METABOLIC ALTERATIONS IN INFERTILE MALE SPERMS

A thesis submitted to the

UPES

For the Award of

Doctor of Philosophy

In

Biotechnology

By

Sujata Maurya

June 2023

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Department of
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UPES, Dehradun – 248007
Uttarakhand

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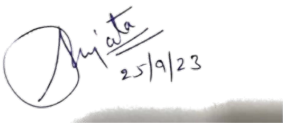
DECLARATION CERTIFICATE

I declare that the thesis entitled "METABOLIC ALTERATIONS IN INFERTILE MALE SPERMS" has been prepared by me under the guidance of **Dr. Dhruv Kumar,** Professor, Department of School of Health Science and Technology (SoHST), UPES, Dehradun. No part of this thesis has formed the basis for the award of any degree or fellowship previously.



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CERTIFICATE FROM GUIDE

I certify that **Miss SUJATA MAURYA** has prepared her thesis entitled "**METABOLIC ALTERATIONS IN INFERTILE MALE SPERMS**", for the award of PhD degree, under my guidance. She has carried out the work at School of Health Science and Technology (SoHST), University of Petroleum & Energy Studies (UPES), Dehradun.

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8th September, 2023

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Abstract

The “inability of a couple to conceive even after one year of unprotected, frequent sexual intercourse” is called Infertility. It affects 15% of couples worldwide, in which male partner are solely responsible in about 20% of the cases and playing a role in another 30% to 40% of infertility occurrences. In this study, we tried to analyze the factors and reasons responsible for male infertility. According to the analysis, we found that there are three major parameters contributing to infertility 1) number of spermatozoa present in semen, 2) sperm movement and 3) sperm morphology. In our study, we tried to focus on flagellar waveforms and propagation of sperm movement using “Computer Assisted Sperm Analysis” (CASA) based on “Convolutional neural network” (CNN) an “Artificial Intelligence” (AI) approach. As the motility of sperm plays a very important role in ovum fertilization, we also tried to find the genes responsible for ATP generation or participating in metabolic pathways in sperm movement. For that we used, *in-silico* tools, to find out the possible responsible genes involved in sperm motility. After identifying APOB as a potential target for sperm motility, we used a structure-based drug repurposing approach to repurpose FDA-approved compounds including marine compounds and metabolites using molecular docking (MD) and molecular dynamics simulation (MS). Structure-based drug repurposing is an extremely time and cost-effective method. In our investigation, 32,000 new compounds were tested against APOB. To the best of our knowledge, this is the first research that should open the door for further experimental investigations to confirm the discovered compound's efficacy as a prospective treatment against APOB and to enhance sperm motility.

Keywords: *CASA, ImageJ, male infertility, metabolically dysregulated spermatozoa, sperm movement, sperm morphology, lipid metabolic genes*

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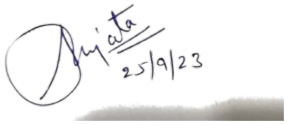

Sujata Maurya

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Abbreviation

µm/s	Micrometers per sec
ACSL	Acyl-CoA synthetase
ADME	Distribution, absorption, metabolism, and excretion
AI	Artificial Intelligence
ALH	Amplitude of lateral head displacement
ART	Assisted reproductive technological
ATP	Adenosine triphosphate
AZO	Azoospermia
BCF	Beat cross frequency
CASA	Convolutional neural network
CM	Comparative modeling
CNN	Convolutional neural network
CPC	Cumulative probabilities of conception
DFS	Dysplasia of Fibrous Sheath
ECM	Extracellular matrix
FAD	Flavin adenine dinucleotide
GO	Gene Ontology
GPx	Glutathione peroxidase
GSH	Glutathione
HB	Hydrogen bond
HED	High-energy diets

MD	Molecular Docking
MD	Molecular dynamics
MFI	Male factor infertility
MM-GBSA	Molecular mechanics-generalized Born surface area
MS	Molecular dynamic simulation
MSA	Multiple Sequence Assessment
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NOA	Non-obstructive azoospermia
NP	Non-progressive motility
NPT	Normal Pressure Temperature
OA	Obstructed azoospermia
OM	Origanum majorana
OS	Oxidative stress
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PGC	Primary Germ Cells
PPARs	Peroxisome proliferator-activated receptors
PPI	Protein-protein interaction
PRDXs	Peroxidoxins
PSA	Polar Surface Area

ROS	Reactive Oxygen Species
SASA	Solvent Accessible Surface Area
SOD	Superoxide dismutase
SP	Standard Precision
TiO2	Titanium dioxide nanoparticles
TRX	Thioredoxin
TTP	Time-to-pregnancy predictions
UniProt	Universal Protein Resource
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
XP	Extra Precision

Chapter 1

Introduction

The word "infertility" is used broadly to refer any type or class of impaired fertility in couples who are unsuccessfully attempting to conceive for the past one year [1]. The terminology used in reproductive medicine is imprecise and misleading [2]. To prevent both over and under-treatment, balanced management of reduced fertility involves the proper scheduling of infertility screenings and the proper timing of treatment initiation [3]. The interaction between the diagnosis of subfertility and its incidence in the general population is a common issue in epidemiological research in reproductive medicine [4]. TTP (Time-to-pregnancy predictions) and CPC (cumulative probabilities of conception) are crucial to discover appropriate exclusions for estimating the prevalence of different subfertility grades. When scheduling regular infertility examinations and beginning therapy in the event of a bad prognosis, these thresholds may be the primary signal. This is essential because an increasing number of couples quickly seek counsel on infertility treatment [5] due to the possibility of test findings being falsely positive and the ensuing overtreatment [6], which may subject women to excessively stressful medical situations and excessive costs. On the other side, premature treatments could signify inadequate care for infertility [7]

Not only women fertility but male fertility is also decline in both emerging and advance nations. Eating disorders, environmental factors and other external variables connected to lifestyle have a devastating effect on spermatogenesis at both the gonadal and central levels [8]. Overconsumption of HED (high-energy diets), smoking, drinking alcohol, late night sleeping, radiations from electronic gadgets affects testicular physiology by affecting its metabolism and bioenergetics capability and influencing the way of male reproductive axis functions. Testicular metabolism displays specific characteristics, in part because of its diversity and the different functions that each cell type performs within the testicular environment. Negative reproductive effects such as sperm abnormalities, spermatogenesis delay or insufficient energy supply to the germ cells result from disruption of the carefully controlled metabolic pathways [9]. Testicular metabolic changes brought on by HED use may also result in mitochondrial malfunction, which is directly related to excessive generation of “OS (Oxidative stress) and ROS (Reactive Oxygen

Species)". Other factors are also there that affects the parameter of spermatozoa that make man infertile.

These days, lipids and spermatozoic DNA are also a cause for worry because they raise testicular ROS, which reduces sperm quality and renders spermatozoa immotile. Recent research revealed that the lipid metabolic gene contributes to male infertility. Understanding how metabolic genes and other external variables contribute to male infertility is crucial [10].

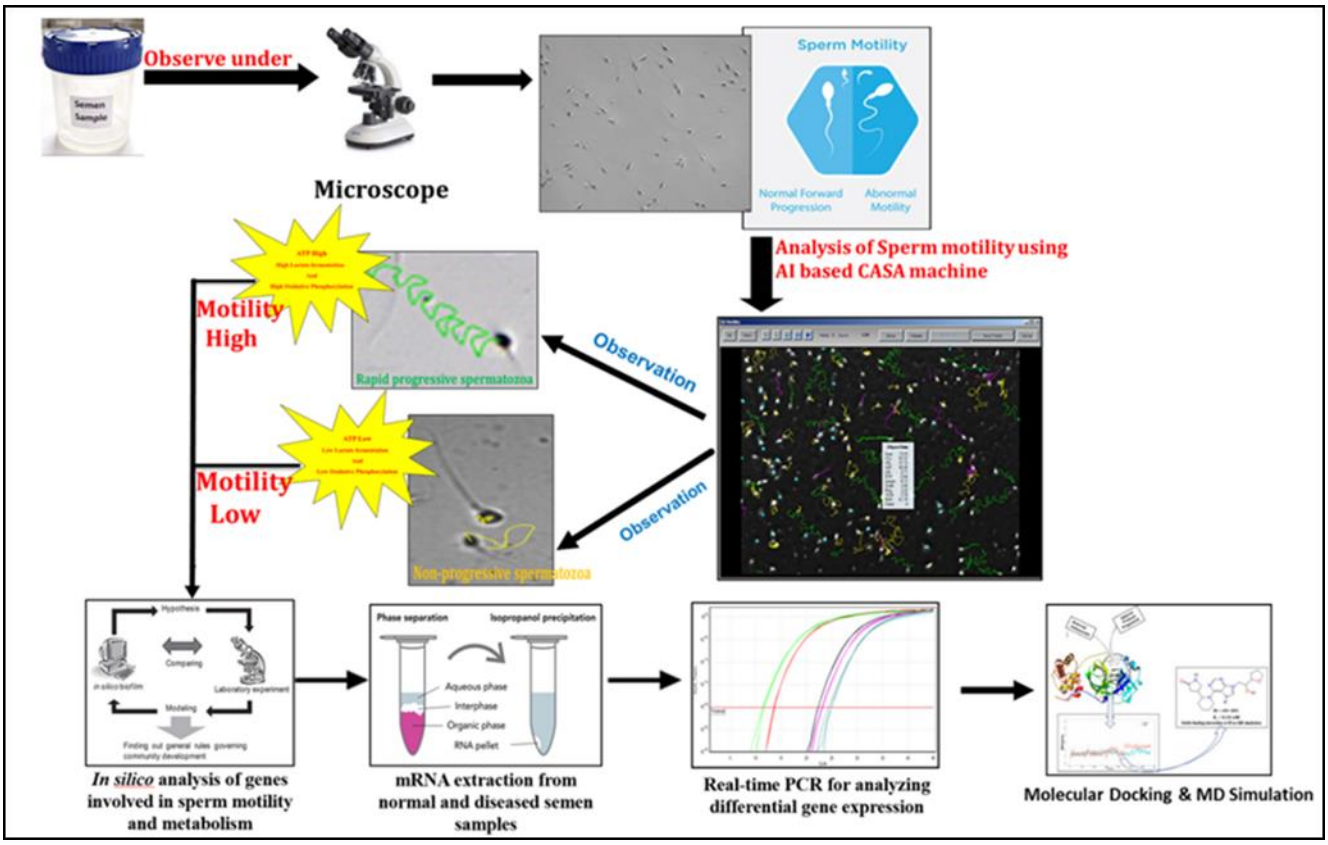
- **Research Gap** – Although there are lots of studies reported on the absence of spermatozoa in semen samples, but there are very few studies dealing with the motility of spermatozoa. Understanding the molecular mechanism of sperm motility is excessively required to improve the fertility of the infertile male population.

- **Hypothesis** – We hypothesize that targeting genes associated with ATP generation and metabolic pathways will help us to enhance sperm motility.

- **OBJECTIVES**

1) **Computational analysis of sperm morphology and their correlation with metabolic activity**
2) ***In silico* identification of specific markers in metabolically dysregulated sperms of the infertile male**
3) **Screening of potential inhibitor(s) for identified markers using *in silico* virtual screening approaches**
4) **Evaluation of identified inhibitor(s) through molecular dynamics simulation**

Overview


Observe under
Microscope
Sperm Motility
Normal Forward Progression
Abnormal Motility
Analysis of Sperm motility using AI based CASA machine
Motility High
Rapid progressive spermatozoa
Observation
Motility Low
Non-progressive spermatozoa
Observation
Hypothesis
Comparing
Laboratory experiment
Modeling
Finding out general rules governing community development
Phase separation
Isopropanol precipitation
Aqueous phase
Interphase
Organic phase
RNA pellet
In silico analysis of genes involved in sperm motility and metabolism
mRNA extraction from normal and diseased semen samples
Real-time PCR for analyzing differential gene expression
Molecular Docking & MD Simulation

Chapter 2
Review of literature

2.1.Infertility

Infertility is also described as "the inability to get pregnant even after involving in unprotected sex within a particular length of time, generally more than one year" [11]. Infertility is often due to disorders in the male or female reproductive system. The medicalization of infertility, which started in the 1950s in the United States with the advent of fertility medicine, has been hastened by assisted reproductive technologies including in vitro fertilization and intracytoplasmic sperm injection [12]. Compared to other disorders, the socialization of sickness and health is possibly much more obvious when it comes to infertility. Firstly, couples do not identify as infertile or seek therapy unless they see motherhood as a desirable societal duty, regardless of how medical professionals may characterize infertility. Secondly, whereas the biological approach views illnesses infertility is often seen as a disease that affects a couple rather than a condition that affects the individual, particularly in industrialized nations, either partner a functional impairment may exist in. As a result, identifying one's infertility involves a discussion between the couple as well as discussions with larger social networks in addition to discussions between the individual and medical specialists [13]. Thirdly, lack of a desired condition is more likely to indicate infertility than the existence of clinical signs. It was described as a "non-event transition" by Koropatnick et al. (1993) [14]. Infertility is clearer when comparing the availability of choices to obtaining a "treatment" to other medical disorders. Alternatives to therapy include consciously choosing not to have children, adoption, fostering, or changing partners. In the contemporary assisted reproductive technological (ART) clinic described by Thompson in 2005 [15], a number of techniques are involved with precise time action (such as hormone injections, sperm ejaculation, and gamete cryopreservation) among interrelated characters such as patients, nurses, and doctors.

A person's age, medications, surgical history, exposure to pollutants in the environment, or genetic abnormalities are just a few of the causes of infertility. By identifying the precise nature of their problems, these and other factors can help us to better understand the main cause of infertility in couples. Additionally, it's crucial to determine whether the relationship is subject to any social

pressure [16]. Male infertility accounts for 20 to 30 percent of cases of infertility, female infertility for 25 to 35 percent, and both genders account for 20 to 40 percent [17].

Infertility is often linked to psychological anguish since it comprises failure to carry out a desired social role. Greil (1997) proposed that infertility has a socio-psychological effect and psychological repercussions made it seem like a catastrophic event. Further contradicting findings from studies investigating the intellectual consequences theory have been obtained. While studies that employed stress and self-esteem measures found meaningful differences between infertile people and others, those that focused on psychopathology did not identify any significant differences between infertile people and others. He discovered evidence that male and female infertility are fundamentally different from one another [18]. Greil also added that the use of cross-sectional methods, non-representative samples, inability to evaluate those who have not sought treatment, impoverished and culturally unique communities, lack of investigation, and inability to address the issue of controls are just a few of the flaws that plagued the psychological distress literature. He observed that the social construction of infertility receives little attention in the psychological distress literature, which discusses infertility as a medical issue with psychological implications [18].

Infertility, which affects 10% of American couples [19] and more [20], has started to get attention from the public as a serious health issue. Sociologists are also recently become interested in this topic even though it is so common and has such a significant psychosocial impact on society. They have examined all the facet of infertility like societal beliefs and the judgments associated with it [21-23], long-term effects [24,25] and patients' opinions on treatment [26].

2.2. Infertility as a Social Process

It's critical to distinguish between infertility as a condition that couples experience on a social level and infertility as a physiological trait that can be diagnosed medically and is frequently referred to as "reproductive impairment" [27]. Medical sociologists, according to one author, should focus on social aspects of sickness, especially how patients perceive disease and how social variables influence this feeling [28]. Infertility follows the same logic. There are other factors at play besides

just one or both partners having a physiological handicap that affect how an infertility journey plays out for a couple. Instead, the "becoming infertile" process is a relational process that involves the way both partners react to, understand, and assign importance to physical signs and physiological situations.

The couple's interpretation of their infertility is influenced by the doctors' diagnosis and treatment recommendations, but they are not explicit about how the husband and wife should view their circumstances. Husbands and wives may disagree with the diagnosis made by doctors, add their own interpretations to the statements made by doctors about their experiences, or try to distort the facts in some other way. The most important choices a couple must make to define and manage their infertility crisis (for example, if to pursue therapy, if to undergo a specific screening assessment, if to stop procedure, if go for the process of adoption, if to research in vitro fertilisation, etc.) aren't even clinical [29]. Between 35 to 64 percent of instances when a reason for the inability to conceive is recognized most of it include a male component, according to studies of clinic populations [30-32]. Mating with an infertile male is the most typical reason for female infertility, as noted by Rothman [33]. The experience of infertility should be scientifically tested because it might appear natural that it would vary greatly based upon whether the spouse has a reproductive impairment.

2.3. Male infertility scenario worldwide

2.3.1 Global scenario

The computed worldwide data suggests that men are responsible for between 20% and 70% of infertility. Furthermore, the proportion of male infertility ranged from 2.5% to 12% among these nations. Central and Eastern Europe (8-12%) and Australia (8-9%) have the highest rates of male infertility. Male infertility occurs at a rate of 4.5-6% on the North American continent. The CDC believes that 9.4 percent of American men are infertile, however, a more precise estimate puts the number closer to 4.5 percent (Figure 2.1). Infertility is often considered to be significant in Sub-Saharan Africa; nevertheless, the rates look modest (Table 2.1), probably owing to underreporting [34].

Table 2.1. Percentage of "male infertility" around the globe

	Men who have been identified as infertile	Infertile partners who have been documented	Partners where the male aspect is one of several contributing aspects
North America	4.5-6%[a]	15%	50%
Middle East	Not known	Not known	60%-70%[b]
Sub-Saharan Africa	2.5%-4.8%[a]	12.5%-16%	20-40%
Europe	7.5%[a]	15%	50% of all infertile couples
Australia	8%; 9%[b]	15%	40%
Central/Eastern Europe	8%-12%	20%	56%
Asia	Not known	Not known	37%
Latin America	Not known	Not known	52%
Africa	Not known	Not known	43%

"[a] Percentages were calculated from data reported on female infertility, using the assumption that 50% of infertility cases are due to females only, and 20-30% are due to male factors only.

[b] Study states that 60-70% of all men presenting to IVF clinics in the Middle East have some involvement in the cause of infertility".

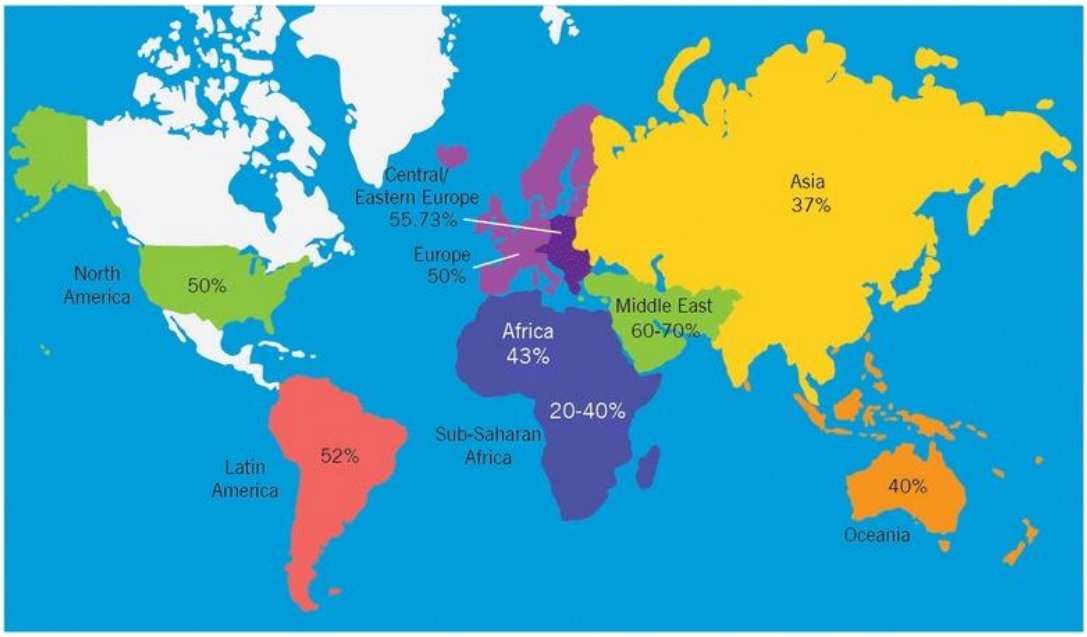


Figure 2.1. Global percentages of instances of infertility caused by male factors by location. Infertility rates for men in each continent (North America, Africa, Asia, Europe, Central/Eastern Europe, Latin America, the Middle East, and Oceania). Adapted from Ashok Agarwal et al. 2015

Primary infertility has a worldwide frequency of 3.9% to 16.8%, according to the WHO. Estimates of infertility also show vast variation amongst the Indian states. While it is estimated at 3.7% in Himachal Pradesh, Uttar Pradesh, and Maharashtra, it rises to 5% in Andhra Pradesh and 15% in Kashmir. Primary infertility rates have also been proven to differ throughout Indian tribes and castes, even amongst those living in geographical proximity to one another. About a quarter of Indian couples who seek help do it because of the man. Nearly half of infertility cases in India are attributable to male reproductive abnormalities or illnesses, according to a new assessment of the prevalence of infertility in the country. In addition, in over 25% of infertility cases, no underlying reason is identified following standard diagnostic procedures, leaving the condition undiagnosed and unexplained [35].

2.3.2 Symptoms of Male Infertility

Not able to bear a child is the main indication of male infertility. There may not be any more assertiveness symptoms or indications. However, in other cases, the signs and symptoms are brought on by an underlying problem, such as a hormonal imbalance, a genetic illness, a disease that inhibits sperm from flowing through, dilated veins around the testicles, or a hereditary illness. Observable symptoms and signs include:

- A very low sperm count
- Lack of sense of smell
- Unusual breast development
- Repeated respiratory illnesses
- Testicular pain, swelling, or a lump
- Loss of body or facial hair, as well as other chromosomal or hormonal abnormalities
- Concerns with sexual function, such as the inability to ejaculate or ejaculate infrequently, a decrease in sexual desire, or trouble maintaining an erection (erectile dysfunction) [36, 37].

2.3.3 Male infertility causes

Like most, including endocrinological disorders, abnormal enlargement of the testicular veins, obstruction/absence of seminal pathways, testicles missing from the scrotum either one or both (cryptorchidism), infections, chemotherapy, alcoholism, or venous plexus in the scrotum (varicocele), can cause male infertility [38]. But genetic changes have also become one of the main reasons for male infertility. The genetic abnormalities frequently found in infertile males include expansions on the Y chromosomes anterior end, single-gene mutations, and polymorphisms, and karyotypic abnormalities Yq microdeletions. These abnormalities, geneticaly hinder the proliferation of male germ cells, prevent the development of the man gonads or the urinary system, or cause the generation of non-functional spermatozoa. Two of the most common genetic reasons of infertility in men, among other issues, are Yq microdeletions and karyotypic abnormalities [39,40]. A few of the conditions that sperms need in order to make males fertile are listed below:-

- **Sperm needs to functional and be motile -** If the motility or function of sperm loses power, it may not be able to penetrate or enter the egg.
- **Generate healthy sperm-** This is first related to the maturation of the male reproductive system throughout puberty. The body must create testosterone and other hormones to start and sustain sperm production, and at least one of the testicles must be healthy.
- **The semen must include an adequate amount of sperm-** If there are few sperm in a male's semen, there is a lesser chance that one of his sperm may fertilize his partner's egg (sperm count). Low sperm counts are defined as 15 million sperm/mL of semen or less than 39 million sperm/ejaculate.
- **Sperm needs to enter the semen-** Sperm are produced in the testicles and are then delivered via tiny tubes to the penis, where they combine with semen and are discharged [41,42].

2.3.4 Medical causes

Male fertility challenges may be brought on by several medical conditions and therapies, including:

a. **Ejaculation problems -** The thin tubes used to convey sperm from the testicles to the penis, where they join with semen and are ejected, are where they are formed. Retrograde ejaculation may be brought on by several medical disorders, including spinal injuries, drugs, diabetes, and surgeries on the urethra (Figure 2.5), bladder, or prostate [43].

b. **Inflammation -** Certain infections may have an impact on the health or production of sperm**,** or they may cause scarring that sperm cannot pass through. These include certain sexually transmitted diseases like gonorrhea or HIV as well as testicular or epididymal inflammation (orchitis). Although certain diseases may permanently harm the testicles, sperm is often still retrievable [44].

c. **Tumors -** Male reproductive organs may have direct effects from malignancies and benign tumors, indirect effects from tissues that produce reproductive hormones, such as the pituitary gland, or unidentified reasons. Male fertility may sometimes be impacted by malignancy treatment methods such as surgery, radiation, or chemotherapy [45,46].

d. **Antibodies directed targeting sperm -** Immune system cells called anti-sperm antibodies interpret sperm for dangerous intruders and work to get rid of them [48].

e. **Imbalances in hormones -** Infertility may result from testicular issues or disorders that impact other hormonal systems, such as the adrenal glands, hypothalamus, thyroid, and pituitary glands. Low testosterone (also known as male hormone) may have several underlying causes. hypogonadism) and other hormonal issues [36].

f. **Undescended testicles -** One or both testicles in certain male fetus fail to penetrate the testicular sac from the abdomen during development (scrotum). Men who have experienced this illness are more prone to have decreased fertility [48].

g. **Chromosome defects** - A person who is born with one Y chromosome and two X chromosomes (instead of one X and one Y) has Klinefelter's syndrome, an inherited condition that results in improper development of the male reproductive system (Figure 2). Cystic fibrosis and Kallmann's syndrome are two other genetic diseases linked to infertility [49].

h. **Errors in the sperm-transporting tubules -** Many pathways exist for sperm to travel. Surgery, infections, trauma, or abnormal development, as in cystic fibrosis or other genetic illnesses, are all possible causes of their obstruction. Any stage of congestion is conceivable, including the testicle itself, the tubes that drain it, the vas deferens (Figure 2.5), close to the ejaculatory ducts, the epididymis, and the urethra [50].

i. **Medications -** Male infertility may be decreased by testosterone replacement treatment, chemotherapy for cancer, certain pharmaceuticals for ulcers and arthritis, long-term anabolic steroid usage, and a few other drugs [51,52].

j. **Celiac disease** - Sensitivity to the gluten protein present in wheat, causes celiac disease, a digestive ailment. Male infertility might be impacted by the disorder. After switching to a gluten-free diet, fertility may improve [53].

k. **Problems with sexual intercourse** - These include ED (erectile dysfunction), premature ejaculation, painful intercourse, physical anomalies like hypospadias, which is the presence of a urethral hole underneath the penis (Figure 2.5), and sex-interfering psychological or interpersonal issues [54,55].

l. **Varicocele** - The testicular veins expand, causing a varicocele. This is the most frequent treatable cause of male infertility. For unknown causes, varicoceles may cause infertility; however, uneven blood flow may be a factor. Varicoceles result in reduced sperm quantity and quality [56].

m. Prior surgeries - Rectal cancer, prostate surgery, testicular or scrotal surgery, and big abdominal surgery for testicular or vasectomy, among other procedures, may all prohibit from having sperm in your ejaculate [57].

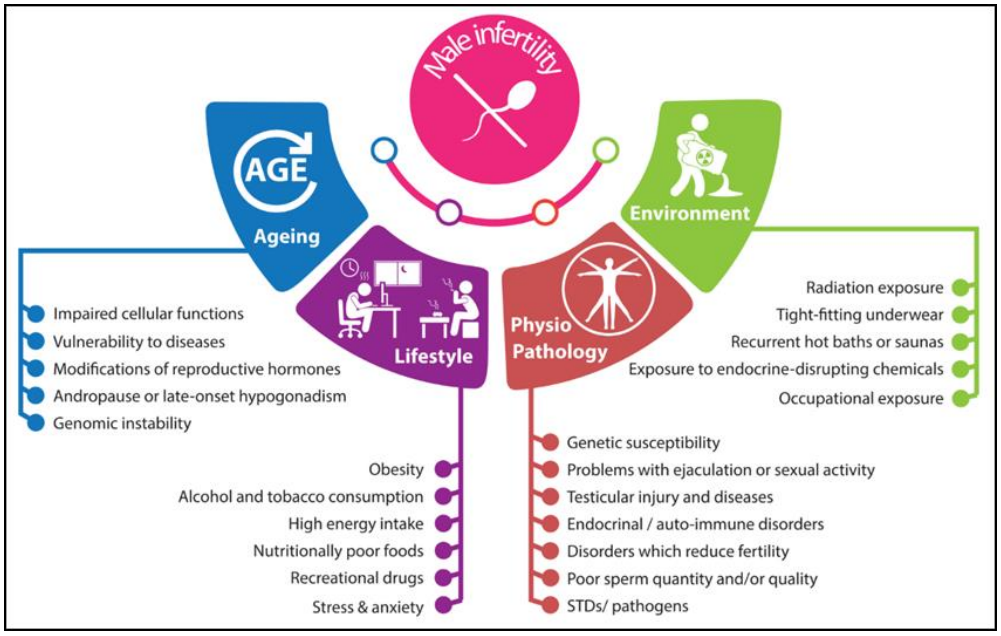


Figure 2.2. Causes of male infertility. Adapted from Mourad Assidi. 2022

2.3.5 The impact of the environment on the risk of male infertility

The environmental dangers to male reproductive function known as "DBCP" (1,2-dibromo-3-chloropropane) that lowered spermatogenesis and infertility were first detected when agricultural workers and pesticide manufacturers were exposed to the nematocide 30 years ago [58,59]. Other chemicals, pesticides and solvent types have now been shown to be toxicants to male reproduction in animal models [60]. Due to their extensive use and potential for environmental leakage, these compounds may pose a danger to male fertility (Figure 2.2). However, the evidence supporting these assertions derives from a tiny number of cross-sectional studies done on occupational groups that were exposed to these compounds at extraordinarily high concentrations. Only a few numbers of drugs have been found to negatively affect human spermatogenesis [61-65].

- **Overheating of the testicles-** High temperatures may harm sperm function and production. Their sperm count may temporarily decline if they often use saunas or hot baths, while research is few and inconsistent [66].
- **Heavy metal exposure-** Infertility may also result from lead or other heavy metal exposure [67].
- **Radiation or X-rays-** Although sperm production may be lowered by radiation exposure, it often ultimately resumes its usual level. High radiation doses have the potential to permanently lower sperm production [68].
- **Industrial chemicals-** Prolonged exposure to certain herbicides, insecticides, organic compounds, and paint supplies may result in low sperm counts [69].

Long hours of sitting, tight clothes, or prolonged use of a laptop computer may all raise the warmth in the scrotum and lower sperm production (Figure 2.2).

There has been a rise in the public's interest in the male reproductive function as a consequence of studies showing an increase in the incidence of various biological issues affecting the male genital tract during the last fifty years [70]. These include an apparent drop in sperm production across the board in the male population, an uptick in the prevalence of sperm defects including hypospadias and cryptorchidism, and an increase in the frequency of testicular cancer. These occurrences have been speculatively related to the widespread usage of endocrine disruptors, or substances having hormonal capabilities [71].

Reduced fertility concentration decreased androgen synthesis and the generation of abnormal spermatozoa are only a few of the repercussions of toxic injury to the testes. Semen analysis, blood hormone measurements, and assessment of prior partner pregnancy success are all part of the non-invasive procedure for determining male fertility in males. Semen analysis allows for direct examination of the male reproductive system as well as research into the relationship between environmental agent exposure and fertility [72,73].

2.3.6 Health, lifestyle, and other factors

Male infertility can also be brought on by:

- **Drug use :** It is used to increase muscular mass and strength; anabolic steroids have the potential to shrink testicles and lower sperm counts. The quantity and quality of your sperm may also briefly reduce if you use cocaine or marijuana [74].
- **Alcohol use :** Alcohol use may reduce testosterone levels, lead to erectile dysfunction, and decreased sperm counts. Fertility issues might result from heavy drinking-related liver damage (Figure 2.2) [75].
- **Tobacco smoking :** Smoking may diminish sperm counts in men compared to non-smokers. Male fertility may also be affected by secondhand smoking [76,77].
- **Weight :** The ability of obesity to affect fertility may be influenced by several factors, including the direct impact on sperm as well as hormone changes that reduce male fertility [78,79].

2.4. Male reproductive biology

Spermatozoa (gamete), androgens, or male sex hormones are generated in the male gonads, also known as the testes. The additional genital organs are the seminal vesicles, epididymides, vasa deferentia, ejaculatory ducts, ductus, and penis [101]. The prostate and bulbourethral (Cowper) glands are two more structures that are present. The primary function of these organs is to transport mature spermatids as they move from the testes to the outside and to secrete substances that are necessary for the development of semen. That’s why having knowledge of detailed description of the anatomy and biology of the male reproductive system is important.

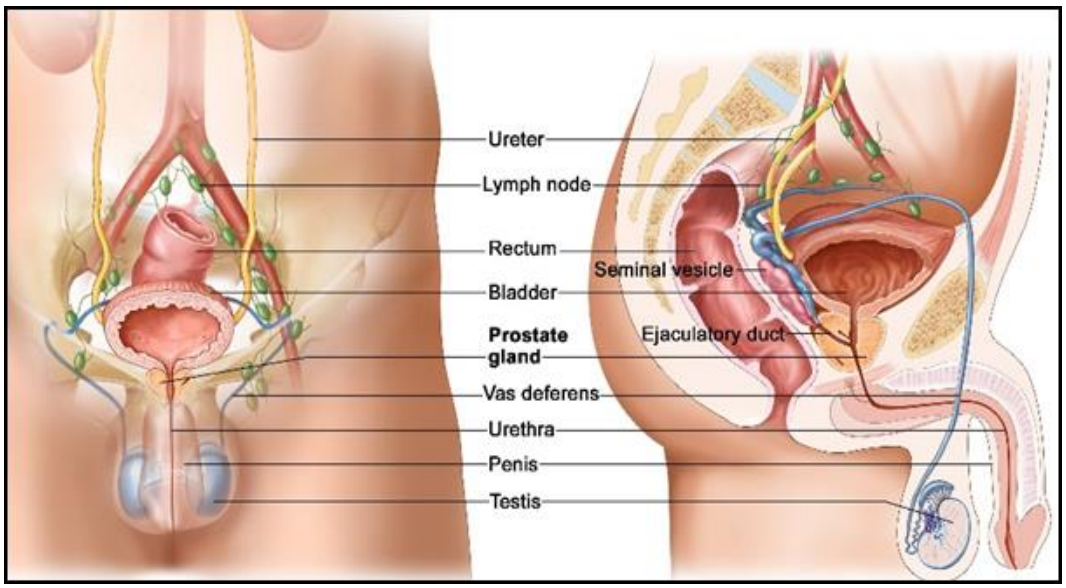


Figure 2.3. Anatomy of the male reproductive and urinary system, including the testicles, prostate, and other organs. Adapted from cancer.gov/publications/dictionaries/cancer-terms/def/reproductive-system

2.4.1. External genitalia

a) Penis

Males have an external and internal penis, which is used during copulation. The penile body, constituting the external portion of the penis, is unrestrained, hanging freely, and entirely enveloped by skin [102]. Its primary structural element comprises cavernous or erectile tissue, which, upon filling with blood, leads to considerable enlargement and erection. Within the penis, there exists a passage called the urethra, responsible for conveying both semen and urine (see figure 2.5).The interior portion of the penis that connects to the bony pubic arch is known as the base.

The "shaft," or penis body, has a triangular cross-section while it is erect but a cylindrical cross-section when relaxed. The left and right corpora cavernosa, the erectile tissue masses situated on the dorsal side of the penis, are closely positioned, leading to a lack of organization. Within the penis, the urethra is housed within a single structure known as the "corpus spongiosum," which occupies a central groove on the underside of the corpora cavernosa [103]. At the outer end of the corpus spongiosum, it expands significantly to form the delicate, cone-shaped, and highly sensitive glans penis. The base of the glans, known as the corona, forms a prominent rim and acts as the

neck of the penis, while the glans itself rests atop the corpora cavernosa groove. The urethra exits the body through a vertical, slit-like external orifice after passing through the glans. The prepuce (also known as the foreskin) is made by thin folding, loosely adhering skin over the penis (which is positioned in the neck) forward over the glans for various lengths. The prepuce's frenulum, a median fold, descends the underside of the glans to a place just below the urethral entrance [104].

The root of the penis is composed of the penile bulb and two crura or projections. The crura and bulb, located at the boundaries of the pubic arch and perineal membrane, are joined together. Each crura is a long, ischiocavernosus-covered structure that extends forward, converges towards the opposite, and joins with one of the corpora cavernosa. The two crura are separated by the oval penis bulb, is surrounded and shielded by the bulbospongiosus muscle. The corpus spongiosum parallel is followed. The urethra can enter through the flattened deep aspect of the bladder, which is next to the perineal membrane. In the corpus spongiosum, it then passes through its contents.

A median fold of the prepuce called the frenulum descends the underside of the glans to a location slightly below the urethral entrance. The erectile tissue of the corpora cavernosa is divided into numerous cavernous sections by a multitude of small fibrous bands. While the penis remains flaccid, it becomes engorged with blood during an erection. In contrast to the corpora cavernosa and similar structured tissues, the corpus spongiosum contains a higher proportion of smooth muscle and elastin. Covering the anatomical components of the penis and attaching it to the pelvic bones just below the midpoint of the pubic arch is a thick fascia or connective tissue sheet called the suspensory ligament [105].

The penis receives a significant blood supply primarily from the internal pudendal artery, which is a branch originating from the internal iliac artery, pelvic region, buttocks, and inner thighs. The cavernous regions become distended with blood during an erection, and the constriction of the local veins prevents the blood from draining away. Autonomic (involuntary) and sensory nerves are abundantly present in the penis. Contraction and dilation of blood arteries are effects of the sympathetic and parasympathetic divisions of the autonomic nervous system, respectively. Conventional explanations place the reason for ejaculation on the sympathetic nervous system, which also keeps sperm from entering the bladder and reduces the need to urinate [106].

b) Scrotum

Located below the pubic symphysis and in front of the upper thighs, there exists a sac-like structure called the scrotum. Within this pouch, the base of the spermatic cord and the testes are situated. The scrotum is divided into two compartments by the scrotal septum, also known as the partition. This ridge or raphe can be observed on the external surface of the scrotum. Before returning to the perineum, the raphe revolves around the base of the penis. [107].

The scrotum's skin is often wrinkled and folded, thin, pigmented, and lacking in fatty tissue. The few hairs and sebaceous glands it has are scattered over its surface. The dartos, a layer of involuntary muscles just beneath the skin, could alter the scrotal contours. As the scrotum is chilled, upon contraction of the dartos muscle, the region adopts a wrinkled and shortened appearance, altering the texture of the scrotum. Conversely, when exposed to warmth, the scrotum relaxes, becoming smooth and flaccid, and loosening its grip around the testes. It's important to note that the fascia underneath the dartos muscle is connected to the covers of the two spermatic cords. The testicular blood and lymph arteries, the arteries to each ductus deferens, and the artery to the cremaster muscle are all contained inside these cords, ensuring that the testes stay securely within the scrotum [108]. The blood supply to the scrotum is provided by the internal pudendal artery and scrotal branches originating from the femoral artery, which is the main artery of the thigh.

c) Testes

The spermatic cords descend from their origin on the abdominal back wall into the scrotum seven months after conception and sustain the two testes, often known as testicles. The testes are enveloped by a fibrous pouch known as the tunica albuginea, which measures approximately 4 to 5 centimeters in length. Surrounding the sac from all sides is the tunica vasculosa, a network of blood vessels, while the tunica vaginalis, an extension of the membrane encasing the abdomen and pelvis, lines the sac (see Figure 2.5). The tunica albuginea exhibits protrusions that serve as partial partitions, dividing each testis (see Figure 2.5) into approximately 250 separate compartments referred to as lobules. [109].

Sperm production takes place within the convoluted tubules, often referred to as thin tubes, present in each lobule. The tubules would be around 70 cm long if they were straight. Beginning with the primordial sperm cells called spermatogonia in the outermost layer, the multistage process

of sperm production in the tubule lasts for around 60 days. Upon leaving the tubules, spermatozoa are not capable of independent movement. Instead, they undergo maturation within the male reproductive system's ducts before entering the female reproductive system after ejaculation. They may even carry on growing thereafter. Sperm maturation in the vaginal tract is referred to as "capacitation." Each spermatozoon is long and thin, with a head, neck, body, and tail. The head of the cell contains the cell nucleus. The tail whips back and forth in a mature spermatozoon to propel the cell.

