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**CLINICAL STUDY FOR HORMONAL VARIATIONS AND THEIR
ASSOCIATION WITH CFTR, FSHR GENES EXPRESSION IN
MALE INFERTILITY**

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**BY
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CLINICAL STUDY FOR HORMONAL VARIATIONS AND THEIR ASSOCIATION
WITH CFTR , FSHR GENES EXPRESSION IN MALE INFERTILITY

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November 2023

We certify that we have read this dissertation and that, in our opinion, it is fully adequate in scope and quality as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

CLINICAL STUDY FOR HORMONAL VARIATIONS AND THEIR ASSOCIATION WITH CFTR, FSHR GENES EXPRESSION IN MALE INFERTILITY

Muntadher Mohammed Jaber JABER

Doctor of Philosophy in Chemistry

Advisor: Prof. Dr. Şevki ADEM

November 2023

Current study is conducted for the period from September 2022 until June 2023 at infertility Unit, Al-Nasiriyah Teaching Hospital, Thi-Qar, Iraq. To examine masculinity and marital infertility, the clinical cases of the patients were collected after being nominated by the urologist, this study aims to evaluate levels of total Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), and Testosterone (T) their relationship to male infertility and investigate the combined effect of FSHR and CFTR gene polymorphism and infertile in case-study men. The present study includes number of 150 samples their age ranging from 20-40 years old, were divided into two groups, first group (healthy group) includes 75 men and patient group include a number of 75 infertile men each group were divided into four groups according the age categories. Present study showed that mean age for patients is 29.28 ± 6.38 years. The results also revealed that the most patients in age groups 26-30 years of age accounted for 28 (37.3 %), present study indicate highly significant increase of median levels of serum FSH in patients with infertility in comparison with healthy control, 3.43 (3.77) versus 2.18 (1.95) respectively, and the difference was highly significant ($P < 0.001$). Also, the median levels of serum Luteinizing Hormone (LH) was increased in patients with infertility in comparison with healthy control, 5.64 (3.76) versus 3.35 (1.86) respectively, and the difference was highly significant ($P < 0.001$). But the median levels of serum testosterone hormone lower in patients with infertility in comparison with healthy control, 3.82 (2.85) versus 5.42 (1.55) respectively, and the difference was highly significant ($P < 0.001$), Testosterone hormone test shows higher sensitivity

(81%) while FSH (72%) and LH (78%). Regarding *CFTR* $\Delta F508$ (C/T) gene in patients with infertility. Although there is no significant difference in the frequency distribution of genotypes between cases and healthy control groups. But there was increased risk of infertility with genotype TT versus CC as Odd ratio 1.54 was TT, with statistically significant and may be considered as etiological fraction for infertility. Also there was increased risk of infertility with genotype CT versus CC as Odd ratio was 1.95, and may be considered as etiological fraction for infertility. Concerning *FSHR* (rs1394205) G/A gene in patients with infertility. In co-dominant mode, there was highly significant difference in the frequency distribution of genotypes between patients and control groups ($P = 0.001$). There was increased risk of infertility with genotype AA versus GG as Odd ratio was 6.26, with statistically significant ($P=0.001$), and may be considered as etiological fraction for infertility, also there was increased risk of infertility with genotype GA versus GG as Odd ratio was 2.35, with statistically significant ($P=0.001$), and may be considered as etiological fraction for infertility. The current results show significant association between *CFTR* $\Delta F508$ (C/T) genotype serum FSH levels, were the FSH levels increased in TT genotype in compared to CC and CT genotype respectively. But the present results show non-significant association between *CFTR* $\Delta F508$ (C/T) genotype serum LH levels. Whereas the present results show non-significant decreased the serum Testosterone Hormone levels in TT genotype in compared to CC genotype and CT genotype respectively. The present investigation show non-significant association between *FSHR* (rs1394205) G/A genotype and serum hormones level. Although increased the serum FSH levels and serum LH levels in AA genotype in compared to GG and GA genotype respectively. But the present results show non-significant decreased the serum Testosterone Hormone levels in AA genotype in compared to GG genotype and GA genotype respectively.

