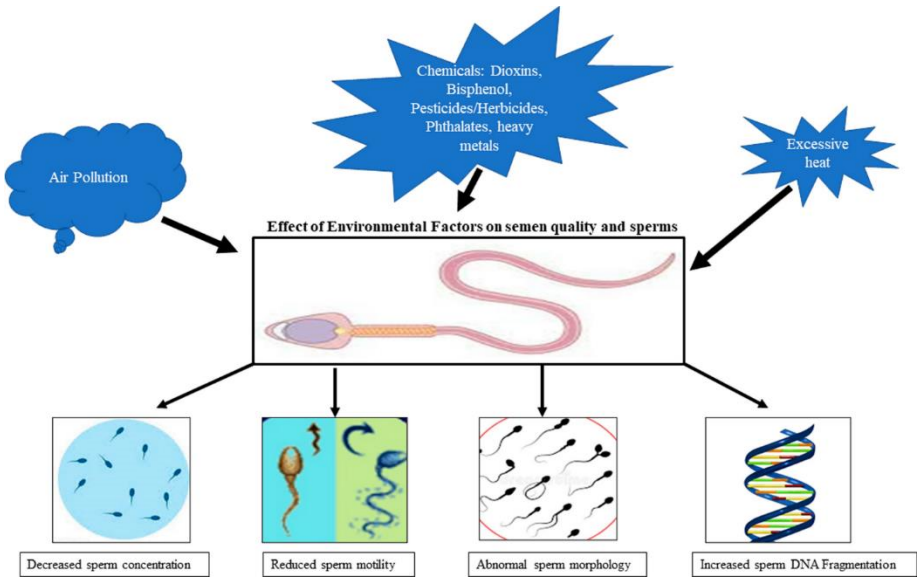


Figure (11):The figure show some common environmental factors that may render a sperm unfunctional [89].

1.3 Therapeutics and Possible solutions

Infertility is a difficult area in therapeutics and medical management, but new technologies, such as the CRISPR Nickase system and organ-on-a-chip technology, are helping to improve treatment and diagnosis. These technologies may play a significant role in addressing genetic factors that contribute to infertility. They also enable a more personalized medicine approach.

1.3.1 Recent advances in understanding male infertility, testing on a chip

Personalized medicine has long been a goal in the field of therapeutics, but traditional animal models have been limited in their ability to predict responses in humans due to differences in molecular cues between species. To address this, a new technology called organ-on-a-chip has been developed. This technology creates a micro-physiological system that can mimic organ-level function and is useful for studying drug action. Specifically, testis-on-a-chip has been gaining attention for its potential to provide new insights into spermatogenesis and therapeutic approaches for male fertility. However, there are concerns about the cost and labor intensity of this technology and its adoption in the scientific community. Despite this, researchers hope that organ-on-a-chip technology will become more widely adopted in the near future as a cost-effective way to achieve personalized medicine [90-94].

1.3.2 Current and recent possible therapeutics and approaches in asthenospermia

Asthenospermia is a condition that can be difficult to diagnose using current panels, as new genes may arise that are not detected by these panels. One proposed solution is to use patient cells to create in-vitro differentiation models, such as a testis-on-a-chip, to study the development of sperm and determine if there is a change due to the loss or presence of a gene. This could be done in parallel with CRISPR-Cas9 modified cells, where the lost gene would be repaired or replenished to restore its function. This approach could provide new ways to treat patients and may have potential applications for other diseases in both males and females. However, prior to this work, a whole exome sequencing and possibly a whole genome sequencing may be needed to have a complete understanding of the patient's condition.

1.3.3 Current and recent possible therapeutics and approaches in azoospermia

Currently, there are few effective ways to treat male infertility, particularly azoospermia. One potential solution is the production of sperm or male gametes in a cell culture in-vitro, by reprogramming cells such as embryonic stem cells, mesenchymal stem cells, spermatogonial stem cells or induced pluripotent stem cells. However, the protocols for producing functional sperm from these cells need further investigation. In-vitro cytoplasmic sperm injection (ICSI) is a technology that can help azoospermic patients father a child depending on the specific nature, presence of sperm and potential for hormonal replacement therapy. Additionally, CRISPR gene editing has become a promising tool that can be used in monogenic cases followed by ICSI. The future hope is that IPSC differentiated into sperm combined with CRISPR-Cas system for gene editing will produce healthy individuals free of azoospermic mutations [95-96].

Objectives

Providing new candidate genes that might be responsible for asthenospermia, as well as for azoospermia, within the Palestinian population, would be considered as a counseling and advisory advantage to a relatively large number of people suffering from infertility in the Palestinian community due to the prevalence of consanguineous marriages, which is set at a rate of around 40% according to the Central Bureau of Statistics in Palestine. There is abundant evidence that infertility has a strong genetic basis, which is still not very well understood [97,98].

In order to gain a deeper understanding of the molecular mechanisms underlying male infertility in the Palestinian population, we conducted a study using whole exome sequencing (WES) on two Palestinian individuals from different families, one suffering from asthenospermia and the other suffering from azoospermia.

The objective of our project was to **identify novel mutations/genes responsible for male infertility due to MMAF or azoospermia.**

- This was achieved by using an exome-based approach in 2 independent patients with a very well-characterized MMAF and azoospermia phenotype.
- The best candidate genes identified by exome were subsequently screened in other affected family members of our cohort by Sanger sequencing.

Literature Review

According to the European Association of Urology (EAU), infertility is defined as the inability of sexually active couples to conceive spontaneously while practicing unprotected sex for 12 consecutive months [99].

WHO declared infertility as a global public health concern [100], even though we still lack an accurate, comprehensive view over global statistics regarding male infertility. [101] It is estimated that globally, one-third of couples suffer from issues related to fertility [102]. Along with estimations that males are responsible for 20-70% of infertility among couples [103].

According to reports from the Palestinian Central Bureau of Statistics (PCBS), it has been found that 8.4% of couples aged between 15 and 49 years suffer from infertility, with male factors owing to 30-40%; female factors 40-55%; 10% are regarded as combined factors of both males and females, and 10% of infertility cases are left unexplained [104]. Male infertility can be caused by several factors, including genetic defects, lifestyle habits, chronic medical disorders, and medications. Genetic causes of infertility are heterogeneous, and their variations represent a challenge when it comes to genotype-phenotype correlation. It is well-established that certain chromosomal anomalies or mutations in certain genes may have a spectrum of phenotypes that rise challenges in clinicogenetic correlations [105,106].

Accordingly, male infertility could be due to many causes, notably multiple morphological abnormalities of the flagella (MMAF). “Asthenozoospermia” is regarded as one of the most severe forms of qualitative sperm defects [107].

Meanwhile, azoospermia in the field of male infertility represents the highest challenge in terms of providing solutions, especially in cases of non-obstructive azoospermia [108]. It is essential to understand the azoospermia population and be able to provide good counseling, and to understand non-obstructive azoospermia causative genes, which might reveal essential factors in spermatogenesis [109].

Chapter 2
Materials and Methods.

2.1 Participants

Following the approval of the ethics committee of Al-Istishari Hospital in Ramallah, and according to the local protocols and the principles of the declaration of Helsinki, an informed consent has been obtained from individuals participating in the study.

Our study was concerned with patients that have non-obstructive Azoospermia or MMAF with/without PCD features and was based on two individuals from two different families. We informed participants that this is part of a research concerning investigating infertility, which is done at Al-Quds University.

Patients have been recruited from the branches of Razan IVF center in Palestine, along with other IVF centers and infertility clinics. Clinical diagnosis has established the patient as infertile, azoospermic, or have spermatozoa with 5 morphological abnormalities for the case of MMAF: short, absent, coiled, bent, and irregular flagella. In order to homogenize the collected clinical data, a standardized clinical form has been designed and filled by the referral doctors. Common causes of azoospermia, such as chromosomal numerical defects and microdeletion of Y chromosome, have been excluded by performing karyotyping and PCR (Deletion testing for AZF-a, AZF-b and AZF-c) respectively. For some patients, results were supplemented with testicular fine-needle aspiration (TEFNA), Testicular sperm extraction (TESE), gonadotropins level and androgen level checkup.