The hormone testosterone, which is responsible for male sex, is produced by leydig cells. During puberty, the hormone responsible for stimulating interstitial cells, known as luteinizing hormone in women, induces a substantial rise in the activity of the tissue. Teenagers' testosterone levels (prostate, seminal vesicles) encourage the development of male accessory sex glands and secondary sex traits in men. Additionally, the hormone is essential for sperm maturation and has the potential to increase a man's sexual drive. The testis also produces some oestrogen, a hormone connected to female sex that supports pituitary activity [110]. Both testes receive blood from the testicular arteries, which arise from the anterior aorta beneath the renal (kidney) arteries. Each artery starts at the top of each testis and passes through the spermatic cord, inguinal canal, and lower back on its way to the lower back. The testis and epididymis are connected by a network of veins that climb into the "spermatic cord". Lymph veins, which also pass through the spermatic cord, carry the lymph fluid to the lateral and preaortic lymph nodes.

d) The sperm canal's anatomy

The sperm canal is formed by the ductus deferens, epididymis (also known as the vas deferens), and ejaculatory ducts, connecting the testicles to the urethra that passes through the prostate. Within the epididymis, around 20 tiny ducts called ductules penetrate its fibrous capsule, transporting sperm from the testis to the head of the epididymis. Initially straight, these ductules progressively enlarge and become highly convoluted, forming distinct compartments near the head of the epididymis. The body and tail of the epididymis are created by a single, extensively branched duct. This duct expands and develops thicker walls as it joins the ductus deferens at the base of the epididymis. The lining of the testicular ductules consists of a thin muscular layer, alternating between clusters of tall columnar cells with cilia and shorter cells without cilia. The cilia assist in

propelling sperm towards the epididymis. The muscular coat is thicker, and the lining of the epididymal duct is rich in non-motile cilia. It is suggested by some evidence that excess fluid and debris are removed by the ductules and the initial segment of the epididymis before the testicular secretions enter these tubes.

Blood supply to the epididymis is provided by a branch of the testicular artery, which reaches the epididymis before reaching the testis. The duct of the epididymis, also known as the vas deferens, continues as the ductus deferens. It extends to the deep inguinal ring, forming the spermatic cord. At the ring, the ductus deferens separates from the blood vessels, nerves, and lymphatic vessels of the spermatic cord, descending through the pelvis to the base of the prostate (see Figure 2.5). The ejaculatory duct is formed when the ductus deferens joins the seminal vesicle. Located near the base of the urinary bladder, the ampulla is a dilated and somewhat twisted segment of the ductus deferens. Peristaltic contractions, driven by well-developed longitudinal muscle fibers, propel sperm into the ampulla. While some cells contain non-motile cilia, the columnar mucous membrane that lines the interior is typically non-ciliated and longitudinally folded. The ampulla, with its thinner walls, likely serves as a reservoir for sperm. Overall, the ductus deferens, with its thick smooth muscle layer, exhibits a distinctive cord-like structure. The movement of sperm into the ampulla is facilitated by peristaltic contractions [111,112].

2.4.2. Accessory organs

a) The prostate gland, bulbourethral and glands-seminal vesicles

The majority of an ejaculate's seminal fluid is formed by secretions from these structures. It is situated anterior to the rectum (Figure 2.5). The prostate has an upward-facing base that reaches directly into the neck of the bladder and is formed like an inverted pyramid. The posterior aspect of the prostate, where the two ejaculatory ducts enter the organ, forms the upper edge. Enclosed within a smooth muscle and fibrous tissue capsule, the prostate exhibits a solid consistency. Its approximate dimensions are 1.6 by 1.2 by 0.8 inches (about 4 by 3 cm by 2 cm). The prostate is composed of glandular tissue surrounded by a muscular framework, with the three lobes unevenly distributed. The main mass consists of two side lobes that extend continuously behind the urethra, connected in front of the urethra by a fibromuscular isthmus that lacks glandular tissue [113]. The third lobe, known as the middle lobe, varies in size, is

relatively smaller, and may not contain glandular tissue. Within the prostate, there are three clinically relevant concentric zones of prostatic glandular tissue surrounding the urethra. The innermost zone comprises small glands that secrete mucus into the urethral channel and are located closest to it. The outer zone consists of glands bordered by tall columnar cells that produce prostatic fluid in the presence of androgens from the testis. This prostatic fluid is a thin, milky liquid with a slightly acidic taste.

Two small sacs called seminal vesicles, each measuring approximately 5 cm in length, are located between the “rectum” and the base of the “bladder.” Most of the semen is composed of these secretions. Each vesicle is composed of connective tissue that wraps around a highly coiled tube, forming a network of diverticula or out pouches. The ejaculatory duct is formed by constricting the lower end of the tube into a straight duct or tube and connecting it to the corresponding ductus deferens. Lower on the cell, the vesicles are grouped, although they are more widely spaced near the individual ducts [114]. The longitudinal and spherical layers of smooth muscle and the mucous membrane-lined chambers of the seminal vesicles, which create the organs' secretions, are what make up these structures. These fluids are released during ejaculation as a result of muscle contractions. The vesicles' ability to operate depends on the testes' ability to produce androgen hormone. The yellowish, dense, adhesive discharge, which is also mildly alkaline in nature contains fructose.

The "Cowper glands," also known as the bulbourethral glands, are small, pea-shaped glands positioned below the prostate gland, near the beginning of the inner section of the penis (see Figure 2.5). These glands are connected to the central, spongy part of the urethra through long, slender ducts which spread outward and inward. With a diameter of approximately 1 cm (0.4 inch), the Cowper glands play a role in contributing secretions to the urethra. Muscle and elastic tissue fibres fill the network of tubules and sacs that make up the glands, giving them the necessary muscular support.

b) Ejaculatory ducts

The ejaculatory ducts, which are derived from the seminal vesicle duct carrying semen secretions and the terminal part of the ductus deferens at the base of the prostate, originate on each side of the midline. Each duct has a length of approximately 2 centimeters (0.8 inches)

and serves as a conduit for both urine drainage from the prostate, through the lateral and median lobes, into the prostatic urethra. Within this section of the urethra, there is a longitudinal ridge known as the urethral crest, located on the floor (or posterior wall). On either side, there is a prostatic sinus that contains the openings for the prostatic ducts. In the center of the urethral crest, a small elevation called the colliculus seminalis houses the opening of the prostatic utricle [115]. The prostatic utricle is a tiny pouch or diverticulum that is lined with mucous membrane and may have analogies to the vagina or uterus in females. The apertures of the ejaculatory ducts can be found near or on either side of the entrance of the prostatic utricle. The ejaculatory ducts are lined with columnar cells and have thin walls.

2.5.The Y chromosome in humans

At least 180 million years ago, autosomes gave rise to the sex chromosomes of mammals. The Y chromosome began to distinguish when the testis-determining gene was acquired. Eventually, there were several inversions and a progressive suppression of recombination between the “X and Y chromosomes” [80,81]. The human “Y chromosome's” history and present state have been thoroughly investigated in recent research. [82].

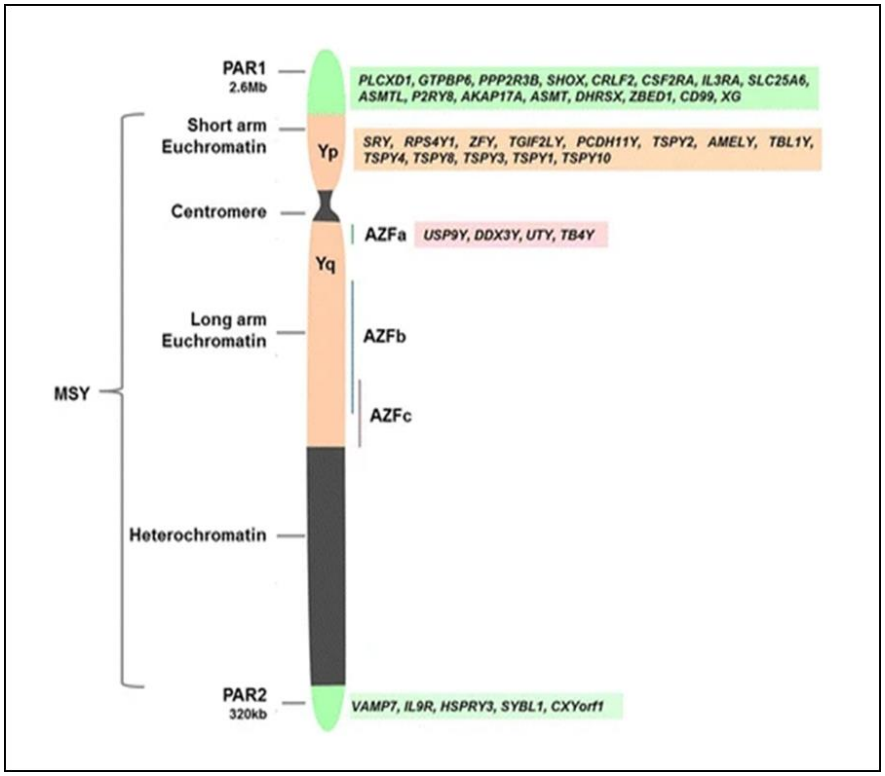


Figure 2.4. The Y chromosome in humans. PAR1 and PAR2 are pseudo autosomal regions on the Y chromosome's extreme ends. Gene coding areas are shown as green boxes. The genes located on the Y chromosome's short arm (Yp) are highlighted in peach box. Both active euchromatin and inactive heterochromatin make up the long arm, Yq. Adapted from Stacy Colaco et al. 2018

The "short arm (Yp) and long arm (Yq)" of the human Y chromosome are made up of two pseudo autosomal regions (PARs) that are separated by centromeres. (Fig. 2.3). Except for the proximal area around the centromere, the long arm is mostly heterochromatic, in contrast to the PARs and short arm, which are euchromatic [83].

The Y chromosome in humans is structured. The Y chromosome's terminal ends are a site for the PAR1 and PAR2 (pseudo autosomal regions). The genes that are encoded in these areas are shown in green boxes. The Y chromosome's short arm, as well as the genes located on Yp, are shown below. Euchromatin and the genetically dormant heterochromatin areas make up the long arm, Yq. The AZFc, AZFa, and AZFb azoospermia factors are in this area. The genes in the AZFa

region. If there are any known genes in the heterochromatin, it is unknown. The MSR is the area on Y that lies beyond the PAR [84].

2.6. Genetic evaluation of infertile men

In the beginning, primary spermatocytes are developed by the mitotic division of spermatogonia, which is followed by a first meiotic division to create secondary spermatocytes. This is the complex biological process known as human spermatogenesis. These secondary spermatocytes create round spermatids, and haploid cells after a second meiosis cycle, which then develop into extending spermatids, and the same develops in spermatozoa via differentiation [85]. The coordinated activities of several hormones, regional secretory elements, and genes specific testes are essential for the spermatogenic process. Any of these flaws might cause an accumulation of faults that impede spermatogenesis and result in infertile males. The World Health Organization defines male infertility as the male partner's inability to conceive or induce conception in a medically normal female. Thirty million men are infertile worldwide, with the highest rates seen in “Central and Eastern Europe” (8–12percent) and Australia (8–9percent) [86]. According to WHO guidelines from 2010, male infertility may be divided into the following groups based on seminograms (Table 2).

Table 2.2. Nomenclature related to semen quality

Asthenozoospermia	Percentage of progressively motile (PR) spermatozoa below the lower reference limit
Asthenoteratozoospermia	Percentages of both progressively motile (PR) and morphologically normal spermatozoa below the lower reference limits
Necrozoospermia	Low percentage of live, and high percentage of immotile, spermatozoa in the ejaculate
Aspermia	No semen (no or retrograde ejaculation)
Azoospermia	No spermatozoa in the ejaculate (given as the limit of quantification for the assessment method employed)
Cryptozoospermia	Spermatozoa absent from fresh preparations but observed in a centrifuged pellet
Haemospermia (Haematospermia)	Presence of erythrocytes in the ejaculate
Leukospermia (Leukocytospermia, Pyospermia)	Presence of leukocytes in the ejaculate above the threshold value
Oligozoospermia	Total number (or concentration, depending on outcome reported) of spermatozoa below the lower reference limit
Teratozoospermia	Percentage of morphologically normal spermatozoa below the lower reference limit

- **Azoospermia** refers to the absence of sperm in the sperm cells. While non-obstructive azoospermia (NOA) refers to the "lack of sperm in the semen as a result of inadequate sperm production". Obstructed azoospermia (OA) refers to the "absence of sperm in the ejaculate as a result of issues with sperm transport". Sixty percent of all instances of azoospermia are caused by NOA [87].
- Normal sperm counts in the ejaculate are known as **normozoospermia**.
- Severe **oligozoospermia**: Ejaculate contains less than five million spermatozoa
- Less than 50% of sperms have **asthenozoospermia**, which is characterized by low motility levels.
- **Aspermia**: Failure in ejaculating semen
- In **teratozoospermia**, only 30% of sperms that survive have normal morphology.
- **Oligozoospermia**: A low spermatozoa count in the ejaculate, which is less than 15-20fiftten to twenty million [88].

About 50% of the time, the male component is the cause of infertility in couples [89]. Oligozoospermia or azoospermia (AZO) are commonly seen in such circumstances. Such infertile individuals exhibit both molecular and genetical methods used to identify abnormalities at the microscopic level and cytogenetic processes used to identify genetic problems at the macroscopic level [90].

The first to claim that chromosome Y contains the genetic code for controlling spermatogenesis was Tiepolo and Zuffardi in 1976 [91]. According to cytogenetic research (Yq), 0.5% of infertile males have macroscopic deletions of the Y chromosome's distal long arm. The azoospermia factor was thought to be located at Yq11.22–23 on the long arm of the Y chromosome [92]. Microdeletions were discovered in the azoospermia factor (AZF) region of oligozoospermic and azoospermic males [93] at a frequency of 5-18% with the development of the PCR (polymerase chain reaction) and the creation of a Y chromosome STS (sequence-tagged site) map [94-99].

2.7. Emerging genetic considerations in male infertility

The connection between minor variations in the genome sequence and male infertility is now receiving a lot of attention. They identify three potential future influences on fertility treatment and

children born via ART (Figure 2.4). Human male infertility is known but the reason behind that is not clear and what exactly caused is also not known. Moreover, genes causing male infertility are also still unknown. Studies below the dotted line are more often connected to male infertility [100].

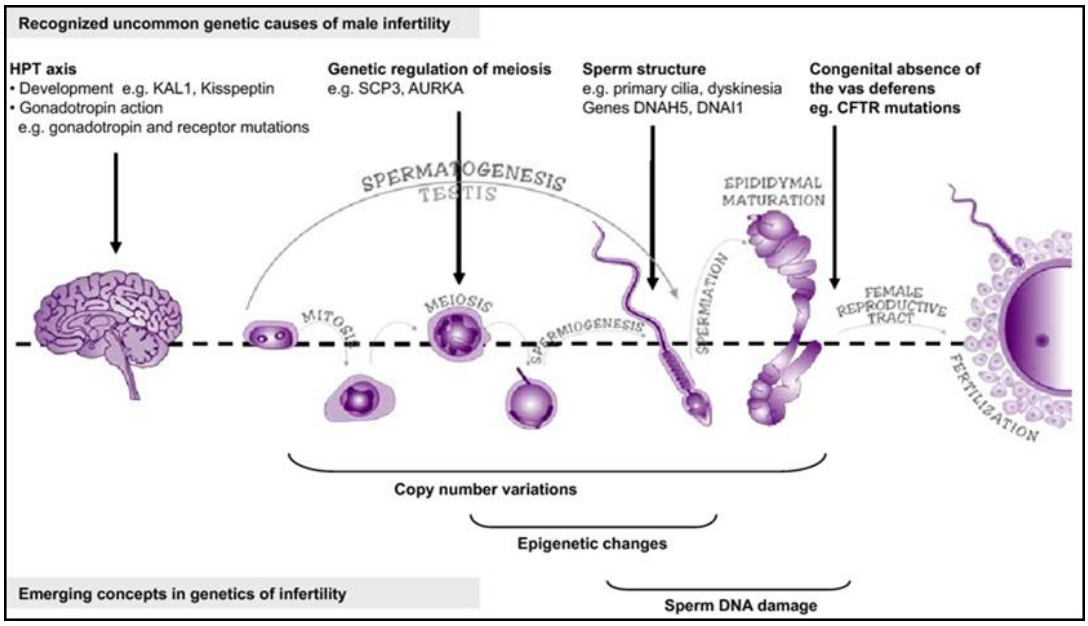


Figure 2.5. Genetic considerations in infertile males. Male infertility is increasingly being linked to fields of study below the dotted line, albeit the exact mechanisms and genes related with infertility have not yet been identified. Adapted from R. McLachlan et al. 2010

2.8. Sperm Development/spermatogenesis

The meiotic reductive divisions are constant throughout all eukaryotic kingdoms, although mammalian sexual dimorphism leads to significant differences in meiosis regulation in males and females. Sperm are produced during spermatogenesis, which is the process of the original germ cells. In a male vertebrate embryo, PGCs (Primary Germ Cells) become a member of the sex cords as they reach the genital ridge. After the organism reaches sexual maturity, the sex cords' epithelium thins out to become the seminiferous tubules, and the sex cords themselves transform into Sertoli cells. During puberty, BMP8B is likely generated by the spermatogenic germ cells, also known as spermatogonia, to regulate the onset of spermatogenesis. The germ cells start to

differentiate when the BMP8B concentration reaches a threshold level. High quantities of BMP8B are produced by the developing cells, spermatogenesis is not started at puberty in mice missing BMP8B [116]. Bone morphogenetic protein 8B (BMP8B) is a growth factor that belongs to the TGFbeta superfamily. Bmp8b appears to be involved in spermatogenesis and reproduction in mice since it is expressed in the trophoblast cells of the placenta and male germ cells of the testis.

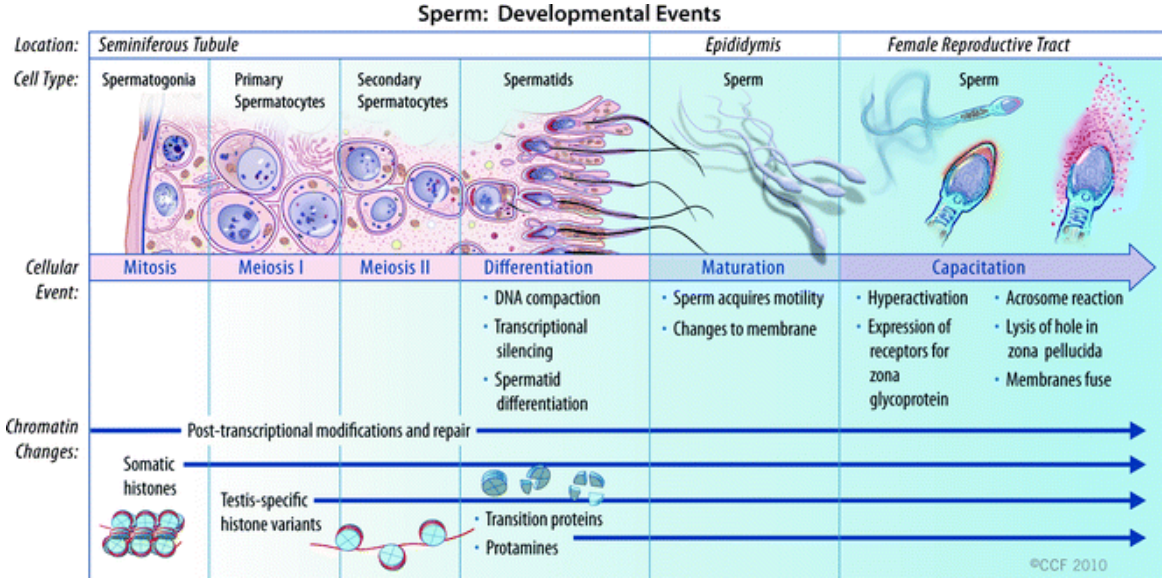


Figure 2.6. The overall process involves in human sperm development. Sequence of events including cell division and chromatin modifications as a germ cell transforms into a spermatozoon and then is released, stored, and travels to the uterus. Adapted from Rakesh Sharma et al. 2011

N-cadherin molecules on both cell surfaces and galactosyl transferase molecules that bind a carbohydrate receptor on the Sertoli cells in the spermatogenic cells hold the spermatogenic germ cells to the Sertoli cells (Figure 2.7). Spermatogenesis the process by which a germ cell develops into a mature sperm takes place in the crevices of the Sertoli cells, which also provide nourishment and protection for the growing sperm cells (117).

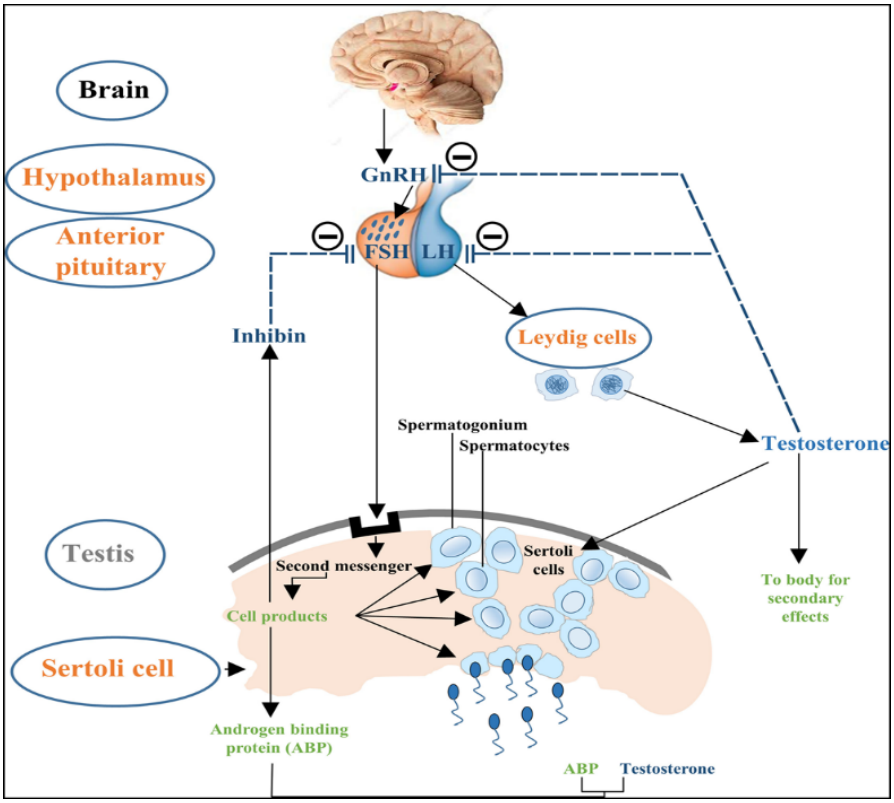


Figure 2.7. Leydig cells, which are engaged in the late Sertoli cell growth stage and are stimulated to secrete testosterone by LH, then undergo negative feedback and limit production of GnRH and LH. In addition to maintaining spermatogenesis, steroid hormones are produced in the seminiferous tubules in response to FSH, namely inhibin. Adapted from Wasim Shah et al. 2021

Type A1 spermatogonia (Figure 2.6) are created by the PGCs' division once they reach the gonad. Its ovoid nucleus, which includes chromatin connected to the nuclear membrane, distinguishes these cells from PGCs, which are smaller in size. The outer foundation membrane of the sex cords is located close to the A1 spermatogonia. These are stem cells, and it is believed that when they reach maturity, they will split to produce both a second kind of cell, the type A2 spermatogonium, which is a lighter color, as well as another form of spermatogonium, the type A1. As a result, every type of A1 spermatogonium is a stem cell with the capacity to both regenerate and create a different kind of cell. The A2 spermatogonia split into the A3 and the A4, which then produce the type A4 spermatogonia. Each of the types A spermatogonium is a kind of stem cell that may self-renew.

Three possibilities are available to the A4 spermatogonium: it may self-renew and create other A4 spermatogonium; it can die via cell death (apoptosis); or it can develop into the intermediate spermatogonium, the first committed stem cell type. To create type B spermatogonia, intermediate spermatogonia undergo one mitotic division, after which they are determined to develop into spermatozoa. Being the final cells in the line to go through mitosis, these cells serve as the spermatocyte precursors. To produce primary spermatocytes (Figure 2.6), which are the cells that begin meiosis, they split only once. It is unknown what drives cells to begin meiotic rather than mitotic division, or what steers spermatogonia towards differentiation rather than self-renewal (118).

Instead, the cells form a syncytium in which communication is carried out via cytoplasmic bridges with a diameter of approximately 1 μm that connect one cell to the others. Each cohort of cells grows simultaneously because of the clones of linked cells created by the subsequent divisions and the ease with which ions and molecules may transverse these intercellular bridges [119]. The spermatocyte nucleus often transcripts genes during this period, the results of which are eventually used to create the axoneme and acrosome. A pair of secondary spermatocytes (Figure 2.6) is produced after each main spermatocyte passes through the first meiotic division, completing the second phase of meiosis. Spermatids are the haploid cells that are produced as a consequence, and cytoplasmic bridges still connect them. Since a gene product generated in one cell may readily diffuse into the cytoplasm of its neighbors, the spermatids joined together in this manner have haploid nuclei but are diploid. The cells migrate steadily closer to the lumen of the seminiferous tubule as the type A1 spermatogonium divides into spermatids and away from its basement membrane. Hence, a specific layer of the tubule contains each kind of cell. The spermatids are located at the lumen's border, where they lose their cytoplasmic connections and transform into sperm cells (Figure 2.6). A mature sperm in a person takes 65 days to develop from a spermatogonial stem cell [120].

2.9. Spermatozoa

The head and flagellum are the two primary components of mature sperm, as depicted in Figure 2.8. The nucleus, acrosome, and a little quantity of cytoplasm are all found in the head. The

flagellum is split into three sections: the midpiece, the main piece, and the end piece. It includes the axoneme, which is a core complex of microtubules encircled by dense outer fibers that stretch from the neck into the main portion. The mitochondria are in the middle component. The "9+2" conserved structure of the axoneme is composed of a core doublet of microtubules encircled by a ring of nine A/B microtubule doublets. [121].

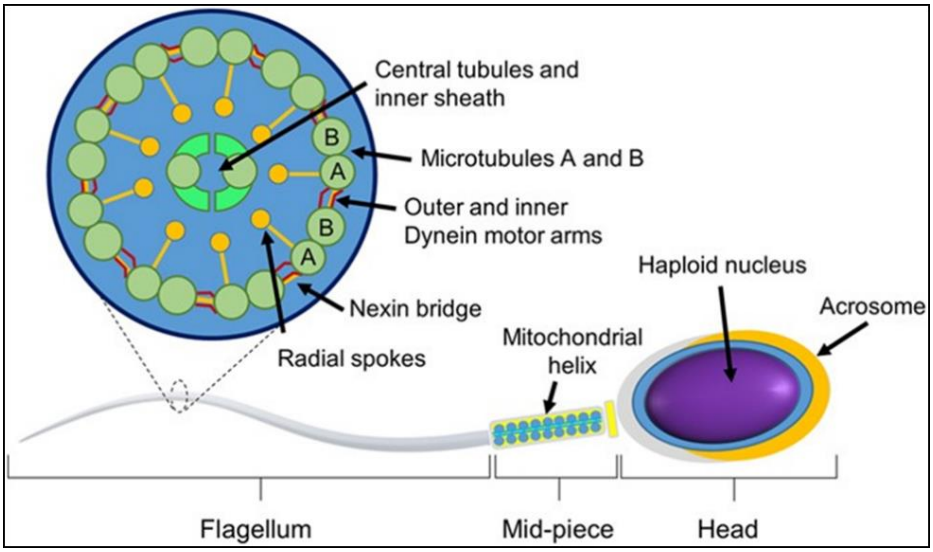


Figure 2.8. Schematic view of human sperm. The flagellum is made up of three parts (i) the head, which has a haploid nucleus and an acrosome on top to dissolve the zona pellucida; (ii) the mid-piece, which is made up of a mitochondrial sheath; and (iii) the tail, in which Microtubules A and B are connected by nexin links and dynein motor proteins. Adapted from Steven Reynolds et al. 2017.

Sperm complete their development in the testis, however, they are still physiologically "immature" at this point. Immotile spermatozoa are generated in the seminiferous tubules and then transferred to the epididymis, where they acquire their motility. Spermatozoa maturation in the epididymis requires androgens. Spermatozoa are passively transferred from the testes to the epididymis through the efferent ducts and rete 4 of the testis. The efferent ducts absorb the majority of the fluid discharged from the testis along with the spermatozoa, which raises the concentration of sperm in the epididymis [122]. There are three sections to the epididymis: the head, the body, and

the tail. Spermatozoa take 10-13 days on average to go through the epididymis in most animals, but just 2-6 days in humans. Spermatozoa isolated from the caudal epididymis tend to be highly motile and advanced, whereas those isolated from the caput epididymis tend to be immotile. To become fertile, sperm must undergo a series of maturational modifications while traveling through the epididymal duct. Modifications to the plasma membrane, nuclear cross-linking of protamines and the outer dense fiber and fibrous sheath proteins, and changes to the outer acrosomal membrane are a few examples. Energy is conserved and long-term survival of spermatozoa is favored by keeping the cells in a quiescent condition when they are stored in the cauda epididymis [123]. The chemicals in semen stimulate motility in spermatozoa when they come into touch with them during ejaculation. Caput sperm and caudal sperm are two types of artificially separated sperm used to examine variations in motility characteristics and metabolism. Despite their mobility, caudal spermatozoa are unable to fertilize an egg. Further maturational changes, including capacitation, hyperactivation, and the acrosome response, must occur in spermatozoa before they may fuse with the female gamete. Once sperm enters the female reproductive system, these alterations start to take place. The cervix plays a key role at the beginning, and possible completion, of capacitation. The plasma membrane, nucleus, metabolism, intracellular ions, and acrosome of sperm all undergo alterations during capacitation. Hyperactivation occurs in the oviduct and aids spermatozoa in navigating the thick fluid of the oviduct. Spermatozoa may break past the egg's protective zona pellucida and unite with its plasma membrane thanks to the acrosome reaction [124,125].

2.10. Sperm RNA

It was initially believed that the significant cellular changes experienced by spermatids during spermiogenesis would lead to the loss or destruction of RNA in mature sperm cells. Therefore, speculation concerning the probable function of sperm RNAs was ignited by the discovery of a diversified and completely intact mRNA population. It was previously believed that the RNA in sperm was inactive. However, numerous investigations have demonstrated that RNA may play active roles both inside the cell and possibly after it enters the egg during fertilisation. The translational activity of sperm is a subject of debate due to findings suggesting their lack of 80S cytoplasmic ribosomal complexes, which are essential for translation. However, the mechanism by which mitochondrial translation of mRNA, which contains 5' recognition sequences targeting 80S

ribosomes, occurs remains unclear. There are reports that the mitochondrial machinery is where sperm RNA is transcribed [273].

The development of the embryo may also be influenced by sperm RNA. Following fertilization, Ostermeier et al. (2004) utilized the discovery of sperm RNA in the zygote as proof that sperm RNA is transported to the oocyte. "A model for epigenetic inheritance through zygotic transfer of RNAs that dysregulated expression of the so-called c-Kit gene, which results in the change of the offspring's phenotypic expression", was presented by Rassoulzadegans et al. (2006). This model demonstrated that, by functioning without reference to the paternal genome, spermatozoa may influence developmental processes. Numerous assays that evaluate sperm function and quality are available in the area of infertility research. Given that at least 50 percent of instances of unexplained infertility include male-factor infertility, there is still a need for improved and more dependable procedures (Hwang et al., 2011). Below are several techniques for evaluating sperm quality, followed by sperm RNA from infertility research.

2.11. Relevance of sperm traits in assessing man infertility

The initial and most important phase in the assessment of infertility, which also involves hormone testing, physical examination, sperm function analysis, and genetic analysis called semen analysis. It also contributes to defining the degree of male factor infertility (MFI) and is regarded as a foundational element of the lab assessment of the infertile man [126]. When the findings of the semen analysis are persistently abnormal by World Health Organization standards, 2021, MFI is diagnosed. Consequently, an anomaly in one or more of the semen characteristics is the root cause of MFI in many cases [127]. Since human spermatozoa exhibit significant variability, even sperm from fertile man may exhibit a range of sperm abnormalities in semen samples [128]. Additionally, a normal spermiogram does not always represent sufficient reproductive potential. Only 40% of afflicted guys seeking help get a correct diagnosis of MFI due to the inherent limitations in the techniques of evaluation [129]. WHO, instructions were released, in 1999, 1992, 1987, 1980, 2005, 2010, and 2021 to harmonize the semen analysis processes used by andrology labs across the globe. The WHO recommended reference values (6th edition, 2021) for evaluating semen characteristics

have been accepted by most human semen analysis labs. Clinical professionals are worried that the present WHO reference levels are too strict.

a) Semen viscosity

The viscosity of the seminal fluid measures flows resistance. The measurement of sperm motility, concentration, and antibody coating may be affected by high viscosity. Semen often begins to coagulate at ejaculation and liquefies within 15 to 20 minutes. Because of their viscosity, sperm struggle to float. Sperm's decreased motility reduces the ovum's likelihood of fertilization. Researchers have examined less well-known associations between semen viscosity and several medical disorders, including infertility [130].

b) Semen pH

The seminal fluid's pH may significantly influence the sperm's ability to function; seminal plasma typically has a pH between 7.2 and 7.8 [131].

c) Semen volume, count, motility, morphology

A normal ejaculation contains 1.5 to 5.0 milliliters of fluid. There are 20 to 150 million sperm in each milliliter. At least 60% of the time, the sperm should go forward properly and have a normal shape.

Sperm motility refers to the ability of sperm to move adequately via the female reproductive canal (internal fertilization) or over the water (external fertilization) to reach the egg. Successful conception also depends on the quality of the sperm, which must be able to properly "swim" in order to reach the egg and fertilize it. Animal sperm mobility also facilitates sperm passage across the zona pellucida and cumulus, two extracellular matrix layers that surround the mammalian egg. Although some slow-moving sperm will speed up when they reach the female reproductive system, this often comprises both fast and slow floaters. It is preferable to have more sperm swimming since they can swim more easily toward the female egg. Depending on the grading system, a sperm's motility may be deemed to be normal if more than 40% of them are floating [132].

Morphology is the proportion of sperm that seem healthy. This is evaluated by examining sperm under a microscope. Morphology is the most difficult semen analysis criterion to grade objectively. The majority of sperm do not have a flawless appearance, and 4% is often considered to be normal (Table 3).

Table 2.3. WHO manual 2010 (5th Edition) and WHO manual 2021 (6th Edition) both contain lower fifth percentiles (with a ninety-five percent confidence range) for the semen parameters from male in relationships.

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

d) Sperm kinetic movement

Sperm kinetic movement refers to the pattern and quality of movement exhibited by sperm cells. Sperm motility is a key determinant of male fertility, and it is assessed in clinical settings using semen analysis, which measures sperm count, motility, and morphology.

Progressive motility and non-progressive motility are the two primary categories of sperm motility. Sperm cells must migrate forward to fertilize an egg, which is known as progressive motility. Movements that are not forward-directed, such as lateral head or tail motions, are referred to as non-progressive motility [133]. Sperm kinetic movement can be further analyzed using "computer-assisted sperm analysis" (CASA), which is a sophisticated imaging technology that tracks and analyzes the movement of sperm cells. CASA measures several parameters of sperm motility, including:

i. **Velocity:** Sperm cell velocity is the rate of movement. Straight-line velocity (VSL), average path velocity (VAP), and curvilinear velocity (VCL) are three further categories into which velocity can be further subdivided [134].
ii. **Linearity:** Sperm cell migration is said to be linear when it is straight. High fertility and greater sperm quality are linked to very linear movement (Figure 2.9) [88].

iii. **The amplitude of lateral head displacement (ALH):** ALH is the term used to describe the side-to-side motion of the sperm head. Higher fertility and better-quality sperm are correlated with high ALH values (Figure 2.9).

iv. **Beat cross frequency (BCF):** The frequency of the sperm tail beats is referred to as BCF. Higher fertility and greater sperm quality are correlated with high BCF values. Beat pattern: The sperm tail's movement pattern and direction are referred to as the beat pattern. Higher fertility and improved sperm quality are linked to normal beat rhythms. (Figure 2.9) [135].

Overall, sperm kinetic movement is a critical aspect of male fertility, and abnormalities in sperm motility can lead to infertility. Advances in imaging technologies such as CASA have allowed for a more accurate assessment of sperm motility (Figure 2.9) and a better understanding of the factors that contribute to male infertility.

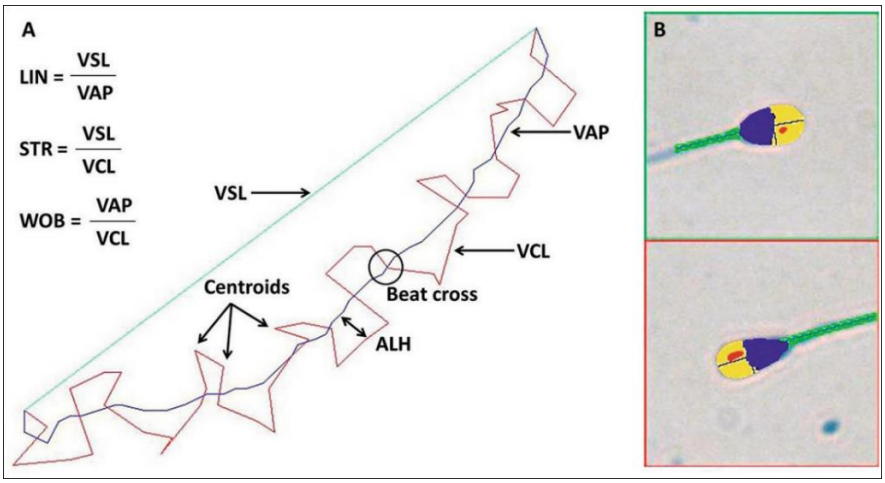


Figure 2.9. The kinematic properties of sperm are measured via computer-assisted semen analysis (CASA). the lateral head's ALH amplitude, the BCF-beat cross frequency, LIN stands for linearity, STR for trajectory straightness, VSL for straight-line velocity, and VAP for temporal average velocity. Adapted from Liana Maree 2021.

2.12. CASA - Computer-Assisted Semen Analysis

Computer-aided sperm analysis is a term for automated systems that use a video capture card, video camera, and computer to create continuous images of spermatozoa, which are then digitally processed and analyzed to accurate yield, precise, and meaningful data about sperm concentration, viability, morphology, and statistical analysis of sperm population. The ideal CASA system requires just routine system maintenance, the insertion of the semen sample into the apparatus, the initiation of the analysis process, and the interpretation of the output data. Nevertheless, both early and modern CASA systems still need human input. The ability of the camera to catch and transform crisp photographs of sperm into digital images is essential for the CASA system to function. Using negative high phase-contrast or dark field pictures, which allow the white sperm heads to be seen against a black background, is the most popular and straightforward method for a CASA device to detect spermatozoa [136].

The computer programmer often began searching for a continuous picture of a "sperm head" at a certain place after computing the sperm coordinate using the sperm head core or the brightest section of the sperm head as a reference point. The region is a circle with a set radius that surrounds a sperm head and represents the greatest possible movement of a spermatozoon during a certain period of time. The coordinate of a sperm's trajectory may be determined after the sperm's head has been recognized in a series of continuous photos. Using the spermatozoon's head as an anchor, we can reconstruct its path and determine its various dynamic properties [137].

The majority of CASA systems developed per this theory are presently used in clinical andrology laboratories, including the WLJY-9000 system, Cell Trak/S system, Cellsoft system, Sperm Class Analyzer, and “Copenhagen Rigshospitalet Image House Sperm Motility Analysis System”. It has also been stated that there are further CASA system variants. One such method is the so-called DNA fluorescence staining computer-aided sperm morphology analysis system, which relies on spermatozoa's capacity to selectively stain by DNA fluorescent dyes and may be separated from non-sperm particles under a fluorescence microscopy, such as the CFT9200 system and others. For very precise measurement of sperm concentration, this kind of CASA technology is unquestionably superior to non-fluorescent staining CASA devices [138].

The CASA system automatically analyze semen samples, including Sperm Quality Analyzer, computer, micro-video, and photovoltaic technologies. To determine sperm motility or concentration, this device employs an optical signal. After being converted from an optical signal to an electric pulse digital signal through digitalization, the associated semen characteristics are then automatically translated from the electric pulse digital signal. Computer-aided sperm morphology analysis is another name for the CASA system for analyzing sperm morphology. It may increase the sperm morphology assessment's objectivity, correctness, and repeatability and quantitatively find the sperm head's long and wide route, its proportion of acrosomes, its area, and its perimeter, among other things. It has been stated that several CASA systems for sperm morphology research have been employed primarily to find the morphology of sperm heads. While software can define sperm midpiece morphology and find more abnormal sperm tails than coiled or kinked tails, the CASA method can detect sperm midpiece and tail morphology [139,140].