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Keywords: Men infertility, FSH, LH, Testosterone, FSHR, CFTR

ÖZET

ERKEK KISIRLIĞINDA HORMONAL DEĞİŞİKLİKLER VE KFTR, FSHR GENLERİ İFADESİ İLE İLİŞKİSİ ÜZERİNE KLİNİK ÇALIŞMA

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Mevcut çalışma, Eylül 2022'den Haziran 2023'e kadar olan dönem için Al-Nasiriyah Eğitim Hastanesi, Thi-Qar, Irak'taki kısırlık Birimi'nde yürütülmüştür. Erkeklik ve evlilik dışı kısırlığı incelemek için, hastaların klinik vakaları ürolog tarafından seçilerek toplandı, bu çalışma toplam Folikül uyarıcı hormon (FSH), Lüteinizan hormon (LH) ve Testosteron (T) düzeylerini değerlendirmeyi amaçlamaktadır. erkek infertilitesi ile ilişkisi araştırıldı ve FSHR ve CFTR gen polimorfizmi ile infertilitenin kombine etkisini vaka çalışması erkeklerde araştırıldı. Yaş kategorilerine göre yaşları 20-40 arasında değişen 150 örnek içeren bu çalışma iki gruba ayrıldı, birinci grup (sağlıklı grup) 75 erkek ve hasta grubu 75 infertil erkek olmak üzere her grup 4 gruba ayrıldı. Bu çalışma, hastalar için ortalama yaşın 29.28 ± 6.38 yıl olduğunu göstermiştir. Sonuçlar ayrıca 26-30 yaş grubundaki hastaların çoğunun 28 (%37.3) olduğunu ortaya koydu, bu çalışma sağlıklı kontrol ile karşılaştırıldığında kısırlığı olan hastalarda medyan serum FSH düzeylerinde oldukça anlamlı bir artış olduğunu gösterdi, 3,43 (3,77) ve sırasıyla 2,18 (1,95) ve fark oldukça anlamlıydı ($P < 0,001$). Ayrıca, sağlıklı kontrol ile karşılaştırıldığında kısırlığı olan hastalarda serum Lüteinizan Hormonun (LH) medyan seviyeleri sırasıyla 5,64 (3,76) ve 3,35 (1,86) arttı ve fark oldukça anlamlıydı ($P < 0,001$). Ancak serum testosteron hormonunun ortanca düzeyleri sağlıklı kontrole kıyasla infertil hastalarda daha düşük, sırasıyla 3,82 (2,85) ve 5,42 (1,55) ve fark oldukça anlamlıydı ($P < 0,001$), Testosteron hormon testi daha yüksek duyarlılık gösteriyor (81%) ise FSH (%72) ve LH (%78). İnfertilite hastalarında CFTR $\Delta F508$ (C/T) geni ile ilgili olarak. Olgular ve sağlıklı kontrol grupları arasında genotiplerin sıklık dağılımında anlamlı bir fark olmamakla birlikte. Ancak, Odd oranı 1.54 TT olduğu için CC'ye karşı

genotip TT ile kısırlık riskinde artış vardı, istatistiksel olarak anlamlıydı ve kısırlık için etiyolojik fraksiyon olarak kabul edilebilir. Ayrıca Odd oranı 1.95 olduğundan CC'ye karşı genotip CT ile infertilite riskinde artış vardı ve infertilite için etiyolojik fraksiyon olarak kabul edilebilir. İnfertilite hastalarında FSHR (rs1394205) G/A geni ile ilgili. Eş baskın modda, hastalar ve kontrol grupları arasında genotiplerin frekans dağılımında oldukça anlamlı bir fark vardı ($P = 0.001$). Odd oranı 6.26 olduğundan, genotip AA ile GG arasında kısırlık riski artmıştır, istatistiksel olarak anlamlıdır ($P=0.001$) ve kısırlık için etiyolojik fraksiyon olarak kabul edilebilir, ayrıca genotip GA ile tek olarak GG ile kısırlık riski artmıştır. oranı 2.35 idi, istatistiksel olarak anlamlıydı ($P=0.001$) ve infertilite için etiyolojik fraksiyon olarak kabul edilebilir. Mevcut sonuçlar, CFTR $\Delta F508$ (C/T) genotip serum FSH seviyeleri arasında anlamlı bir ilişki olduğunu göstermektedir; FSH seviyeleri, sırasıyla CC ve CT genotipine kıyasla TT genotipinde artmıştır. Ancak mevcut sonuçlar, CFTR $\Delta F508$ (C/T) genotip serum LH seviyeleri arasında anlamlı olmayan bir ilişki olduğunu göstermektedir. Mevcut sonuçlar, sırasıyla CC genotipi ve CT genotipine kıyasla TT genotipindeki serum Testosteron Hormon seviyelerinin anlamlı olmayan bir şekilde azaldığını göstermektedir. Mevcut araştırma, FSHR (rs1394205) G/A genotipi ile serum hormon düzeyi arasında anlamlı olmayan bir ilişki olduğunu göstermektedir. AA genotipinde serum FSH seviyeleri ve serum LH seviyeleri sırasıyla GG ve GA genotipine göre artmasına rağmen. Ancak mevcut sonuçlar, sırasıyla GG genotipi ve GA genotipine kıyasla AA genotipindeki serum Testosteron Hormon seviyelerinin anlamlı olmayan bir şekilde azaldığını göstermektedir.