1. Case suffering asthenospermia:

Our asthenospermia probands have been defined as asthenospermia based on diminished sperm motility and not associated with other clinical problems. Based on this, the case was selected for further investigation. Unfortunately, we could not get any information regarding other family members from our asthenospermia proband in order to fully segregate our candidate genes because we lost contact with the patient.

2. Case suffering from azoospermia:

In this study, we adopted a patient suffering non-obstructive azoospermia, which is a condition characterized by the absence of sperm in the semen due to a problem with sperm production rather than a blockage in the reproductive tract. The Patient with non-obstructive azoospermia that was considered in the study met specific criteria related to the diagnosis and presentation of the condition.

2.2 Extraction of Genomic DNA

Five mL of peripheral blood has been collected from each participant. We have 3 participants in the study (the asthenospermia case, the azoospermia case, and his brother); DNA has been extracted as follows, which is according to the manufacturer instructions:

DNA was extracted using the MACHEREY-NAGEL, Nucleospin blood XL by following the company instructions. The protocol briefly was carried out by mixing 5 ml of blood with protease K and buffer BQ1. Then 5 ml of ethanol was added. The lysate was loaded into a NucleoSpin Blood L column and centrifuged at 4000 x g for 3 minutes, and the process was repeated twice to improve the precipitation quality. The silica membrane was washed twice with BQ2 at 4000 x g for 10 minutes. The pure DNA was then eluted by adding preheated buffer BE at 70°C, followed by incubation for 2 minutes and centrifugation at 4500 x g for 2 minutes. The quality of the DNA was then checked using Nanodrop, and was stored at 4°C until needed for further experiments.

2.3 Whole exome sequencing (WES)

WES with a coverage of 45 Mb of exonic content were captured on NextSeq 500 (Istishari Arab Hospital, Ramallah, Palestine). The gDNA was quantified using Qubit V.3 and quality was checked by gel electrophoresis. Library preparation was carried out using TruSeq Capture Exome Kit (Illumina). The probe set was designed to enrich 214,405 exons. After Sequencing, data were uploaded onto a server, and reads were aligned to the reference human genome (hg19) using BWA aligner. Prior to variant calling by GATK (Genome Analysis ToolKit), mapped reads (BAM format) went through preprocessing steps by removing PCR duplicates, realigning around indels, and recalibrating base quality. The final list of variants was annotated by ANNOVAR using several databases of minor allele frequency such as 1000G as well as variant effect predictors such as SIFT, Polyphen2, and CADD. Variants

with low coverage, synonymous, predicted benign, MAF 1% on GnomAD and 1000G were filtered out.

2.4 Polymerase Chain Reaction (PCR)

A. The following protocol describes the steps taken to perform Polymerase Chain Reaction (PCR) for the amplification of the CCDC40 gene. After DNA extraction, the PCR was performed using Promega's Master Green Mix (Lot #: 0000410912, Ref# M7128) and primers designed to detect mutations of interest. The primers were normalized by mixing them with RNase-free water from Qiagen (Lot#: 142326457) at a 1:20 ratio. The primers used in the PCR reaction were as follows:

Reverse Primer: 5'ACGTTAGAGGGTCACAAAGTT3'

Forward Primer: 5'CCCTTGACCCAGCTTTTACC3'

The PCR program used for the amplification of the CCDC40 gene is represented in table 4 :

Table 4: PCR Program for CCDC40 Gene Amplification

Temperature (°C)	Duration
98	30 Seconds
98	10 Seconds
60	10 Seconds
72	1 Minute 15 Seconds
72	5 Minutes
10	Infinity

The PCR reaction mix consisted of the following components:

1. 10 µL of Promega's GoTaq Master Green Mix (Lot #: 0000410912)
2. 2 µL of primers (1 µL of reverse primer and 1 µL of forward primer)
3. 2 µL of patient DNA
4. H2O was added to bring the final volume to 20 µL.

B. The following protocol outlines the steps for performing (PCR) to test for Y-chromosome microdeletions. The PCR program used for this test is presented in Table 5. The primers used in the PCR reaction were taken from Peter et al (Methods Mol Biol, 2013) and were designed to amplify the genes ZFY-SRY-SY86-SY254-SY127-SY84-SY134-SY255 table 5 explains the cycle program.

Table 5: PCR Program for Y-Chromosome Microdeletion Testing

Temperature (°C)	Duration
95	5 Minutes
57	1 Minute 30 Seconds
72	1 Minute
Cycles Done X34	Cycles Done X34
72	5 Minutes
12	Infinity

The PCR reaction mix consisted of the following components:

1. 10 µL of Promega's GoTaq Master Green Mix (Lot #: 0000410912)
2. 2 µL of primers (1 µL of reverse primer and 1 µL of forward primer)
3. 2 µL of patient DNA
4. H2O was added to bring the final volume to 20 µL.

The gel was prepared using 1 gram of agarose and 100 ml of TEA buffer, which was boiled in a microwave and then cooled with the addition of Ethidium bromide. The gel was cast into a tray with combs and used for gel electrophoresis at 120 volts for 30 minutes.

2.5 Sanger sequencing

Sequencing has been carried out on the ABI 3500 (Applied Biosystems) at the Ishtishari Arab Hospital, we converted AB1 file into FASTA through using a downloaded free version of Chromas software.

Sequencing primers for *MDM1*: c.1876G>A:p.Ala626Thr variant were designed using primer3web version 4.1.0 . and were as follow:

Forward primer: 5’ TGAGATAAGCTGTTCAGTGGC 3’.

Reverse Primer: 5’ ACCACTTTACTCCCAGCTTACT3’.

Chapter 3

Results

3.1 Demographic, Clinical, and Biological Information

Our study was focused on patients with non-obstructive azoospermia or MMAF without PCD features, and was based on two individuals from two different families, and both are below the age of 40 years, who were recruited mainly from the branches of Razan IVF center in Palestine. Clinical diagnosis had established the patients as infertile categorizing as azoospermic, or having spermatozoa with 5 morphological abnormalities for the case of MMAF. Clinical data, was supplemented by laboratory tests.

3.1.1 Asthenospermia case

The asthenospermia patient that was evaluated in this study, is a male of Palestinian origin, age below 40 years. Biological information for this patient included Seminogram, that revealed diminished sperm motility. Further lab investigations, could not be performed because we lost contact with the patient.

3.1.2 Azoospermia case

A patient with non-obstructive azoospermia that was included in this study. Clinical information showed that the patient had a history of primary infertility and a diagnosis of non-obstructive azoospermia. As shown in Figure 12, the male individuals with non-obstructive azoospermia are represented by squares and are highlighted with black and the arrow denote our proband. This pedigree, mostly suggests an autosomal recessive pattern, meaning that an individual must inherit two copies of the genetic variant (one from each

parent) in order to be affected by the condition.