2.12.1 Role of CASA in assessing sperm motility

CASA is an artificial sperm tracking technique that can evaluate a number of distinct motility parameters that characterize the motions of spermatozoa. With the advantage of providing more objective, repeatable, and comprehensive data on sperm motility, CASA is an artificial intelligence and can assess a range of particular sperm motility parameters that define the movements of spermatozoa. Numerous studies have shown that computer-aided sperm morphology analysis provides a direct quantitative determination of sperm motility in cases when human spermatozoa are capable of fertilizing in vitro, but the standard semen profile does not. Computer-aided sperm morphology analysis has been used to identify hyperactivation and estimate the number of hyperactivated sperm in a sample because hyperactivation results in a characteristic non-progressive star-spin pattern of movement [141].

2.12.2 CASA Algorithm

Simulation models offer several advantages in the development and evaluation of CASA algorithms. They provide a controlled environment where the parameters of the simulated image

are precisely known. This allows for accurate measurements of algorithm performance and facilitates the identification of areas for improvement. Simulations also make it feasible to generate large-scale datasets, which can be challenging to obtain from real semen samples. Moreover, simulations enable researchers to explore diverse scenarios and evaluate algorithms under various conditions, enhancing their robustness and generalizability (Figure 2.10).

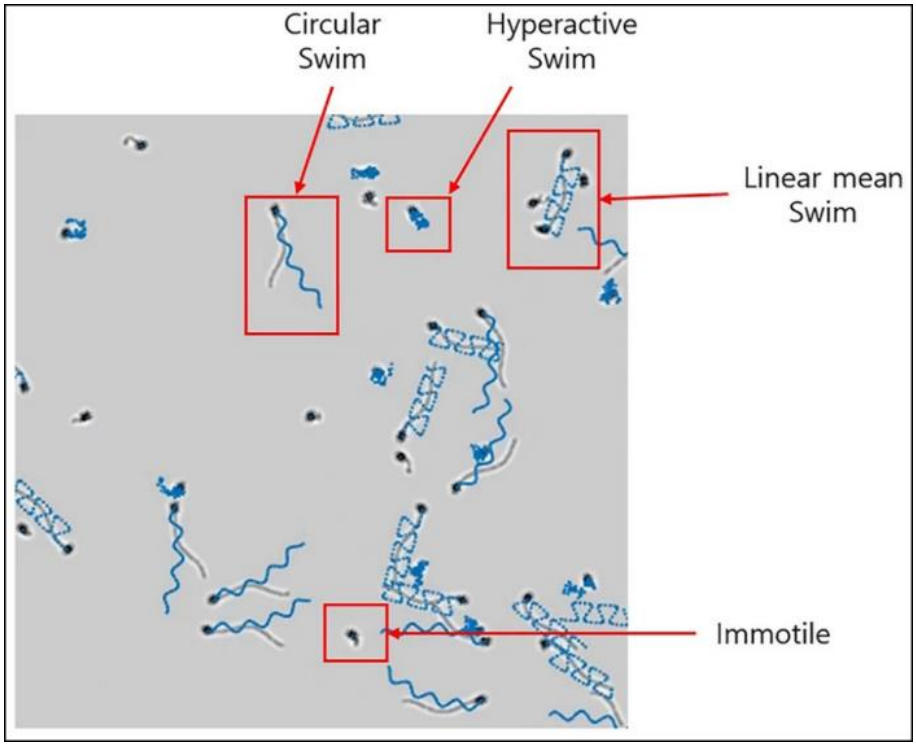


Figure 2.10. A simulated picture with the track of each cell is highlighted in blue. Adapted from Ji-won Choi et al. 2022

2.12.3 The algorithm behind CASA for assessing sperm motility

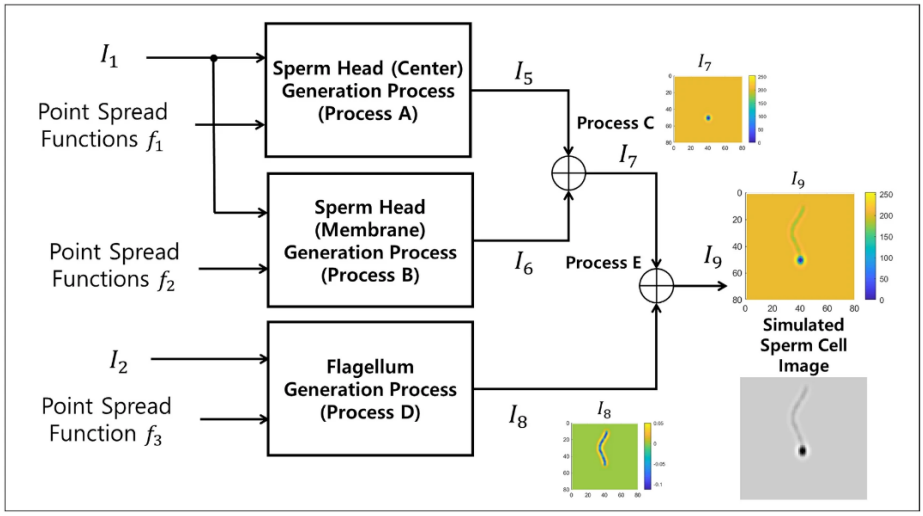


Figure 2.11. Process flow diagram for creating sperm images. The inputs consist of the images I1 and I2 and the point spread functions F1, F2, and F3. The image of a fake sperm can be found in output I9.Adapted from Ji-won Choi et al. 2022

$$I_2(x,y,t) = \begin{cases} 255 & (x,y) \in \{(x_{tail_C}(k,t), y_{tail_C}(k,t)),\ (x_{tail_L}(k,t), y_{tail_L}(k,t)), \\ & \quad (x_{tail_H}(k,t), y_{tail_H}(k,t)),\ (x_{tail_I}(k,t), y_{tail_I}(k,t))\},\ k = 1, \\ & 2, 3, \ldots, M, \\ 0 & \text{otherwise.} \end{cases}$$

Here, (xtailC,ytailC), (xtailL,ytailL), (xtailH,ytailH), and (xtailI,ytailI) are the points along the sperm flagellum's curve for circular swimming, linear mean swimming, hyperactive swimming, and immotile swimming, respectively. Each flagellum has M points, often 200. "Swimming models" refer to the precise placement of the sperm head ((xHC,yHC), (xHL,yHL), (xHH,yHH), (xHI,yHI), and the positions along the curve of the sperm flagellum ((xtailC,ytailC), (xtailL,ytailL), (xtailH,ytailH), (xtailI,ytailI)) as well as hyperactivated, immotile, or dead (Figure 2.11).

2.13. Mechanics of flagellar motility

The motile machinery and energy metabolism must both be activated for sperm flagellar movement to occur. The movement of the flagella is mediated by the motor activities of the axonemal dynein arms operating against stable microtubule doublets. For the flagellar waveform to begin, the axonemal dynein must be phosphorylated. The activation of the dynein ATPase follows phosphorylation. The microtubules glide past one another because of the force created when ATP is hydrolyzed and released. The process is subsequently reversed by calcineurin, a calmodulin-dependent protein phosphatase, dephosphorylating dynein. Phosphorylation and dephosphorylation of the dynein arms occur asynchronously in a circular pattern and along the length of the axoneme, leading to their simultaneous activation and inactivation. The time and placement of the active dynein arms are controlled by the axoneme to propagate bends in both directions. Calcium from inside the cell influences how much the center pair of microtubules slides [142,143].

2.14. Pathways involve in sperm motility

Human sperm motility is a complex process that involves the coordination of many molecular and cellular pathways. Here are some of the key pathways involved in human sperm motility:

- **Calcium signaling:** Sperm motility is significantly regulated by calcium ions. When calcium levels in the sperm rise, this triggers a series of events that result in the flagellum (the tail of the sperm) beating faster and more strongly, leading to increased motility. Calcium signaling is controlled by several proteins, including CatSper channels, which are located on the surface of the sperm (Figure 2.12).
- **Cyclic AMP signaling:** Cyclic AMP (cAMP) is a second messenger molecule that is involved in many cellular processes, including sperm motility. When cAMP levels in the sperm rise, this leads to increased motility by activating several downstream signaling pathways (Figure 2.12).
- **Protein phosphorylation:** Phosphorylation is the process of adding a phosphate group to a protein, and it plays a critical role in regulating sperm motility. Dynein, a motor protein that

propels the flagellum's movement, is one of several proteins phosphorylated during sperm motility.

- **Mitochondrial respiration**: The flagellum of the sperm is powered by energy generated by the mitochondria, which are organelles that produce ATP through oxidative phosphorylation. Mitochondrial dysfunction can lead to reduced sperm motility and infertility.
- **Signal transduction pathways:** Signals are transferred from the surface of the sperm to the inside of the cell via a sequence of chemical events known as signal transduction pathways. These pathways involve several proteins, including G-proteins and protein kinases, which are activated by ligand binding (such as neurotransmitters or hormones) and lead to changes in intracellular signaling (Figure 2.12).

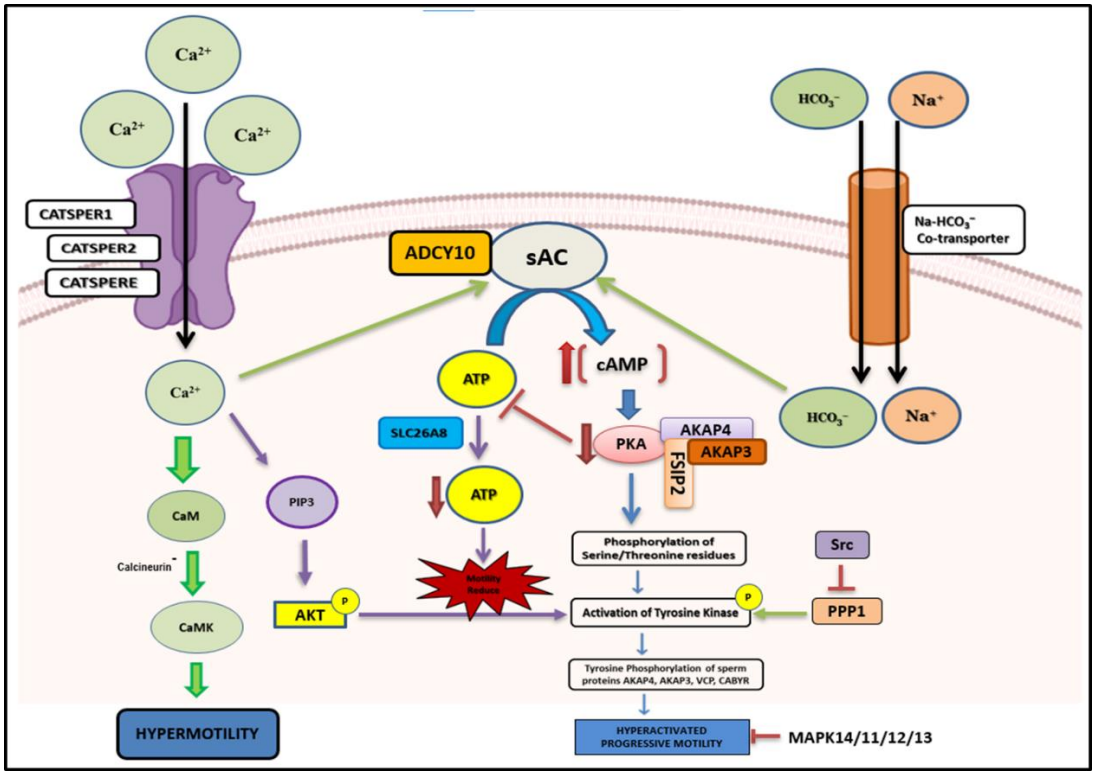


Figure 2.12. Calcium and the cAMP/PRKA signaling pathway are the two major signaling mechanisms that control its motility. Catsper channels, which are triggered by either progesterone or the alkalinization of sperm cytoplasm, allow the inflow of Ca^{2+} ions. The simultaneous rise in Na^{+} and HCO^{3-} ion concentration caused by Na^{+} and HCO^{3-} co-transporter helps to keep spermatozoa's pH stable. Intracellular soluble adenylyl cyclase (sAC) is activated by both Ca^{2+} ($Ca^{2+)}$ and HCO^{3-} to raise the levels of cAMP. Sperm motility requires the activation of sAC and a steady supply of cAMP [145].

- **Ion channels**: Ion channels are proteins that bridge the cell membrane and permit ion movement inside and outside of the cell. Many ion channels, including potassium, calcium, and chloride channels, are present in sperm cells. These channels are crucial for controlling membrane potential, intracellular calcium levels, and flagellar beating (Figure 2.12).
- **Redox signaling:** Reactive oxygen species (ROS) and antioxidants can coexist in an unbalanced state, which can lead to changes in oxidative stress that cells can detect and react to. Due to the high sensitivity of sperm cells to oxidative stress, impaired motility and infertility might result from redox signalling dysfunction (Figure 2.16).
- **Interactions with the extracellular matrix:** Extracellular matrix (ECM), a complex network of proteins and other chemicals, is the substance that surrounds and supports cells. In the female reproductive tract, sperm cells engage in interactions with the ECM that may have an impact on sperm motility and fertilization.

2.15. Sperm energy metabolism

Throughout their lives, spermatozoa's highly energetic cells experience dynamic changes in their energy requirements. Various processes, such as spermatozoa production, transit through the female reproductive system, capacitation, and fertilisation, result in these modifications. In order to keep the sperm axoneme engine running, both the male and female reproductive tubes must have an ongoing supply of ATP. During epididymal maturation, as well as during capacitation and hyperactivation in the female reproductive tract, spermatozoa have different ATP requirements. Reactive oxygen species (ROS) are inevitably produced as a result of the numerous oxidation-reduction events involved in sperm's energetic metabolism. Maintaining proper sperm function requires proper management of ROS generation. Therefore, furthering our understanding of male factor infertility in human sperm biotechnologies in both people and animals requires research into the interplay between metabolism and redox homeostasis. In sperm, both glycolysis and oxidative phosphorylation pathways can contribute to ATP production (Figure 2.13). However, the glycolytic route is specifically compartmentalized and plays a major role in enabling modifications in the motility pattern known as hyperactivation. The exact preference for ATP production pathway

in spermatozoa bodies is still unknown and an active area of research. Understanding the preferred pathway for ATP production in spermatozoa is important for developing strategies to improve sperm function and fertility. Investigating the metabolic and redox processes in sperm can provide valuable insights into the underlying mechanisms of male infertility and assist in the development of novel biotechnological approaches for human and animal reproductive health [144].

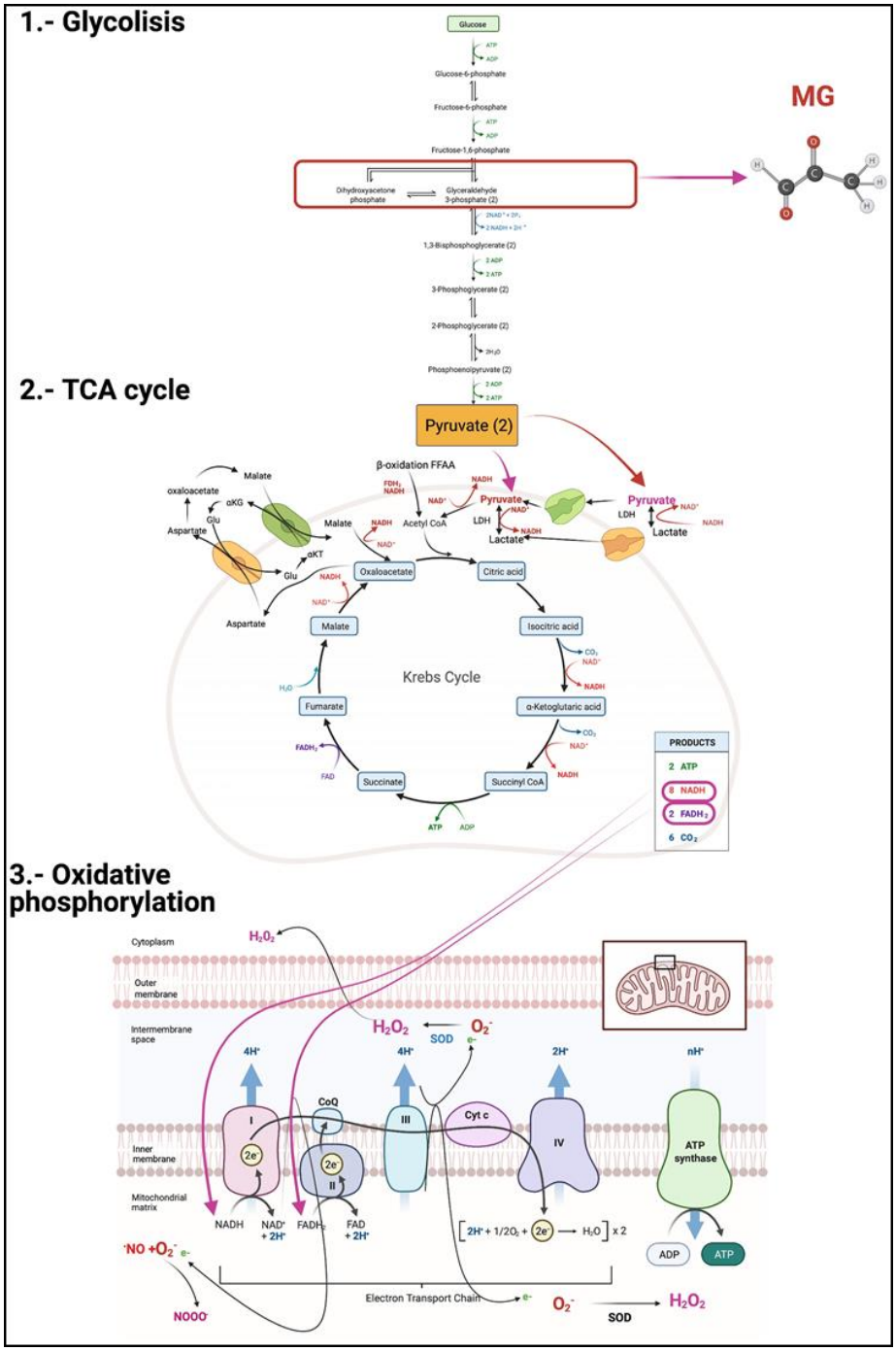


Figure 2.13. Overview of the interaction between glycolysis, the TCA cycle, and oxidative phosphorylation in spermatozoa. Pyruvate, which is created during glycolysis, fuels the TCA cycle, which in turn creates NADH and FDAH2, which are used as electron transporters in the ETC, where they ultimately lead to the creation of ATP. Glycolysis produces the 2-oxoaldehydes MG and G, whereas the ETC produces ROS as a consequence. The relationships between metabolism and redox homeostasis need to be properly controlled for sperm to function at their best. Adapted from Fernando J. Peña et al. 2022

2.15.1 Glycolysis: Glycolysis refers to the metabolic process through which glucose ($C_6H_{12}O_6$) is converted into pyruvate. During this process, free energy is converted into the high-energy molecules adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). The term "glycolysis" refers to a set of ten chemical reactions that need enzymes to complete. Glycolysis is an alternative oxygen-free metabolic pathway (In anaerobic conditions pyruvate is converted to lactic acid).Glycolysis often occurs in various species, which suggests that it is an old metabolic route [154].

The glycolysis pathway can be separated into different phases:

i) Glycolysis Phases

The two phases of glycolysis are the investment phase and the reward phase. The process uses energy as ATP, and ADP and NADH molecules are net synthesized during the payment phase. The payment phase generates 4 ATP from a total of 2 ATP in the investment phase, leaving a net total of 2 ATP [155]. Substrate-level phosphorylation is the term for the processes that lead to the production of fresh ATP.

ii) Investment Phase

At this stage, glucose is given two phosphates. Hexokinase phosphorylates glucose to produce G6P at the start of glycolysis (G6P). At this phase, a phosphate group is transferred for the first time, and ATP is used for the first time. Moreover, this is a permanent action. Since the glucose molecule cannot easily penetrate the cell membrane, this phosphorylation keeps it within the cell. G6P is then isomerized by phosphoglucose isomerase into fructose 6-phosphate (F6P). Phosphofructokinase follows by incorporating the second phosphate (PFK-1). When PFK-1 phosphorylates the F6P with the help of the second ATP, fructose 1,6-bisphosphate is produced. At this stage, the rate-limiting mechanism is also irreversible. “Fructose 1,6-bisphosphate” is subsequently transformed by fructose-bisphosphate aldolase into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate after being split into two molecules (G3P). Triosephosphate isomerase is the enzyme that changes DHAP into G3P. DHAP and G3p are in equilibrium with one another as they alternately change [156].

iii) Payoff Phase

It is crucial to remember that each glucose molecule has two total 3-carbon sugars at the start of this phase. Glyceraldehyde-3-phosphate dehydrogenase transforms G3P into 1,3-diphosphoglycerate by turning NAD+ into NADH. After a phosphate group loss interaction with phosphoglycerate kinase, the 1,3-diphosphoglycerate transforms into 3-phosphoglycerate, which is subsequently phosphorylated at the substrate level to provide ATP. At this time, each 3-carbon molecule has created one ATP, totaling two. Phosphoglycerate mutase converts 3-phosphoglycerate to 2-phosphoglycerate, which is subsequently converted by enolase into phosphoenolpyruvate (PEP). Pyruvate kinase converts PEP into pyruvate in the penultimate step and phosphorylates adenosine di-phosphate into adenosine tri-phosphate by substrate-level phosphorylation, producing two more adenosine triphosphate. This action cannot be undone. In all, one glucose molecule requires 2 ATP as an input, and 4 ATP, 2 NADH, and 2 pyruvate molecules are produced as a result [157].

Inside cells, NADH must be regenerated back into NAD^+ for glycolysis. Without NAD^+, the payout phase will come to an end, which will slow down glycolysis. In aerobic cells, NADH is transformed back into NAD^+ by the process of oxidative phosphorylation. It takes place via fermentation in aerobic cells. Lactic acid fermentation and alcohol fermentation are the two forms of fermentation [158].

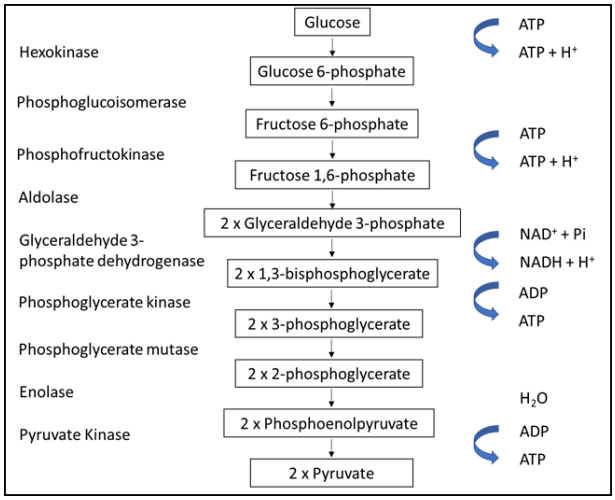


Figure 2.14. The process involves Glycolysis. Adapted from Raheel Chaudhry et al. 2022

2.15.2 Oxidative phosphorylation

Oxygen reduction is used in a biological process known as oxidative phosphorylation to create the high-energy phosphate link adenosine triphosphate (ATP). ETC stands for electron transport chain, a sequence of protein and lipid complexes found inside mitochondria, transports electrons between nicotinamide adenine dinucleotide (NAD) + hydrogen (H) and flavin adenine dinucleotide and oxygen (O2) during energy production. The catabolic cellular processes that produce NADH and FADH2, which are used by the electron transport chain [159].

The Mechanism of Oxidative Phosphorylation:-

i) **COMPLEX I**: Due to its function in oxidizing NADH to NAD+, the initial complex in the ETC is known as "NADH dehydrogenase." An electron pair may be transferred from NADH to ubiquinone the complex's enzymatic activity (Q). Flavin mononucleotide, an accompanying prosthetic group (produced from riboflavin, or vitamin B2, is the first to transfer an electron pair from NADH into NADH dehydrogenase. The complex's electron pair is transported to complex II after being transformed from ubiquinone to ubiquinol by a series of successive transfers from one iron-sulfur cluster to another. When electrons are transferred from NADH to ubiquinone, the complex generates energy, which is then utilized to push four H+ ions across the membrane. The intermembrane gap enzyme activity products include Nicotinamide adenine dinucleotide, ubiquinol, and 4 H+ ions [160].

ii) **COMPLEX II**: Complex II is called when "succinate dehydrogenase" because of its function in the oxidation of succinate to fumarate in the TCA. Moreover, electron transport occurs there through $FADH_2$. This enzyme is responsible for the electron transport chain that moves them from $FADH_2$ to ubiquinone. The compact structure contains "iron-sulfur" clusters and their related cofactor, FAD. In both forms, electrons are shuttled from FADH2 to ubiquinone. Nevertheless, this action expends too little energy, and as a consequence, no H^+ ions are displaced from this complex into the intermembrane space. NADH produces a larger output of ATP (about 2.5 ATP molecules) than $FADH_2$ because FADH2 skips complex I and enters complex II directly

(about 1.5 ATP molecules). Ubiquinone (in the form of ubiquinol) transports electrons from complex I and II to complex III [161].

iii) **COMPLEX III**: Cytochrome c reductase is the name given to the third complex (complex III), which reduces cytochrome c. Numerous molecules, including cytochrome B and several Fe-S clusters, make up this complex. These molecules are all necessary for the movement of electrons to cytochrome c. Enzymatically, complex III pumps four hydrogen ions into the intermembrane gap after transferring electrons from ubiquinol to cytochrome c. Cytochrome c can only consume one electron, but ubiquinol can provide two. Cytochrome c reductase is a dimeric enzyme that can fit two cytochrome c molecules. The Q cycle is how complex III reduces cytochrome c [162].

iv) **COMPLEX IV**: Cytochrome c oxidase is the term given to the fourth complex which is responsible for oxidising cytochrome c. Several heme groups, cofactors, and subunits compose this complex molecule. An oxygen atom is trapped within the complex by the copper and iron metal ions in the heme groups. Complex IV is an enzyme necessary for the transfer of electrons from cytochrome c molecules to oxygen, the final electron acceptor. As oxygen is broken down, two hydrogen ions are drawn out of the surrounding matrix and transferred to the reduced O_2 atom, where they combine to produce water. At simultaneously, the complex releases $4H^+$ ions into the intermembrane gap. The electrons are passed from one complex to the next in a linear fashion because the electronegativity of each successive complex increases linearly [164].

v) **COMPLEX V**: The multi-subunit structure of the enzyme enables the transformation of potential energy into chemical and mechanical energy when the “F1” subunit spins in response to the force of protons attempting to re-enter the matrix.

ATP synthase is given to the fifth complex, which uses the proton motive force to produce ATP. F0 and F1 are two of the many protein subunits that make up ATP synthase. A route for H+ ions to flow through so they may reach the F1 section is present in the hydrophobic, and phospholipid bilayer-anchored F0 portion. The primary catalytic site is the hydrophilic F1 section, which has the appearance of a stick joined to a cylinder. It has a gamma subunit at its centre and an alpha and beta ring around it that spins. Hydrogen ions go from the F0 region to the F1 region, forcing the F1 region to rotate and catalysing the binding of Adenosine di-phosphate and inorganic phosphate (Pi) to create Adenosine tri-phosphate. This process is known as the binding-change phenomenon [165].

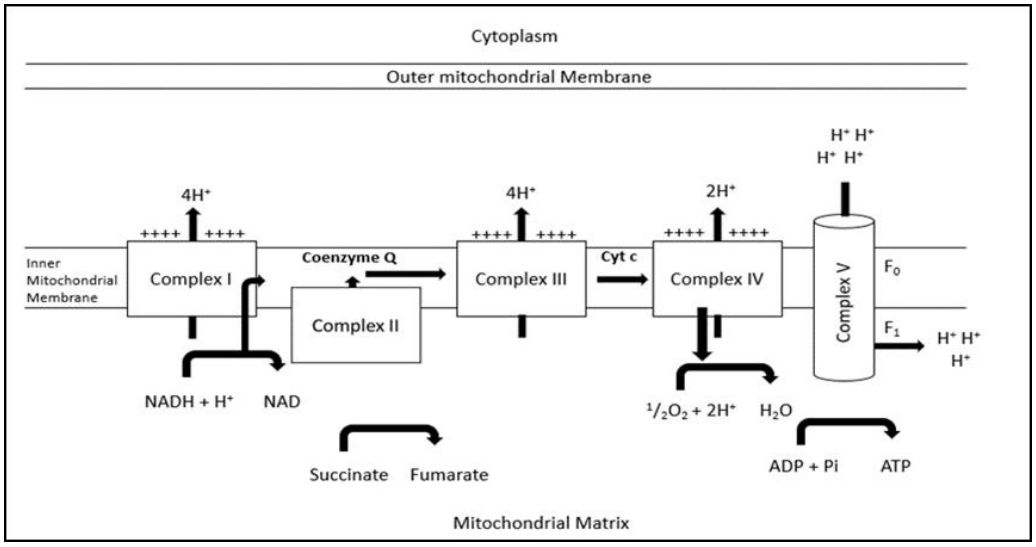


Figure 2.15. The process involves in Oxidative phosphorylation. Adapted from Ojas A. Deshpande et al. 2022

2.15.3 Lipid metabolism

Lipids are essential components of sperm cell membranes and are involved in various aspects of sperm function, including motility. Lipid metabolic genes play an important role in regulating lipid metabolism in sperm cells, and abnormalities in lipid metabolism can lead to impaired sperm motility and male infertility. Several studies have identified specific lipid metabolic genes that are important for sperm motility, including:

- Phospholipase A2 (PLA2): PLA2 is an enzyme that catalyzes the hydrolysis of phospholipids, which are important components of cell membranes. Studies have shown that inhibition of PLA2 activity in sperm cells leads to reduced motility and impaired fertility [166].
- Fatty acid desaturase (FADS): FADS is a family of enzymes that catalyze the conversion of polyunsaturated fatty acids (PUFAs) to long-chain PUFAs. PUFAs are important components of sperm cell membranes, and studies have shown that disruption of FADS gene expression leads to reduced sperm motility and impaired fertility [167].
- SREBPs (sterol regulatory element-binding proteins) are transcription factors that control lipid production and metabolism. Studies have shown that disruption of SREBP expression in sperm cells leads to reduced motility and impaired fertility [168].
- Acyl-CoA synthetase (ACSL): ACSL is an enzyme that catalyzes the conversion of fatty acids to acyl-CoA, which is a key step in fatty acid metabolism. Studies have shown that inhibition of ACSL activity in sperm cells leads to reduced motility and impaired fertility [169].
- Peroxisome proliferator-activated receptors (PPARs): PPARs are a family of nuclear receptors that regulate lipid metabolism and energy homeostasis. Studies have shown that disruption of PPAR expression in sperm cells leads to reduced motility and impaired fertility [170].

2.16. About APOB gene

An apolipoprotein called apoB helps break down lipids. Receptors are involved in the elimination of low-density lipoprotein particles from the bloodstream, and ApoB is their ligand. The 2p23–24 region of the chromosome has been identified as the location of the human apoB gene. The gene is very diverse, with two alleles encoding apoB signal peptides of various lengths (27 and 24 amino acids, respectively). A structural gene connects the B apolipoproteins APOB-100 and APOB-48 [171]. They are essential for both the metabolism of lipoproteins and the transit of plasma lipids. While APOB-48 is required for the formation of chylomicrons in the gut, APOB-100 is required for the liver's assembly of very low density lipoprotein. Despite a study showing that male mice

with the ApoB heterozygosity were fertile, knockout trials on ApoB gene deletion showed severely reduced fertility in the ApoB heterozygous male mice. A polymorphism linked to an erroneous translocation of the APOB protein that results in the protein's signal peptide losing three amino acids [172].

The most prevalent apolipoprotein found in chylomicrons, VLDL, IDL, and LDL particles (commonly referred to as "bad cholesterol" in the context of both heart disease and vascular disease generally) is apolipoprotein B. It is in charge of transporting lipids including cholesterol to all cells in all tissues throughout the body. Even while the full extent of ApoB's functional roles within low density lipoprotein particles are still mostly unclear, it is the primary structural protein component of the particles and is unquestionably required for their synthesis. Other evidence points to an interaction between LDL receptors on a number of cells throughout the body and ApoB on the LDL particle [173].

Plaques in vascular disease (atherosclerosis) are caused primarily by high levels of ApoB, especially when associated with greater low-density lipoprotein particle concentrations; atherosclerosis's numerous systemic consequences, such as heart disease and stroke, only become apparent decades into the illness's course [174]. There is a lot of data to suggest that ApoB concentrations, particularly the NMR assay, are better markers of vascular/heart disease-related physiology than total cholesterol or LDL-cholesterol. Despite the fact that other lipid tests for atherosclerosis risk are available, cholesterol and estimated LDL-cholesterol by calculation remain the most often marketed lipid test, in part owing to past cost/complexity difficulties. ELISA or nephelometry immunoassays are often used to test apoB. Measurement differences between the various ApoB particles are possible because to automated and sophisticated NMR techniques [175].

2.16. Role of APOB gene in sperm motility

The apolipoprotein B protein, which is encoded by the APOB gene, is essential for the body's metabolism of lipoproteins. While some evidence points to a potential involvement for APOB in sperm motility, the specifics of this association are yet unclear. According to studies, human spermatozoa contain the APOB protein, and the expression of this protein is associated to sperm

motility. Infertile men with reduced sperm motility have been shown to express APOB at lower levels, which suggests that sperm motility may be regulated by APOB. Nevertheless, it is still unclear exactly how APOB influences sperm motility. Due to its involvement in the transport and metabolism of lipids, a significant source of energy for spermatozoa, some studies hypothesise that APOB may have a function in the energy metabolism of sperm. In accordance with other research, APOB may have a role in the control of calcium ion channels in the sperm cell membrane, which are crucial for the start and upkeep of sperm motility [176]. Variations in the APOB gene may be linked to male infertility, particularly in males with poor sperm motility, according to recent research that have examined the function of APOB in male fertility. A 2020 study, for instance, indicated that specific genetic variations in the APOB gene were strongly connected with male infertility, including decreased sperm motility. APOB may be used as a biomarker for male infertility, according to some research [177]. According to a 2019 study in the Journal of Urology, males with infertility had considerably lower levels of APOB expression in seminal plasma than fertile controls. As a result, it is possible that APOB could be used as a diagnostic indicator for male infertility. To ascertain the clinical importance of APOB as a biomarker for male infertility and to comprehend the underlying processes by which APOB impacts sperm motility, additional research is nonetheless required. This could result in the creation of novel diagnostic methods and therapies for the widespread and frequently challenging problem of male infertility [178].

APOB expression and sperm motility may be impacted by causes other than genetic differences in the APOB gene. For instance, sperm motility may be impacted by lifestyle factors including nutrition and exercise that control APOB expression and lipid metabolism. According to a 2016 study in the Journal of Clinical Endocrinology & Metabolism, men who were more physically active than sedentary males had higher levels of APOB expression in their semen. This shows that exercise may affect sperm motility by controlling APOB expression and lipid metabolism in the male reproductive system. According to several other studies, food may have an impact on sperm motility and APOB expression. For instance, a 2017 study published in Andrology reported that mice fed a high-fat diet had decreased APOB expression and poor sperm motility. This implies that dietary elements may also affect how sperm motility and APOB expression are regulated in people. Overall, despite the fact that the function of APOB in sperm motility is still not fully understood, mounting evidence points to a potential role for lipid metabolism and APOB

expression in male fertility. To completely comprehend the mechanisms underlying this association and create successful treatments for male infertility, more study is required [179].

2.17. Oxidative stress in infertile male

The main cause is DNA damage in poor sperm function, which is mostly caused by OS. A prevalent underlying cause of male infertility, repeated miscarriages, complex neuropsychiatric problems, and childhood malignancies in children whose fathers had faulty sperm cells may be high levels of OS (oxidative stress), which harm sperm DNA, RNA transcripts, and telomeres. Spermatozoa have a single, restricted method for detecting and repairing DNA damage, which makes them very susceptible to oxidative stress. Most of the lifestyle-related variables that contribute to OS (oxidative stress) are controllable. Both antioxidant regimens and lifestyle changes may be viable treatment options for alleviating the burden of oxidative stress-induced male factor infertility. Oxidative stress is brought on by several controllable variables, and by using simple measures, the incidence of both infertility and complicated disorders in the children may emerge could be decreased.

Oxidative stress, characterized by the presence of reactive oxygen species (ROS) and oxygen-derived oxidants, is a known indicator of cellular damage. In the context of male infertility, the susceptibility of human spermatozoa to oxidative stress has been identified as a contributing factor [180]. ROS play a dual role in normal sperm function, as they are involved in essential processes such as capacitation, hyperactivation, acrosomal response, and sperm-oocyte fusion when present in low levels. However, elevated ROS production can lead to oxidative stress, resulting in pathological changes in spermatozoa [181]. The detrimental effects of ROS on spermatozoa primarily manifest through peroxidative damage to the cell membrane, impaired sperm motility, and oxidative damage to DNA [182].

Studies have shown that a significant proportion of infertile males, ranging from 20% to 40%, exhibit high levels of seminal ROS [183]. Consequently, understanding the impact of oxidative stress on the pathophysiology of sperm function is crucial in the study of human fertility.

There is substantial evidence supporting the role of oxidative stress as a significant factor in male infertility, as abnormal spermatozoa often display signs of oxidative stress, such as increased ROS levels and reduced antioxidant capacity [184,185].Furthermore, it has been shown that irregular spermatozoa and male infertility are linked to a high quantity of oxidatively damaged DNA (Figure 2.16).

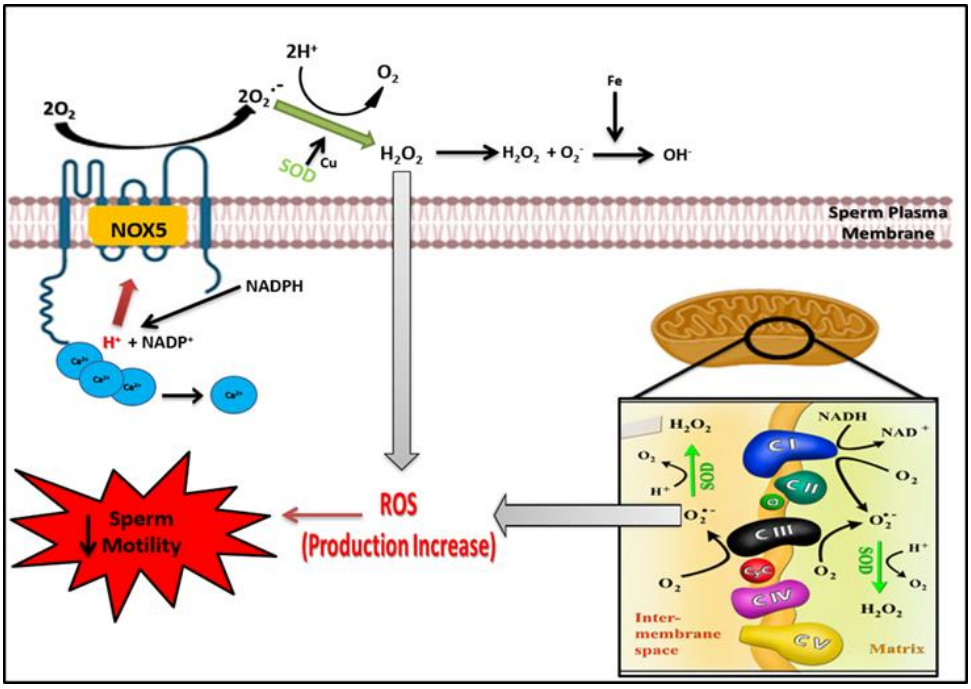


Figure 2.16. Pathway participates in the generation of ROS that lower sperm motility [145].