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Anahtar Kelimeler: Erkek kısırlığı, FSH, LH, Testosteron, FSHR, CFTR

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LIST OF SYMBOLS

μL	Microliter
mg/mL	Milligram per milliliter
mL	Milliliter
ng/L	Nanogram per liter
nm	Nanometer



LIST OF ABBREVIATIONS

ABP	Androgen-binding protein
AR	Androgen receptor
ARMS-PCR	Amplification-refractory mutation system
ART	Assisted reproductive technology
AZF	Azoospermia factor
CAG	Cytosine–adenine–guanine
CBAVD	Congenital bilateral absence of the vas deferens
CFTR	Cystic fibrosis transmembrane conductance regulator
DSB	Double-strand break
ECD	Extracellular hydrophilic domain
ECLIA	Electrochemiluminescence Immunoassay
EDTA	Ethylene diamine tetra acetic acid
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GPHRs	G-protein-coupled receptors of transmembrane proteins
HPG axis	Hypothalamus-Pituitary-Gonadal axis
ICSH	Interstitial cell-stimulating hormone
ITT	Intra-testicular testosterone
KiSS	Kisspeptin
KNDy	Kisspeptin-neurokinin B-dynorphin
KNDy	Kisspeptin-neurokinin B-dynorphin
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MtDNA	Mitochondrial DNA
OS	Oxidative stress
PPT	Protein precipitation
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
SCO	Sertoli cell-only syndrome
SNPs	Single-nucleotide polymorphisms
SRY	Sex-determining region
STDs	Sexually-transmitted diseases
T	Testosterone
TMDs	Transmembrane domains
WHO	World health organization

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1. INTRODUCTION

Infertility is the condition that occurs when a clinical pregnancy is not attained following at least one year of regular, unprotected sexual interaction (Zegers-Hochschild *et al.* 2009). Because men account for over 15% of infertile couples and infertility is almost always caused by a male factor, male infertility and subfertility are important issues in public health (Huhtaniemi *et al.* 2018). Male infertility is a complicated disease that accounts for about one-third of all infertility occurrences worldwide, (Joseph *et al.* 2021). Male infertility may be caused by a number of things, such as abnormal sperm activity or obstructions that hinder ejaculation. It can also be caused by things like illness, accidents, chronic illness, and lifestyle choices, among other things (Agarwal *et al.* 2021).

Hormone problems, physical problems, sexually transmitted diseases, the environment and lifestyle, and genetics are the most common reasons why men can't have children (Krausz *et al.* 2018). The three primary endocrine centers that make up the hypothalamic-pituitary-gonadal (HPG) axis control both puberty and fertility hormonally, about 15% of couples have decreased fertility, the causes of reduced fertility, however, differ from case to case (Rosati *et al.* 2020).

The control of hypothalamic, pituitary, and gonadal secretions is necessary for both the beginning of puberty and the maintenance of fertility, changes in hormone levels may lead to infertility and improper reproductive function (Guijarro *et al.* 2006). Conventional criteria, such as sperm shape, DNA damage, and seminal fluid characteristics, as well as omics-driven indications, such as single-nucleotide polymorphisms (SNPs), proteins, transcripts, mutations, and active chemicals, need to be included in these biomarker sets, these biomarker sets are required to be comprehensive (Assidi *et al.* 2022).