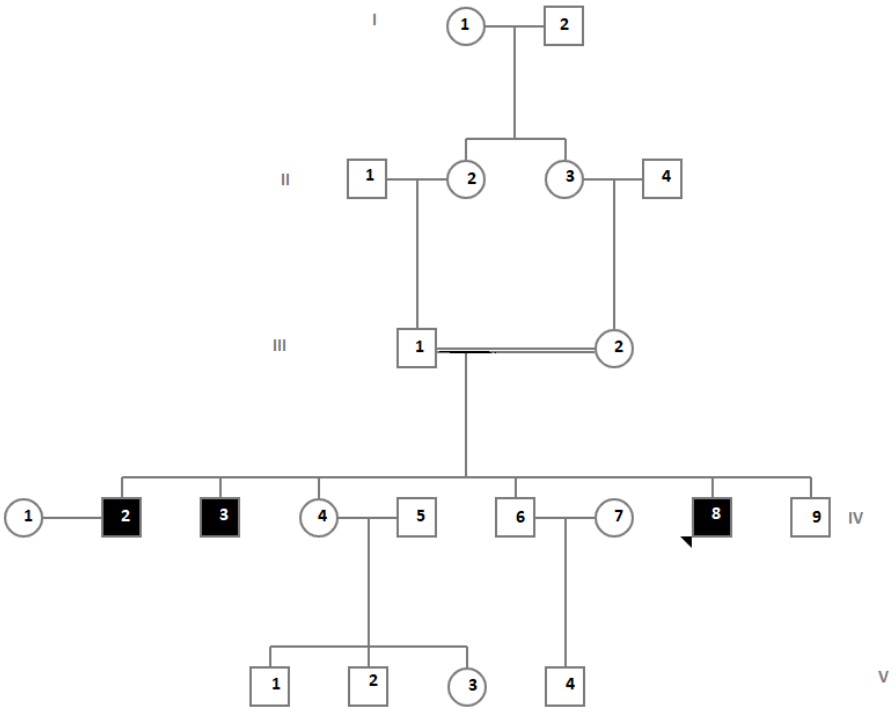


Figure (12) : A pedigree for a family with three brothers suffering NOA, with the arrow indicating our proband. The figure above represents a familial case of non-obstructive azoospermia, that is most probably caused by genetic factors that are inherited within the family.

Clinical data for the proband (III-8), which was supplemented by laboratory tests for gonadotropin levels, and androgen level checkup presented in table 6 along with karyotyping, testicular fine-needle aspiration (TEFNA), testicular sperm extraction (TESE). The results showed normal levels of LH, FSH, Prolactin, and TSH, but a low level of total testosterone. The testicular sperm extraction and testicular fine needle aspiration results were not efficient, as no spermatozoa were available.

Table (6): Azoospermia patient clinical and biological characteristics, As we can see from the table that testicular sperm extraction was negative for sperm retrieval also fine aspiration was negative androgens and gonadotropins were normal.

Hormone/Test	Result	Normal Range
LH	2.79 mIU/ml	0.57-12.07
FSH	7.3 mIU/ml	2.0-18.7
Prolactin	8.70 ng/ml	3.46-19.4
TSH	1.21 mIU/L	0.39-4.0
Total testosterone	3.4 ng/ml	2.4-8.7
Testicular sperm extraction	Not efficient (no sperms present)	N/A

Testicular fine needle aspiration	No spermatozoa available	N/A

3.2 Chromosomal Investigations

In azoospermia, the cause could be chromosomal anomalies, which can be identified through karyotyping. On the other hand, in asthenospermia, the reduced motility of sperm typically is not associated with chromosomal abnormalities and is instead thought to be related to genetic and/or environmental factors.

The methodology for chromosomal analysis in our study involved getting karyotyping profile to understand the patients' chromosomal structure and identify any chromosomal anomalies. The results of the karyotyping showed that the azoospermia patient involved in our study had a normal 46-XY karyotype, which is the typical karyotype for male individuals. This normal karyotype was considered a significant finding since chromosomal anomalies are a common cause of azoospermia. No abnormal chromosomal findings were detected in the azoospermia patient, which suggests that infertility is likely due to other factors. These findings highlight the importance of chromosomal analysis to exclude chromosomal causes of infertility.

3.3 Y-Chromosome Analysis

In addition to the chromosomal findings, our study also included a Y-chromosome microdeletion PCR investigation . The methodology involved performing PCR tests to detect any microdeletions on the Y- chromosome. The results of the Y-chromosome tests were normal as we can see from the figure 15,16,17, with no microdeletions detected on the Y-chromosome of azoospermia patient. This is a significant finding as Y- chromosome microdeletions have been associated with azoospermia cases. Our study investigated several specific Y-chromosome microdeletions, including ZFY-SRY-SY86-SY254-SY127-SY84-SY134-SY255, according to the methods described by Peter et al. in Methods Mol Biol (2013). A normal Y- chromosome microdeletion test result suggests that the individual being tested should be further investigated for other causes of infertility. The importance of Y-chromosome microdeletion analysis was to identify and/or exclude Y- chromosome microdeletions as a causative reason for the azoospermia case.

As evident from the gel electrophoresis the Y-chromosome microdeletions does not exist the images were visualized by GENIUS camera.

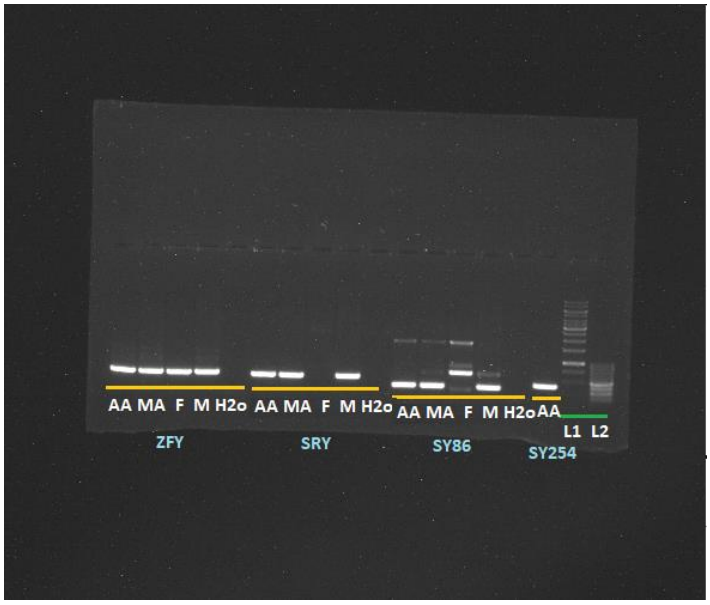


Figure (13): Visualizing Y-Chromosome Microdeletion through Gel Electrophoresis. PCR products of ZFY, SRY, SY86, SY254:18 wells. AA is a male in our research, MA is a brother of AA, F: female, M: normal Male, H2O: Water, L1:ladder1, L2:ladder2. Size of the products range between 300-500 bp.

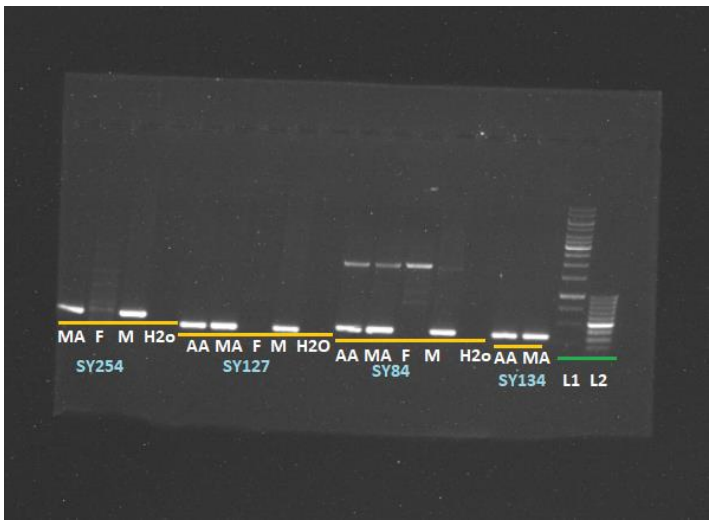


Figure (14): Visualizing Y-Chromosome Microdeletion through Gel Electrophoresis, PCR products SY254, SY127, SY84, SY134: we have 18 wells. AA is a male in our research, MA is a brother of AA, F: female, M: normal male, H2O: Water, L1: ladder1, L2: ladder2. Size of the product ranges between 250-400 bp.