2.18. **mRNA isolation**

Sperm purification must be done correctly in order to retrieve sperm RNA and prevent interference from other somatic cells in seminal plasma. There are various techniques for sperm purification. The most popular techniques include density gradient centrifugation, double swim-up, swim-up, and swim-down. Sperm can be separated from semen using magnetic activated cell sorting, electrophoresis and fluorescence cell sorting techniques. The methods used to extract RNA can be broadly categorized into three categories: direct cell lysis, organic extraction and spin basket methods. Direct liquid-liquid extraction 2 is done using the guanidinium-isothiocyanate-phenol-chloroform extraction method. N. Sacchi and P. Chomczynski, 1987. Commercially, a solution of

phenol, chloroform, and isoamyl alcohol is produced under the trade name Trizol for the extraction of RNA [228]. Because phenol has a higher density than water, it creates the bottom organic phase while trizol separates RNA into the upper aqueous phase. Proteins are denatured by phenol, and guanidium isothiocyanate, a chaotropic substance, is also a denaturer of proteins. Proteins contain hydrophobic interiors and hydrophilic exteriors; nevertheless, when phenol is present, the hydrophobic inside is exposed, leading to the denaturement of the protein. At neutral pH, DNA retains its water solubility and separates into the interphase, while at acidic pH, DNA loses its solubility and separates in the phase of aqueous solution. Effective sperm extraction from semen must be carried out in order to anticipate a higher and more acceptable RNA yield. It is essential to separate the spermatozoa from the seminal plasma in order to estimate the quantity of sperm cell RNA and prevent infection by other somatic cells such as leucocytes, and cell debris. Techniques for separating sperm from semen include density gradient centrifugation, double swim-up, swim-down, and swim-up. The RNases' pervasiveness makes isolation a far more challenging approach. RNases renature when the mixture cools down hence they cannot be inactivated by autoclaving micropipette tips or centrifuge tubes. RNases can be rendered inactive by treatment with the RNase inhibitor Diethyl pyrocarbonate (DEPC). Because it is required for following processes like RT-PCR, RLM-RACE, cDNA synthesis, microarray analysis, and RNA sequencing, high-quality RNA must be obtained [229].

2.19. RT- qPCR

Quantitative reverse transcription PCR (RT-qPCR) is a molecular technique used to identify and quantify RNA molecules. It involves the conversion of RNA or mRNA into complementary DNA (cDNA) using the enzyme reverse transcriptase. The specific targets within this cDNA are then amplified and measured using quantitative polymerase chain reaction (qPCR) or real-time PCR. During this process, various fluorescent compounds are utilized to continuously monitor the amplification of DNA during each PCR cycle. Common fluorescent agents include hydrolysis probes like TaqMan® probes or double-stranded DNA binding dyes like SYBR® Green dye, which generate a fluorescent signal [230].

When selecting a fluorescent chemical for RT-qPCR, factors such as usage, cost, and test type should be considered. DNA-binding dyes are preferred for singleplex assays with low throughput

as they are easier to use, require less setup time, and are more cost-effective. On the other hand, fluorescent probes are increasingly employed in high-throughput, multiplex testing scenarios where enhanced selectivity is required. A fluorescent reporter molecule is added to each reaction well to enable real-time detection of PCR products. When the quantity of DNA increases, the fluorescence of the reporter molecule increases. Chemistries used fluorescence dyes for this purpose that bind to DNA and fluorescently labelled primers or probes that are unique to certain sequences. The fluorescence signal is monitored as it increases using fluorescence detector modules on specialized heat cyclers. The overall amount of amplicon is proportional to the measured fluorescence; the amount of amplicon created in each cycle is calculated using the change in fluorescence over time [231].

Real-time PCR offers a significant advantage over conventional PCR by allowing sensitive and precise quantification of the initial copy number of the template DNA, covering a wide dynamic range. This technology, also known as quantitative real-time PCR or qPCR, enables both quantitative (determining the copy number) and qualitative (detecting the presence or absence of a sequence) results. In contrast, conventional PCR is typically only semi-quantitative [140]. One of the key benefits of real-time PCR is that it eliminates the need for gel electrophoresis, saving time and increasing throughput. The analysis of real-time qPCR data can be performed directly without the additional steps of gel electrophoresis. Additionally, real-time qPCR reactions are conducted in a closed-tube system, reducing the risk of contamination and avoiding post-amplification modifications during the analysis process. These features enhance the efficiency and reliability of qPCR analysis. [232].

2.20. Cure of male infertility

Antioxidants are frequently made up of substances that inhibit the effects of ROS or neutralise, consume, and stop their production. The primary antioxidants consist of vitamin A, tocopherols, and tocotrienols (Vitamin E), beta-carotene, trace minerals, and vitamin C. Dietary supplements such as selenium, zinc, carnitine, arginine, and vitamin B-12 have been shown to enhance sperm motility and count, leading to improved fertility [186]. Antioxidants like vitamin C, Coenzyme Q, glutathione, and vitamin E have received recognition for their potential in treating male infertility

(refer to Figure 2.18). Humans have developed a complex and well-structured antioxidant defense system to combat reactive oxygen species (ROS) and protect immunological tissues and organ systems. To counteract the effects of free radicals, the system uses both endogenous and external components [187]. Both enzymatic and non-enzymatic substances, such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase are examples of endogenous antioxidants (Figure 18). Exogenous antioxidants, such as vitamin C and E, carotenoids and polyphenolsare mostly derived from diet. Redox equilibrium is kept and restored by the interaction of endogenous and exogenous antioxidants. One well-known instance of this is the replenishment of tocopherol (vitamin E), glutathione (GSH), and vitamin C to halt the reaction of lipid peroxidation [188]. The SOD, CAT, and GPx enzyme trio is referred to as the primary antioxidant in the semen. There will also be discussion of the peroxidoxin family of antioxidant enzymes.

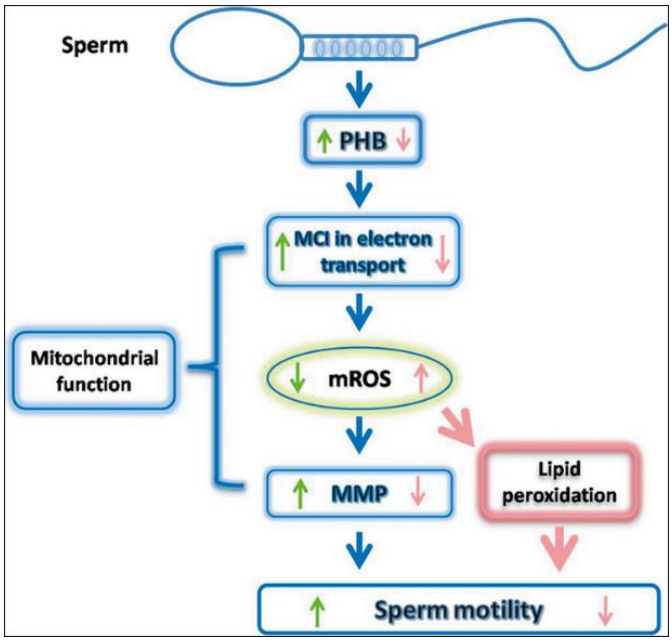


Figure 2.17. Pathway depicting how Prohibitin controls sperm motility in humans. Prohibitin (PHB) keeps sperm motile under normal circumstances (green arrow) by controlling the amount of reactive oxygen species (ROS) generated by mitochondrial cytochrome oxidase (MCI) and mitochondrion matrix metalloproteinase (MMP). When PHB is down-regulated (indicated by the pink arrow), it results in mitochondrial dysfunction, characterized by increased mitochondrial reactive oxygen species (mROS) production caused by the inhibition of MCI's electron transport activity. This dysfunction leads to compromised sperm motility, as evidenced by impaired mitochondrial membrane potential (MMP) and lipid peroxidation. Here, MCI refers to mitochondrial complex I, and MMP refers to mitochondrial membrane potential. mROS = mitochondrial reactive oxygen species. Adapted from Ran-Ran Chai et al. 2016

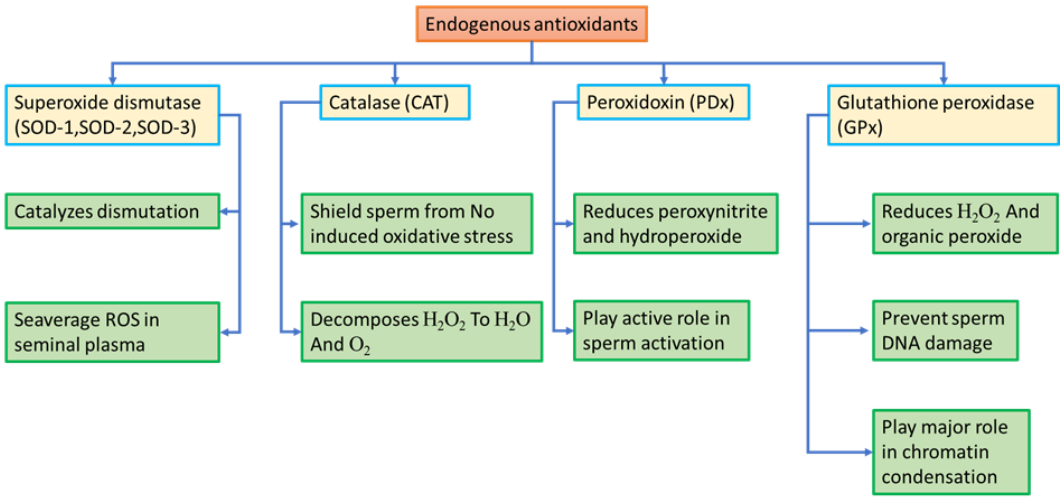


Figure 2.18. Antioxidants in spermatogenesis.

2.20.1 Already approved drugs for the cure of male infertility or for increasing of sperm motility:

i. **Peroxidoxins:** Many living organisms have high levels of peroxidoxins (PRDXs). They are acidic proteins that rely on SH and have a molecular mass of 20–31 kDa. PRDXs possess a limited number of cysteine residues (cys) at their active site, lacking heme or selenium. To enhance their capacity for eliminating both organic and inorganic hydroperoxides and peroxynitrites (as depicted in Figure 2.18), they form a complex with the thioredoxin (TRX) reductase system. With their sulfhydryl (SH) group, PRDXs promptly target H2O2 and undergo rapid oxidation in cells with low H2O2 levels. PRDXs play a vital role in cellular H2O2 removal due to their widespread distribution across multiple cellular compartments, including the endoplasmic reticulum (ER), plasma membrane (PM), mitochondria, cytosol, and nucleus. It's fascinating to note that research has shown that human spermatozoa have a similar pattern of dispersal. In each subcellular compartment, only two of the PRDX members shown. Their importance as essential antioxidants in sperm is shown by the uneven distribution across all components. Significantly, PRDX6 is quite extensive, and this only found in all the organelles of human sperm which interacts with H2O2 at concentrations that cause spermatids production. As a

result, it has been proposed that PRDX6 may be essential for mediating sperm activation [189].

ii. **Exogenous Antioxidants:** Polar chemicals known as carnitines are very common in nature. The two primary sources for satisfying every need in humans are nutrition and endogenous biosynthesis, and highly carnitine is found in the male genital tract of spermatozoa and epididymis [190]. Carnitine facilitates fatty acid admission and usage inside the mitochondria, enhancing cellular energy. Moreover, it restores the mitochondrial membrane's phospholipid composition by preventing oxidation in fatty acid. For illustration, the metabolism of sperm by spermatozoa uses the energy given by carnitine and acetyl carnitine, which has a good effect in spermatogenic process. Carnitines protect the cell membrane and sperm against Deoxyribose Nucleic Acid's breakage and death that are brought on by ROS. Studies have revealed that frequent use of carnitine and acetyl carnitine improves the quality and functionality of sperm. In fact, it has been proposed that low levels of carnitine are one of the causes of sperm abnormalities including azoospermia and asthenospermia [191].

iii. **Vitamin E:** A significantly relevant antioxidant molecule found in the cell membrane is vitamin E. It is proposed that it prevents peroxidation of lipid and free radicals get soluble and generated by univalent reduction of molecular oxygen as well as by oxidative enzymes acting normally. Low motility is the consequence of the phospholipids in the sperm mitochondria being peroxidized because of the radicals' production [192]. Most probably vitamin E enhances the production of antioxidant enzymes which scavenge free radicals. Suleiman et al. (1996) found that supplementing with vitamin E may considerably lower lipid peroxidation (Figure 2.17) in seminal plasma, enhance sperm motility, and increase the probability of becoming pregnant [193,194]. Similarly, sperm motility significantly increased, and the proportion of damaged spermatozoa decreased in an experiment where vitamin E and selenium were given together as a combined therapy for six months [195,196].

iv. **Vitamin C:** It is 6-C keto-lactone which is synthesized and secreted in the liver. But because humans are unable to generate these essential vitamins, they must either eat foods that contain it or take supplements. Several important enzymes need vitamin C as a cofactor.

It contributes to the metabolism of tryptophan, tyrosine, and folic acid. The well-known benefits of vitamin C for tissue development and wound healing are well demonstrated. Additionally, it is very effective in scavenging ROS [197]. Vitamin C supplements of 100-200 mg daily boosted sperm counts by 112-140 percent, respectively, in research involving 30 healthy but infertile males. It's noteworthy to notice that the serum concentration is 10 times lower than the seminal plasma concentration [198]. By counteracting the detrimental effects of hydroxyl, superoxide, and H2O2 radicals, PRDXs serve as important protectors of human spermatozoa, shielding them from oxidative damage. A very low content of vitamin C is linked with increased ROS in semen samples [199]. Additionally, vitamin C and E act to protect spermatozoa against peroxidative damage and DNA breakage.

v. **Carotenoids:** Fruits and vegetables contain carotenoids by nature. Since humans are unable to produce carotenoid, they must get it from food sources like plants and fruits. Lycopene is the carotenoid family's most significant chemical. Lycopene is one of the top carotenoids for quenching oxygen only, however carotenoid's mixture is more effective than a single component [200]. According to certain studies, the testes have the highest concentration of lycopene in the body. Its antioxidative action during spermatogenesis may be the cause of this [201]. Similarly, a little amount of lycopene was found in the human seminal plasma of male infertile [202]. Furthermore, it was shown that supplementing with lycopene enhanced broiler sperm motility. These suggest that lycopene contributes to the preservation of sperm integrity.

Without a doubt, improving fertility requires a balanced diet. Exogenous enzymes are derived from plants and may promote the growth of endogenous enzymes, whereas endogenous enzymes are created in cells and tissues to prevent ROS formation. They can be obtained as supplements and used to treat infertility and lessen oxidative stress. The physiology of the sperm depends on these enzymes. For instance, sperm are supplied energy by carnitines and are protected from DNA damage by vitamin C as well as vitamin E.

2.20.2 **Herbal Treatment for Infertility in Men:-**

i. **Eurycoma longifolia Jack:** The plant is recommended for a wide range of uses, including male infertility, antimalarial, anticancer, and antibacterial properties [203]. The ability of the plant to increase blood testosterone levels has been also noted by several researchers [204,205]. EL extract has recently been shown to have both androgenic and pro-fertility effects in an in vivo study.

ii. **Cardiospermum halicacabum:** Rheumatism, snake bites, and bleeding piles are all treated with it. However, it has been discovered that the plant known as balloon vine increases testosterone production. After 30 days of CH therapy, there was a significant increase in caput and epididymal sperm count as well as sperm motility. Additionally, the plant increased blood testosterone levels, which are linked to saponin in CH. The extensive range of phytocompounds in CH, primarily the flavonoids, which are renowned for their antioxidative properties, may have an impact on sperm characteristics [206].

iii. **Grape Seed Extract:** Extract from grape seeds has been found to have anti-inflammatory, antioxidant, and antibacterial properties. Furthermore, it possesses hepatoprotective, neuroprotective, and cardioprotective properties. A study revealed that aluminium chloride-induced testicular dysfunction which than used to improve sperm motility, viability, and count it also indicated that grape seed extract boosted these characteristics. Like this, they shielded the sperm cell from DNA deterioration. By decreasing the activities of nitric oxide synthase, the extract reduced the invasion of the testis by nitric oxide (NO). Moreover, grape seed is known to reduce the death of germ cells brought on by testicular torsion or detorsion [207-209].

iv. **Marjoram Essential Oil:** Marjoram is obtained naturally from Origanum majorana's dried leaves and blossom tips. Several bioactive substances, including as flavonoids, terpenoids, sitosterol, and phenolic glycosides, which are present in O. majorana (OM) [210]. In conventional medicine, marjoram extracts are used to treat headaches, gastrointestinal issues, nausea, and cramping [211]. In an experiment where a high-fat diet caused degenerative changes in seminiferous tubules, marjoram showed the ability to enhance both spermatogenic and sperm cells. Along with a rise in androgens, the lipid profiles of the serum and testes both improved. The weights of the testis, epididymis, and sex organs were

decreased by ethanol administration, but were regenerated by OM and grape seed extract. Serum testosterone levels in the animals given the mixed formulation significantly increased as well.

v. **Syzygium aromaticum:** Sexual dysfunction and poor libido have traditionally been treated with SA [212]. A manganese chloride overdose was utilised by Boudou et al. (2013) to induce reversible infertility in Wistar rats. The testis and body weight in the negative groups that received primarily manganese chloride and were significantly decreased. The degeneration of seminiferous tubules, lack of sperm or a low sperm count, a wide interstitial gap, and deficiencies in the Leydig and basement membranes were also categorised as belonging to the manganese group. In contrast, the seminiferous tubules of the group treated with SA had histological sections that are densely populated, appeared healthy, and exhibit clear evidence of regeneration [213].

vi. **Nigella sativa:** The seeds contain immunomodulatory, anti-inflammatory, and antiviral properties. To illustrate how NS affects spermatogenesis, Haseenah et al. (2015) employed diabetic rats that were induced with streptozotocin. In diabetic rats, testosterone and luteinizing hormone levels were as low as predicted at the end of the experiment, but they had significantly increased in the NS-treated groups. Subfertility in diabetic males has been identified as being characterised by decreased sperm concentration and motility as well as increased aberrant morphology [214].

vii. **Lycium barbarum:** The protective effect of LBP on reproductive organs during a 24-hour exposure to heat (43 °C) was examined in the study using 36 rats. Six male Kong Ming mice were killed, and their testicular cells were removed for in vivo tests. Testicular cells were pretreated with different dosages of LBP and then subjected to hydrogen peroxide to increase DNA damage. Using 46 males and 46 females in a second experiment, the scientists looked at how LBP affected the sexual behaviour of rats. At the end of the study, it was discovered that heat-exposed testes had degenerative symptoms, aberrant seminal tubules, no spermatids or sperm, and several spermatocyte abnormalities. However, biochemical, and histological results indicate a partial restoration of the seminiferous tubule's morphological integrity in the testis of rats given LBP treatment [215].

viii. **Tribulus terrestris:** The substance can cause oxidative stress and testicular damage. Rats in the negative control group that only administered sodium valproate had decline in their testes and seminal vesicles weight. Low blood levels of testosterone, FSH, and LH were detected by biochemical testing. Additionally, low semen quantity and quality were found. SOD, GPx, and CAT levels were impacted by the SVP's effect, as well as other antioxidant enzyme levels. In testicular histopathological sections, edoema, necrosis, and clearly atrophic seminiferous tubules are seen. However, the weight of the testes and seminal vesicles rose after METT treatment. Additionally, in a dose-dependent way, it enhanced the amount and quality of semen. It also raised the levels of LH, FSH, and testosterone. METT therapy resulted in a partial improvement [216].

ix. **Asteracantha longifolia:** A. longifolia, a member of the Acanthaceae family, it has a long history of use as a medicine in India. A. longifolia (AL) seeds can act as both androgenic and aphrodisiac agents. The capacity of AL to preserve the testicles of rats given a lethal amount of cadmium chloride, was the subject of a study conducted by a team of researchers in 2015, India [217,218]. When given the dose of CdCl2, all stages of germ cells had a considerable reduction in diameter. In contrast, A. longifolia seed powder increased the micrometric measures of spermatogonia, primary and secondary spermatocytes, as well as spermatids, reducing the impact of CdCl2 toxicity [219]. Cadmium chloride has been proven in studies to reduce testosterone production and alter the hypothalamus pituitary-gonadal axis's regulatory system [220,221].

x. **Polycarpea corybosa:** According to a recent study, ethanol extract from Polycarpea corymbosa boosted sperm motility and density while reducing faulty sperm. Additionally, when compared to the control group, the total plant product of P. carymbosa raised blood levels of LH and testosterone. Compared to untreated rats, there was a significant increase in the number of females giving birth to P. carymbosa-treated paternal rats' pups. Additionally, the testicle, epididymis, vas deferens, ventral prostate, and seminal vesicle masses all increased as a result of the impacts of the plant extract [222].

2.20.3. **Role of Curcumin for male infertility**

The main curcuminoid present in turmeric, also known as 1,7-bis [4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione, is thought to be its most potent component. Curcumin is a medicinal substance with various positive activities, and its most notable attribute is that it has no negative effects. Studies have shown that curcumin may protect tissues from oxidative damage. Curcumin boosts the production of semen testosterone and thus helps a great deal in infertility issues. Due to its peculiar conjugated structure, which consists of two methoxylated phenols and an enol form of -diketone, curcumin is an antioxidant. As a chain-breaking antioxidant, this substance traps free radicals. It can considerably reduce ROS production both in vitro and in vivo. Animal studies have demonstrated that curcumin may prevent testicular damage from oxidative stressors such cisplatin, ischemia/reperfusion injury, sodium arsenite, and di-n-butyl phthalate [223].

Curcumin, an antioxidant found in the rhizome of the Curcuma longa plant, is a yellow phenolic pigment. Antioxidant, anti-inflammatory, anti-cancer, and anti-mutagenic are only few of its many biological and pharmacological uses. Curcumin functions as an antioxidant in two separate ways: one is based on its chemical make-up, and the other is related to its ability to encourage the production of antioxidant enzymes. Curcumin has a variety of effective antioxidant groups, carbon-carbon double bonds, such as β-diketo groups, and phenyl rings. Curcumin enhanced in vitro and in vivo fertilization, acrosome response, and capacity in sperm cells. By controlling the levels of the transcription factor Nrf2, this antioxidant may enhance semen characteristics in people with asthenoteratospermia and boost sperm motility in those with leucocytospermia [224]. Curcumin has a preventive effect against anomalies in spermatogenesis brought on by “titanium dioxide nanoparticles” (n-TiO2), in addition to healing testicular damage brought on by alcohol, ischaemia reperfusion, cisplatin, metronidazole, and cadmium exposure in mice. Additionally, it lessens the testis and epididymis' histological changes brought on by monosodium glutamate, leading to an increase in rats' sperm counts. Curcumin supplementation has been shown to have favourable benefits on sperm freezing medium in several animal models, including Wistar mice, Angora goats, bulls, and buffalos. In compared to the control, the antioxidant supplementation increased the proportion of sperm acrosome integrity and provided

significant protection for sperm mitochondrial function. Curcumin exhibited protective effects on frozen-thawed sperm parameters in rams at various dosages (Figure 2.19) [225].

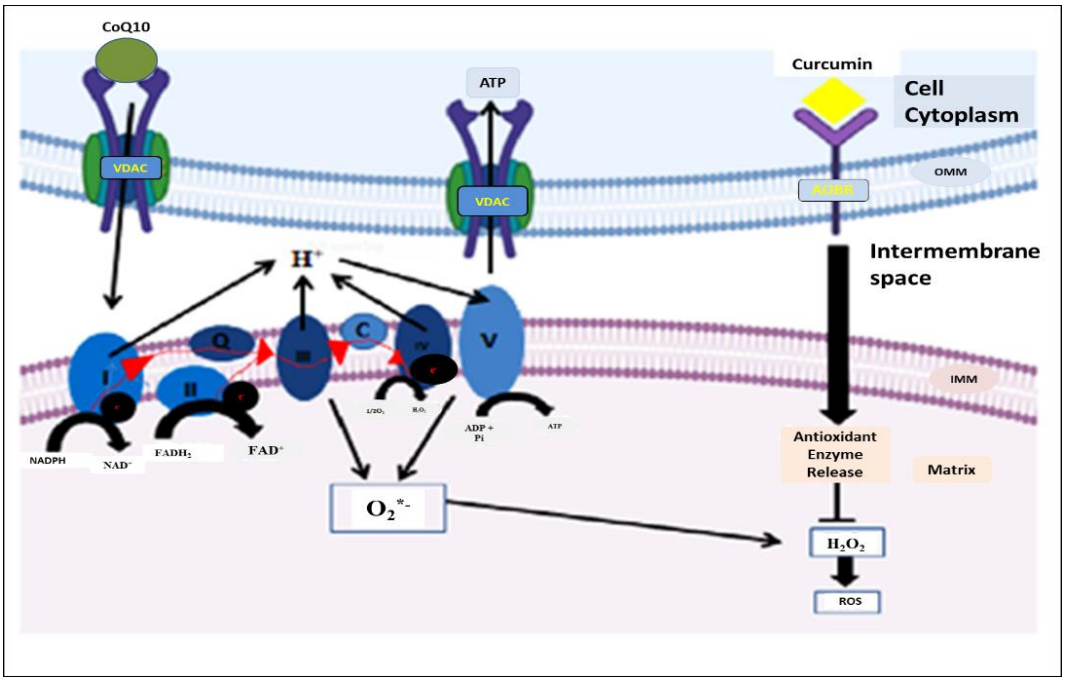


Figure 2.19. Coenzyme Q10 and curcumin work together in the sperm mitochondria to reduce ROS, which increases sperm motility [145, 274-275].

2.21. Future scope of new detecting methods and drug discovery for male infertility

Conventional molecular methods like restriction fragment length polymorphism and single nucleotide primer extension will be phased out as more mutations linked to male infertility are discovered in favour of non-gel-based solution detection systems, which do not need time-consuming post-PCR processing and gel analysis when analyzing point mutations and small insertions and deletions. Genetic testing must ultimately accommodate quick, high throughput systems and retain a high accuracy and dependability as additional mutations connected to male infertility are discovered [226]. Hence, gene chips, which use oligonucleotide microarray technology, are poised to become the standard in genetic testing because of their capacity to provide the simultaneous exploration of several loci within the genome. Recent technology advances have made it possible to accurately and reproducibly assemble up to 1,000,000 different

oligonucleotides on a glass slide. The groundwork has been laid for an exciting genomics revolution that will improve knowledge of male reproductive health and illness and provide new treatment opportunities for infertility. This revolution will be centered on the development of a male infertility gene chip that can precisely detect genetic variation and assess deviation from normal testis gene expression. High-end gene chips are predicted to soon make whole genome and tissue transcript profiling. The practitioner will have access to previously unheard-of technologies to evaluate illness risk and put the best plans and treatments in place to manage the patient's health and fertility from conception on [227].

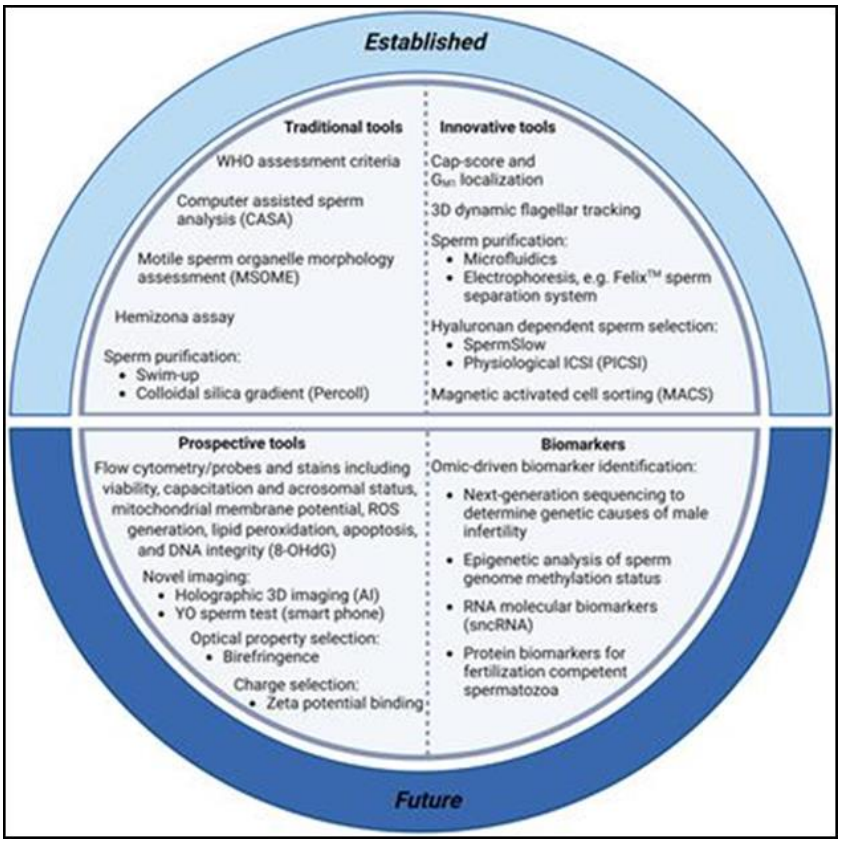


Figure 2.20. A summary of the methods using nowadays and in the future to choose sperm and make a clinical determination of male infertility. Adapted from Brett Nixon et al. 2023

Chapter 3

Materials and Methods

3.1. Collection of sample and preparation for microscopic examination

Male patients who came to the Indira IVF Hospital in Patel Nagar, Delhi, were asked to participate in the study by filling out the consent form and provide their demographics details. A total of 30 participants were included in the current study where 5 are control (normozoospermia patients) and 25 were diseased (asthenozoospermia patients). Each subject was older than 18 years old and provided the semen samples for semen analysis. Even after engaging in unprotected sexual activity for more than a year, subjects were still having reproductive issues. Prior to sample collection, subjects were told to abstain from sexual activity for 72 hours. Masturbation was used to gather sperm into sterile 50 ml bottles. Samples of semen were incubated for 30 minutes at 37°C with 5% CO2 to liquefy the samples. After the samples had liquefied, the volume of the samples was measured with a pipette, and a drop of the sample was then dropped onto the Makler chamber (Figure 3.1) [243].

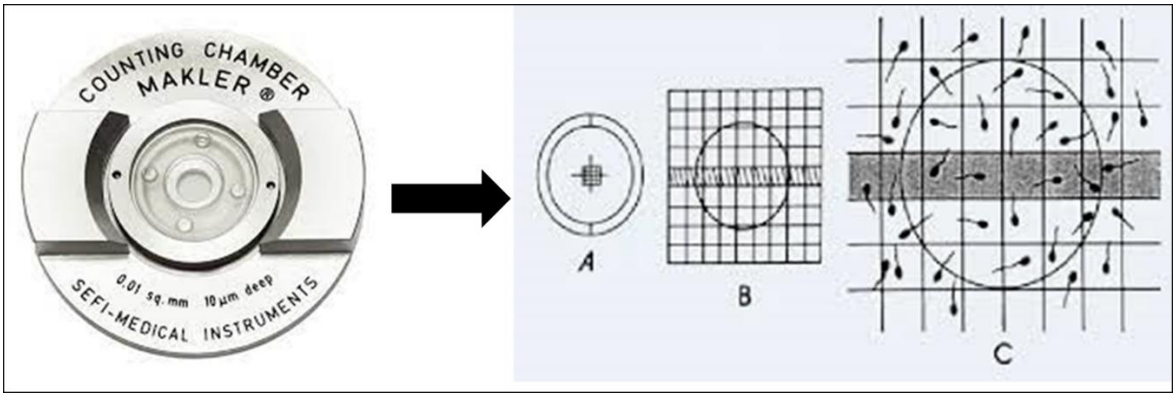


Figure 3.1. Makler Sperm Counting Chamber used during IVF and treatments connected to andrology to count the number of sperm in a sample of pure semen. Spermatozoa concentration in millions/mL is shown by the number of spermatozoa detected in any strip of 10 grid squares.

3.2. Sperm movement analysis through CASA

The MMC SPERM CASA Machine was used to examine the Makler chamber for its count and motility [233]. To assess sperm morphology and motility, CASA uses a categorization, detection, and backtracking methodology. Following the Dip staining approach, these non-progressive or immotile spermatozoa were evaluated morphologically [234,235]. Convolution neural networks (CNN) with three layers are used to classify the head, midpiece, and tail of human sperm.

3.3. Analysis of sperm motility with ImageJ

Graphs were created using ImageJ to analyses the spermatozoa's motions captured by MMC SPERM CASA version 2.4.078.0 and to better comprehend the distance travelled by metabolically active and dysregulated spermatozoa [236].

3.4. Collection of asthenozoospermia genes from different databases

From existing databases and published works, genes related to "asthenozoospermia," "low sperm motility," "no sperm motility," and "dysregulated sperm motility" were gathered, databases like GeneCards and DisGeNET (http://www.disgenet.org) were used for it. The GeneCards database [237] provides comprehensive coverage of all annotated and predicted human genes in easily searchable detail. The DisGeNET database, which serves as a platform for discovery, has one of the largest publicly available collections of genes and variants connected to human disorders.

3.5. Network analysis of protein-protein interactions (PPI)

The common genes acquired from GeneCard and DisGeNet were uploaded to the STRING database (https://string-db.org/, version11.0) for PPI network research. The interacting proteins with a confidence level of less than 0.900 were picked to generate the PPI network visualisation [238]. The proteins that have a favourable relationship with the glycolysis gene, the sperm flagellum and spermatogenesis were chosen for pathway analysis and gene ontology.

3.6. Pathway analysis and Gene Ontology (GO)

Understanding the metabolic pathways in which particular genes are involved is possible using the Reactome Pathway Database (https://reactome.org) [239,240]. To learn about the biological importance of the gene set and comprehend the functions of the genes, the Enrichr tool (found at https://maayanlab.cloud) and the DAVID bioinformatic database (found at https://david.ncifcrf.gov/tools.jsp) were used for gene ontology [241,242]. The GO keywords and pathways that support the relationship of the specified genes with male infertility were chosen based on a high count and a P value of 0.05.

3.7. Collection and preparation of semen samples for mRNA extraction

25 semen samples from individuals diagnosed with asthenozoospermia and 5 semen samples from healthy individuals were collected for mRNA extraction. The samples were obtained through masturbation into sterile containers after a period of 3-7 days of sexual abstinence. Sperm quality analyzers were used to assess various parameters of the semen, including morphology, concentration, motility, viability and spermatozoa counts. The diagnostic criteria for asthenozoospermia were based on the standards provided by the World Health Organization (WHO 2021, Sixth Edition). According to these criteria, asthenozoospermia is characterized by a low percentage of rapidly forward progressive motile sperm (grade A <15%) and a low percentage of total progressive motile sperm (grades A+B <42%) in fresh ejaculate. Semen samples were considered normal if they met the following parameters: total sperm count ≥ 39 × 10^6 per ejaculate, semen volume ≥ 1.4 ml, pH ≥ 7.2, progressive motility (PR) ≥ 30% and immotile sperm <20%. Additionally, normal morphology of spermatozoa was defined as 4%. The age range of both the asthenozoospermia patients and the normal healthy controls was between 18 and 40 years old.

3.8. Trizol reagent-based mRNA extraction from collected semen samples

mRNA extraction from semen samples of asthenozoospermia patients (n=25) and normal healthy controls (n=5) was performed using Trizol reagent. Firstly in a 1.5 ml centrifuge tube, 250 µl of sperm suspension was mixed with 2 ml of PBS and homogenized by pipette mixing. The mixture was centrifuged at 8000 rpm for 5 minutes, and the supernatant was discarded. This washing step was repeated three times. The pellet obtained from the previous step was mixed with 500 µl of Trizol and incubated for 5 to 10 minutes at room temperature. The liquid was then vigorously hand-mixed for 15 seconds with 200 µl of chloroform. The tube was subsequently chilled on ice for 15 minutes. Now, the contents were centrifuged at 10,000 rpm for 15 minutes at 4°C to separate the phases. As a result, three separate layers were created. The RNA-containing colorless upper aqueous phase was carefully pipetted and moved to a fresh centrifuge tube devoid of RNAse. 500 ml of ice-cold isopropanol were added to the aqueous phase to precipitate the RNA. A deep freezer was used to incubate the mixture for a whole night at -20 C after forcefully mixing it by hand. The RNA pellet was washed with 75% ethanol by adding it to the pellet and centrifuging at 7500 g for 5 minutes at 4°C the following day after the tube had been inverted to discard the supernatant. The RNA pellet was air-dried at room temperature after the ethanol supernatant was removed. After this dissolved the pellet 30 µl of DEPC-treated, nuclease-free water.

Quantification and purity analysis of the RNA were performed using a NanoDrop 1000 Spectrophotometer. A 2 µl volume of sample was pipetted into a microplate reader, along with a blank of DEPC-treated water that had not been exposed to nucleases. The absorbance at different wavelengths provides information about the presence of RNA, DNA, proteins, phenols, carbohydrates, and salts in the sample. The yield and purity of the extracted RNA were assessed by measuring the absorbance at 260 nm, 280 nm, and 230 nm using a spectrophotometer [244].

3.9. cDNA synthesis

With a minimal modification to the manufacturer's instructions, the High Capacity cDNA Reverse Transcription Kit (Thermo ScientificTM, K1621) was used to reverse-transcribe whole RNA. By

using the kit and 20 µl of the entire reaction mixture, reverse transcriptase-PCR was used to create the cDNA template. The following components were added to a 0.2 ml PCR tube to create a 5X RT reaction master mix.

Table 3.1. cDNA 5X master mix preparation

Components	
5x Buffer	4µl
10mM dNTPs	2 µl
RNAse Inhibitor	1 µl
Revert Aid	1 µl
Random Primer	1 µl
Template RNA	1000ng(x)
NFW (Nuclease Free Water)	11-x
Total	20 µl

In a thermal cycler, polymerase chain reactions were carried out at denaturing, annealing, and extension temperatures of 25°C, 42°C, and 70°C for five minutes, sixty minutes, and five minutes, respectively. Before usage, cDNA was kept at - 20° C [245].

3.10. Differential gene expression analysis using qPCR

Following the manufacturer's instructions, the quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was carried out using the Applied Biosystems 7900HT equipment (Applied Biosystems) and a SYBR Premix Ex TaqTM kit (Thermo). Steps taken for qRT-PCR procedure are mention below:

1. Primers Design: Primers for amplifying the target mRNA were designed using Primer BLAST (Table 3.2), an online tool provided by the National Center for Biotechnology Information (NCBI). The primer sequences were synthesized by Lac Gene company.
2. RNA Template: The cDNA obtained from the previous reverse transcription step was used as the template for qPCR analysis.

3. RT-qPCR Setup: The SYBR Premix Ex Taq™ kit from Thermo was used, and the reaction mixture was prepared according to the manufacturer's instructions. This typically includes the SYBR Green master mix, forward and reverse primers, and the cDNA template. The reaction mixture was aliquoted into PCR tubes or plates.
4. Thermal Cycling: The RT-qPCR was performed using the Applied Biosystems 7900HT system, which allows for precise temperature control during the PCR process. The thermal cycling conditions, including denaturation, annealing, and extension temperatures and times, were set according to the specific primer design and the manufacturer's instructions.
5. Data Analysis: After the RT-qPCR run, the system generated amplification curves for each sample, representing the fluorescence signal over the course of the PCR cycles. The threshold cycle (Ct) value, which indicates the cycle at which the fluorescence signal reaches a significant level, was recorded for each target gene and the reference gene (GAPDH). The mRNA expression level was quantified using the $2^{\wedge(-\Delta\Delta CT)}$ method, where $\Delta\Delta CT$ represents the difference in Ct values between the target gene and the reference gene.
6. Replicates and Experimental Repetition: Each sample was run in triplicate, meaning that RT-qPCR was performed three times for each sample to ensure reproducibility. The entire qPCR experiments were repeated at least three times (n=3) to validate the results and obtain reliable data [246].

Table 3.2. Primer sequences of selected genes for qPCR

Gene Name	**Forward Sequence**	**Reverse Sequence**
CatSper1	CACTGGTGGGTGAATGAGCC	GGGCTTGTAGCCAGAAATGG
CatSper4	GGCACACCTTTTCCATGCTAA	AAGGACATCCGCCAGATGTC
APOA2	CTCTCCCTCTCCACAACTGAAG	CTGGCTAGGTAAGATAAGGAGGC
APOB	AAGCCACACTCCAACGCATA	GGCCAAGGTCAGGGAAATCA
LDHC	TGACCTAAGGAAGGGAATCCTGA	CCAGGGCTCAAAACTGTGTC

3.11.

a) Protein sequence retrieval and structure modelling

As there is no crystal structure of APOB is available on RCSB PDB (Protein Data Bank) database or on any other protein structure database. That's why the protein sequence is obtained from Universal Protein Resource (UniProt), a publicly available database that houses the information on proteins. Through their accession number (**P04114**), the necessary protein sequence was obtained in FASTA format. Further this sequence is used for analysis of primary and secondary structure of protein using PSIPRED and also in protein modelling [247,248]. Because of unavailability of protein structure, different online servers like Swiss modelling [249], Phyre2 [250], I-TASSER [251], Alpha fold, Alpha fold colab [252] and Robetta [253] was used for protein modelling on the availability of the template sequence.

b) Preparation of the protein through MS

The protein was once more prepared using the wizard tool in Maestro version 2022-1 due to lack of confidence in the protein model created using the web servers. The incomplete side and back chains were added during this procedure. Preparation and refining were the two processes that made up by the tool. The protein molecules' X-ray crystallography structures occasionally revealed interactions with water molecules, leading to entanglement. As the protein structure obtained from online servers was in open conformation and was not suitable for the ligand docking, hence, MD (Molecular Dynamic) simulation was done to get other conformation of protein structure for docking study. Consequently, at this step, the processes of protein structure optimization and minimization were finished [254].