Pulsatile gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and supplied to the anterior pituitary gland via the hypophyseal portal (Sussman *et al.* 2008).

The hypothalamus controls the secretion of stress hormones and gonad sex steroids, which form a functional relationship between the hypopituitary, adrenal glands, and gonads (Selvaraj *et al.* 2020). When blood levels of sex steroid hormones, especially testosterone in men, drop, the hypothalamus releases gonadotropin-releasing hormone (GnRH), which controls the HPG axis. In reaction to this, the anterior pituitary releases follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Kandasamy *et al.* 2019).

It is known that hereditary factors are responsible for 30–50% of instances of male infertility (Rhouma *et al.* 2018). Depending on their frequency and severity, genetic diseases may induce various types of infertility, ranging from small modifications in spermatogenesis to full failure of the gonads, the process of spermatogenesis is regulated by a precise cascade of on/off gene expression (Laan *et al.* 2021).

Male infertility may be brought on by the chromosomal or monogenic defects, mutations in the mitochondrial DNA (mtDNA), deletions of the Y-chromosome, multifactorial diseases, imprinting disorders or genetically based endocrine problems, the spermatogenesis process depends on around 10% of the genome's genes. 15–30% of male infertility instances are caused by genetic abnormalities, which might result in permanent partial or complete spermatogenesis cessation (Zhang *et al.* 2020).

The expression of the FSHR gene is essential for reproduction because it regulates the activity of spermatogenesis cells, which includes the creation of follicle-stimulating hormone (FSH) (Jardon *et al.* 2018). Class A subfamily glycoprotein hormone receptors include FSHR, LHR, and TSHR. Single nucleotide polymorphisms (SNPs) in FSH and FSHR work together to predict infertility risk and treatment success. However, little is known about the impact of FSHR gene expression on male infertility (Simoni and Casarini 2014).

The gene known as cystic fibrosis transmembrane conductance regulator (CFTR) spans 230 kb and is situated on the long arm of chromosome 7q31.2. The gene's normal allele code for a protein with 1480 amino acids that is bound to the cell membrane; the gene's

size is 6.5 kilobases. its protein functions as a regulated chloride channel in many kinds of epithelial cells; the CFTR gene is in charge of its function (Hwang *et al.* 2010).

1.1 Aims of Study

The study was designed to evaluate levels of total FSH , LH , and Testosterone (T) and their relationship to male infertility and investigate the combined effect of FSHR and CFTR gene polymorphism in infertile men using the following objectives :

- Identification of the serum levels of FSH, LH, and total testosterone in fertile and infertile males.
- Detection of FSHR (rs1394205) G/T Polymorphism and Detection of CFTR Δ F508 (C/T) Polymorphism in infertile men.
- Investigate the association between serum FSH, LH, and T testosterone levels, and the FSHR and CFTR polymorphism.

2. LITERATURE REVIEW

2.1 Male Infertility

Male infertility is a complex disorder that affects approximately one-third of all infertility cases globally. It is caused by a variety of defects, including low sperm quantity or quality, which can be attributed to a number of chromosomal abnormalities with single genes or multiple genes (Joseph *et al.* 2021).

A clinical pregnancy cannot be achieved after at least one year of regular, unprotected sexual intercourse; this condition is known as infertility (Zegers-Hochschild *et al.* 2009). Because men account for over 15% of infertile couples and infertility is almost always caused by a male factor, male infertility and subfertility are important issues in public health (Huhtaniemi *et al.* 2018). Moreover, both the quantity and quality of sperm are now declining globally (Niederberger *et al.* 2018).

The subtypes of male infertility may be classified according to abnormalities in the quantity and movement of spermatozoa in seminal ejaculate: Approximately 70% of male infertility cases can be resolved with the help of relevant medical history, physical examination, seminal and hormonal assessment, and other tests. Azoospermia is the absence of sperm, oligospermia is a sperm count of 20 million/mL, asthenospermia is a sperm count of normal but motility of 50%, and teratospermia is a lower proportion of sperm with normal morphology. Testicular azoospermia cannot be cured, and most cases of pre-testicular azoospermia are caused by endocrine problems, which are characterised by low androgen levels and changed gonadotropin levels. There are three main categories of azoospermia based on aetiology: pre-testicular, testicular, and post-testicular. A low testosterone level and an abnormal gonadotropin level characterise pre testicular azoospermia (Gudeloglu *et al.* 2013).