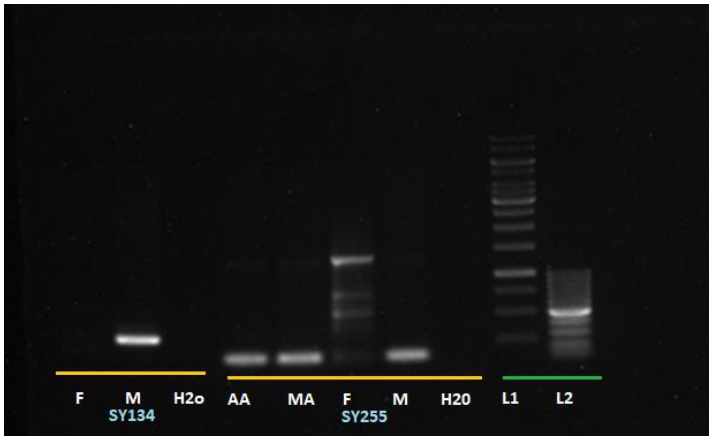


Figure (15): Visualizing Y-Chromosome Microdeletion through Gel Electrophoresis, PCR products SY134, SY255: we have 10 wells. AA is a male in our research, MA is a brother of AA, F: female, M: normal Male, H2O: Water, L1: ladder1, L2: ladder2. PCR products size~120-300 bp

3.4 Whole exome sequencing

Whole exome analysis was performed on two individuals with infertility phenotypes, asthenospermia and non-obstructive azoospermia. The analysis revealed several candidate genetic variations that may contribute to the observed phenotypes.

Our WES analysis was based on finding variations that are homozygous (Autosomal recessive) that may cause loss of function and at a population frequency of =<0.01.

3.4.1 The WES of asthenospermia case

WES analysis led us to the identification of variants in five candidate genes that may contribute to the observed asthenospermia phenotype.

Five candidate genes (CCDC40, MROH8, MUC4, FADS6, TAS2R43) were identified with variants that may explain the suspected MMAF phenotype. these variations are summarized in table 7. However, due to a lack of contact with the patient, we were unable to investigate other family members which is essential in order to identify a more specific candidate gene or to rule out the possibility of compound heterozygosity as a cause of the asthenospermia. More information was needed to expand the scope of search in a plausible way.

Table (7): The best candidate genes which were obtained from the asthenospermia WES data analysis.

Asthenospermia	filtering WES of the asthenospermia case.
Ensembl Gene ID:	Candidate genes , name and mutation :
ENSG00000141519	CCDC40, coiled-coil domain containing 40. *CCDC40*: NM_001243342: exon18: c.3065_3095del.
ENSG00000101353	MROH8 , maestro heat like repeat family member 8. *MROH 8* ,NM_152503:exon1:c.93+1->AGTGCCGGCCGCGGGGCCCTGTCTATAAG;NM_213631:exon1:c.93+1-.
ENST00000463781	*MUC4*, Mucin glycoproteins. *MUC4*: NM_018406: exon2: c.11011dupT.
ENSG00000255374	TAS2R43, taste 2 receptor member43, *TAS2R43*:NM_176884:exon1:c.761_762insAA.
ENSG00000172782	FADS6,predicted to be an integral part on cell membrane. Slight expression in testes. *FADS6*:NM_178128:exon1:c.17_18insGATGGAACCTACGGAGCCCATGGAACCTACGGAGCCCATGGAACCTACGGAGCC.

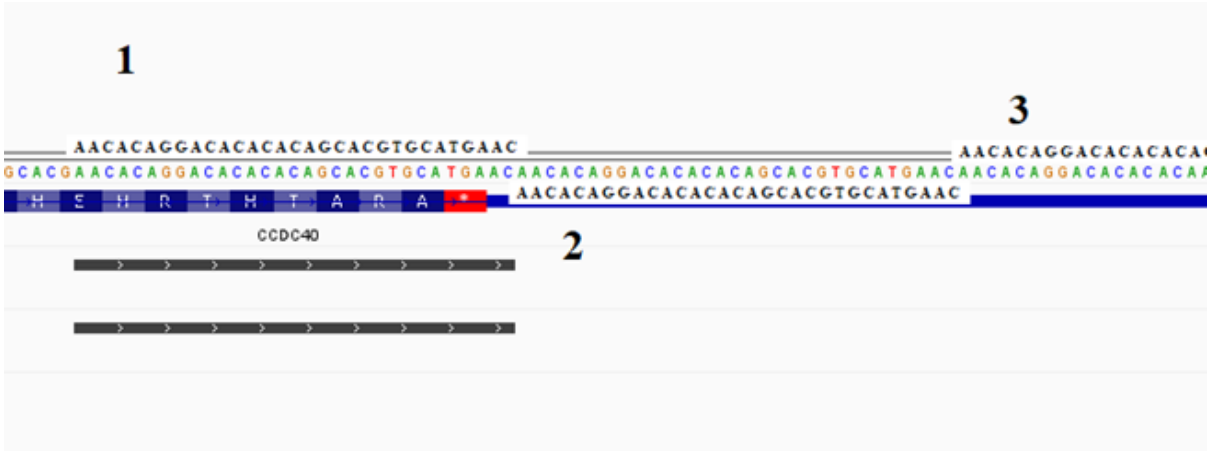


Figure (16) : Using the IGV we blast in *CCDC40*: NM_001243342: exon18: c.3065_3095del: p.E1022E , The following sequence (AACACAGGACACACACAGCACGTGCATGAAC) represent the loss between c.3065-3095 del in *CCDC40* NM_001243342, this loss is being make up for by the repeats that follow the loss replacing segment 1 for segment 2 or segment 3. If the first two is lost replacing an E for an E on position 1022 of the protein amino acid sequence.

CCDC40 was previously classified as PCD gene. Based on this we wanted to segregate *CCDC40* mutation to see mother and father status in this respect. So, we started by ameliorating the experimental set ups and results were as follow.

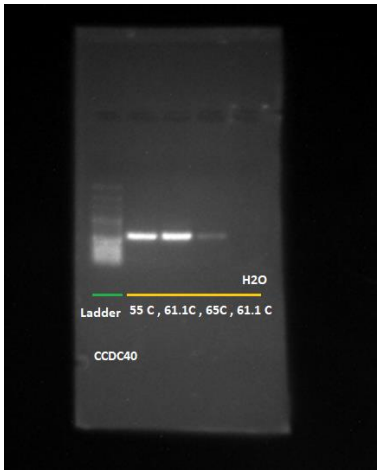


Figure (17): Optimization of experimental set ups, as shown in the gel the CCDC40 primers were testes against different temperatures, to optimize visualization of the bands using our PCR machine, this was done before we lost contact with the patient, and so we stopped here in the asthenospermia case.

3.4.2 The WES of azoospermia case

WES analysis led to the identification of five candidate homozygous missense variations (MAGEC1, OSBP2, NAT10, CD248,MDM1) in the azoospermia case that may also play a role in NOA ,these variations are summarized in table 8.

Table (8): The best candidate genes which were obtained from the Azoospermia WES data analysis.

Azoospermia	filtering WES of the azoospermia case.
Ensembl Gene ID:	Candidate genes, name and mutation :
ENSG00000111554	MDM1, Mdm1 nuclear protein , *MDM1*: NM_017440.4: c.1981G>A.
ENSG00000174807	Cd248, CD248 molecule, *Cd248*, NM_020404.2: c.908C>T.
ENSG00000135372	Nat10, N-acetyltransferase 10. *Nat10*, NM_024662.2: c.1624C>T.
ENSG00000184792	Osbp2, oxysterol binding protein 2, *Osbp2*, NM_030758.3: c.2018G>C.
ENSG00000155495	MAGEC1, MAGE family member C1. *MAGEC1* , NM_005462.4:c.2522G>T .

Notably, a homozygous missense variation in the *MDM1* gene (NM_017440.4: c.1981G>A: p.Ala661Thr) has been observed and highlighted, which may explain the observed spermatogenesis failure. The affected brother of the proband was also found to be homozygous for the same variation in the *MDM1* gene, providing further support for its role in the azoospermia observed in this family., Our findings provide insights into the genetic basis of infertility in the Palestinian community, where consanguineous marriages exist at a relatively high frequency and may aid in identifying unique signatures associated with infertility.