3.12. Drug library preparation

Screening libraries for lead-like compounds are available in the free resources Pubchem [255], Drugbank [256], and Comprehensive Marine Natural Products Database (CMNPD) [257,258]. These databases also include tools and resources for research in the domains of early drug

development, medicinal chemistryand biology. From the above-mentioned libraries, a signature library in ".sdf" format with 32,000 molecules was downloaded. The ligands in this collection were made using the LigPrep module of Maestro (Schrodinger suite, LLC, New York, NY, 2020-1). The low-energy isomer of the ligand was created in this phase by optimisation using the OPLS 2004 force field. The protein's active site was then docked with all of the ligand molecules that were created in the complex structure for input [259].

3.13. Virtual screening

a) Receptor grid generation and Molecular docking

Following ligand preparation, molecular docking was conducted using a virtual screening workflow (VSW) in combination with the GLIDE module of Maestro, a software package in the Schrodinger suite. Docking was performed in two modes: Standard Precision (SP) and Extra Precision (XP). These modes allow for the exploration of different levels of accuracy and efficiency in the docking calculations. Using Glide's Receptor grid generating panel, a grid was created on the protein's active site predicted from site map based on OPLS-4 force field. On the same location, the prepared library was screened and put through several filtering steps of the screening operation [260].

b) Binding energy calculation

The geometry of total 361 docking compounds structures has also been improved using post-docking minimizations. The Prime Molecular mechanics-generalized Born surface area (MM-GBSA) module was used to post-process the final postures from the VSW. The outcomes of the docking were then quantified using the Prime MM-GBSA energy and the consensus of the docking scores. The relative binding affinity of ligands to the receptor is determined using the MM-GBSA technique [261]. A greater negative number indicates a better binding since the MM-GBSA binding energies are estimates of binding free energies (ΔG bind).

c) Structural interaction fingerprinting (SIFt)

The SIFt method, which transforms three-dimensional interaction patterns into one-dimensional fingerprints that are simple to Scan, evaluate and store is used to examine the protein-ligand complexes of 361 compounds. The binding region of a receptor's binding region contains residues that can be used to identify whether a particular type of interaction between a set of ligands and those residues has occurred or not [262].

3.14. Post-processing analysis for filter hit like molecules

a) ADME properties

The distribution, absorption, metabolism, excretion, and other ADME-related features for the control and library of 361 substances were identified using Maestro Schrodinger's QikProp module. The following ADME descriptors for the present study were anticipated: molecular weight (MW), number of hydrogen bond donors (donorHB) and acceptors (accptHB), solvent accessible surface area (SASA), predicted octanol/water partition coefficient (QPlogPo/w), number of rotatable bonds (rotor), predicted aqueous solubility (QPlogS) and percentage of human oral absorption. There is also a limit on how many rule five and three violations can result from hits [263]. The toxicity of a ligand is taken into consideration to be important for it to act as an effective medicine in novel drug development and QikProp generates physically relevant descriptions.

b) Molecular dynamic (MD) simulation

The top ten hit complexes (Ligand+Protein) were chosen for MD-simulation using the Maestro, Schrodinger suite, 'Desmond' module. The orthorhombic simulation box with particular dimensions and an explicit solvent system that was simulated under various circumstances was utilized in the Desmond module. Based on the findings from ADME analysis, XP docking, and binding interactions, complexes were chosen for MD simulation. System builder, minimization and MD simulation were the three phases that were used to complete the simulation. The docked protein-ligand complex was put through a system builder utilizing a predetermined T3P solvent

solution in the first stage. Orthorhombic boundary conditions are mostly created using the NTP solvent system. Additionally, the model's negative charge was balanced by the injection of sodium ions. The resultant model was then minimized and balanced at 1 bar pressure using a normal pressure-temperature ensemble (NPT ensemble) at the pressure of 300 K. A 100ns frame was recorded and stored into the trajectory during the 20-ns MD simulation. 5000 frames in total were recorded throughout the MD simulation [264].

c) Analysis after MD simulation

After the MD simulation, the RMSD of the protein and the ligand was calculated, and the interactions shown by the ligand during the whole simulation were examined. The average change in displacement is determined by the RMSD between a frame and a reference frame for a set of chosen atoms. For frame x, the RMSD is:

$$\mathrm{RMSD}_X = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(r_j'(t_x) - r_j(t_{ref})\right)^2}$$

where N is the selection's atoms' total number. r is the location of the chosen atoms in frame x, and t ref the reference time [265].

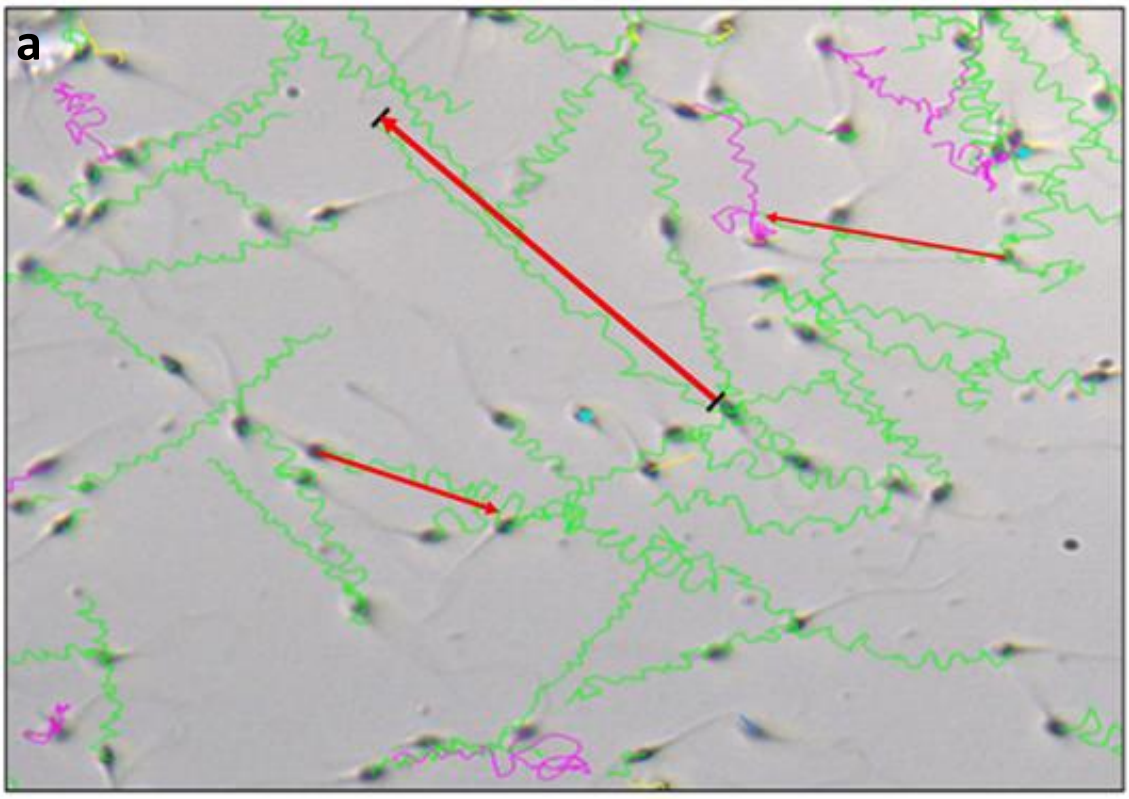
3.15. Figures and graphs: Maestro and VMD [266] were used to make the images. XMGRACE [267] was used to plot the graphs.

Chapter 4

Results and Discussion

4.1. CASA tracks of human spermatozoa

Human sperm samples taken from patient samples and analyzed using CASA tracks revealed alterations in motility patterns. Observations made during the investigation led to the division of the samples into three groups. First, semen samples with motility of greater than 50% at a concentration of between 80% and 50% (Figure 4.1a). Second, semen samples with concentration between 80% and 50% and motility less than 39% (Figure 4.1b) and third, semen samples with no motility at all between 80% and 50% of concentration (Figure 4.1c). It was found that semen sample having motility more than 50% are covering more distance and forward progressive path. Their movement energy is very high, they are highly active and showing vigorous movement. While those semen samples whose motility was less than 39%, they are not travelling progressively but they are showing non progressive movement. They just move around short distance or just show zigzag movement at the nearby its place. And in third type of semen samples, no movement at all in spermatozoa was seen. Sperms are live but immotile (have no energy for the movement).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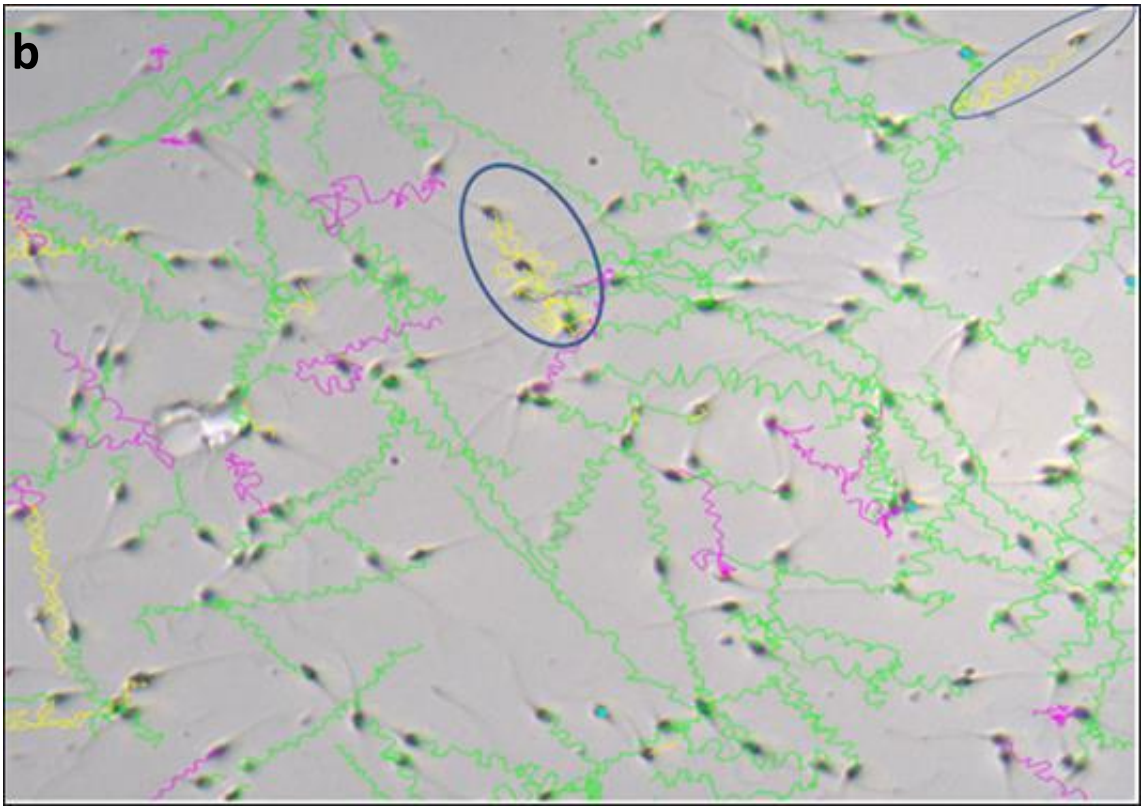
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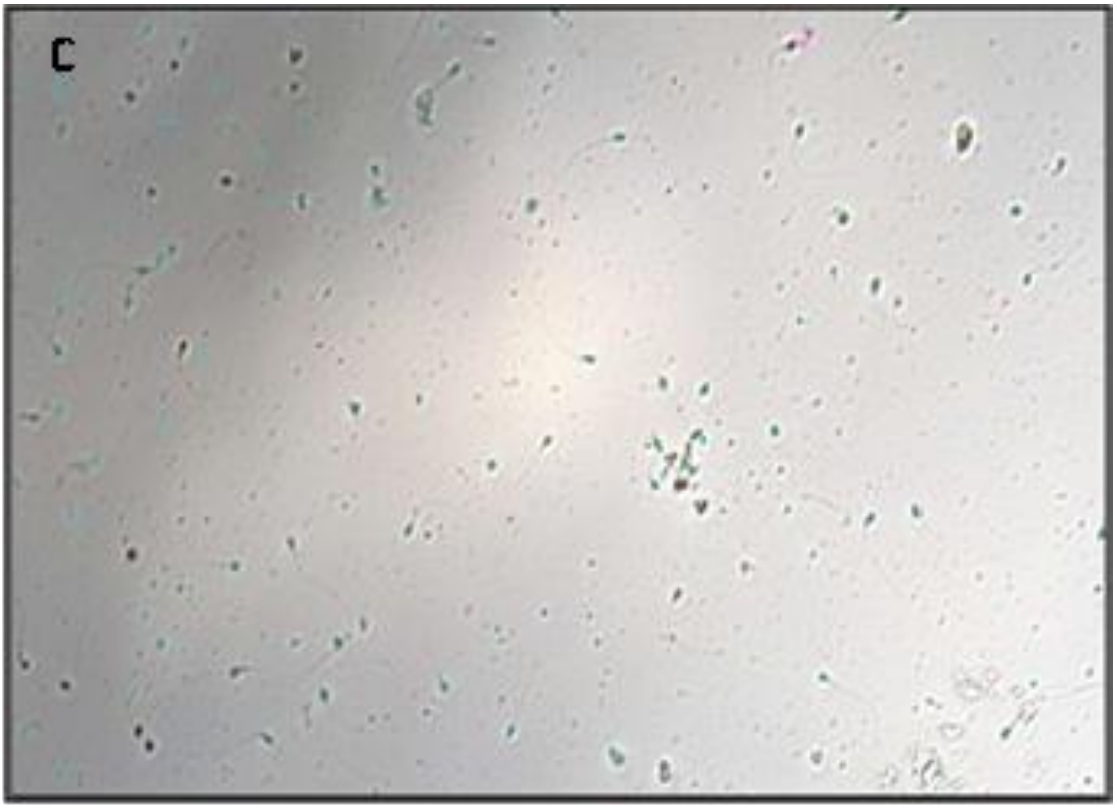


Figure 4.1. Human spermatozoa are tracked by CASA using backtrack approach. a) The route taken by metabolically active spermatozoa is shown by the squiggly green lines. b) The route (yellow line) taken by metabolically dysregulated spermatozoa took. c) Sperm immotility is shown by blue dots [268].

4.2. CASA-derived changes in sperm motility track

According to new World health organization guidelines 6th edition for semen analysis, a semen sample's progressive motility must be more than 32%. Progressive motility of at least 25 µm/s micrometers per sec is required for the effective transit of spermatozoa through cervical mucus. Here, our investigation shows that the progressive spermatozoa motility rate in the semen sample is more than 40 µm/s, or motility >50% (Table 4.1) (Figure 4.2a). Contrarily, non-progressive spermatozoa in semen are intended to be less motile. The sample has a motility rate of 0–35 µm/s (or motility of 39%) (Table 4.2) (Figure 4.2b). This data signifies that spermatozoa covering a

distance of more than 40 µm/s are rich in ATP production and are metabolically active whereas spermatozoa covering a distance less than 40µm/s have less ATP production in it and are metabolically dysregulated.

Table 4.1. ImageJ analysis of highly motile spermatozoa showing a forward fast progressive movement.

Spermato	Area	StdDev	Angle	Length
1	18.049	33.783	-94.792	54.767
2	18.476	31.512	56.125	56.286
3	16.34	41.132	68.827	49.764
4	16.019	45.293	70.346	48.582
5	16.233	44.72	-7.97	49.498
6	16.981	48.461	47.311	51.573
7	23.602	33.505	-23.883	71.837
8	16.874	43.395	152.292	51.308
9	20.398	40.81	-175.764	61.934
10	17.942	39.799	-35.02	54.669

Table 4.2. ImageJ analysis of slow motile spermatozoa showing a non-progressive (NP) movement.

	Area	StdDev	Angle	Length
1	7.583	40.991	29.745	5.269
2	7.796	56.718	-45	6.47
3	11.214	52.992	99.462	7.951
4	11.534	35.243	-20.556	8.376
5	19.01	42.36	-34.778	14.323
6	30.971	34.247	20.462	23.37
7	34.282	33.092	110.726	25.856
8	39.942	15.494	170.028	30.195
9	41.544	29.204	124.019	31.542
10	47.952	33.093	48.621	36.584

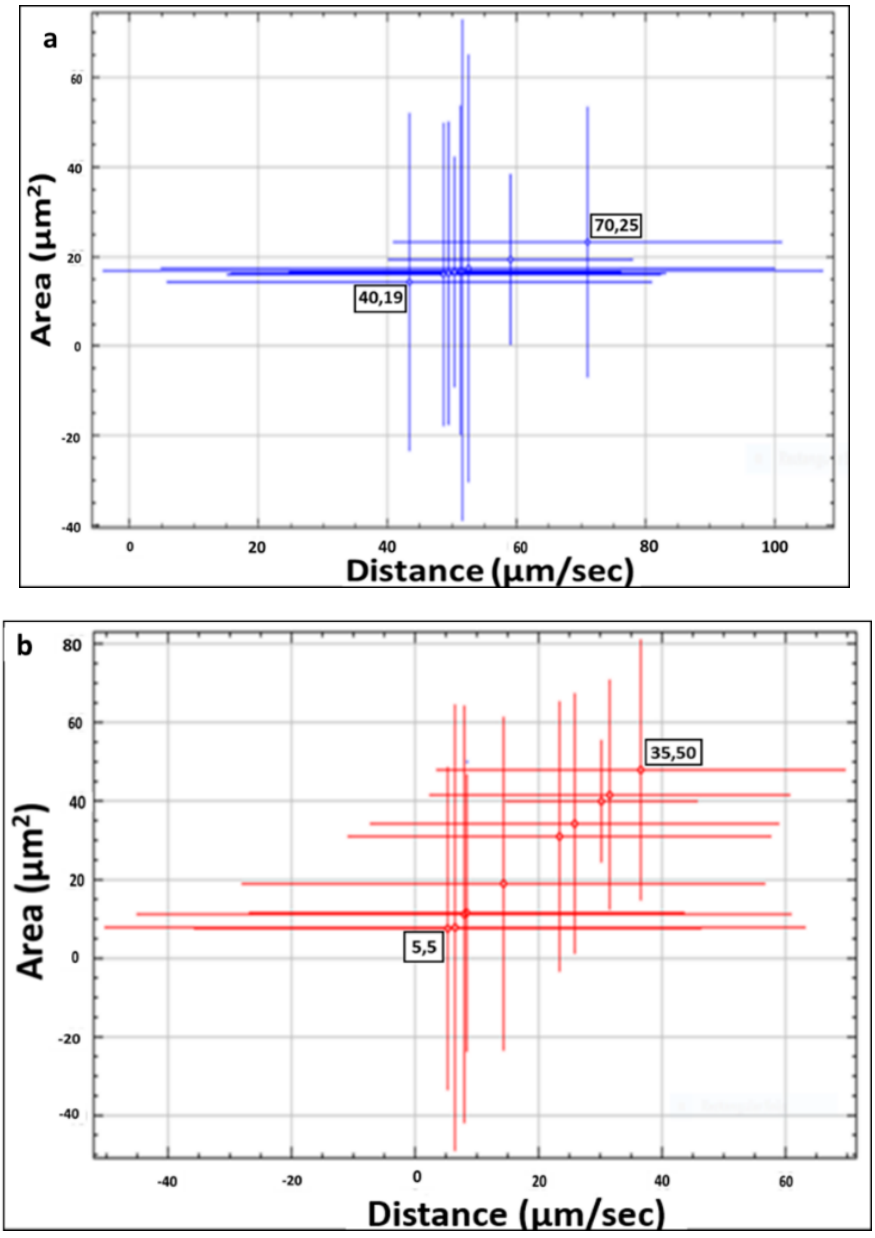


Figure 4.2. Sperm motility track in semen samples using ImageJ. a) A highly motile/hyperactive semen sample (Motility = more than 50%) moves at a pace of more than 40 µm/sec, both in terms of distance travelled and surface area covered, according to the graph. b) The graph depicts the movement of low motile/non-progressive semen samples (Motility = less than 30% or Rapid Progressive = less than 15%) over a specific area at a speed between 0 and 40 m/sec [268].

4.3. Morphological evaluation of spermatozoa with altered metabolic regulation

A reduction in sperm quality has been recorded in around 40% of males who are of reproductive age. Men with defective sperm morphology have also received attention as a key factor contributing to male infertility. A key indicator of the quality of the sperm in a semen sample is evaluated by its morphology. When we examined the less mobile sperm samples, we discovered sperms with broken, double, and coiled tails as well as some with terminal droplet spermatozoa (Figure 4.3). The improper seminal fluid conditions or the presence of microorganisms have revealed coiled-tail sperm. These sperm are unable to swim because their tails have been damaged. Researchers have found a link between smoking and coiled-tail sperm. Dysplasia of Fibrous Sheath (DFS) or Stump tail spermatozoa are characterized by abnormally short tails and low or no motility. This tail shortage develops during late spermiogenesis, or the generation of sperm cells in the testis [270]. Some DFS patients also have immotile cilia syndrome-related chronic respiratory conditions. An autosomal recessive genetic disorder known as stump-tail or DFS is linked to faulty genes that encode for proteins including the sperm neck centrin 1 protein. Higher rates of sperm aneuploidies or defective chromosomes have been associated with DFS [271].

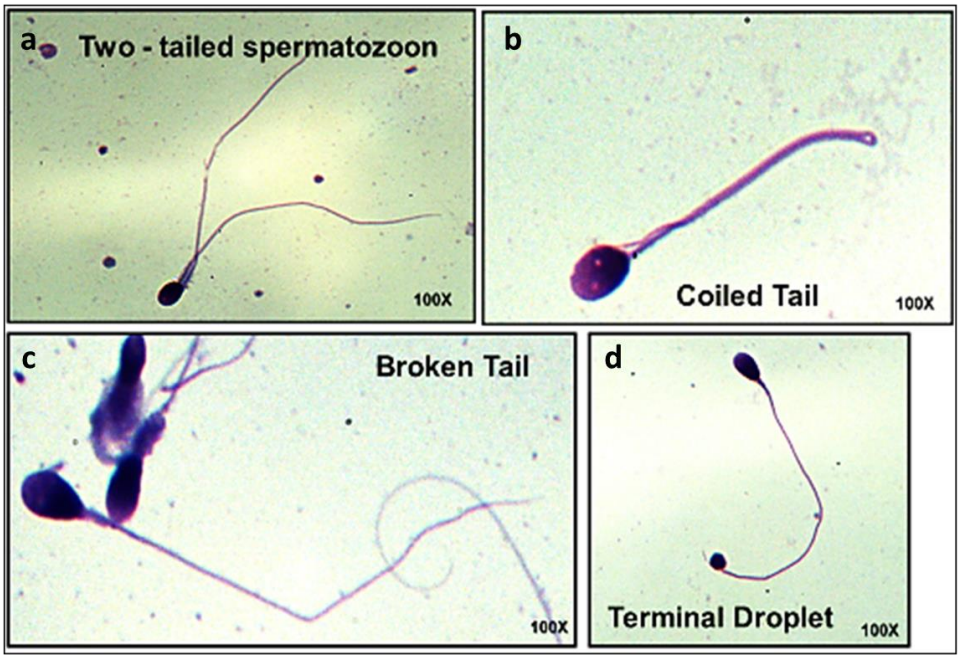


Figure 4.3. Morphological examination of samples having inactive sperm found in semen (a) two-tailed (b) coiled tail (c) broken tail (d) terminal droplet. Images obtained in 100x resolution [268].

4.4. Collection of genes from literature review

From a literature review and databases, the genes "CatSper1, CatSper2, CatSper3, CatSper4, GAPDHS, PGK2, LDHC, PGM4, PGM2" were chosen because they have been linked to asthenozoospermia or severe asthenozoospermia.

4.5. PPI Network analysis

The STRING database was used to create a PPI network using the nine chosen possible targets. Proteins and their interactions with other proteins are represented in the network by the nodes and edges, respectively. This clearly illustrates the interaction of the sperm motility gene with metabolic genes and the significance of the metabolic pathway in sperm motility in Figure 4.4 where “CATSPER1, CATSPER2, and CATSPER3” show their interaction with glycolytic proteins (i.e., GAPDHS and PGK2) as well as their involvement in the sperm flagellum.

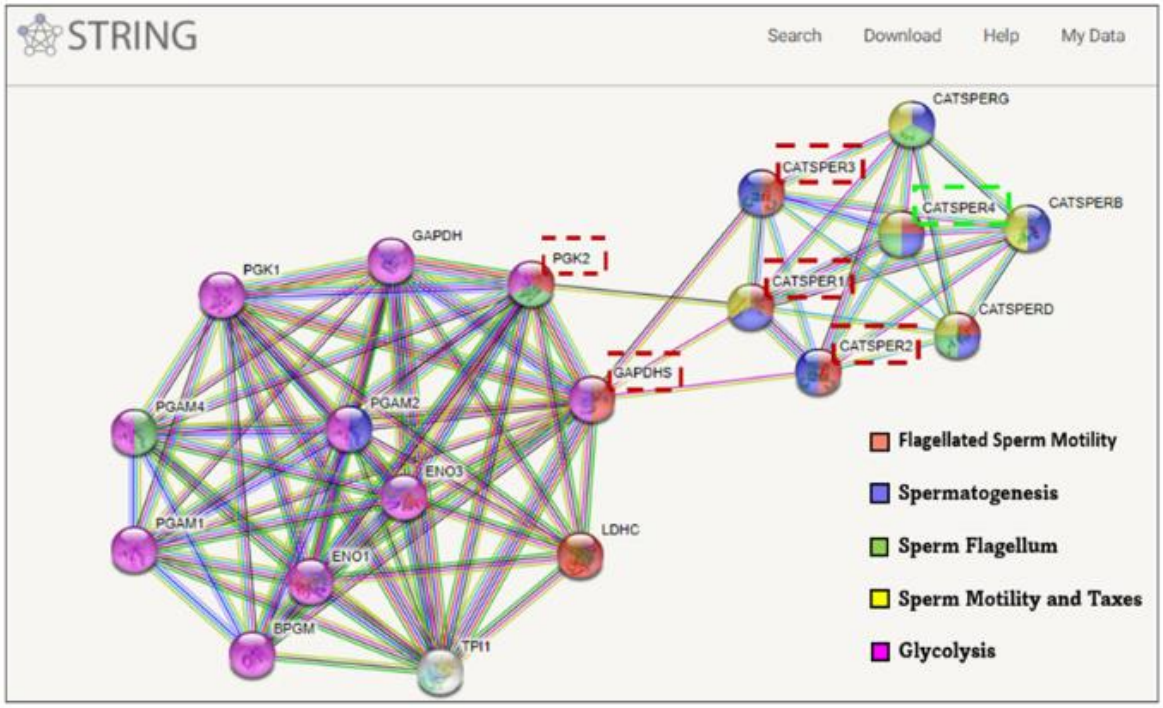


Figure 4.4. A PPI network study of 9 selected genes (mention in 4.4) shows that red indicates flagellated sperm motility, blue represents spermatogenesis, green represents the sperm flagellum, yellow represents sperm motility and taxes, and pink represents glycolysis [268].

4.6. Pathway analysis

The CATSPER1, CATSPER2, and CATSPER3 proteins were chosen from the Reactome database to better understand their function in the metabolic pathway. Figure 4.5 shows that the CATSPER1 protein is engaged in three different metabolic pathways, and Figure 4.6 shows that it interacts with the APOA2 gene with a score of 0.781. This discovery once again led us to investigate protein-protein interactions (PPIs) in our quest to understand the function of lipid metabolic genes in sperm movement.

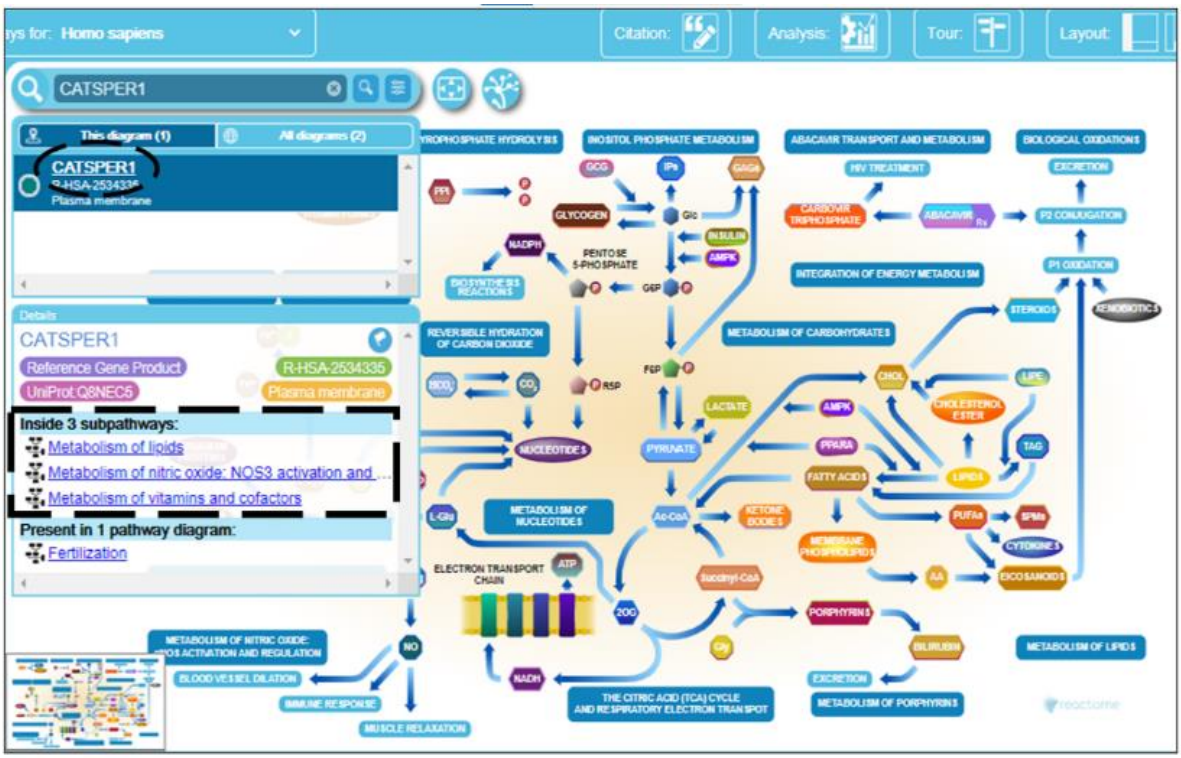


Figure 4.5. CatSper1 gene expression in three distinct metabolic pathways. Lipid metabolism, NOS3 activation for nitric oxide metabolism, and vitamin and cofactor metabolism [135].

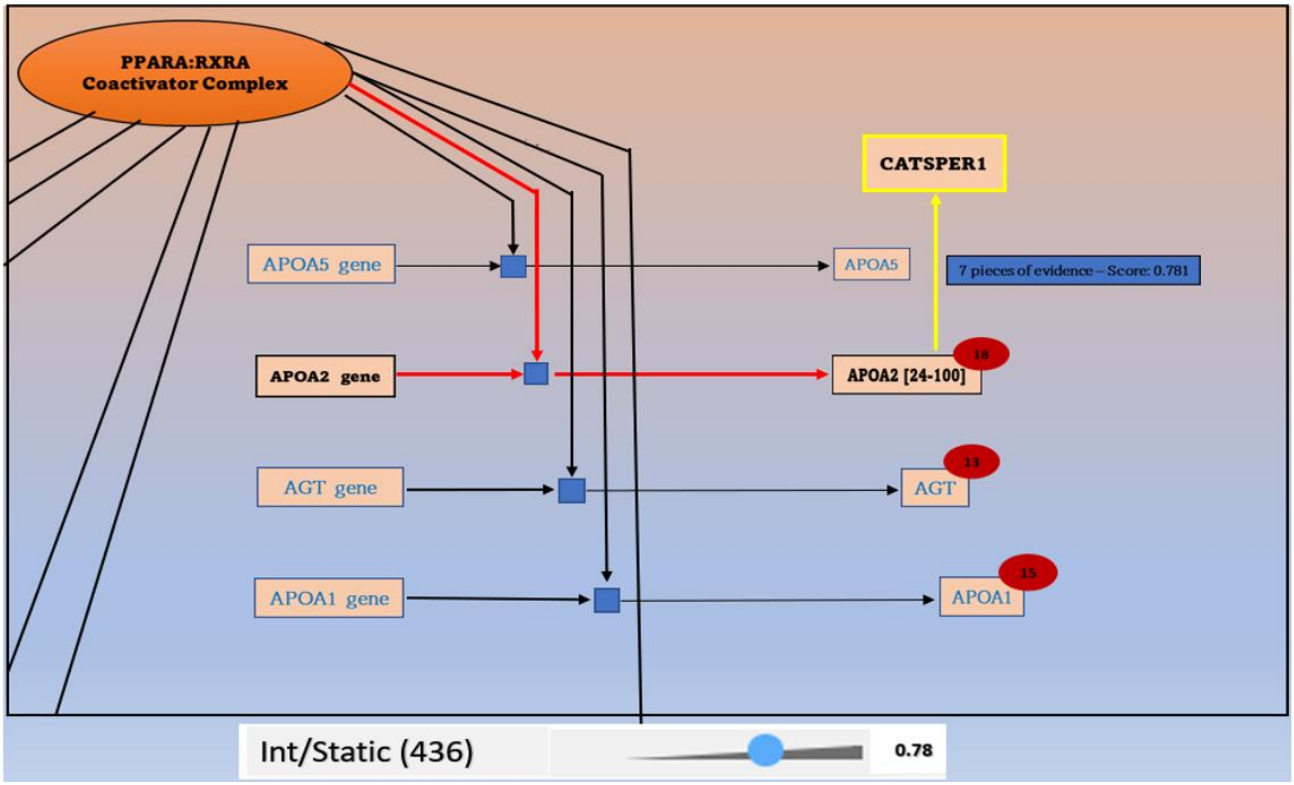


Figure 4.6. With an interacting score of 0.78, the gene APOA2, which is involved in lipid metabolism, demonstrated its contact with the gene CatSper1 [135].

4.7. Selected Genes Associated with Lipid Metabolism and APOA2 Genes through PPI Network Analysis

After "CATSPER1" and a lipid metabolic gene interacted, the PPI investigation continued and it was found that the APOB gene, which is involved in lipid metabolism, is connected to human flagellated sperm motility and spermatogenesis (Figure 4.7).

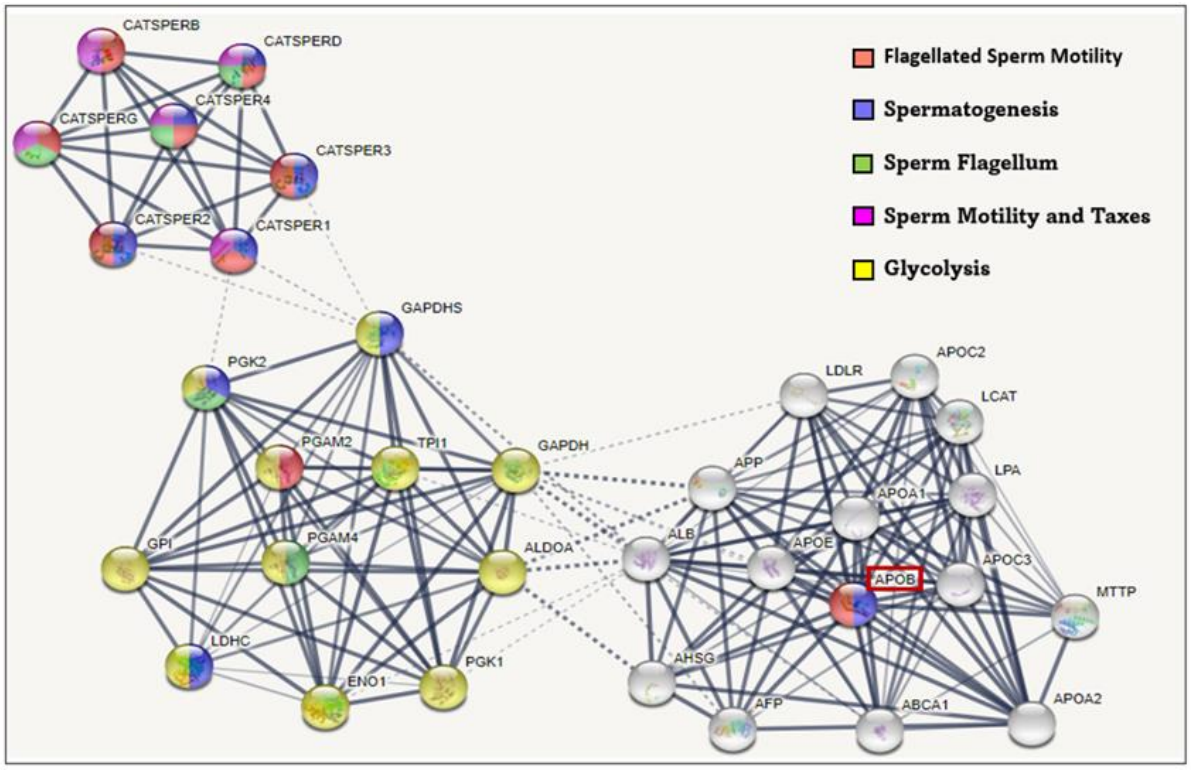


Figure 4.7. PPI analysis demonstrating the link between sperm motility and spermatogenesis and APOB. Flagellated sperm motility is represented in red, spermatogenesis is depicted in blue, the sperm flagellum is depicted in green, glycolysis is depicted in yellow, and sperm motility and spermatogenesis are represented in pink. [268].

4.8. Gene Ontology

Gene ontology was used to verify the results of the PPI network and pathway analysis, and the results showed that APOB is associated with asthenozoospermia (Figure 4.8) and sperm motility (Figure 4.9), respectively. Additionally, the Enrichr tool revealed that the metabolic protein GAPDHS is associated in male infertility with a p-value of 3.502e-8 (Figure 4.10), and that the sperm protein CATSPER4 is present (Figure 4.11).

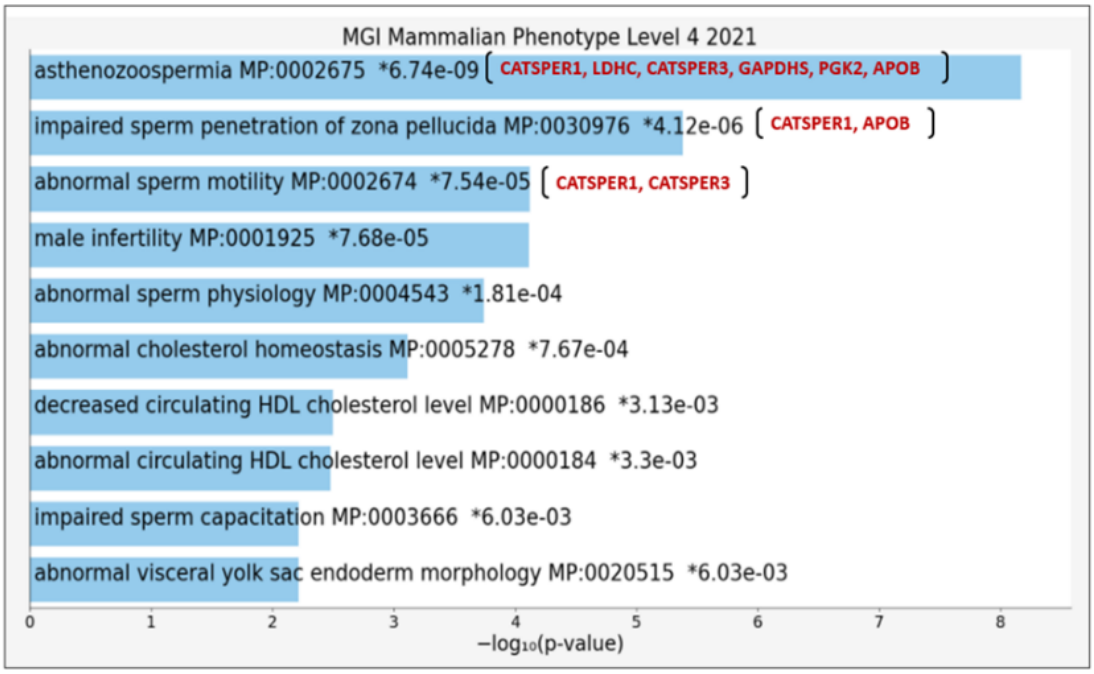


Figure 4.8. With a p-value of 6.74e-09, the Enrichr result demonstrates the role of APOB and glycolytic genes in asthenozoospermia [268].