Testicular azoospermia is caused by problems with sperm production that are either born with the person or come up later in life ,these problems can be present from birth

like Kallmann syndrome, develop over time (like hypothalamic or pituitary disorders), or be a side effect of something else, like a drug (Kohn *et al.* 2018).

Congenital testicular causes include things like anorchia which means the absence of one testicle, abnormalities germ cell aplasia, cryptorchidism genetic and sperm cell maturation arrest, Infections, drugs, trauma, radiation exposure, renal failure, surgery and varicoceles are all examples of acquired causes of testicular cancer, other causes include testicular tumors.(Santana *et al.* 2019).

The post-testicular causes of azoospermia include obstructions of the ejaculatory ducts which prevent spermatozoa from leaving the testis, these obstructions can be acquired as a result of surgery, infection ,vasectomy or they can be congenital and result from the congenital bilateral absence of the vas deferens (CBAVD), infertility in male can also be categorized based on defects involving the hypothalamus related accessory sex glands or poor sperm transport caused by conditions in the testicles, men who are infertile frequently exhibit azoospermia and abnormal sperm characteristics, such as a low sperm count, however, this condition can also be brought on by a number of congenital diseases, such as congenital bilateral agenesis , of the vas deferens, also known as CBAVD, Sertoli cell-only syndrome, also known as SCO, and hypospermatozoid, these are just some of the congenital diseases that can bring on this condition (Kirakoya *et al.* 2014).

Essential reproductive hormone endocrinology for diagnosing and treating endocrine disorders identification is crucial because the precise hormonal analysis is frequently successful, not only is the synthesis of testosterone required for the process of spermatogenesis, but it is also essential for the maturation of acceptable sexual behavior and secondary sexual traits, understanding reproductive physiology is essential since endocrinological reasons of male infertility are infrequent, accounting for fewer than 2% of cases (Anawalt *et al.* 2019).

An important neurohormone in reproductive control is kisspeptin. Infertility can be caused by mutations in the kisspeptin gene or its receptor. Its pulsatile secretion triggers

the secretion of gonadotropin releasing hormone (GnRH), which in turn stimulates the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Nabi *et al.* 2015).

It is vital to extend our understanding of the processes behind the beginning and maintenance of the process of spermatogenesis, as well as the pathophysiology of its abnormalities, in order to implement appropriate treatments, spermatogenesis is a process that is little understood, maintaining the differentiation and proliferation of sperm cells is dependent on a complex network of paracrine, endocrine and metabolic interactions that are mediated by Leydig peritubular, Sertoli, and germ cells (Figure 2.1), these interactions are required for the regulation of spermatogenesis, in order to get an understanding of the underlying mechanics, the hypothalamic-pituitary-gonadal axis, sometimes known as the HPG axis, a regulatory mechanism that carries the greatest weight, it is responsible for the coordination of important processes via the action of the heterodimeric glycoproteins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), additionally, it is responsible for the maintenance of a high concentration of intra-testicular testosterone (ITT) concentration, these hormones are made up of a subunit that is shared by all of them and a subunit that is unique to each hormone (McLachlan *et al.* 2002).

To begin and continue the process of spermatogenesis in humans, there must be an increase in the production of LH during puberty, this promotes a significant number of Leydig cells to proliferate and differentiate, which is essential for the preservation of sperm quality, Sertoli cells are vulnerable to the combined activities of T and FSH, which delimit the seminiferous tubule walls and stimulate the formation of sperm cells (Dube *et al.* 2013).

Numerous autocrine, paracrine, and other hormonal signals and nutrients interact sequentially to stimulate the development of germ cells, these biological processes include mitotic development, genetic material recombination during meiosis, and morphological sperm maturation (Ehmcke *et al.* 2006).