3.5 Further WES Data Analysis

The results of the WES analysis in the present study led to the identification of several candidate genetic variations in two independent individuals with different forms of infertility: asthenospermia and non-obstructive azoospermia. For the asthenospermia case, we identified variants in four candidate genes (*MROH8, MUC4, FADS6, TAS2R43*) that may explain the MMAF phenotype. In the azoospermia case, we identified a homozygous missense variation in the *MDM1* gene (NM_017440.4: c.1981G>A: p.Ala661Thr), which may explain the observed spermatogenesis failure. Protein alignment analysis of multiple different systems showed that Ala 662 is conserved in mammals (Figure 20).

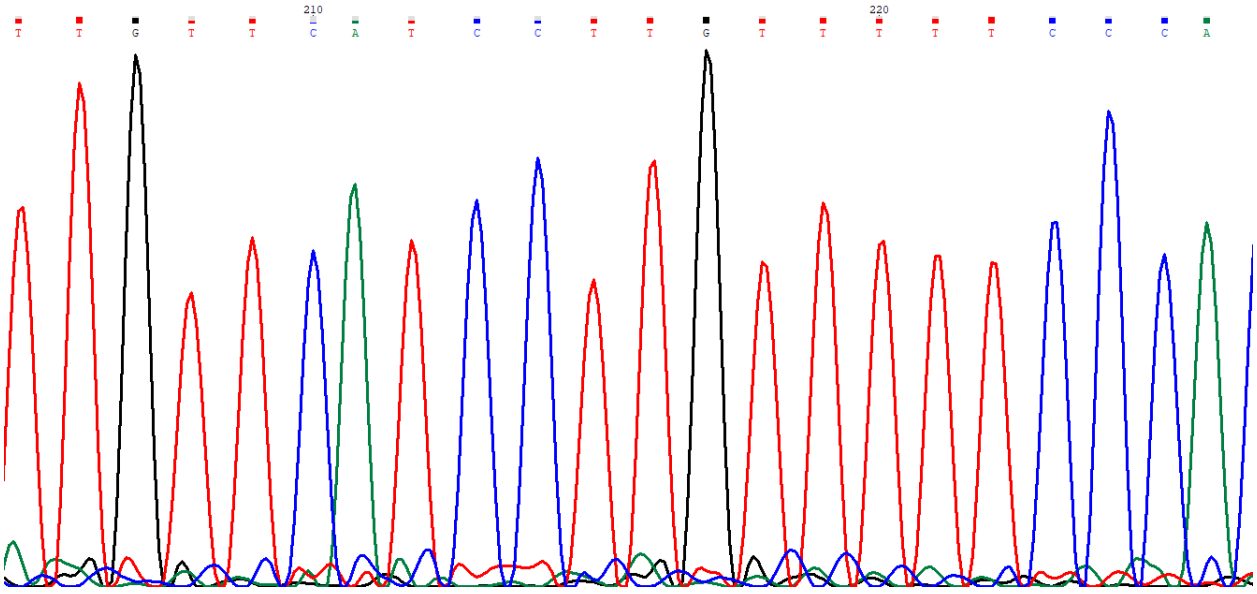
Family segregation of the *MDM1* candidate variant in the azoospermia case:

The *MDM1* variant was very tempting especially when we knew it is related to ciliated cells, so it was further confirmed by Sanger sequencing which was performed on the patient and his brother. In fact, we identified the p. Ala661Thr variation in exon 1 of *MDM1* in the homozygous state in the affected brother.

Exporting data, we obtained:

- For >Our_Proband_*MDM1*_R

ACAGGGTTTCAAGGATCTAATGAAAAATCTGTGGAAGTACTTCATAAGCTATAA
AGTGCTAATCAAATGTAAAGCCATTATACTCTCATAGACTAGAACTCAAACTTTT
ATGTTATGTGACTACTGAAATACCTTCATCATTAAAGGCTTCATGTTGAGGTAAC
TGCAAATTGTTCATCCTTGTTTTTCCCACTTAAAAAAAAAAAAGGCARAAWAAAT
TATCCTTCAAAASC



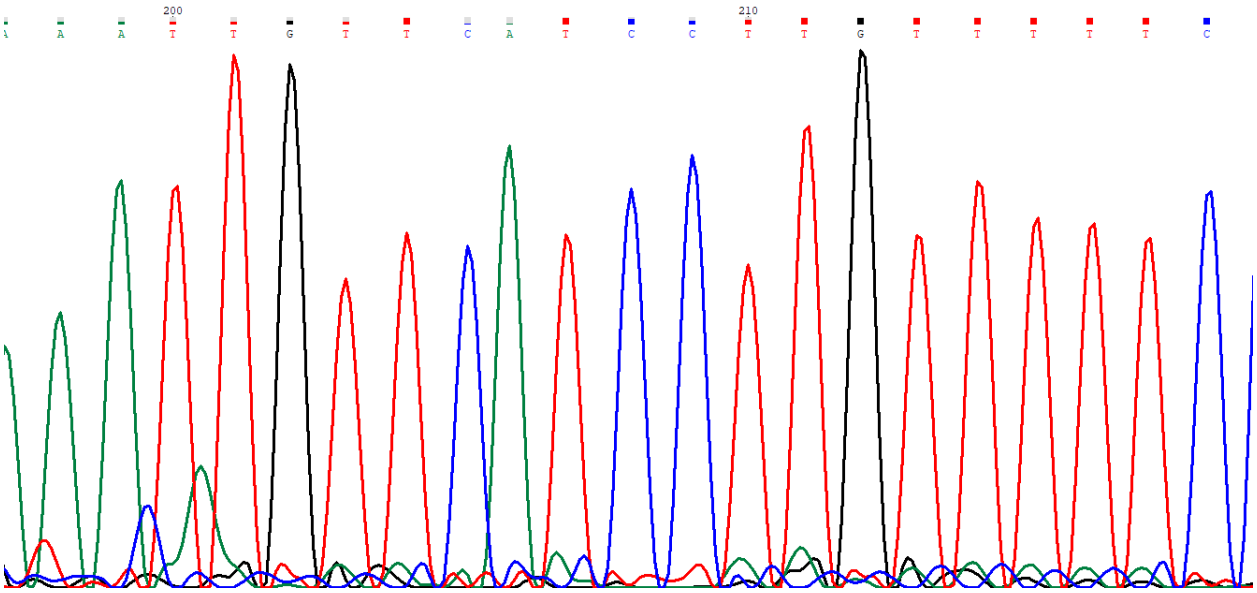
Position 218 on the graph, appears to be homozygous. Chromatogram obtained from chromas software.

Figure (18): Chromatogram obtained from Sanger sequence showing the sequence in letters and peaks. Position 218 refers to the position in the graph and is not related to the gene.

- For >Our_proband_brother_MDM1_R

CAGGGTTTCAAGGATCTAATGAAAAATCTGTGGAAGTACTTCATAAGCTATAAA
GTGCTAATCAAATGTAAAGCCATTATACTCTCATAGACTAGAACTCAAACTTTTA

TGTTATGTGACTACTGAAATACCTTCATCATTAAAGGCTTCATGTTGAGGTAACT
GCAAATTGTTCATCCTTGTTTTTCCCACTTAAAAAAAAAAAAGGCARAAWAAATT
ATCCTTCAAAASCCMCTGAACA



Position 213 on the graph, appears to be homozygous. Chromatogram obtained from chromas software

Figure (19): Chromatogram obtained from Sanger sequence showing the sequence in letters and peaks for our proband brother. Position 208 refers to the position in the graph and is not related to the gene.