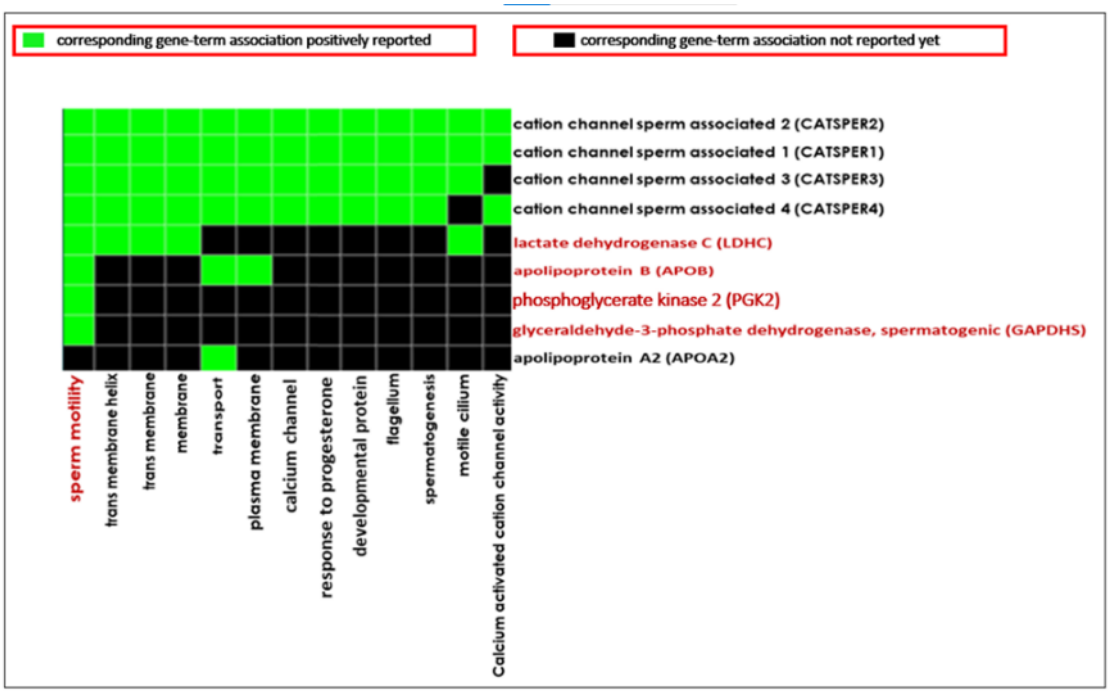
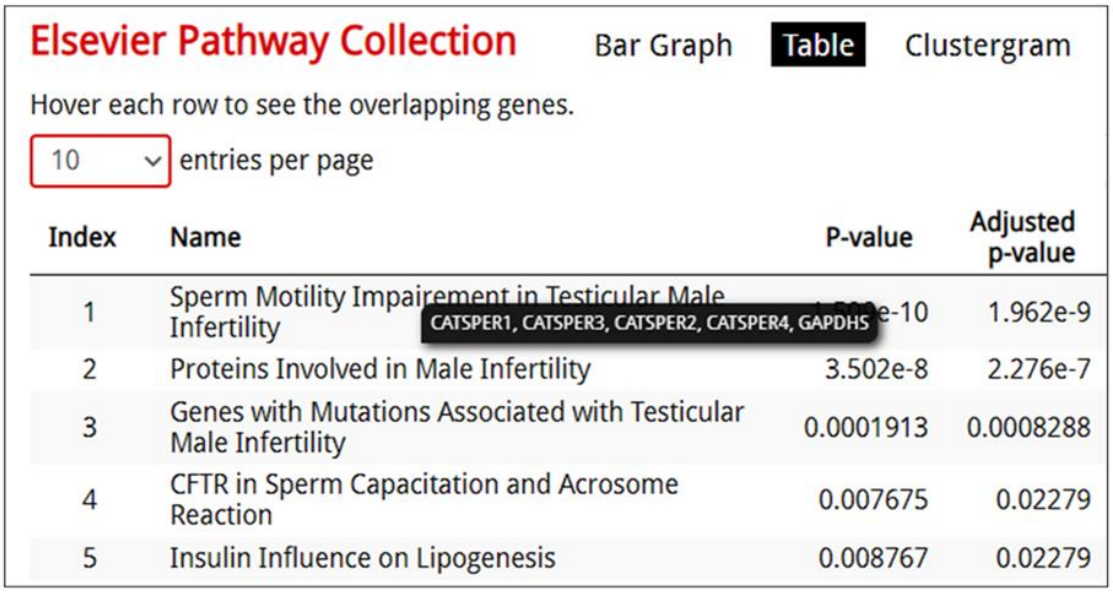
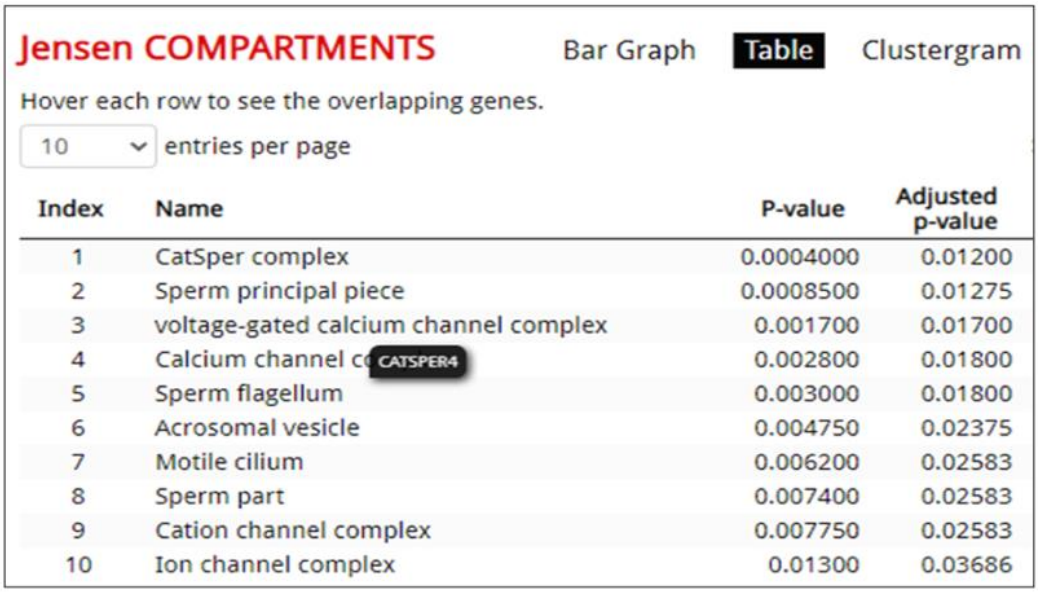


Figure 4.9. Functional clustering results from the DAVID database demonstrating the role of APOB and glycolytic genes in sperm motility [268].



Index	Name	P-value	Adjusted p-value
1	Sperm Motility Impairement in Testicular Male Infertility	e-10	1.962e-9
2	Proteins Involved in Male Infertility	3.502e-8	2.276e-7
3	Genes with Mutations Associated with Testicular Male Infertility	0.0001913	0.0008288
4	CFTR in Sperm Capacitation and Acrosome Reaction	0.007675	0.02279
5	Insulin Influence on Lipogenesis	0.008767	0.02279

Figure 4.10. An Enrichr result suggests that GAPDHS and the CATSPER family of proteins are related to male infertility [286].



Index	Name	P-value	Adjusted p-value
1	CatSper complex	0.0004000	0.01200
2	Sperm principal piece	0.0008500	0.01275
3	voltage-gated calcium channel complex	0.001700	0.01700
4	Calcium channel c	0.002800	0.01800
5	Sperm flagellum	0.003000	0.01800
6	Acrosomal vesicle	0.004750	0.02375
7	Motile cilium	0.006200	0.02583
8	Sperm part	0.007400	0.02583
9	Cation channel complex	0.007750	0.02583
10	Ion channel complex	0.01300	0.03686

Figure 4.11. CATSPER4 protein involvement in the sperm flagellum is shown in an Enrichr result with a p-value of 0.003000 [268].

4.9. Relative sperm mRNA transcription levels

Five specific genes' relative mRNA transcript levels in sperm samples from asthenozoospermic and normal patients were examined. The five genes that were chosen play roles in spermatogenesis, energy metabolism and sperm motility. Figure 4.12 displays the relative transcript levels for these genes.

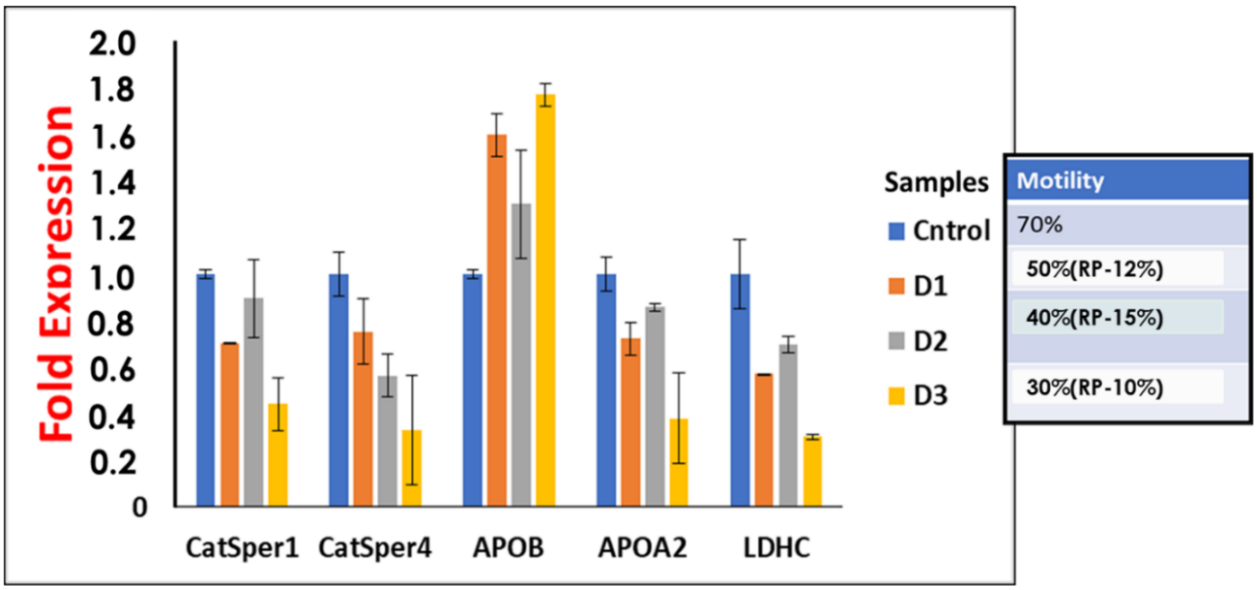


Figure 4.12. Relative mRNA expression levels of sperm motility and energy metabolic genes involved in asthenozoospermic and in normal semen samples assessed by qPCR. Data is present as log2 transformed $2^{-\Delta\Delta Ct}$ values. The error bars show the standard error of the mean while the bars show the average fold change.

4.10. Molecular modelling and structural validation

a) Sequence retrieval and secondary structure prediction

Through protein accession number (**P04114**), the FASTA sequence of APOB protein was obtained from the UniProt database, the necessary protein sequence of 3001-3500bp was taken for protein modelling and total 10 amino acid long sequence were taken as active site for ligand docking (Figure 4.13a and 4.13b). Through PSIPRED, the secondary structure of protein sequence predictions were compared. In the sequence structure of APOB protein, α-helices, β sheets, and

coils are distributed throughout the secondary structure. Coils (50.5% of the total secondary structure), α helix (45.9%), β-sheets (3.5%) all were the next-most common components (Figure 4.14).

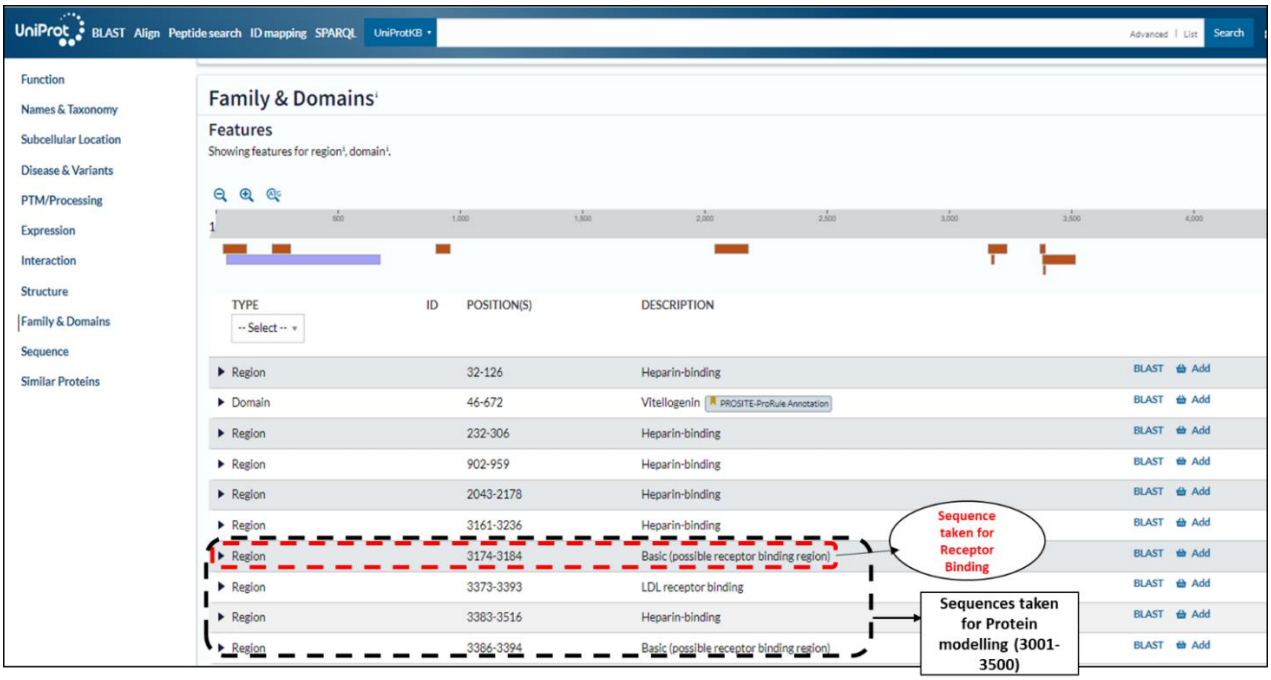


Figure 4.13 a. (UniProt P04114, http://www.uniprot.org/uniprot/P04114)

VLTAKGMALFGEGKAEFTGRHDAHLNGKVIGTLKNSLFFSAQPFEITA STNNEGNLKVRFPLRLTGKIDFLNNYALFLSPSAQQASWQVSARFNQY KYNQNFSAGNNENIMEAHVGINGEANLDFLNIPLTIPEMRLPYTIITTPP LKDFSLWEKTGLKEFLKTTKQSFDLSVKAQYKKNKHRHSITNPLAVLC EFISQSIKSFDRHFEKNRNNALDFVTKSYNETKIKFDKYKAEKSHDELP RTFQIPGYTVPVVNVEVSPFTIEMSAFGYVFPKAVSMPSFSILGSDVRVP SYTLILPSLELPVLHVPRNLKLSLPDFKELCTISHIFIPAMGNITYDFSFK SSVITLNTNAELFNQSDIVAHLLSSSSSVIDALQYKLEGTTRLTRKRGLK LATALSLSNKFVEGSHNSTVSLTTKNMEVSVATTTKAQIPILRMNFKQE LNGNTKSKPTVSSSMEFKYDFNSSMLYSTAKGAVDHKLSLESLTSYFSIE SSTKGDV

Figure 4.13b. The 500bp sequence annotation taken for APOB (protein) modelling. (UniProt P04114, http://www.uniprot.org/uniprot/P04114)

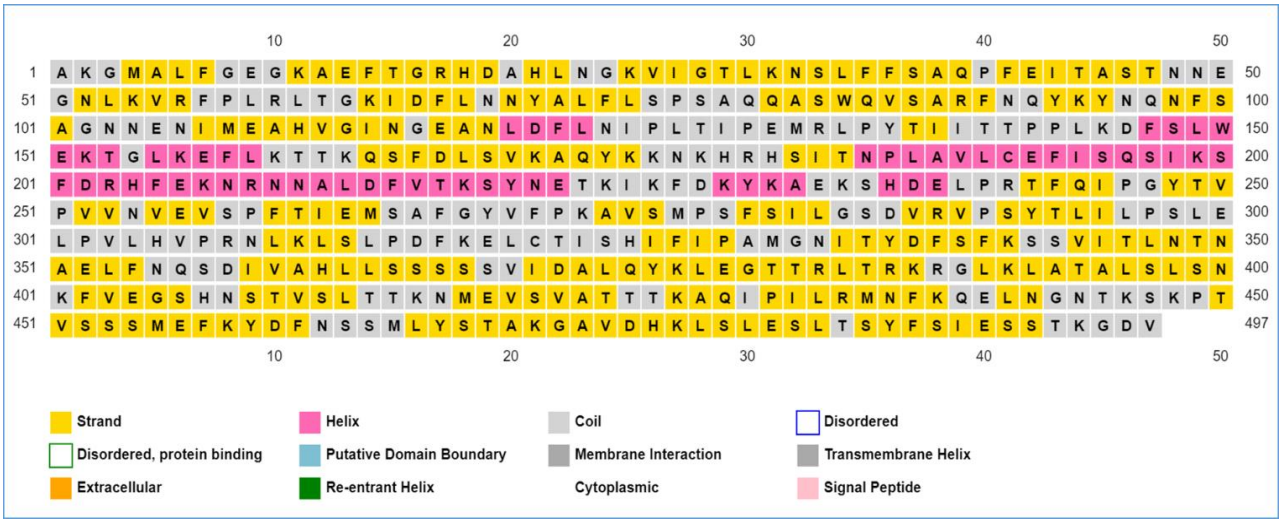


Figure 4.14. PSIPRED was used to analyze the secondary structure of protein chains and provide predictions about the α-helix, extended strand, random coil, and β-turn.

b) Protein structure prediction using an online Server

We used five online servers for the prediction of protein structure: (1) SWISS-model, (2) Phyre2, (3) I-TASSER, (4) Alphafold Colab, and (5) Robetta. The result obtained from the SWISS Model, Phyre2, Alphafold Colab, I-TASSER shows the limited and disordered structure, unclear grooves, not well-defined structure and 2-D structure was obtained (Figure 15a). Although the 3D model of APOB was obtained from Robetta using deep learning-based methods, RoseTTAFold and TrRosetta with paired (Multiple Sequence Assessment) MSA or comparative modeling (CM) (Figure 4.15b). Model generated from Robetta was considered for Molecular docking (MD) and Molecular dynamic simulation (MS). Also, the disoderness of the structure sequence was checked by using the PSIPRED server and Robetta also.

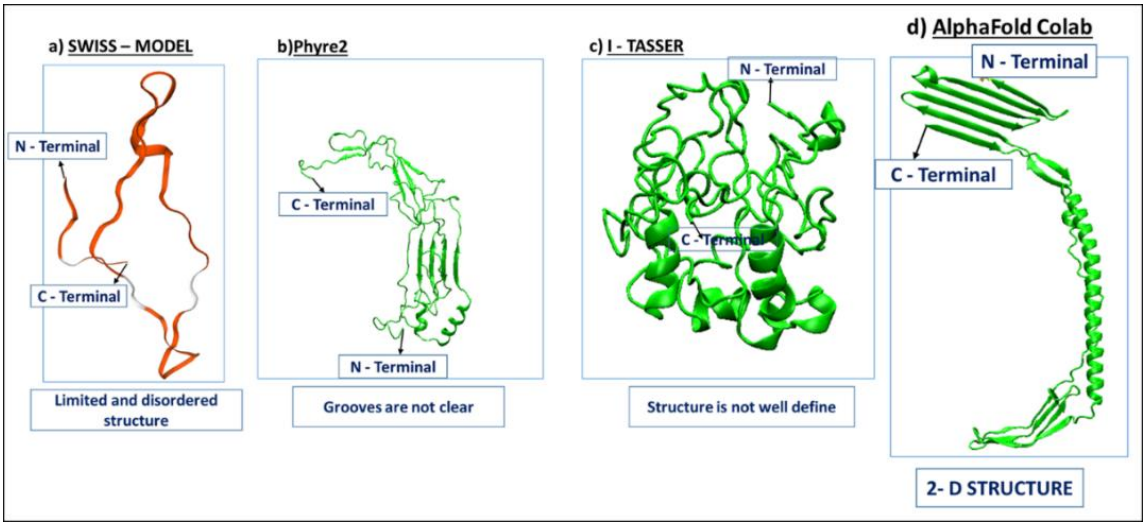


Figure 4.15a. Exploring the model of a protein using different online protein modelling servers.

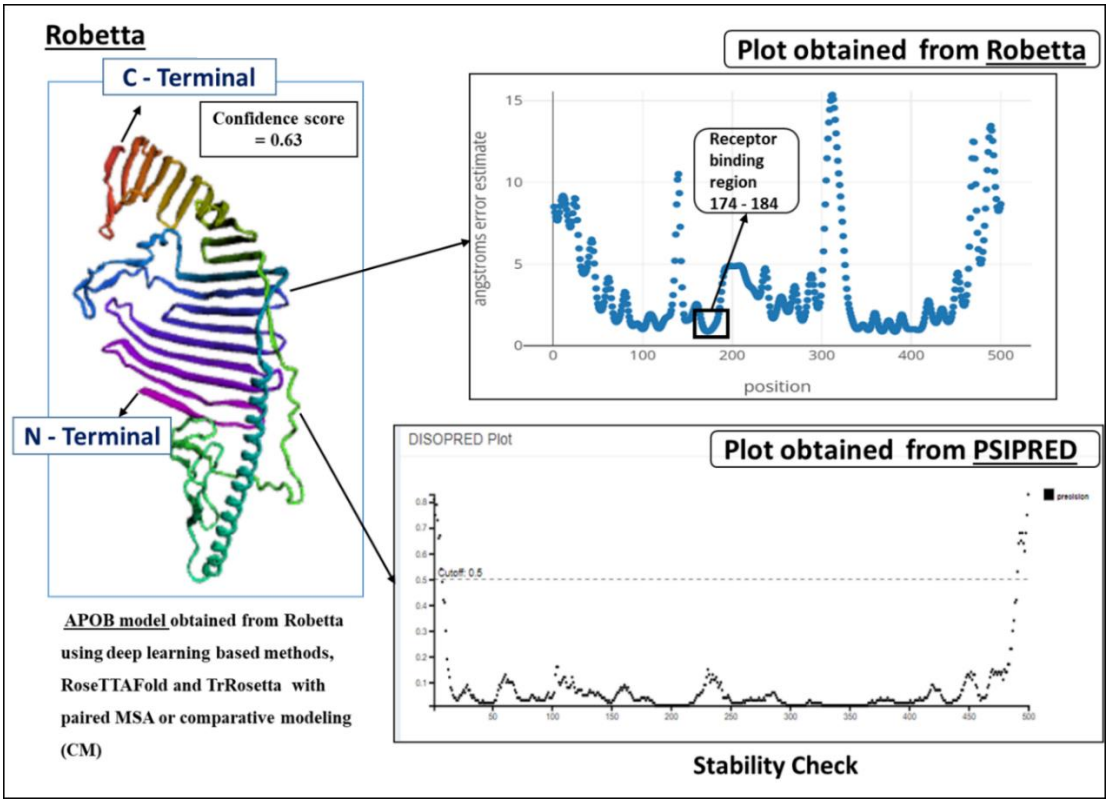


Figure 4.15b: Final model of APOB protein obtained from Robetta with confidence score = 0.6 and domain analysis using Robetta and PSIPRED showed obtained model is lie in the ordered region.

c) Modeled protein structure validation

Robetta's APOB model, which was developed by structural modeling, has excellent sequence identity with the template and hence good overall stereochemical quality. No residues were found in the outlier area for the APOB model, whereas 90.3% were found in the preferred region. A, B, and L are used to denote the most popular areas. a, b, l, and p are the extra permitted areas. Prolines and non-glycine residues are all shown as filled black triangles, whereas non-glycine residues are depicted as filled black squares. Red is the color for disallowed residues of modeled protein (APOB) structure (Figure 4.16).

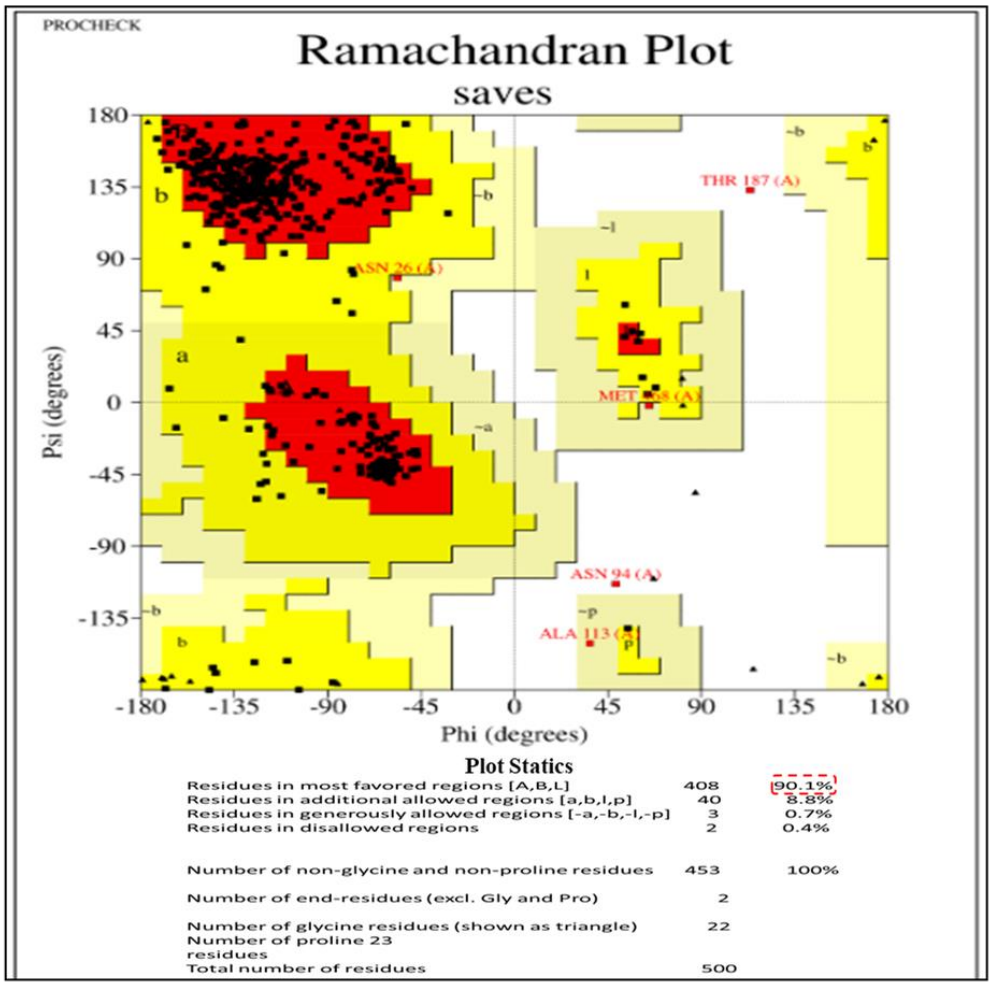


Figure 4.16. Stereochemical analysis using Ramachandran Plot for APOB protein. The PROCHECK, plot's red (core), yellow (allowed), and beige (generously allowed) filled 'boxed' sections denote the areas utilized for confirming the modelled protein structure.

d) Molecular Simulation (MS) of the APOB-APO model

A total of 300 ns of molecular simulation of the APO model was run in which 15000 frame of the model was obtained. During MS, three conformations of protein was seen. 1) The open conformation of protein at 0 ns which is 1st frame of the model. 2) The fold conformation of protein where RGB pocket (protein fold to create pocket) was formed at 120 ns which is the 6999th frame of protein and 3). The closed conformation, where the protein was completely closed and makes globular structure. This starts from 165 ns of MS which is the 10000th frame of protein (Figure 4.17). The structural motions of protein from its initial frame to variation in the 15000th frame were very high in their area cover. The three residues L62, HIE 182, and VAL 500 were selected to find the area difference covered by three different conformations of the apo model (Figure 4.18).

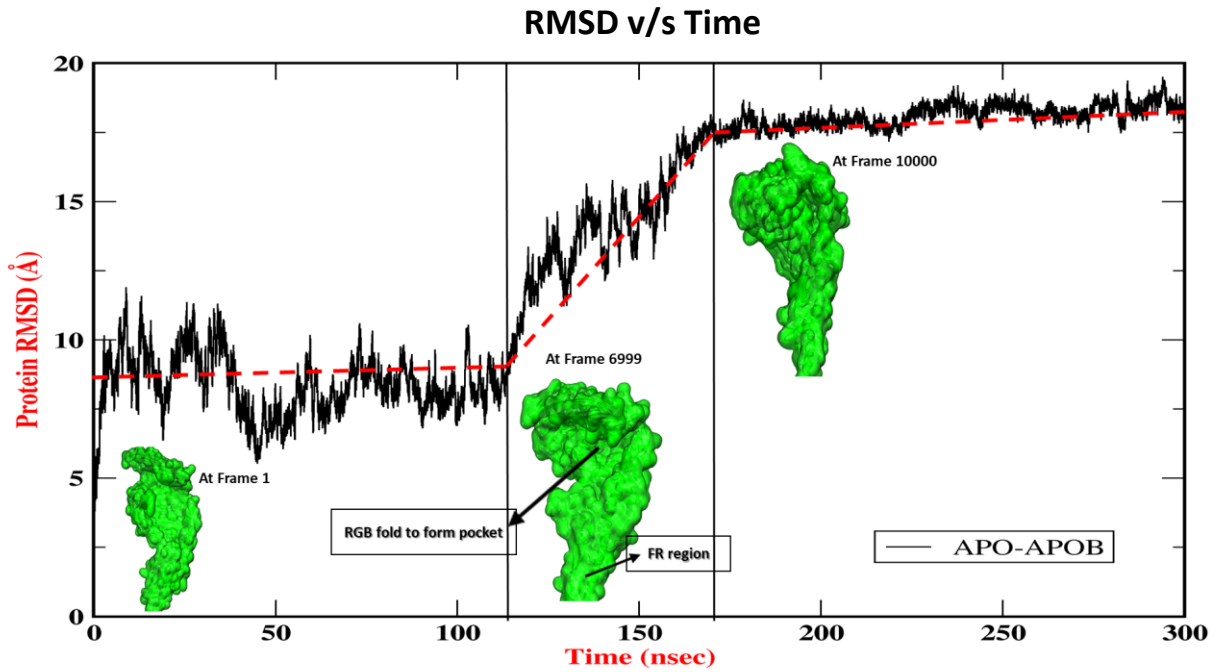


Figure 4.17: Time development of the Root Mean Standard Deviation (RMSD) of C-atoms with respect to the starting structure for APOB-APO (Protein) in molecular dynamic (MD) simulations.

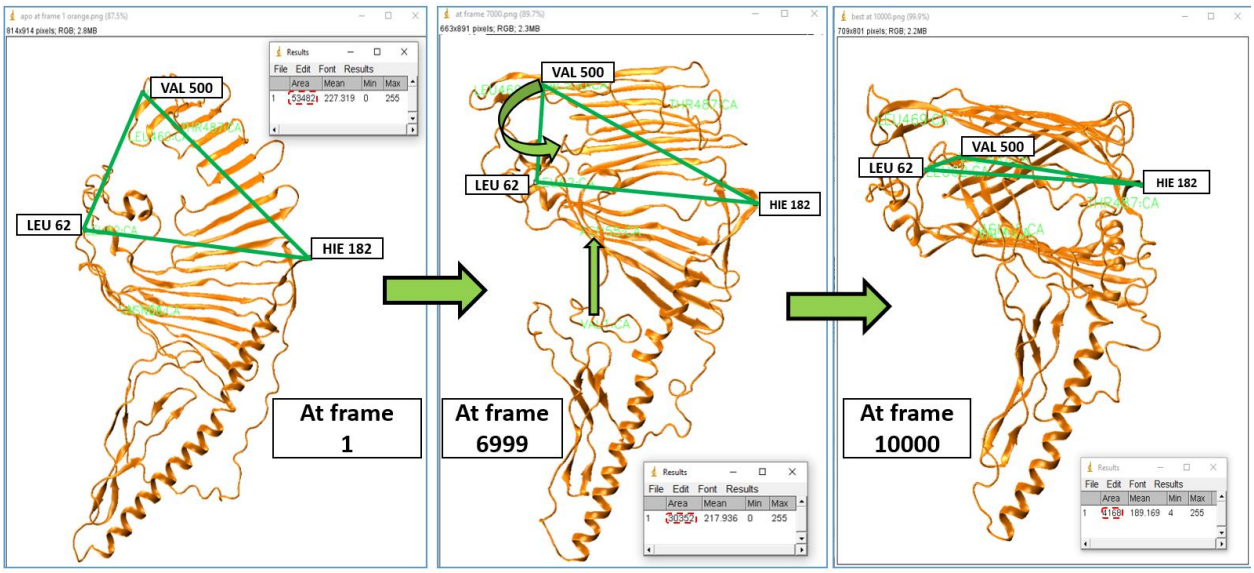


Figure 4.18. Conformational changes occurred by the APO model during MD simulation. Residue L62, HIE 182, and VAL 500 were showing significant changes in the area at different time intervals.

4.11. **Active site identification and Molecular docking**

After analyzing the apo model, Grid formation – using Glide (Software) in Maestro was done. 10 bp long amino acid residue was found as an active site of protein where the pocket was formed during MS of the apo model(Figure 4.19).

After this, screening of prepared drug from lig prep was done. About 32000 compounds were docked and out of that 361 compounds were successfully shown their binding to the active site of the protein (Fig 4.20). H_2 bond interactions between medicinal molecules and the protein pocket are shown by this method. The molecular docking strategy generates ligand docking scores by calculating the "Hydrogen bond" distance between the ligand and the target, and it also calculates the glide energy between the two molecules. In this computational study, Gemfibrozil (control) is employed as a benchmark against which the apo-protein, re-docked, and IFD postures may be compared (Figure 4.21). The docking score of control was -3.33. Dabigatran M636 has a higher docking score of -15.255, than that of the other ligand molecules (Table 4.3).Additionally, calculations of the MMGBSA binding free energies were also conducted on all 361 compounds.

The highest binding energies was -68.91 kcal/mol of Dabigatran M636, were better than control Gemfibrozil compounds. As its binding energy was -27.51.

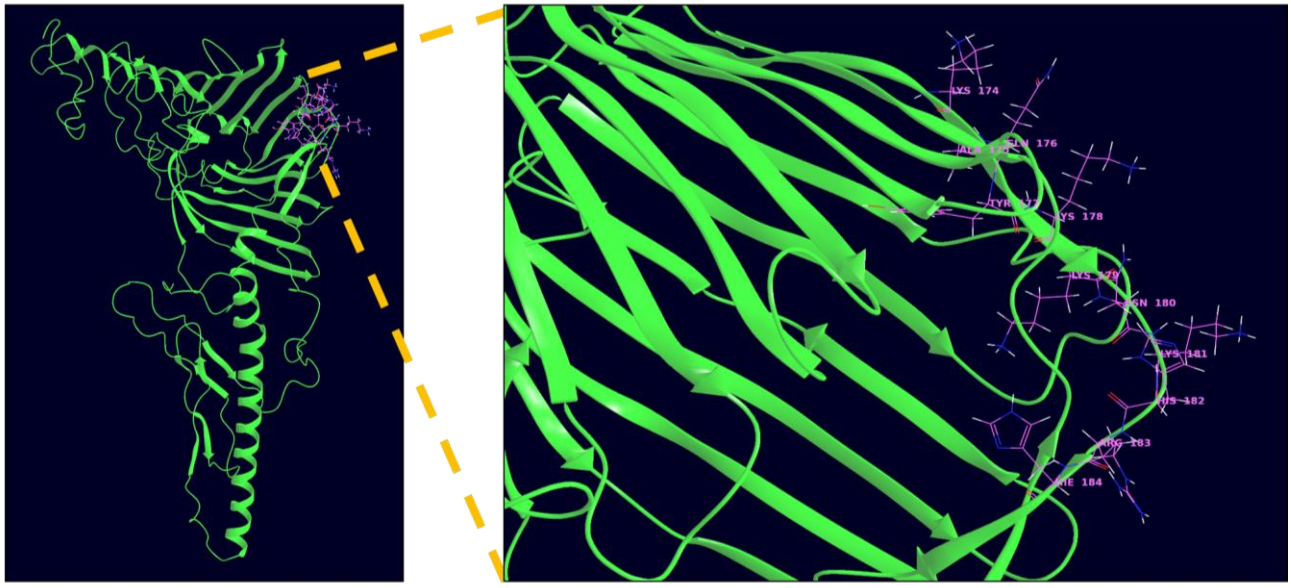


Figure 4.19. Amino acid residue from Lys (147) to His (184) as active site of protein used for ligand docking.

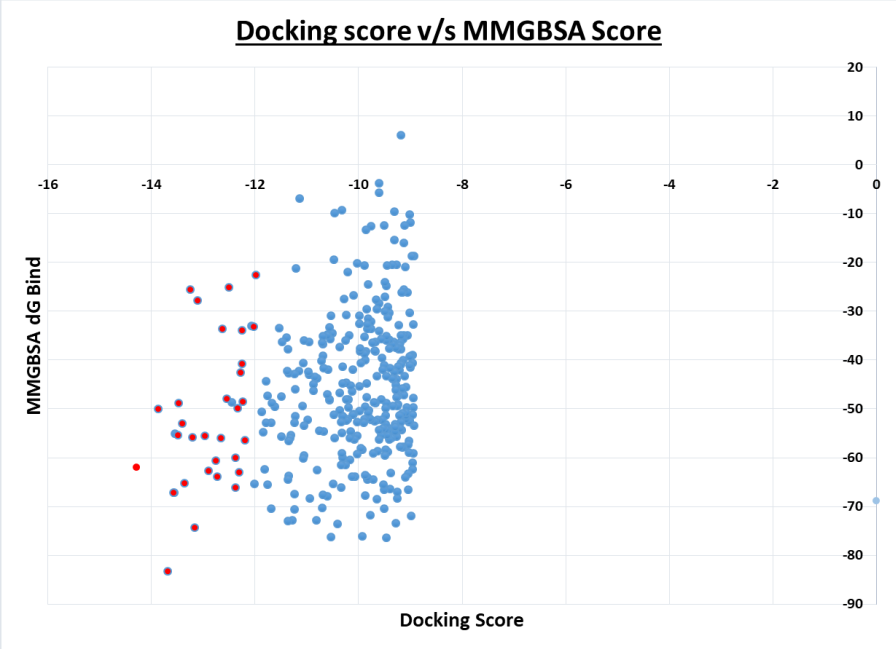


Figure 4.20. 361 VSW hits (red in colour) stand out among 32,000 ligand library hits in a scatter plot utilising docking scores (x-axis) and estimated MM-GBSA binding free energies (kcal/mol) (y-axis).

Table 4.3. List of selected hits on the bases of the Docking Score and free energy binding score.

	Docking Score	**MMGBSA Score**
Control	-3.333	-27.51
Hit 1	-15.255	-68.91
Hit 2	-13.562	-67.18
Hit 3	-11.622	-49.54
Hit 4	-10.531	-76.26
Hit 5	-10.32	-54.96
Hit 6	-9.547	-52.31
Hit 7	-9.707	-48.68
Hit 8	-9.29	-53.11
Hit 9	-9.216	-51.42
Hit 10	-9.222	-32.93

4.12. Top hits' ADMET characteristics and Lipinski's filter

To further narrow down the 361 compounds, we investigated their ADME characteristics. Compounds that adhere to the RO5 and RO3 are more likely to be drugs with high oral bioavailability. Lipinski's RO5 suggests the following ranges for molecules: (a) molecular weight 500 g/mol; (b) HBD 5; (c) HBA 10; and (d) logPo/w 5. A value of -5.0 or less is preferred for the logS. Important factors for the assessment of orally active substances are the polar surface area (PSA) and the number of rotatable bonds. The optimal ranges for rotatable bond count and PSA are (10) and (140). With these considerations in mind, we have selected 10 molecules by using the criteria of having no more than one rule of three violation and no more than one rule of 5 violation. The ADME properties of the selected 10 hits are mentioned in Table 4.4.