The production of follitropin-releasing hormone (FSH) and luteinizing hormone (LH) by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus is essential for the generation and maintenance of both quantitative and qualitative spermatogenesis. GnRH regulates the development, maturation, and function of the gonads, and its secretion is a necessary step in this process. To do this, it triggers the anterior pituitary's gonadotrope cells to secrete gonadotropin in discrete pulses, which are then released into the bloodstream (Ramaswamy *et al.* 2014). In addition to its psychological and anabolic benefits, spermatogenesis and secondary sexual characteristics and functions are dependent on T, which is increased during the production of LH due to its crucial role in the process (Huhtaniemi *et al.* 2018).

The interaction between the two hormones is crucial for the adult testis to retain its fertility and virility. Testosterone (T) is the master switch of spermatogenesis, while FSH affects the quantity and quality of sperm. FSH also preserves the spermatogenesis-supportive function of sertoli cells, which leads to an increase in T activity (Oduwole *et al.* 2014). Genes for follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) are located on chromosome 2p21, suggesting that these genes were passed down from a common ancestor to each of the different species. T is produced by Leydig cells and can enhance functional responses necessary for spermatogenesis through the androgen receptor AR, which is expressed in Sertoli cells (Rousseau *et al.* 1993). The identical thyroid-stimulating hormone receptor, on the other hand, remained dispersed across the genome until it was found on 14q31 (Gross *et al.* 1991).

It is possible that the gonadotropin receptor genes evolved from a proto-receptor gene encoding the receptor trans-membrane domain and a leucin-rich repeats gene located in the extracellular domain. This gene stands in contrast to other G protein-coupled receptor genes, which have shorter extracellular domains without introns (Misrahi *et al.* 1998).

The diagnosis of male infertility is based on the conventional testing of sperm. Typically, abnormal sperm parameters are associated with the following issues: The

World Health Organisation (WHO 2010) defined the terms related to sperm quality as follows: (sperm concentration, morphology, motility, and vitality) (Figure 2.1):

- A. The Azoospermia
- B. Oligospermia : 15*10, mL, sperm concentration
- C. Asthenozoospermia : 32% of spermatozoa that move more slowly
- D. Teratozoospermia : 4% spermatozoa with normal morphology
- E. Oligosthenoterataspermia :Disruption of morphological characteristics and concentration, motility together
- F. Cryptozoospermia : Spermatozoa were not present in the fresh sample, but were detected in the centrifuged pellet.
- G. Aspermia.