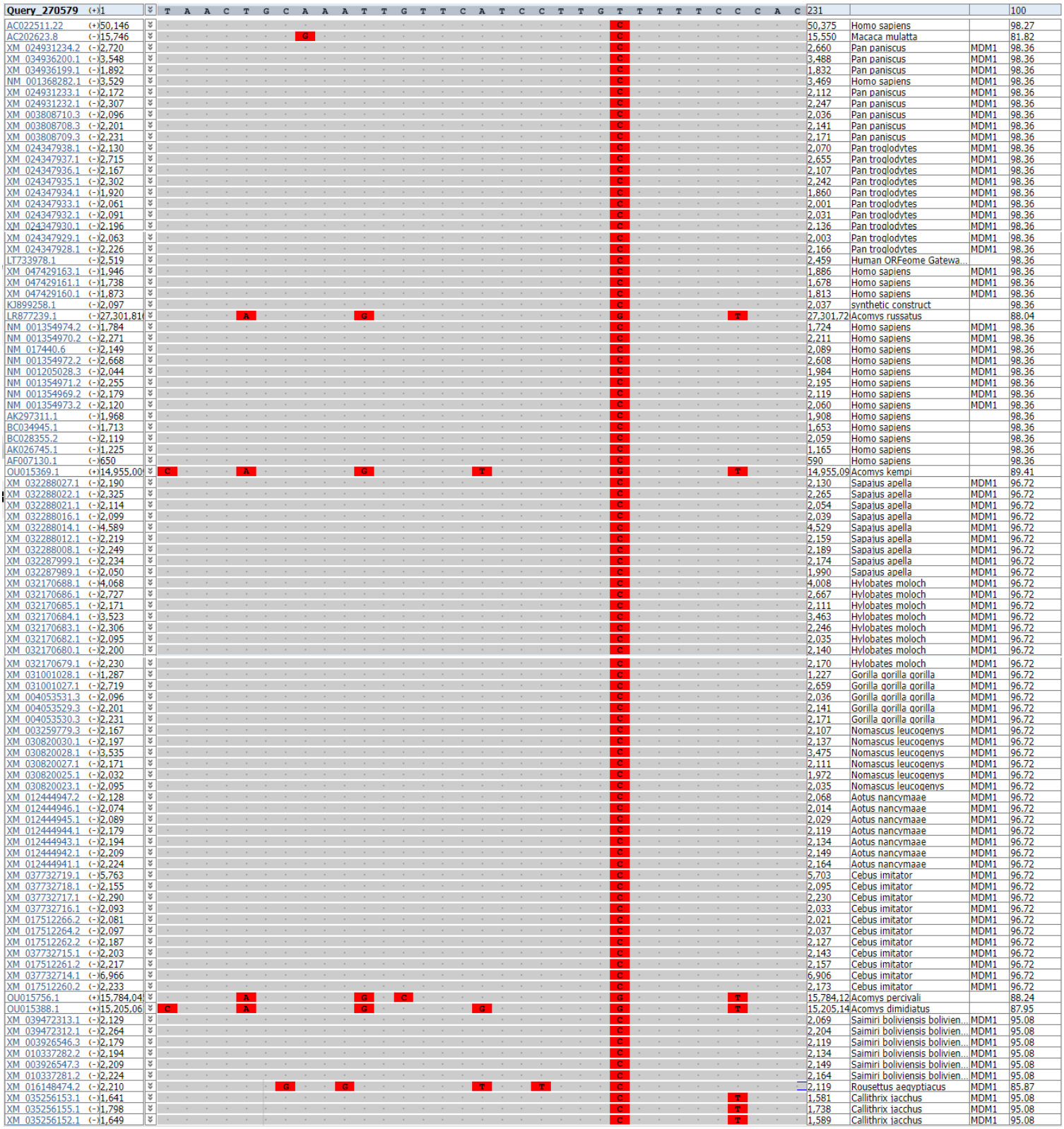


Figure (20): Multiple Sequence Alignment Viewer 1.22. The site in which our mutation is present is very conserved among multiple systems and in multiple variants of *MDM1*.

As a conclusion, it has been found that both brothers have the same mutation, which in turn promotes the idea that MDM1 could be a gene responsible for testicular azoospermia. A very interesting work would be to investigate the mother and the father. Though this investigation between brothers reveals primarily that the mutation is stable, heritable, and replicable, revealing in some sense a recessive Mendelian mutation in MDM1 that may be a cause for non-obstructive azoospermia.

This further supports its potential role in the azoospermia observed in this family. Sanger sequencing was used to confirm the presence of the homozygous missense variation in the affected brother, demonstrating the usefulness of this tool for gene segregation analysis.

A summary of the WES analysis for both patients is listed in table 9.

Table (9): A summary of WES findings and in silico investigation in terms of mutation status, expression site, conservation status through evolution and Polyphen 2 predictions, taking into consideration that PolyPhen-2 predicts the functional significance of an allele replacement from its individual features by Naïve Bayes classifier trained using supervised machine-learning.

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Case	Candidate gene		Expression status in testis	Conservation status in evolution
Asthenospermia case		Mutation Status		
	CCDC40	Deletion mutation	Expressed in testis	Mutation site conserved
	MROH8	Deletion mutation	Expressed in testis	Mutation site conserved
	MUC4	Duplication mutation	Expressed in testis	Mutation site conserved
	TAS2R43	Insertion mutation	Expressed in testis	Mutation site conserved
	FADS6	Insertion mutation	Expressed in testis	Mutation site conserved
Azoospermia case		Polyphen 2 score		
	MDM1	0.003	Expressed in testis	Mutation site conserved
	CD248	0.957	Expressed in testis	Mutation site conserved
	MAGEC1	0.969	Expressed in testis	Mutation site conserved
	Nat10	1	Expressed in testis	Mutation site conserved
	Osbp2	0.938	Expressed in testis	Mutation site conserved

Chapter 4

Discussion

Infertility is a multifactorial condition that can arise from various causes, some of which may overlap. Protein-altering mutations are among the contributors to infertility, with potential implications for the developmental sequence of cells destined to become sperm. These mutations may disrupt cellular differentiation at different stages, from primordial germ cells to sperm, thereby impacting the functionality of the resulting sperm. In this thesis, we sought to identify the genetic basis of primary male infertility in two distinct families, utilizing WES analysis as a potent tool for identifying inherited genetic diseases, including male infertility.

In our investigation of candidate genes associated with asthenospermia in the patient from the first recruited family, we identified CCDC40 as the first candidate gene. Initially, we were optimistic because CCDC40 is strongly associated with PCD. However, our patient did not show any clinical evidence of PCD, so we cautiously proceeded with further validation of the identified variant. Sanger sequencing and in silico and bioinformatic analysis revealed that the CCDC40 variant is located in a repetitive region and has been classified as benign in ClinVar. Therefore, we concluded that the CCDC40 variant we identified is unlikely to be the cause of asthenospermia in the patient from the first family. Our findings underscore the importance of careful validation of genetic variants, especially when investigating potential gene-disease associations outside the established clinical spectrum.

The second candidate gene was MROH8. This gene is highly expressed in the testis, in our proband the mutation was an exonic mutation along with a splicing mutation, which might cause disturbance in the integrity of the produced MROH8 protein, accordingly causing loss of fidelity in sperm production. Following this, we need further functional studies and more in-depth investigation in this respect to rule out this claim. The function of MROH8 is not yet known, as for MROH8 the highest expression in testis is in the early and late spermatids which is the stage of tail formation, suggesting motive and capacity for further investigation.

The third candidate gene was MUC4. As a protein, MUC4 contains more than 5400 amino acids, which, if we conclude, represent a loss of function mutation since the mutation in our proband involves a substitution due to duplication causing a frameshift on position 367 causing a stop codon after the 6th amino acid after the frameshift. Following our conclusion in the respect of MUC4 protein loss, we have data that support further investigation in the

respect of asthenospermia as causal gene. It is also possible that MUC4 has paralogous genes, meaning that it has closely related genes that have similar functions, which could compensate for the loss of function caused by the mutation in MUC4. This could make the mutation in MUC4 less significant in terms of disease pathology.