Table 4.4. List of Pharmacokinetic properties of selected hits, calculated by QikProp.

No. Of Hit	Name Of Drug	#rotor	mol_MW	SASA	donorHB	accptHB	QPlogPo/w	QPlogS	PSA	Rule Of Five	Rule Of Three
Hit 1	Dabigatran M636	22	635.675	964.99	9	18.5	0.114	-3.37	238.548	3	2
Hit 2	Dabigatran 4-0-acylglucuronide	16	647.643	945.69	8	18.8	0.232	-4.097	257.749	3	2
Hit 3	Motuporamine A	8	297.526	641.88	3	4.5	2.429	-1.073	40.7	0	0
Hit 4	Alpha-hydroxysalmeterol	20	431.571	891.92	5	9.05	3.092	-4.165	102.469	0	1
Hit 5	Gabexate mesylate	10	321.375	672.51	4	6.5	1.803	-4.256	139.116	0	0
Hit 6	Parazoanthine C	8	329.358	625.36	5	6.25	1.248	-3.719	148.74	0	0
Hit 7	Bunodosine 391	6	391.223	599.12	3.25	5.75	2.088	-3.614	121.279	0	1
Hit 8	Pre-pseudomonine	6	330.343	612.17	3	9.45	0.418	-2.468	115.991	0	0
Hit 9	Leuchagodine B	5	330.346	608.57	2	5.5	2.399	-4.53	127.487	0	0
Hit 10	PROTOKYLOL	9	331.368	591.61	4	6.2	1.56	-1.986	95.992	0	0
Control	Gemfibrozil	6	250.337	558.97	1	2.7	3.85	-4.282	52.434	0	0

4.13. **SIFt analysis of the filtered hits using structural interaction fingerprinting**

Along with the Control molecule - Gemfibrozil, we carried out the protein-ligand interaction fingerprinting for these ten compounds. We have examined the residues at their binding sites that come into contact with them in any way. All of the filtered hits have the greatest number of interactions with the binding site's 10 amino acids and another protein residue. Considering the threshold of the number of residue interactions >10, the considerably involved residues with all hits and the control are L25, K28, N72, G83, F102, M334, F341, S368, and Y379 (Figures 4.21a and 4.21b).

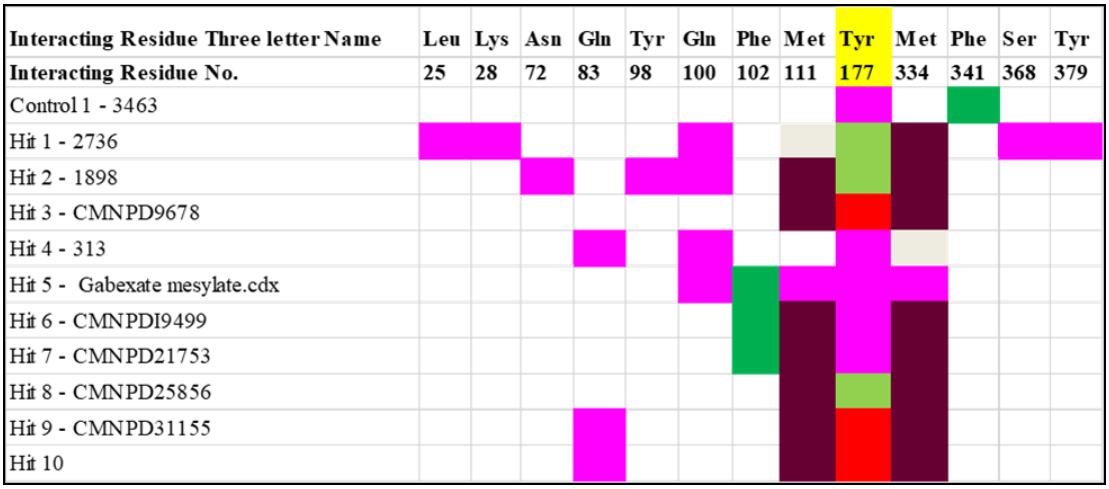


Figure 4.21a. Virtual drug library screening and SIFt of filtered hits.

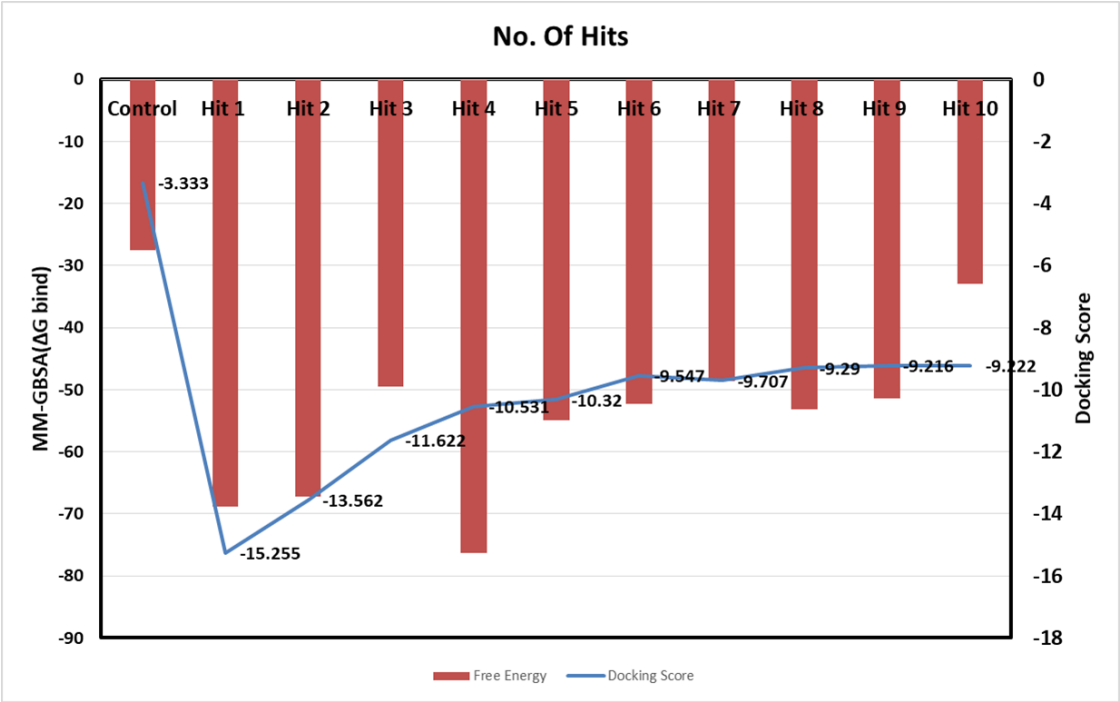


Figure 4.21b. Molecular docking score and binding free energy analysis. Control:- Gemfibrozil = Docking score = -3.26,Free energy = -27.51

4.14. MD simulation of selected 10 hits

Desmond was accustomed to simulating the control and 10 selected ligand complexes using molecular dynamics, which allowed him to see how the molecules behaved dynamically in a solvent environment. The complexes' MD simulations were ran for 100 ns. The convergence of Figure 22's RMSD graphs for the selected complex and control systems demonstrates that the MD trajectories have stabilised with respect to the starting conformation of each frame that was structurally reduced. All complexes showed temporal trends with high RMSD. After inspecting the trajectories, we selected two metabolites (i.e., Dabigatran M636 [Hit1] and Alpha-hydroxy salmeterol [hit4]) and marine compounds (i.e., Parazoanthine C[Hit6] & Bunodosine [Hit7]) for additional simulation interaction studies as well as the protein-ligand interactions that remained constant across the MD simulations (Figures 4.23a and 4.23b). In Figure 24, the APOB (protein) contacts with selected ligands were shown in the form of stacked bar charts which are normalized throughout the 20 ns trajectory. Hydrogen bonds, ionic bonds, hydrophobic interactions, and water bridges are the primary types of these connections. Hydrophobic contacts, hydrogen bonds, and water bridges predominated throughout the simulation, as seen by the bar chart. Throughout the whole simulation, the H2 bond with residue Y177 was maintained. Water-bridged interactions also relied on the same residues. The residues Y177 and A175 were also shown to interact hydrophobically. We also found that Y177, A175, L179, and H184 form water-bridged interactions. As a result, the Y177 residues were singled out as pivotal in the complex's development.

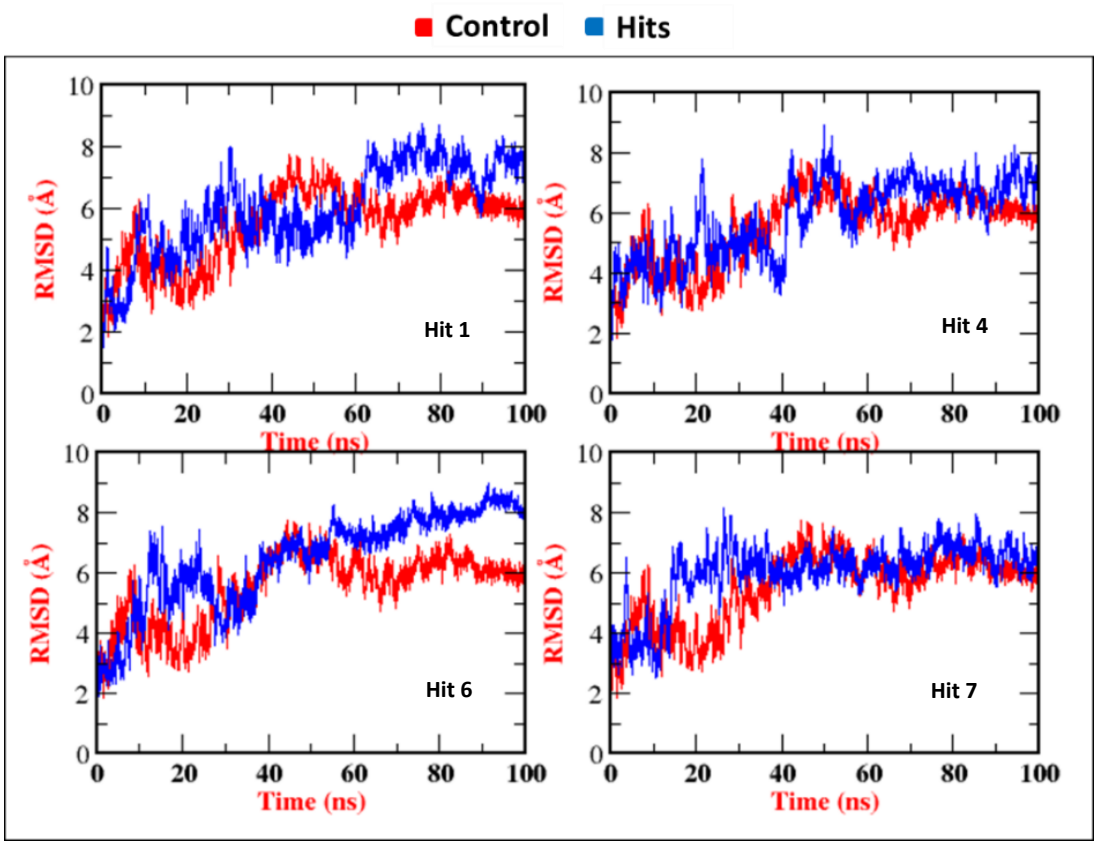
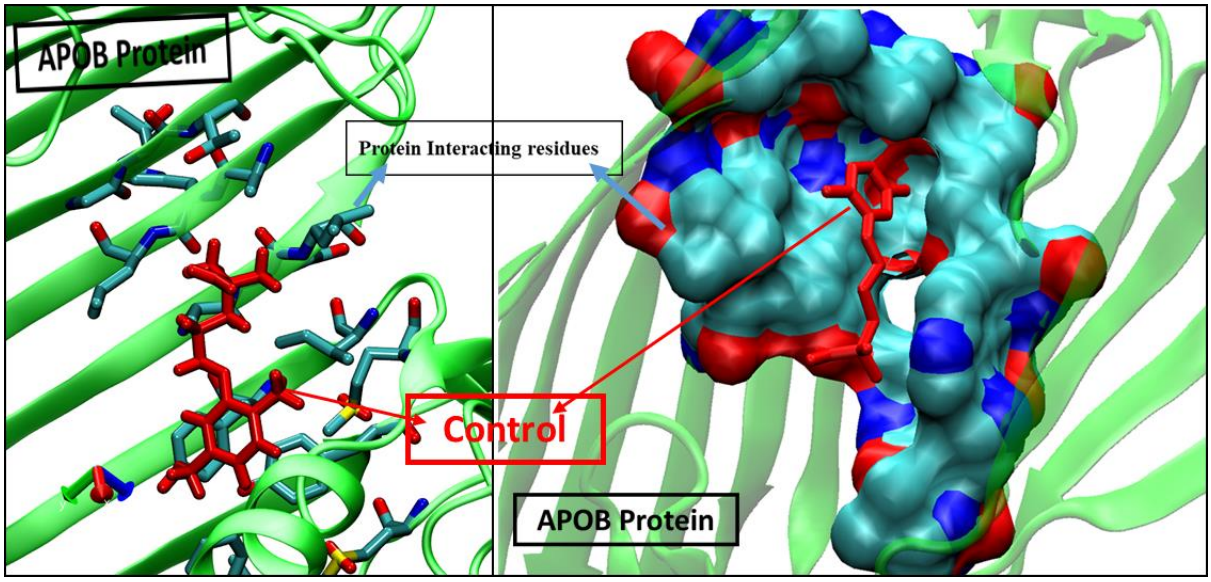
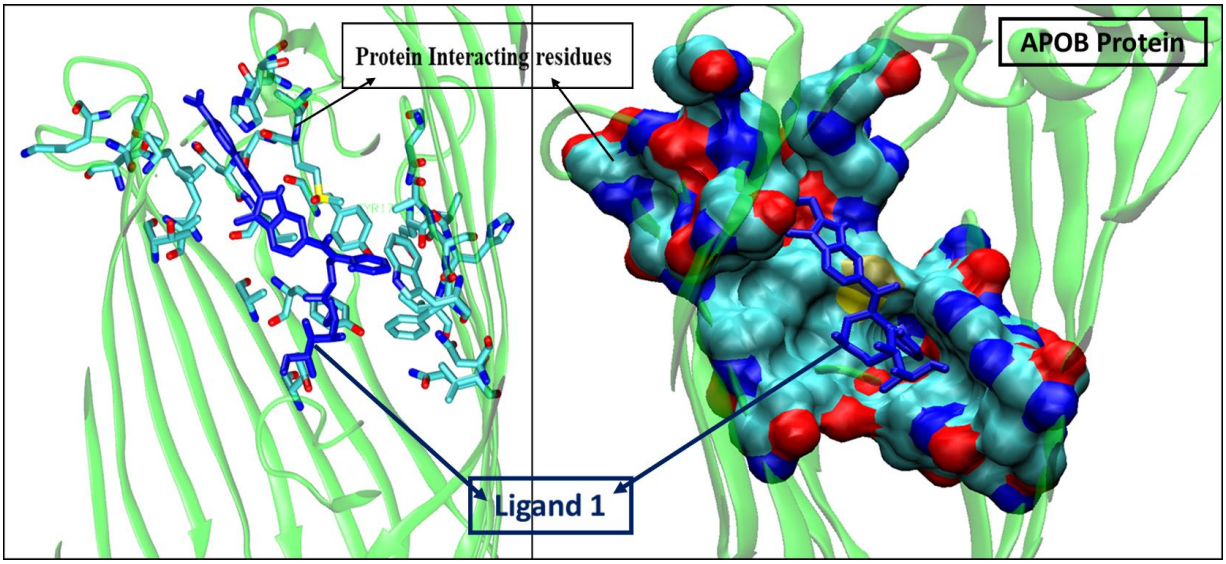


Figure 4.22. The filtered hits' dynamic properties during the MD simulations. The panel displays the RMSD (in ns) with the temporal development of a few selected hits for simulated interaction studies out of a total of 10 hits.

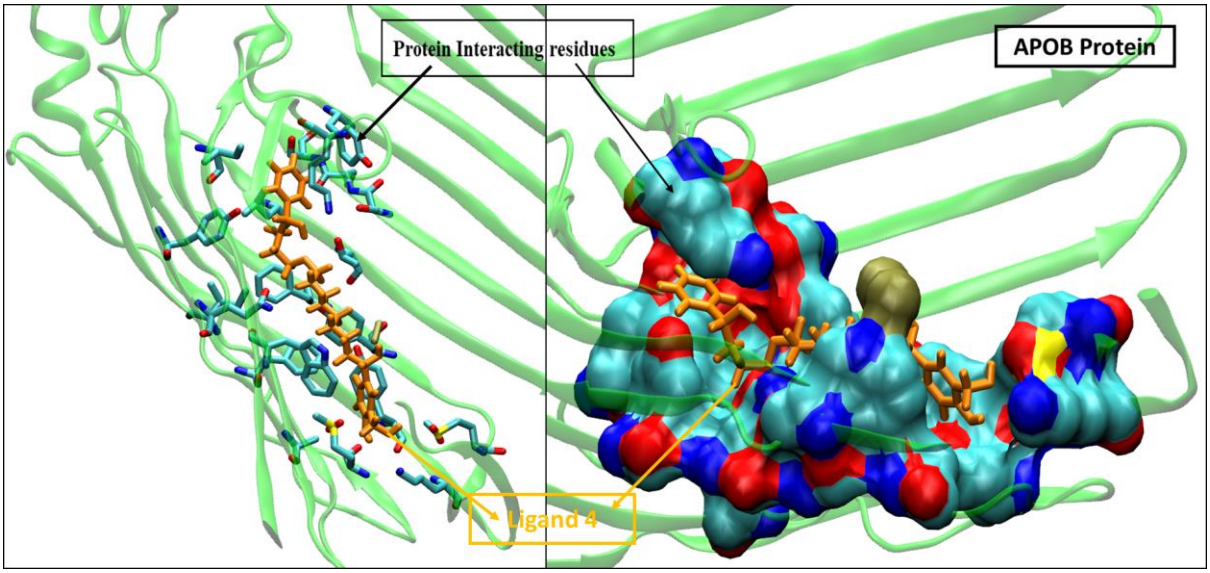
a)


APOB Protein
Protein Interacting residues
Control
APOB Protein

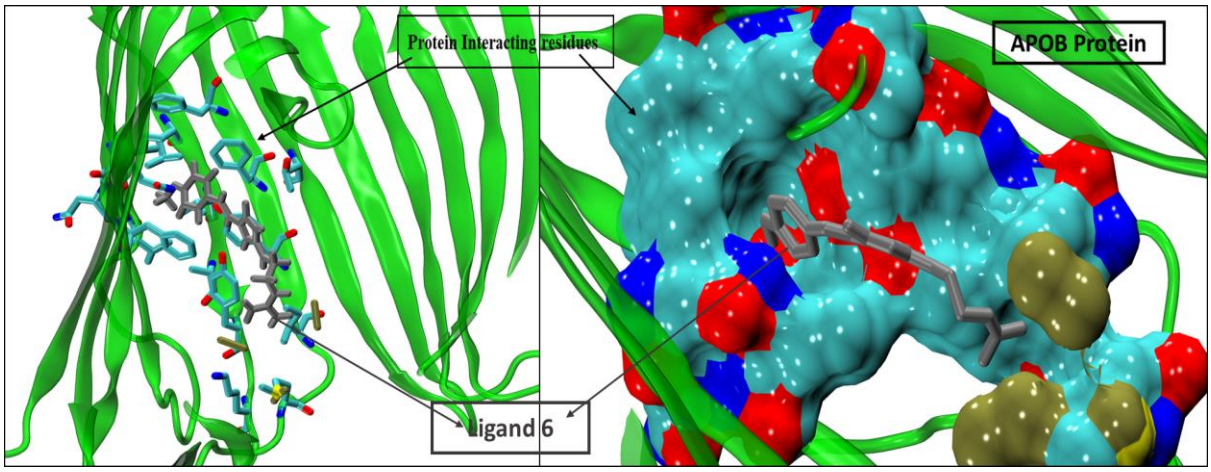
b)


Protein Interacting residues
APOB Protein
Ligand 1

c)


Protein Interacting residues
APOB Protein
Ligand 4

d)


Protein Interacting residues
APOB Protein
Ligand 6

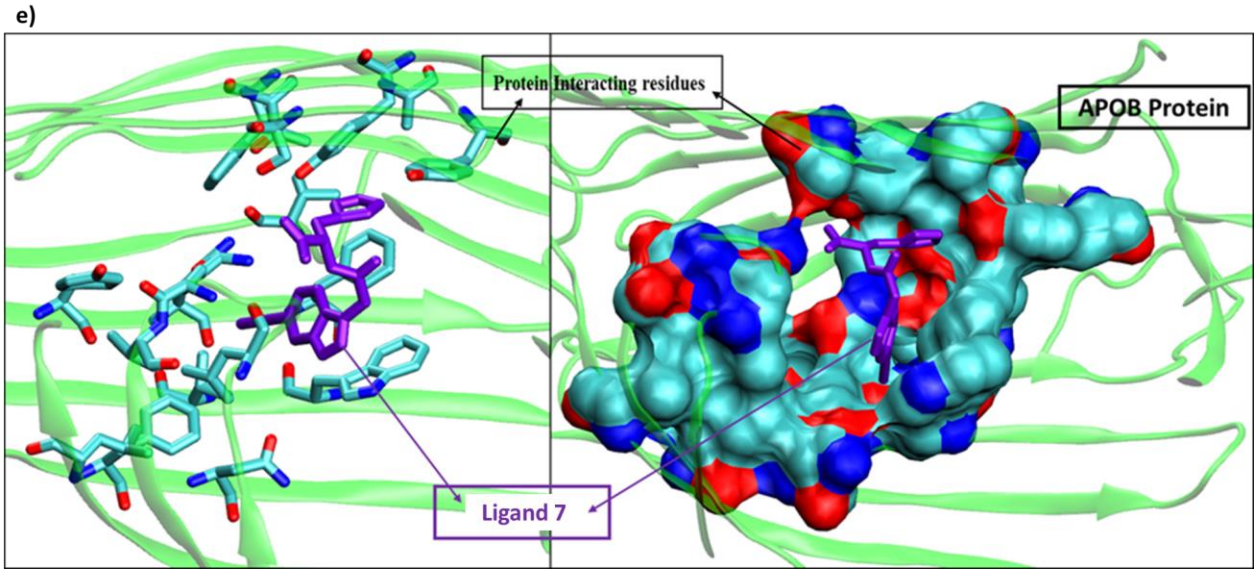
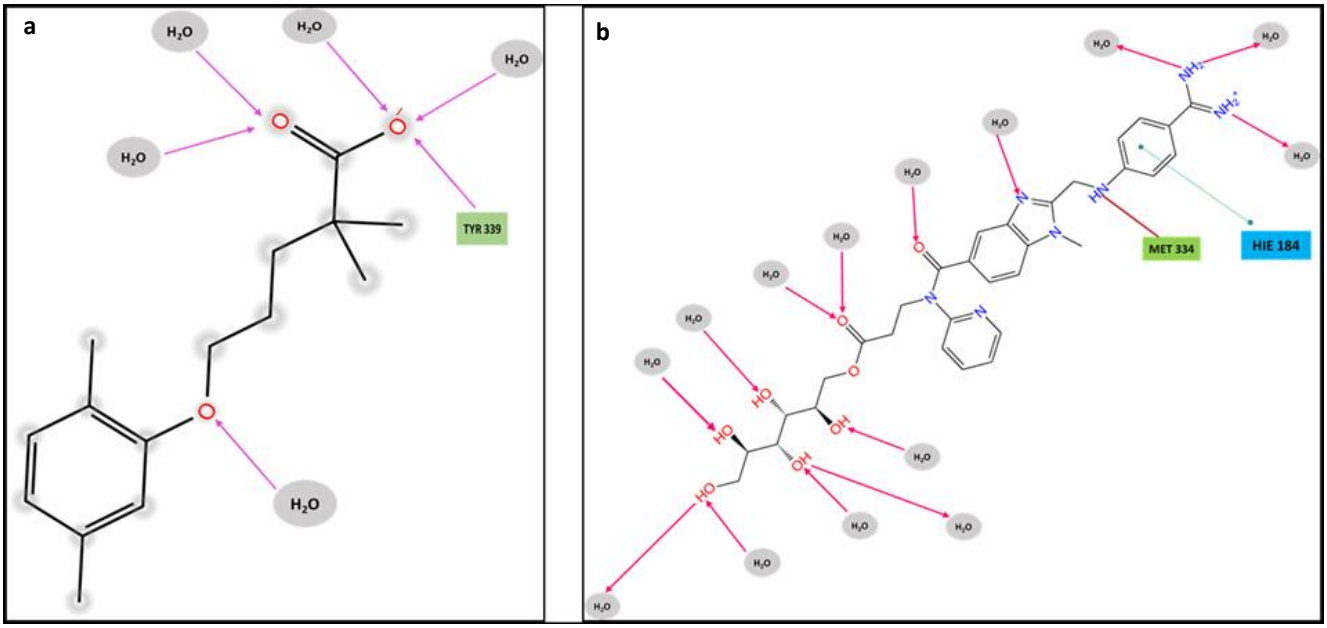


Figure 4.23a. Docked poses and molecular interactions of control, Ligand 1, 4, 6, and 7 in the active site of APOB (green ribbon)). "3D-Ligand interaction and 3D molecular surface diagrams of (a) control (red), (b) Hit 1 (blue), (c) Hit 4 (orange), (d) Hit 6 (grey) and (e) Hit 7 (purple).



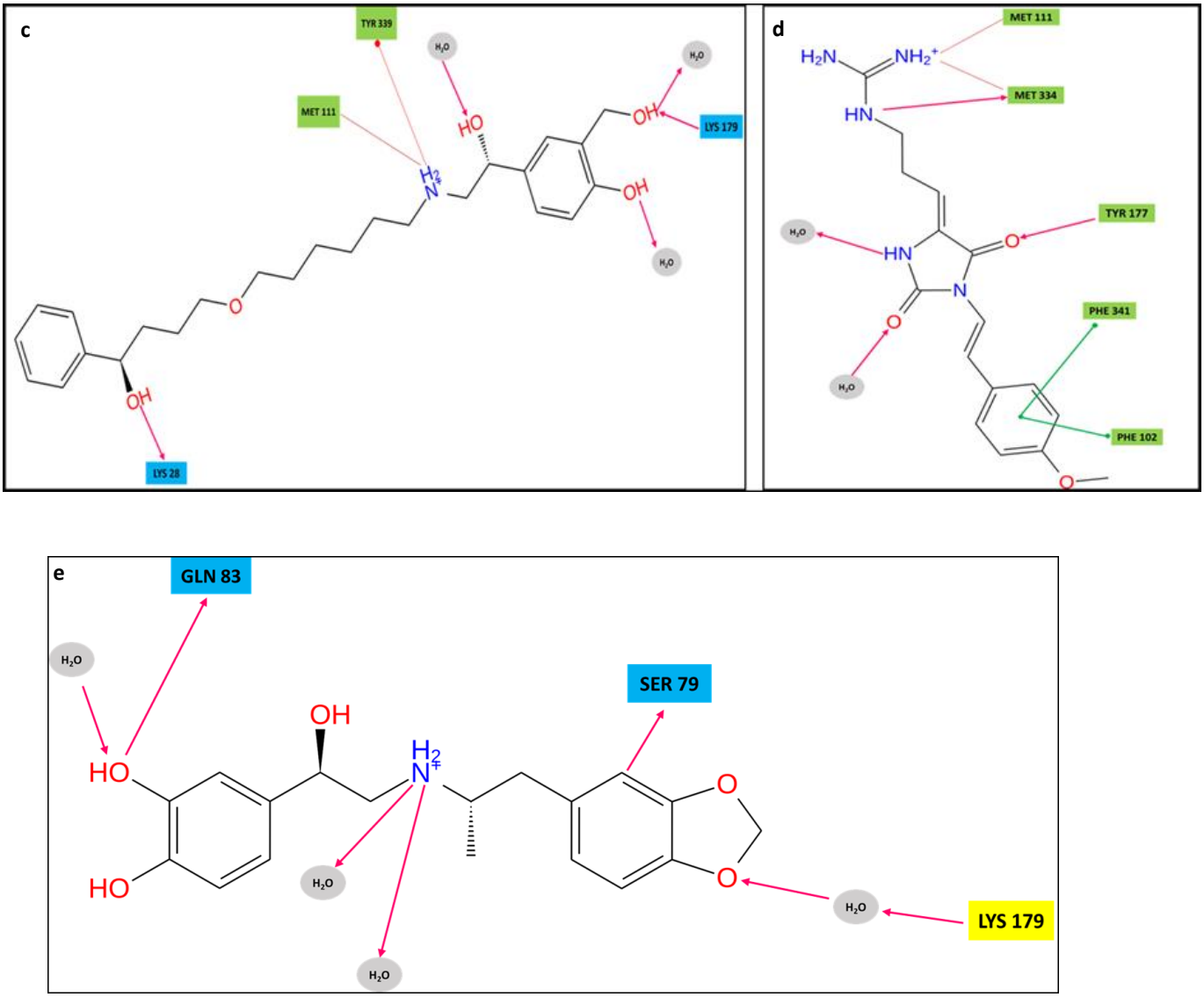
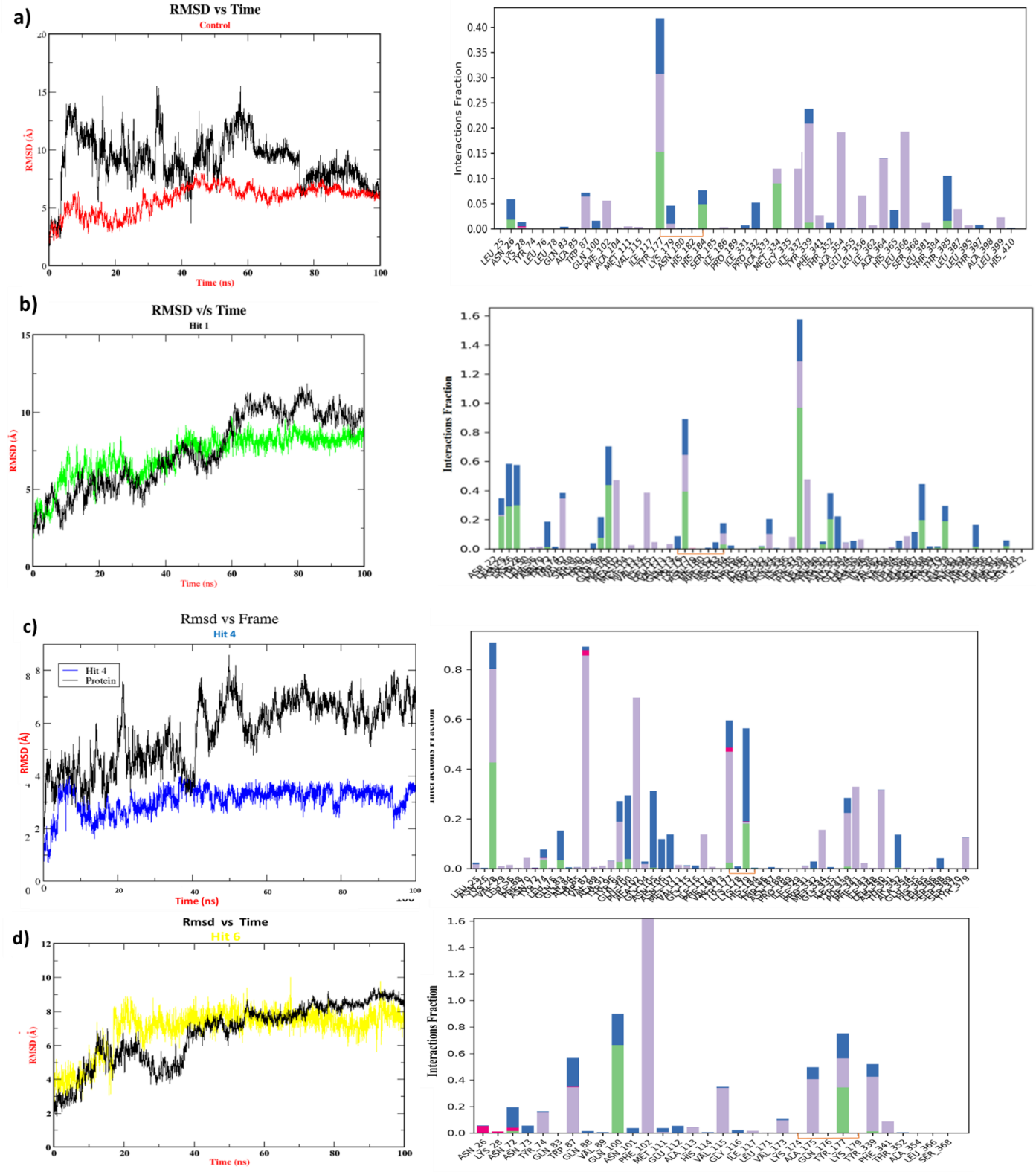


Figure 4.23b. Panels depict protein-ligand interactions that persisted during MD simulations. (a) Control, (b) Hit 1 – Dabigatran M636, (c) Hit 4 – Alpha-hydroxy salmeterol, (d) Hit 6 - Parazoanthine C (e) Hit 7 - Bunodosine. Hydrogen bonds, pi-pi interactions, and pi-cations are all examples of the several kinds of interactions shown by the pink arrow, green lines, and red lines, respectively, in protein-ligand complexes.


a)
RMSD vs Time
Control
RMSD (Å)
Time (ns)
Interactions Fraction
b)
RMSD v/s Time
Hit 1
RMSD (Å)
Time (ns)
Interactions Fraction
c)
Rmsd vs Frame
Hit 4
Hit 4
Protein
RMSD (Å)
Time (ns)
d)
Rmsd vs Time
Hit 6
RMSD (Å)
Time (ns)
Interactions Fraction

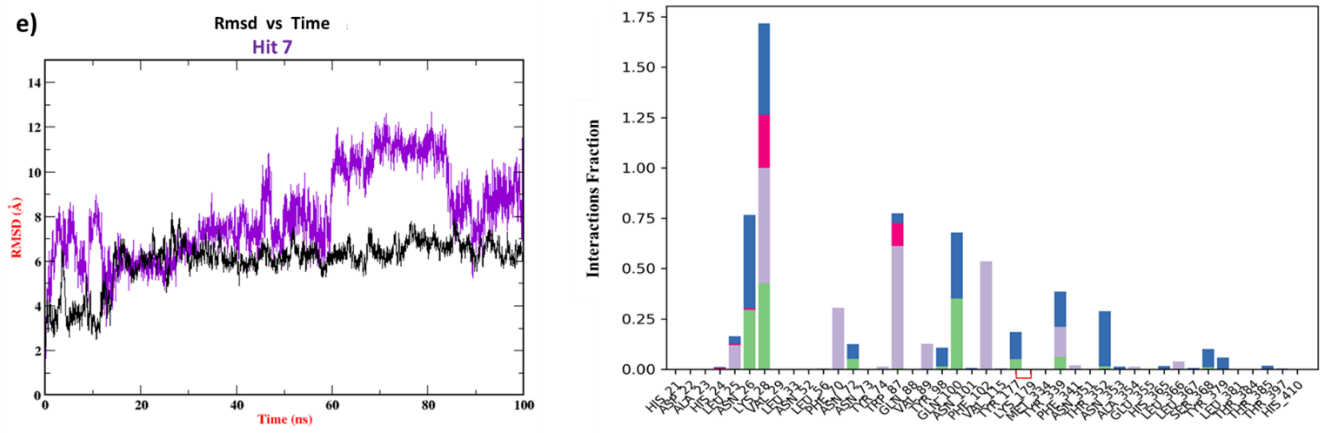
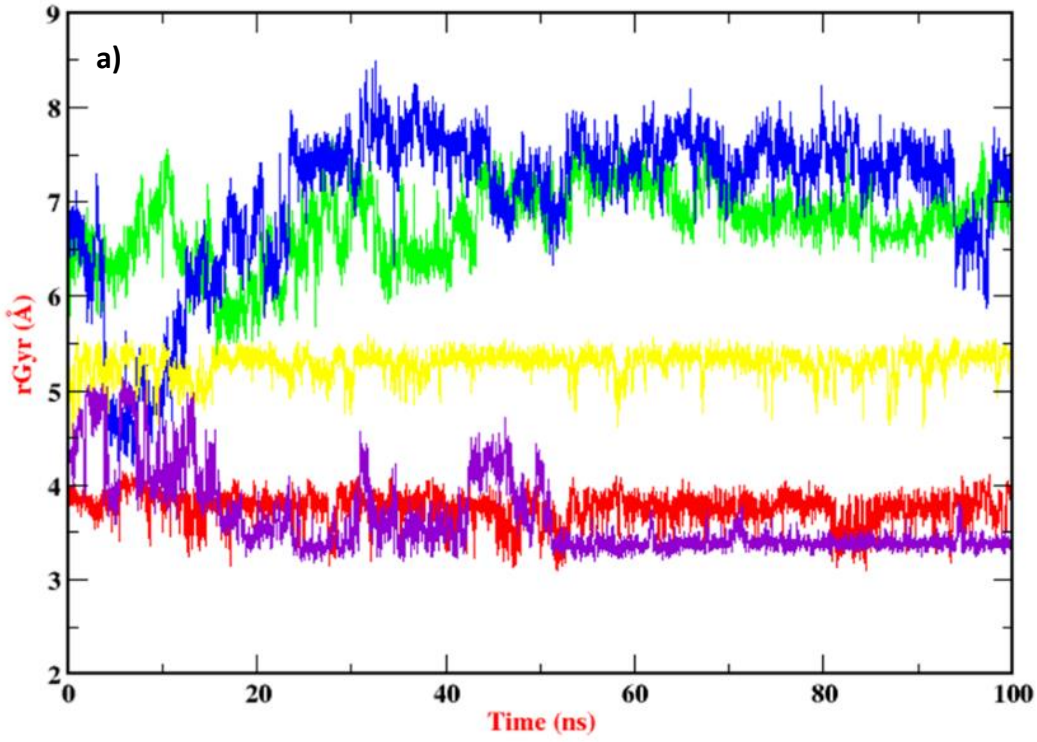


Figure 4.24. Analysis of RMSD, molecular interaction, and type of contacts between APOB (protein) and selected ligand during MD simulation. Normalized stacked bar chart of active site residues interacting with (a) Control- Gemfibrozil showing its higher fraction of interaction with Y177 of protein active site residue via hydrogen bond (green), hydrophobic bond (grey) and water bridges (blue) whereas with L179 it is showing hydrophobic and water bridges only and with H184 it is showing h-bond and water bridges. (b) Hit 1 – Dabigatran M636 shows its higher fraction of interaction with Y177 of protein active site residue via hydrogen bond (green), hydrophobic bond (grey), and water bridges (blue) whereas with R183 it is showing water bridges only and with H184 it is showing small fration of interaction with H-bond, hydrophobic bond and water bridges. (c) Hit 4 – Alpha-hydroxysalmeterol showing its higher fraction of interaction with Y177 of protein active site residue via hydrophobic bond (grey), then small fraction of interaction with water bridges (blue), H-bond (green) and ionic interaction (pink) whereas with L178 it is showing water bridges only and with Y179 it is showing again interaction with H-bond, water bridges and very small interaction fraction with ionic bond. (d) Hit 6 - Parazoanthine C showing its interaction with A175 of protein active site residue via hydrophobic bond (grey) and water bridges and with Y177 it is showing small fraction of interaction with H-bond (green), hydrophobic bond and water bridges (blue) and (e) Hit 7 Bunodosine 391 showing its interaction with Y177 of protein active site residue via H-bond (green) and water bridges (blue) and there is no interaction is shown with L179.

To see how well the selected ligands held their positions in the active site, we calculated the solvent surface area of molecules using a 1.4 Å probe radius, counted the number of intramolecular hydrogen bonds also, counted the solvent surface area of molecules that were accessible to water molecules, and counted the solvent surface area of molecules that were contributed solely by O and N atoms. (Figures 4.25a and 4.25b).