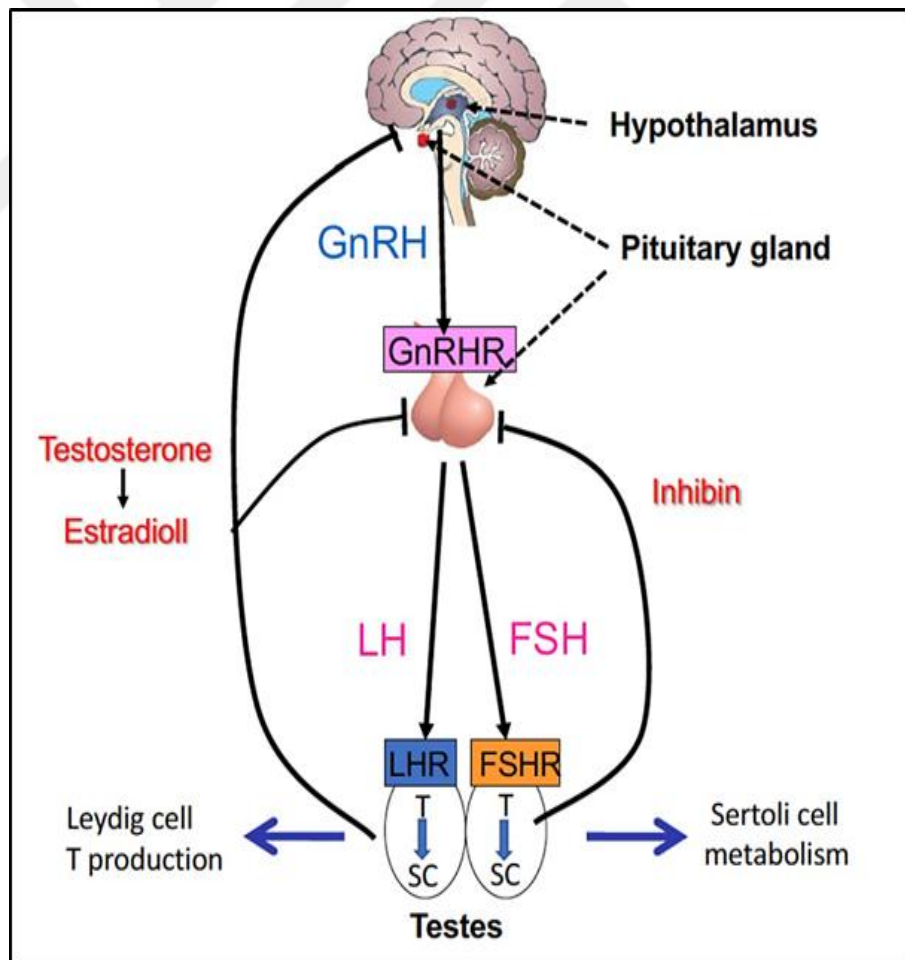


Figure 2.1 Hormonal control of spermatogenesis (Oduwole *et al.* 2021)

2.2 Etiology of Male Infertility

Male infertility may be caused by a number of things, such as abnormal sperm activity or obstructions that hinder ejaculation. It can also be caused by things like illness, accidents, chronic illness, and lifestyle choices, among other things (Agarwal *et al.* 2021).

This intricate developmental process is defined by both mitotic and meiotic divisions, significant biochemical and morphological differentiation, and the formation of an adult spermatozoan. It is also characterised by the fact that it is very difficult to reproduce normally, which is why spermatogenesis—the production of spermatozoa in the testes from spermatogonia—is so important in determining male fertility (Turner *et al.* 2020).

Infertility in males can be caused by a number of causes, (Figure 2.2) including hormone imbalances, physical difficulties, lifestyle concerns, psychological issues, sex issues, chromosomal abnormalities, single-gene flaws and hereditary factors (Babakhanzadeh *et al.* 2020). Semen analysis is the primary method used to, diagnose male infertility, unusual sperm characteristics include sperm concentration, morphology, and motility (De Kretser *et al.* 1999).

Infertility caused by male factor abnormalities accounts for 30% of all occurrences of infertility globally (Agarwal *et al.* 2015). Chromosomal abnormalities or single-gene changes are responsible for around 15% of instances of male infertility, despite the fact that the clinical symptoms of male infertility are numerous and complex (Asero *et al.* 2014). Male infertility genes include those involved in spermatogenesis, such as steroid hormone signaling, and steroid hormone receptor signaling, male reproductive system development such as FSHR and CFTR (Cui 2017, Mitchell 2012).