The fourth candidate gene was FADS6, expressed in the testis. Though, fatty acid desaturase 6 seems to act as a predisposing factor to sperm motility, as sperm membrane lipids homeostasis might be involved in a bunch of vital processes such as sperm motility, capacitation, along with the structural integrity of the sperm [110]. Still, our mutation needs further evaluation in terms of functionality.

The last candidate gene in the asthenospermia case was TAS2R43. It was very interesting to know that the taste receptor 2 family could be expressed in the testis and not only in the oral cavity, which raises questions of where these receptors are involved. Some groups suggest a possible involvement in gametogenesis and/or successful reproduction [111]. For instance, according to Luddi, A. et al. (2019), genetic deletions and polymorphisms of taste receptors may impact male fertility. Also, according to Ali, M. A. et al. (2021), it is worthy to investigate the roles of odorant and taste receptors in sperm chemotaxis because activation of these receptors can cause an increase in intracellular calcium concentration and result in hyperpolarization of the sperm cell, both of which are important for sperm chemotaxis. The gene was not further deciphered because we lack the capacity of segregation.

We think that if it were possible to analyze other family members of the proband family, we would have been able to define on a deeper level the cause, because it could be compound heterozygosity that causes the phenotype or may be a spectrum of PCD. In compound heterozygosity, we expect to find two different genetic mutations or variations, or alleles, at the same gene locus in an individual, one inherited from each parent. Recently, Xu, Yingjie et al. showed that a compound heterozygotic form of CCDC40 mutation have been a causative gene in MMAF which extends the spectrum of MMAF (with classical PCD manifestation) [112].

In our azoospermia case, we will start talking about the last candidate gene, which is MDM1. It has been found to be a very tempting candidate gene that might be causing azoospermia. This was taken into consideration because MDM1, as a gene, has been observed as active during the differentiation of multiciliated cells. and its close proximity to centrioles, which play an important role in stabilizing the cellular microtubular structure [113]

In our azoospermia case, MDM1 is a very promising candidate gene that may be causing azoospermia. This is due to MDM1's role as an active gene during the differentiation of multiciliated cells and its close proximity to centrioles, which play an important role in stabilizing the cellular microtubular structure. Furthermore, centrioles have critical and essential functions in sperm formation and development, and mutations in genes related to centrioles or their function may result in a spectrum of phenotypes, including reduced or no spermatozoa in semen [114].

MDM1 has also been found to have high relative expression in spermatocytes, the cells that undergo division and differentiate into the next stage of development. A study by Y. Son, et al. shows that MDM1 protein is located between the nucleus and centriole in the sperm neck. It is important to note that, while this research found that germ cells still exist even when MDM1 is knocked out, it is unknown if these sperm are mature and capable of being present in ejaculate. Additionally, the study used mice as subjects, so the findings may not necessarily apply to humans.

The second candidate gene in our azoospermia case was MAGEC1, which has been found to be an expected damaging mutation according to polyphen-2. Notably, the molecular role of MAGEC1 in male infertility will be enhanced using cell-based assays and animal models, since Melanoma Antigen Protein Magec1 Mutation has been identified by Pastuszak, AW et al. as a gene responsible for Familial Non-Obstructive Azoospermia [116]. MAGEC1 is tempting to be investigated in terms of our mutation to check the possible role in spermatogenesis since it is expressed highly in the testis, which reflects functionality in sperm nurturing and development.

The third candidate gene in the azoospermia case is OSBP. A functional study done on the OSBP protein family found that a mutant male drosophila model is sterile [117]. Still, though our mutation is expected to be damaging by polyphen-2 and the mutation site is evolutionarily conserved. The mutation finding could be supported by evidence of drosophila model, we need to study it more and see the effect in humans. Perhaps, a testis-on-a-chip model could give a good indication over the status of the mutation found in our proband.

The fourth candidate gene in the azoospermia case is NAT10-mediated N4-acetylcytidine, which is reported to be an essential part of meiosis entry in spermatogenesis [118]. Our proband mutation Nat10 is expected to be a damaging mutation according to polyphen-2, and

the mutation site is evolutionarily conserved. Based on this, our mutation could be a very good candidate as a causal gene of NOA, which arrests the development of sperms in a certain immature stage.

The fifth candidate gene in the azoospermia case is CD248, which is expressed at a 0.8 nTPM in late spermatids, and this gene is located in the extracellular exosome. In case of being mutated, it could cause miscommunication between cellular compartments, which emphasizes the study of intercellular trafficking caused by this gene. Our CD248 proband mutation is expected to be a damaging mutation according to polyphen-2, and the mutation site is evolutionarily conserved. This suggestion needs further evaluation and another frame of study.

Though genes could be filtered out in our infertility cases, what we have found is a needle in a haystack regarding male infertility as a field of research. We believe the model on which a functional study will be carried out later should cope to a very close extent with the human cellular niche and microenvironment. We think that an organ-on-a-chip model and organoids modeling is a very promising tool that gives us good indications of a mutation status.

Organoid models are three-dimensional structures derived from stem cells that mimic the organization and structure of real tissues and organs. They are more advantageous than traditional cell and tissue culture models, as they better imitate the microenvironment found in vivo, have the ability to mimic the cellular diversity of a particular tissue or organ, can be propagated for a long-term period, and can be used to study both normal tissue development and disease progression. Additionally, organoids have the ability to self-organize and can be used in personalized medicine. They have the potential to revolutionize the field of in vitro cell and tissue culture, despite having their own limitations.

In summary, our analysis of the proband's WES data revealed several candidate genes for the asthenospermia case, including CCDC40, MROH8, MUC4, FADS6, and TAS2R43. Further functional studies are needed to fully evaluate the potential causative role of these genes in the proband's asthenospermia. Additionally, the results of the WES analysis in our azoospermia case have highlighted several promising candidate genes, including MDM1, MAGEC1, OSBPD, NAT10, and CD248. While these genes have been found to be associated with azoospermia, the possibility of paralogous genes compensating for the loss of function caused by the mutations in these genes cannot be ruled out in this study. Further functional studies and in-depth investigations are needed to confirm the causality of these

genes in azoospermia and to better understand the role of paralogous genes in sperm development. This could be facilitated by many means, e.g., animal models where we might knock out a gene in case of loss of functionality or a knock-in in case of predicted gain of functionality. The possibility of compound heterozygosity, where two different genetic mutations or variations are present at the same gene locus in an individual, should also be considered. Overall, the field of male infertility research is complex and multifaceted, and the use of organoid models and other advanced technologies may help to uncover new insights and potential therapeutic targets.

Conclusion

As a conclusion, MDM1 was a notable candidate gene for azoospermia in this thesis. The variation identified in MDM1 may lead to azoospermia due to its critical position and function as a microtubule-binding nuclear protein. This suggests the need for further studies at the protein level. Also, due to the lack of definitive cutting evidence explaining our azoospermia case, we hypothesize that the variation in MDM1 may act as a predisposing factor associated with other factors resulting in azoospermia, probably along with a variation in another gene.