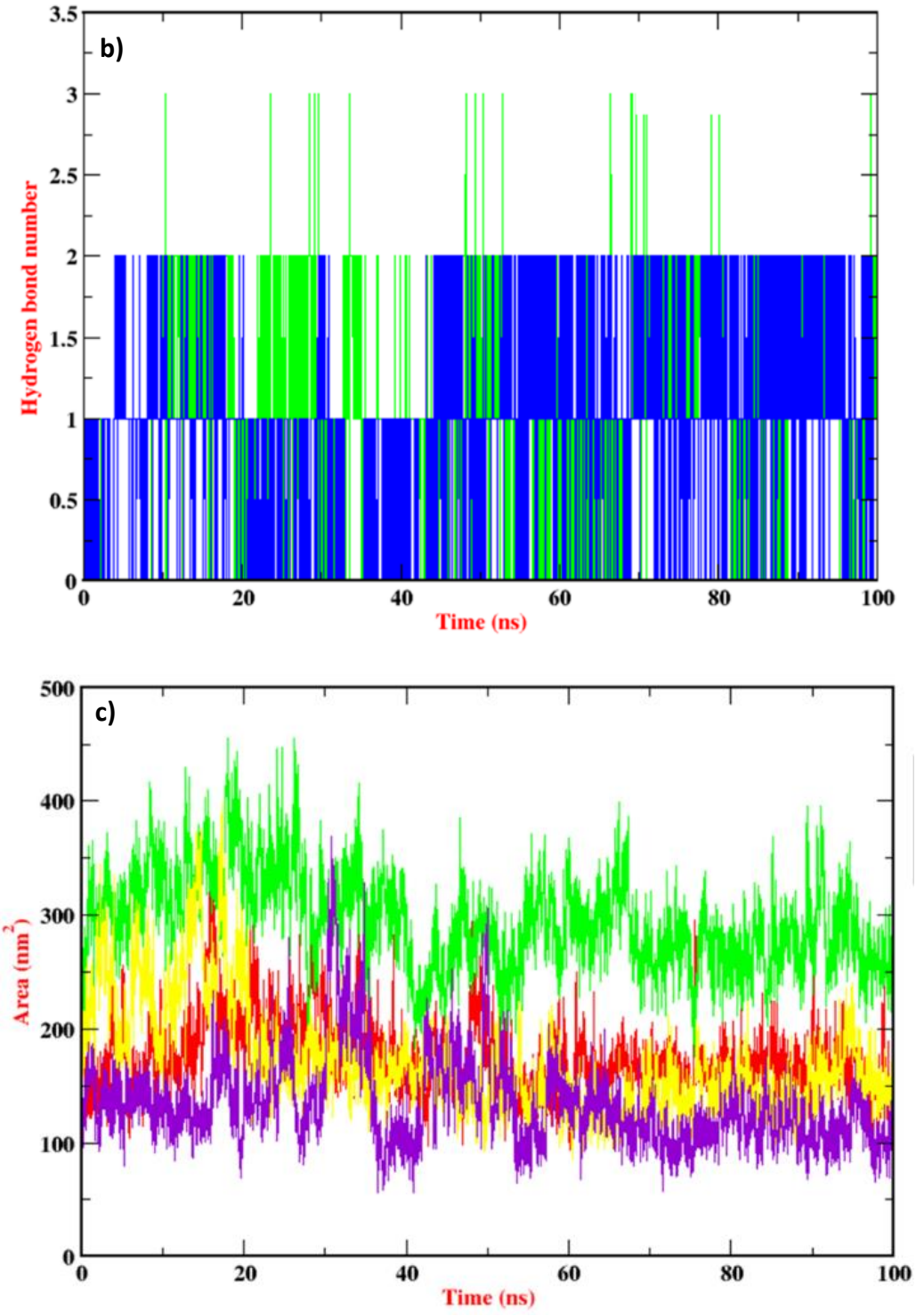
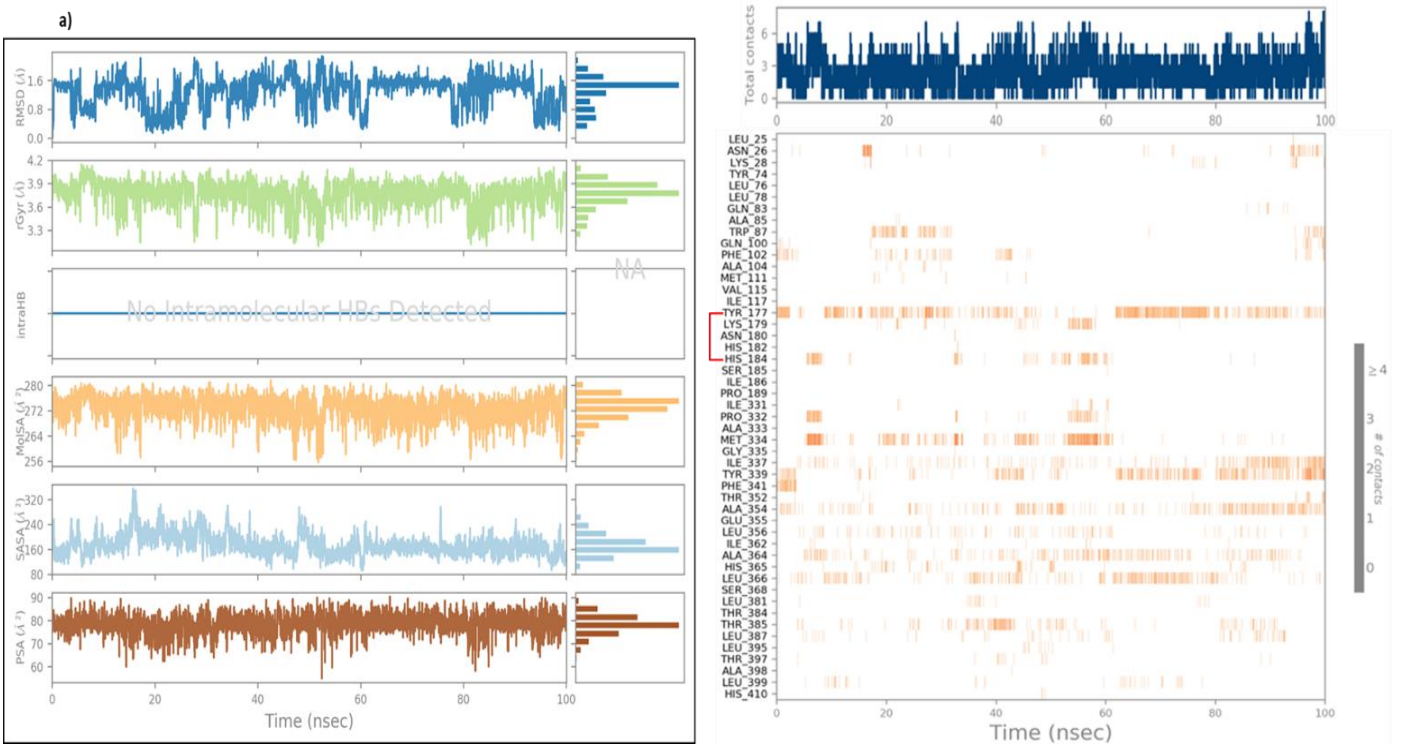
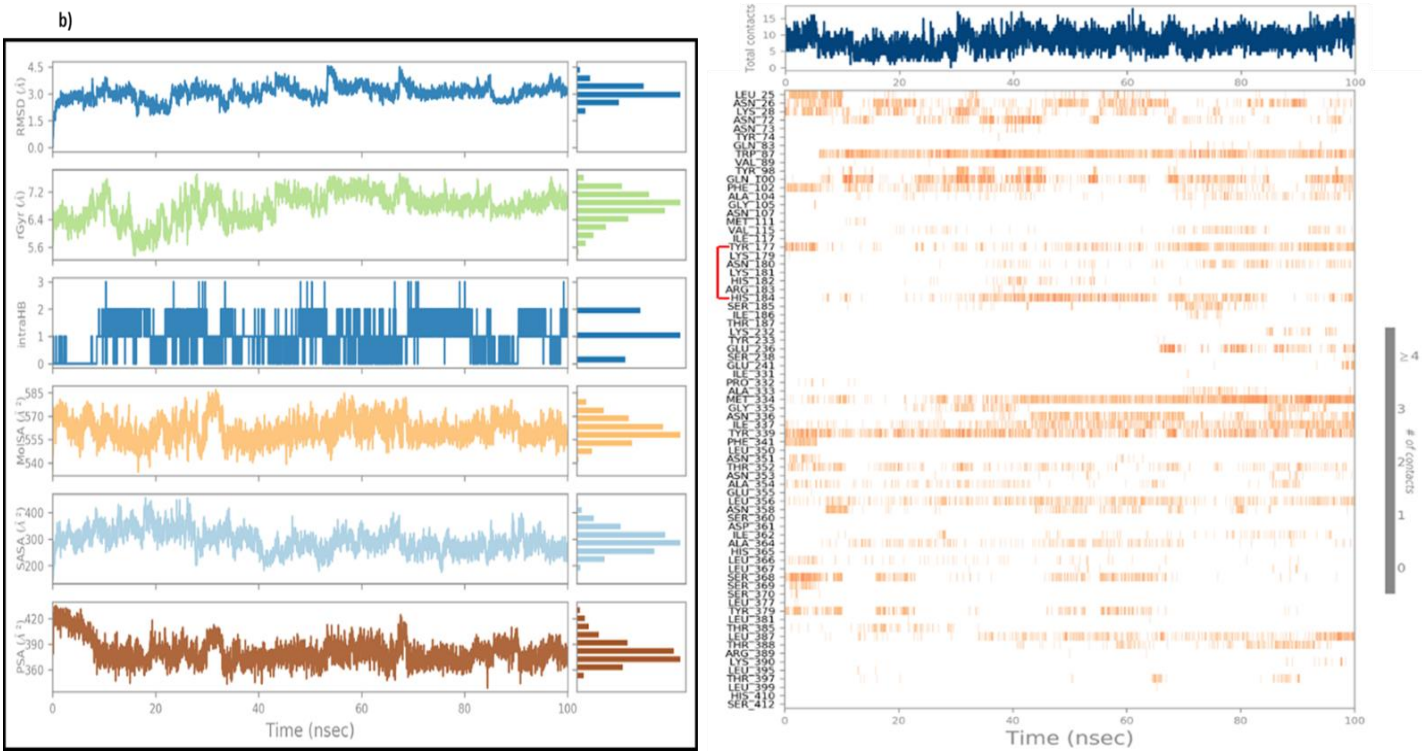
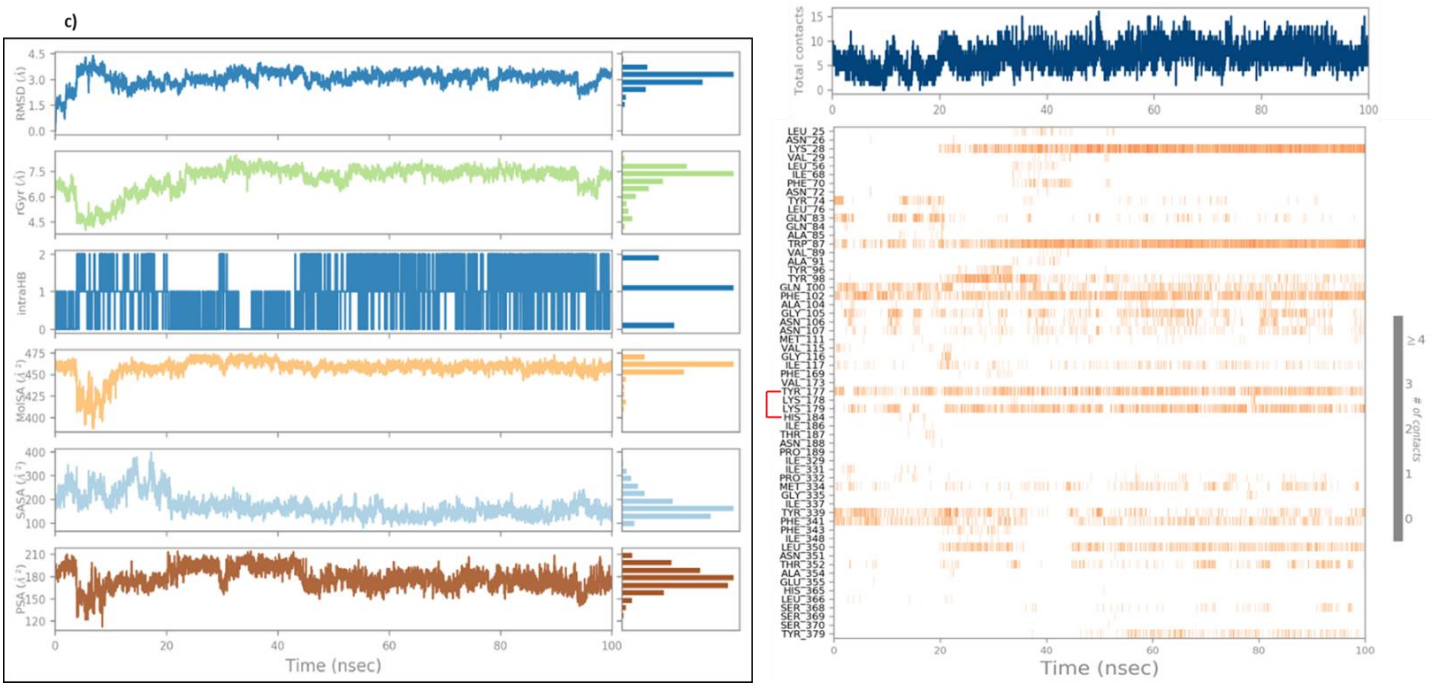
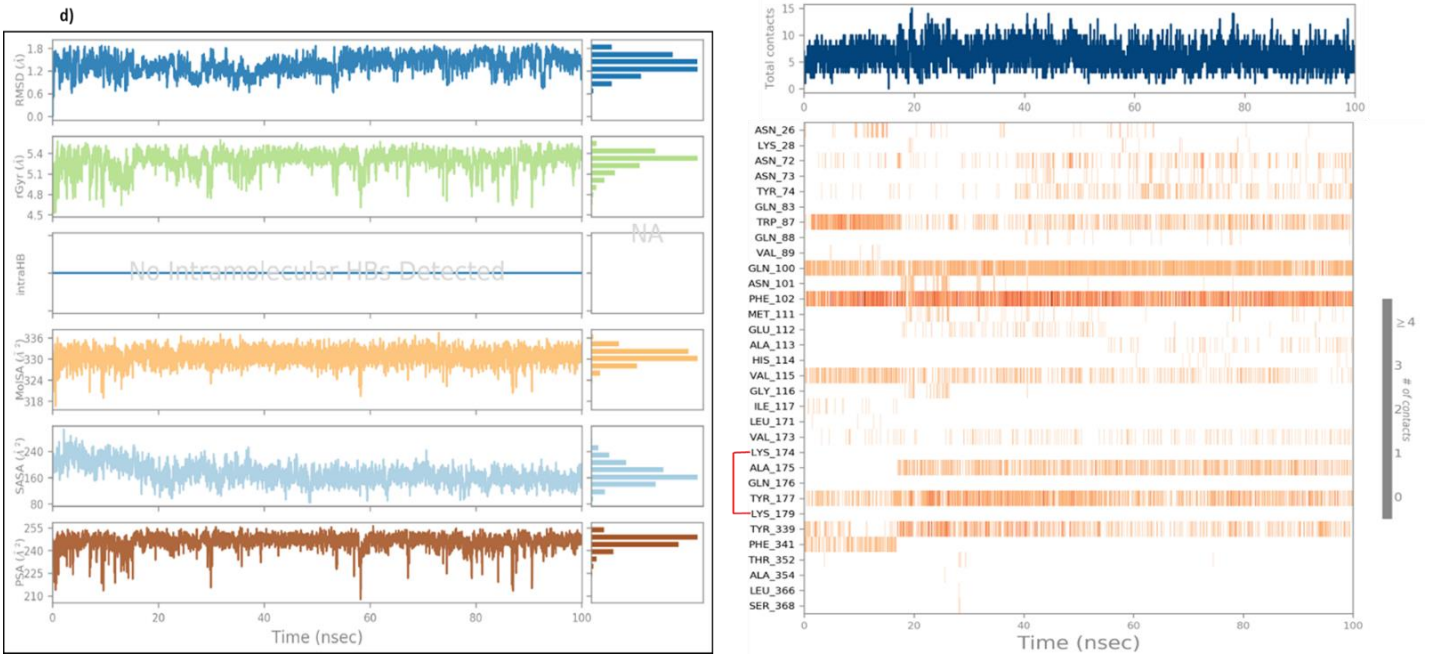


Figure 4.25a. Analysis of a) radius of gyration, b) hydrogen bonding and c) SASA of APOB protein with control and selected drugs. Where red is control, green is hit 1, blue is hit 4, yellow is hit 6, and violet is hit 7.


a)
RMSD (Å)
rGyr (Å)
intraHB
No Intramolecular HBs Detected
NA
MolSA (Å²)
SASA (Å²)
PSA (Å²)
Time (nsec)
Total contacts
# of contacts
Time (nsec)


b)
RMSD (Å)
rGyr (Å)
intraHB
MolSA (Å²)
SASA (Å²)
PSA (Å²)
Time (nsec)
Total contacts
# of contacts
Time (nsec)


c)
RMSD (Å)
rGyr (Å)
intraHB
MolSA (Å²)
SASA (Å²)
PSA (Å²)
Time (nsec)
Total contacts
LEU_25
ASN_26
LYS_28
VAL_29
LEU_56
ILE_68
PHE_70
ASN_72
TYR_74
LEU_76
GLN_83
GLN_84
ALA_85
TRP_87
VAL_89
ALA_91
TYR_96
TYR_98
GLN_100
PHE_102
ALA_104
GLY_105
ASN_106
ASP_107
MET_111
VAL_115
GLY_116
ILE_117
PHE_169
VAL_173
TYR_177
LYS_178
LYS_179
HIS_184
ILE_186
THR_187
ASN_188
PRO_189
ILE_329
ILE_331
PRO_332
MET_334
GLY_335
ILE_337
TYR_339
PHE_341
PHE_343
ILE_348
LEU_350
ASN_351
THR_352
ALA_354
GLU_355
HIS_365
LEU_366
SER_368
SER_369
SER_370
TYR_379
Time (nsec)
# of contacts


d)
RMSD (Å)
rGyr (Å)
intraHB
No Intramolecular HBs Detected
NA
MolSA (Å²)
SASA (Å²)
PSA (Å²)
Time (nsec)
Total contacts
ASN_26
LYS_28
ASN_72
ASN_73
TYR_74
GLN_83
TRP_87
GLN_88
VAL_89
GLN_100
ASN_101
PHE_102
MET_111
GLU_112
ALA_113
HIS_114
VAL_115
GLY_116
ILE_117
LEU_171
VAL_173
LYS_174
ALA_175
GLN_176
TYR_177
LYS_179
TYR_339
PHE_341
THR_352
ALA_354
LEU_366
SER_368
Time (nsec)
# of contacts

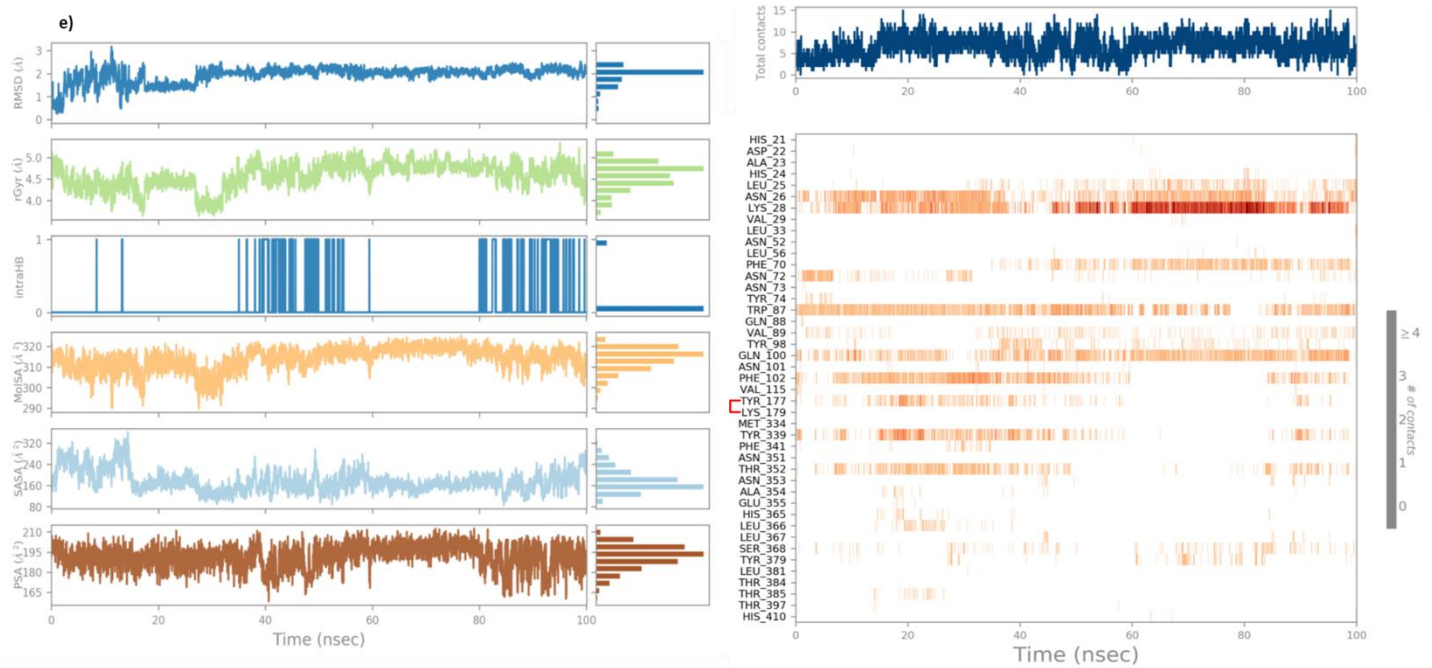
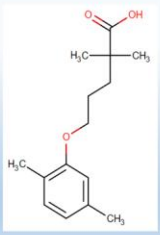
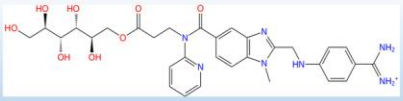
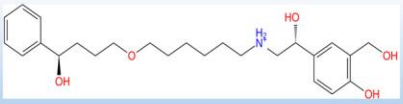
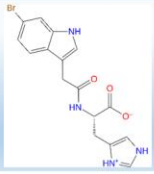


Figure 4.25b. The simulation lasted 100 ns, and the data showed that the ligand's characteristics changed with time. (a) Control, (b) Hit 1 (c) Hit 4. (d) Hit 6 and (e) Hit 7.

Table 4.5:- List of control and selected hits for drug repurposing

Hit No.	Compound name Drug Bank/ PubChem ID IUPC Name	2D Structure	Mechanism of action	Mechanism of action with APOB
Control	**Gemfibrozil** **Drug Bank ID** - DB01241 **IUPC Name** - 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid		It increase plasma high density lipoproteins (HDL) and to decrease plasma triglycerides (TG) in a wide variety of primary and secondary dyslipoproteinemias.	Activates peroxisome proliferator-activated receptor-α (PPARα), which alters lipid metabolism. This activation leads to increased HDL, apo AI, apo AII, lipoprotein lipase (LPL), inhibition of apo B synthesis, peripheral lipolysis, decreased removal of free fatty acids by the liver, and increased clearance of apoB. Upregulated LPL reduces plasma triglyceride levels. Decreased hepatic removal of fatty acids decreases the production of triglycerides. The effects on apoB synthesis and clearance decrease VLDL production which also reduce plasma triglyceride levels.
Hit 1	**Dabigatran M636** **Drug bank ID** - DBMET03389 **IUPC Name** - 2,3,4,5,6-pentahydroxyhexyl 3-[1-(2-{[(4-carbamimidoylphenyl)amino]methyl}-1-methyl-1H-1,3-benzodiazol-5-yl)-N-(pyridin-2-yl)formamido]propanoate		Dabigatran (Pradaxa) reversibly binds to the active site on the thrombin molecule, preventing thrombin-mediated activation of coagulation factors.	ApoB is an important cofactor in atherogenesis and an elevated apoB-to-apoA1 ratio has been associated with cardiovascular disease. In the Heart publication, an analysis of RE-LY is presented, showing that the use of dabigatran is associated with a reduction in plasma apoB levels, suggesting an unexpected pleiotropic side effect with potential clinical consequences.
Hit 4	**Alpha-hydroxysalmeterol** **PubChem CID** - 197627 **IUPC Name** - 4-[1-hydroxy-2-[6-(4-hydroxy-4-phenylbutoxy)hexylamino]ethyl]-2-(hydroxymethyl)phenol		Beta-2 adrenoceptor stimulation causes relaxation of bronchial smooth muscle, bronchodilation, and increased airflow. Salmeterol is hypothesized to bind to 2 sites on the beta-2 adrenoceptor.The saligenin moiety binds to the active site of the beta-2 adrenoceptor.The hydrophilic tail of salmeterol binds to leucine residues in the exo-site of the beta-2 adrenoceptor almost irreversibly, allowing salmeterol to persist in the active site, which is responsible for it's long duration of action	Not Known
Hit 6	**Parazoanthine C** **PubChem CID** - 4687 **IUPC Name** - 1,7-dimethyl-3H-purine-2,6-dione		Paraxanthine is a natural product found in **Mus musculus, Citrus maxima.** Parazoanthine act as antagonists of adenosine receptors A1 and A2a similar as caffine. Despite these similarities in mechanisms, it has been reported that there are potential differences in adenosine receptor binding affinity, as paraxanthine exhibited higher binding potencies for adenosine A1 and A2A receptors in equine forebrain and a stronger locomotor activating effect in rats relative to caffeine. Furthermore, paraxanthine, but not other methylxanthines (caffeine, theobromine, theophylline) has been shown to potentiate nitric oxide neurotransmission; which has been associated to increased blood flow and subsequent changes in aerobic exercise performance and cardiovascular health.	Not Known
Hit 7	**Bunodosine 391** **PubChem CID** - 52936986 **IUPC Name - (2S)-2-[[2-(6-bromo-1H-indol-3-yl)acetyl]amino]-3-(1H-imidazol-5-yl)propanoic acid**		It is an analgesic acylamino acid from the venom of the sea anemone Bunodosoma cangicum.	Not Known

Chapter 5
Conclusion

High-quality sperms in a semen sample are mostly attributable to the existence of metabolically active spermatozoa, which are essential for male fertility. Because spermatozoa are so specialized and have so many different roles to play, their metabolism must be regulated by the expression of certain metabolic genes. In our study, we used CASA to understand the different movements of spermatozoa and found that a motility rate above 40µm/s is important for sperm to fertilize the ovum. These type of samples with a motility rate above 40µm/s shows fast and forward progressive movement. This completely signifies that ATP generation is very high in these samples but when compared with semen samples having motility less than 39%, we observed a non-progressive movement of spermatozoa covering a distance of less than 40µm/s. In these types of samples, ATP production is less, that's why spermatozoa are not able to cover large distances and progressive movement. And we also observed a third type of semen sample, in which there was no movement shown by spermatozoa. All sperm are alive but are not able to move, from this we concluded that there is no ATP generation at all in this type of spermatozoa that's why no movement was observed. From this we conclude that appropriate amount of ATP generation is required for sperm motility. That's why further we performed a systematic *in-silico* analysis to find the key metabolic genes and metabolic pathways involved in sperm motility and ATP generation in spermatozoa body. For this, we used STRING, Reactome pathway database, DAVID and Enrichr database to validate the presence of novel protein APOB in spermatogenesis and sperm motility. The observations confirmed the involvement of APOB in asthenozoospermia and its role in the sperm metabolic pathway. Data from the DAVID database validated the role of the APOB gene in "sperm motility".

Hence, we suggest here APOB be a possible target for further analysis. After this the data obtained from qPCR also shows the unregulated expression of APOB gene and down regulated expression of LDHC in diseased semen samples when compared with normal one. From all this *in-silico* and *in-vivo* analysis, we were confirmed to take APOB as a potential target for drug development. We used a combination of computer-aided drug design (CADD) tools to quickly search the FDA, Drugbank, and CMNPD databases for promising compounds. Out of 32000 compounds, 10 compounds were identified as potential hit molecules based on thermodynamic profiling, targeted docking, binding mode analysis, and interaction analysis. The SIFt and ADME of these hits were also monitored. Then, we ran MD simulations on selected hits. Further, the pharmacological activity of these selected novel compounds for potential usage as therapeutic medicines was determined with the use of the ADME prediction. Four of these molecules were shown to form a stable complex in the active region of the APOB protein during MD simulations. We have proposed four novel potential molecules (i.e., H1, H4, H6, and H7) for the inhibition of APOB. When compared to the currently marketed medicine (Gemfibrozil), which had superior pharmacokinetic features, selected 4 molecules showed significantly improved binding modes and affinities. Inhibitory efficacy, binding affinities and cell-based functional tests are required to confirm these results, although these new inhibitors are anticipated to be examined as possible therapeutic options. These novel chemical entities could potentially act as starting points for lead optimization and hit-to-lead conversion, accelerating the process of finding new APOB inhibitors.

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Publications

1. **Research Paper :-** Maurya S, Raj S, Bhoi NR, Mani N, Rathi B, Kumar D (2023). Morphological analysis of metabolically dysregulated spermatozoa using Artificial Intelligence based approach. *Journal of Integrated Science and Technology*, *11*(4), 569. https://pubs.thesciencein.org/journal/index.php/jist/article/view/569

2. **Research Paper :-** Maurya S, Bhoi NR, Kesari KK, Roychoudhury S, Kumar D (2022). In Silico Analysis of CatSper Family Genes and APOB Gene Regulation in Male Infertility. Adv *Exp Med Biol*, 1391:323-332. doi: 10.1007/978-3-031-12966-7_18. https://pubmed.ncbi.nlm.nih.gov/36472830/

3. **Review Paper** :- Maurya S, Kesari KK, Roychoudhury S, Kolleboyina J, Jha NK, Jha SK, Sharma A, Kumar A, Rathi B, Kumar D (2022). Metabolic Dysregulation and Sperm Motility in Male Infertility. *Adv Exp Med Biol*, 1358:257-273. doi: 10.1007/978-3-030-89340-8_12. PMID: 35641874. https://pubmed.ncbi.nlm.nih.gov/35641874/

4. **Review Paper** :- Sujata Maurya et al. Exploring state-of-the-art advances in targeted nanomedicines for managing acute and chronic inflammatory lung diseases. Nanomedicine (Lond). 2022 Dec;17(30):2245-2264.. Epub 2023 Mar 28. PMID: 36975758. https://doi.org/10.2217/nnm-2021-0437

5. **Book Chapter :-** Maurya, S., Mishra, M.K., Rathi, B., Kumar, D. (2022). Lipid Nanocarriers: Applications in Biomedical Research and in Drug Delivery. In: Gopi, S., Balakrishnan, P., Mubarak, N.M. (eds) Nanotechnology for Biomedical Applications. Materials Horizons: From Nature to Nanomaterials. Springer, Singapore. https://doi.org/10.1007/978-981-16-7483-9_2

6. **Book Chapter :-** Raj, S., Chandel, V., Maurya, S., & Kumar, D. (2020). Role of Macrophages in Solid Tumor Metabolism. In (Ed.), Macrophages. IntechOpen. https://doi.org/10.5772/intechopen.93182

7. **Book Chapter :-** OMICS IN CANCER: FROM DIAGNOSTICS TO THERAPEUTICS (2020) Sujata Maurya, Vaishali Chandel, Sibi Raj, Atul Kumar Upadhyay, Chanderdeep Tandon and Dhruv Kumar. Recent Trends in ‘Computational Omics'Publisher: Nova Science Publishers, Inc.

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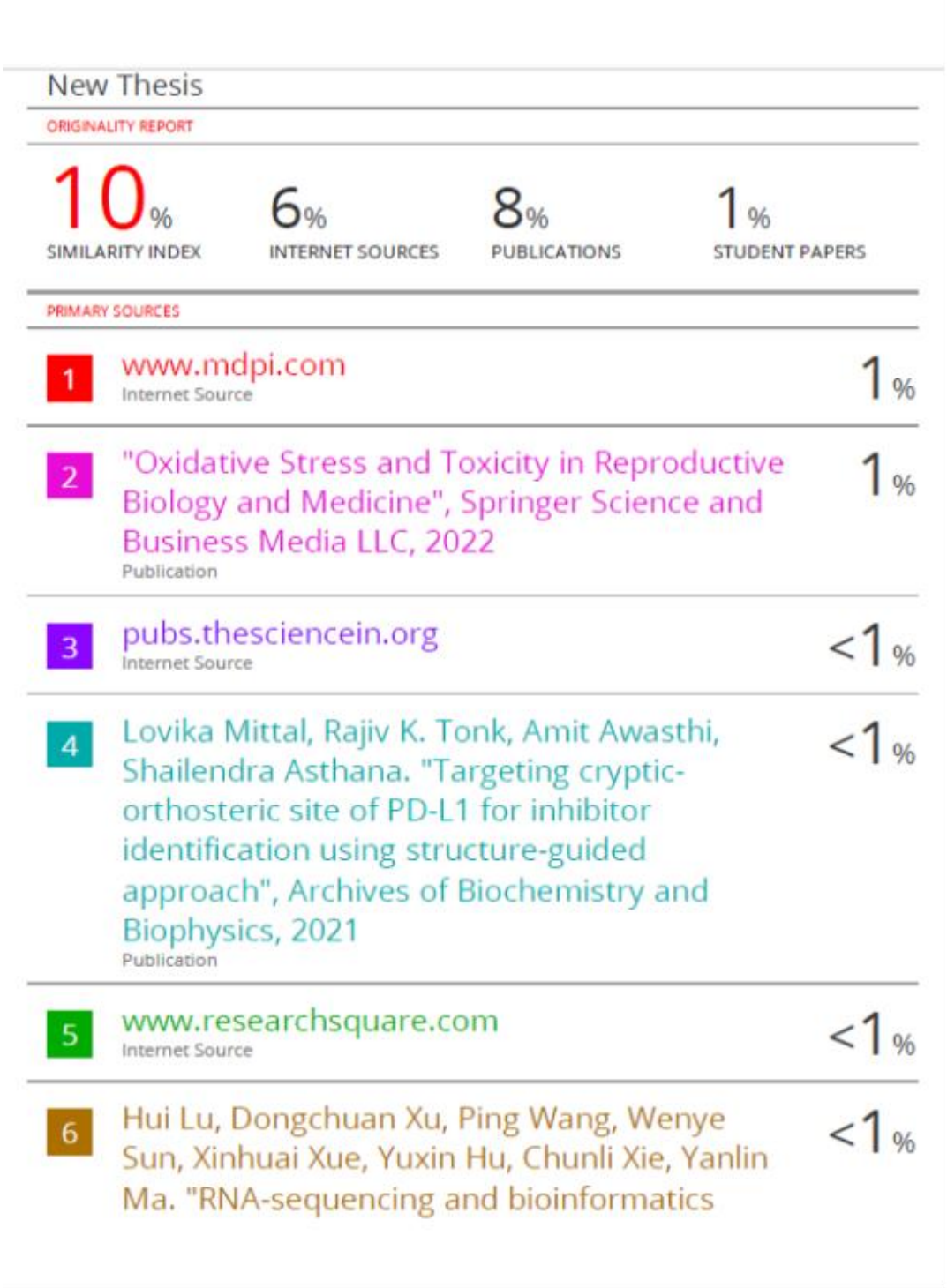

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Annexure 1 – Ethical Clearance Certificate

INDIRA IVF
HOSPITAL INSTITUTIONAL ETHICS COMMITTEE
Reg. No.: ECR/1636/Inst/DL/2021

To,
Miss Sujata Maurya
Phd Scholar,
Amity Institute of Molecular Medicine and Stem cell Research,
Amity University, Sector – 125, Noida,
Uttar Pradesh – 201301 India.*

Dear Miss Sujata Maurya,

The Indira IVF Hospital Ethics Committee (Delhi) has reviewed the research proposal submitted by you under the supervision of Dr Dhruv Kumar (Principal Investigator) entitled

Study title: "Metabolic Dysregulation in Infertile Male Sperm"

The following documents were reviewed:

a) Protocol
b) Patient Information Sheet
c) Consent Form
d) CV of Investigator and Scholar

Considering the importance of research and extending our continuous efforts to support the same, we at Indira IVF Hospital Institutional Ethics Committee-Delhi have reviewed this study proposal over an online meeting (Microsoft Teams Platform).
The following ethics committee members were present at the conference video call meeting held on 08-Apr-2022 at 04:00 pm.

S. No.	Name of Member	Qualification	Role	Gender
1.	Dr Pooja Sharma	MBBS, MD (Obstetrics and gynaecology)	Chairperson	Female
2.	Dr Anju Saxupriya	MBBS, MD(Anaesthesia), DM (Cardiac Anaesthesia).	Member Secretary	Female
3.	Dr Jyothi Gupta	MBBS, MD (Obstetrics and gynaecology)	Clinician	Female
4.	Dr Sagarika Aggarwal	MBBS, MD (Obstetrics and gynaecology)	Clinician	Female
5.	Dr Ritu Punhani	MBBS, MS (Obstetrics and gynaecology)	Clinician	Female

REGD. OFFICE INDIRA IVF HOSPITAL ETHICS COMMITTEE 49, Opp. Metro Pillar-203, South Patel Nagar, West Delhi, Delhi - 110008 India

S. No.	Name of Member	Qualification	Role	Gender
6.	Dr Lalit Kanodia	MBBS, MD (Pharmacology) MBA (Health Care Management)	Medical Scientist	Male
7.	Mr Deepak Ranjan	B Com, MA in Buddhist studies, Diploma in Pali Language	Social scientist	Male
8.	Mr Ram Chander Singh	B. Com, LLB	Legal expert	Male
9.	Mrs Jyothi Jain	BCA, MCA	Lay Member	Female

The Ethics Committee approved the study on 08 April 2022.

The Indira IVF Hospital Institutional Ethics Committee functions by ICH GCP and New Drugs and Clinical Trial Regulations-2019. The Ethics Committee is to be informed about any protocol changes, informed consent and provided with a copy of the final report.

Yours sincerely
Member Secretary
Indira IVF Hospital Institutional Ethics Committee

REGD. OFFICE INDIRA IVF HOSPITAL ETHICS COMMITTEE #9, Opp. Metro Pillar-203, South Patel Nagar, West Delhi, Delhi - 110008 India

Annexure 2 – Consent Form

INDIRA IVF Hospital Pvt Ltd, South Patel Nagar, New Delhi
&
AMITY INSTITUTE OF MOLECULAR MEDICINE & STEM CELL RESEARCH (AIMMSCR), AMITY UNIVERSITY, Sector 125, Noida, U.P.

Consent Form for Research Work

I, Mr. _____ consent to donate (volunteer) my semen for research work for the project tittle **"Metabolic Dysregulation in Infertile Male Sperm"** at Indira IVF Hospital Pvt Ltd, Patel Nagar, New Delhi in collaboration with Amity Institute of Molecular Medicine & Stem Cell Research (AIMMSCR), Amity University, Noida.

I had a full discussion with Dr. **Arvind Vaid** (Centre Head) and **Miss. Sujata Maurya (Ph.D. Scholar)** regarding their research work and completely agree with them.

I have been counselled very well and understand that there will be no direct or indirect effect of sperm donation on my health, and my personal identity will not be disclosed in their work.

Signature:
Patients ID:

Signature of the Witness from the clinic

Mr. Imran Khan (Lab in-charge):

Signature of the Doctor:
Date:

Annexure 3 – Patient Details Form

Patient Details

Name or ID :-

Age :-

Year of Marriage :-

Year of Infertility :-

1^0 or 2^0 Infertility :-

Daily Habits :-

Smoking		**Tea/ Coffee**		**Exercise**	
Drinking		**Use of Laptop**		**Work/ Occupation**	

Absence Time :-

Previous Heath issue :- (1) Any Accident -

(2) Any Surgery –

Illness in past 1 year :-

Other medical condition :-

(Diabetes/Thyroid/Obesity etc)

Current Medication :-

Infertility History in family :-

Case of male infertility in family

Analysis report :- 1) Volume -

2) Count -

3) Motility – Total - **PR -** **NP-**

4) Morphology –

5) Any Infection -

Hormones Result :- LH - **FSH-** **Progesterone-**

Date :- **Signature :-**

Collection Time :- Proceeding Time :-