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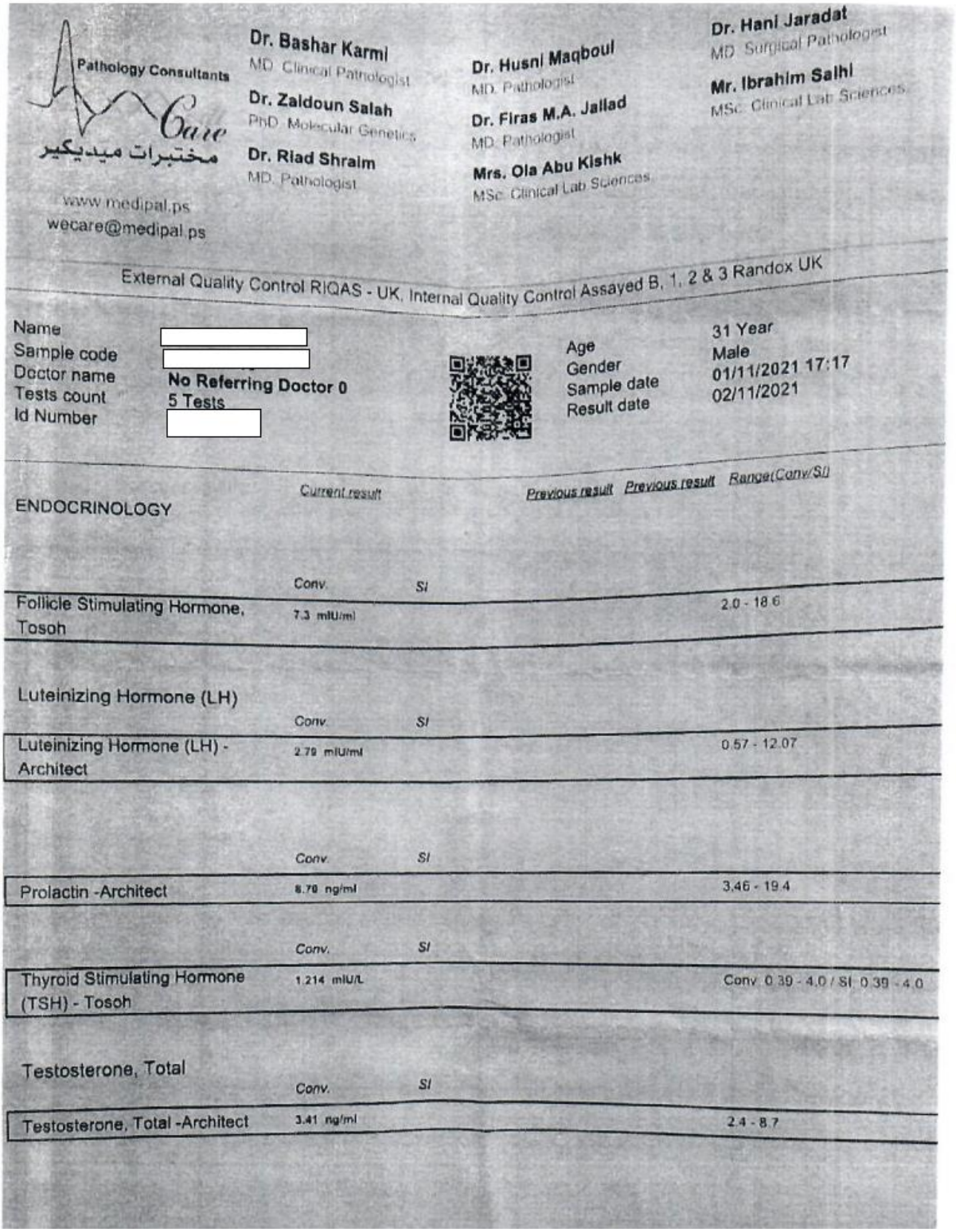
Supplementary data (standardized clinical form for all patients)



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External Quality Control RIQAS - UK. Internal Quality Control Assayed B, 1, 2 & 3 Randox UK

Name	[redacted]	Age	31 Year
Sample code	[redacted]	Gender	Male
Doctor name	No Referring Doctor 0	Sample date	01/11/2021 17:17
Tests count	5 Tests	Result date	02/11/2021
Id Number	[redacted]		

ENDOCRINOLOGY	Current result		Previous result	Previous result	Range(Conv/SI)
	Conv.	SI			
Follicle Stimulating Hormone, Tosoh	7.3 mIU/ml				2.0 - 18.6
Luteinizing Hormone (LH)					
	Conv.	SI			
Luteinizing Hormone (LH) - Architect	2.79 mIU/ml				0.57 - 12.07
	Conv.	SI			
Prolactin -Architect	8.79 ng/ml				3.46 - 19.4
	Conv.	SI			
Thyroid Stimulating Hormone (TSH) - Tosoh	1.214 mIU/L				Conv. 0.39 - 4.0 / SI. 0.39 - 4.0
Testosterone, Total					
	Conv.	SI			
Testosterone, Total -Architect	3.41 ng/ml				2.4 - 8.7

Testicular Biopsy Report

H. Name :
W. Name :
H. Age : 27yrs.
W. Age : 27yrs.
Date : 7.4.18

Diagnosis : Azoospermia
Done for : Diagnostic
File No. : 102/16
Consultant : Dr. Karam

Procedure : PESA ☐ MESA ☐ TEFNA ☐ TESE ☐

Procedure

Findings

Site: Left testis	Sperms Motile	Sperms Non-Motile	Conc. mill\ml	Others	Site: Right testis	Sperms Motile	Sperms Non-Motile	Conc. mill\ml	Others
L 1	—	—	—	RBCs	R 1	—	—	—	RBCs
L 2					R 2				

Procedure : TEFNA

Findings

Site: Left testis	Sperms Motile	Sperms Non-Motile	Sp.tides	Sp.cytes	Tissues	Site: Right testis	Sperms Motile	Sperms Non-Motile	Sp.tides	Sp.cytes	Tissues
L 1						R 1					
L 2						R 2					
L 3						R 3					
L 4						R 4					
L 5						R 5					
L 6						R 6					

Lt 1-2 Rt 1-5

PESA: Percutaneous Epididymal Sperm Aspiration. MESA: Microsurgical Epididymal Sperm Aspiration. TEFNA: Testicular Fine Needle Aspiration. TESE: Testicular Sperm Extraction.

Note: U-Small tissues from $Rt_1 - Rt_{15}$ & $Lt_1 - Lt_{15}$ were received to Lab.

Results: Rare spermatides & spermatocytes ~~are~~ found unfortunately, no sperms were seen

Al-Hiba IVF Center

→ WES

→

→ Karyotying for both

[redacted]

Dr. Fawaz

المستشفى الإستشاري العربي
ISTISHARI ARAB HOSPITAL

Moleular Genetics Lab
Whole Exome Sequencing
Clinical Form

Identity of the patient:
Last Name: [redacted]
First Name:
Date of Birth:
Phone:

Place of Birth: [redacted]
Sex: Male / Female / Ambigious
Referred By: Dr. Fawaz
Blood Collection Date: 1/3/2022
Arabic Name: [redacted]

Family History:
Consanguinity: Yes / No
Geographical origins: Father [redacted] Mother [redacted]
Clinical Information:
Symptoms:

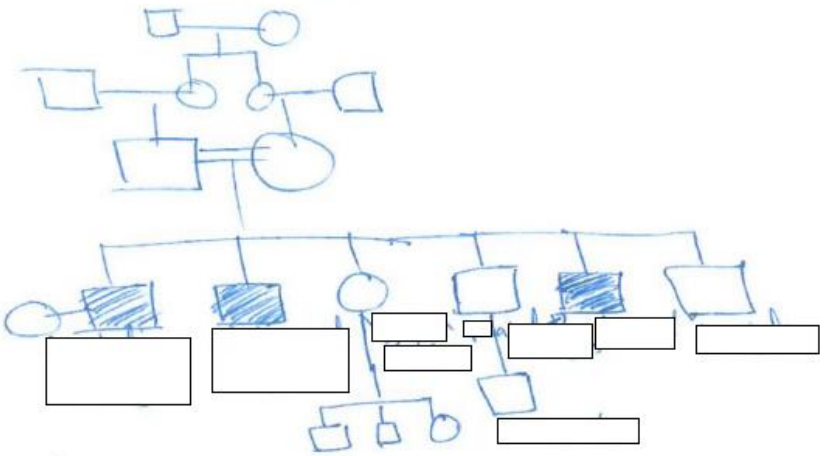
→ azoospermia (non-obstructive)

→ Apart from azoospermia, no other clinical proplems

Age of Onset: primary
Treatment:
Additional Information:

[redacted] is married since 2015

Pedigree:



[redacted]

[redacted] married for 3yrs. He is divorced now.
TEFNA and TESE were done but no sperms.

[redacted]

→ Karyotyping. WES for [redacted]. MDY for both