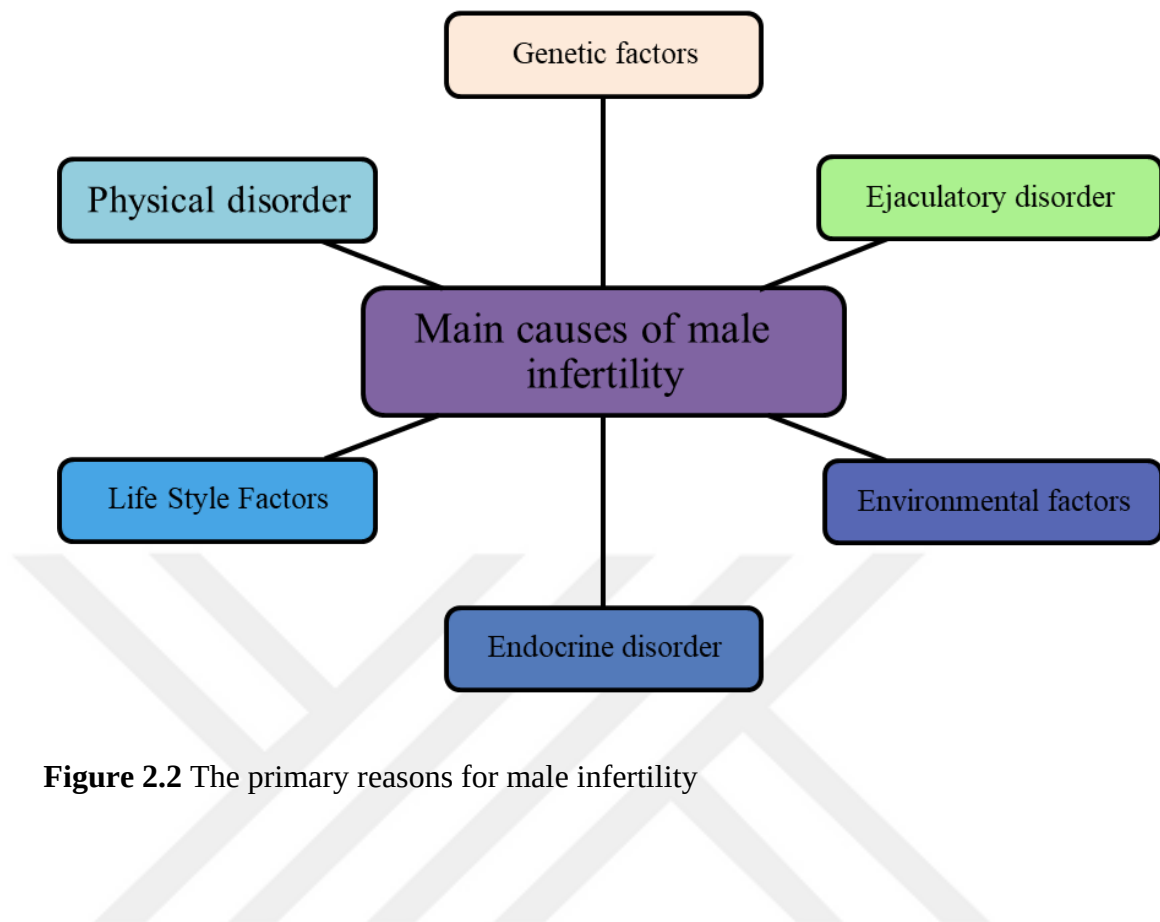


Figure 2.2 The primary reasons for male infertility

Infertility can be caused by problems with the way the sperm look or how many of them there are. Several hereditary reasons for male infertility have been identified, found through cytogenetic methods, microarrays, and sequencing of particular panels of male infertility genes (Gunes *et al.* 2021). But for 40% of male infertility cases, the genetic cause is still unknown (Krausz *et al.* 2018). It's essential, to comprehend the, hereditary origins of male, infertility because improving prognoses, treatments and figuring out how likely it is that a genetic flaw will be passed on through natural or assisted reproduction (Hanson *et al.* 2018).

Hormone problems, physical problems, sexually transmitted diseases, the environment and lifestyle, and genetics are the most common reasons why men can't have children (Krausz *et al.* 2018). Due to the complicated reasons why men can't have children, several factors may affect each other, in the age of personalized medicine, more complete sets of biomarkers were needed to figure out the molecular and physiological causes of male infertility. Conventional criteria, such as sperm shape, DNA damage,

and seminal fluid characteristics, as well as omics-driven indications, such as single-nucleotide polymorphisms (SNPs), proteins, transcripts, mutations, and active chemicals, need to be included in these biomarker sets, these biomarker sets are required to be comprehensive (Assidi *et al.*2022).

2.2.1 Endocrine and systemic disorders

Concerns about the trend of male infertility's rising prevalence and its ambiguous etiopathogenesis have spread around the world (Sengupta *et al.* 2017). Understanding the fundamental processes behind the cause of male infertility is crucial given that over half of all instances of infertility worldwide include male factors (Barratt *et al.* 2017). The endocrine system (Table 2.1) is the principal regulator of male reproductive processes, which are dependent on a complex interaction of hormones (Singh *et al.* 2019).

In response to gonadotropin-releasing hormone (GnRH) released into the bloodstream by the brain, the anterior pituitary gland secretes luteinizing hormone and follicle-stimulating hormone (FSH), which stimulate the Sertoli cells to promote spermatogonial growth (Dutta *et al.* 2019). Despite luteinizing hormone (LH) being responsible for testosterone synthesis and release, normal spermatogenesis in Leydig cells requires substantially higher levels of intratesticular testosterone compared to serum testosterone. This intratesticular testosterone indirectly promotes germ cell maturation via Sertoli cells (Lipshultz *et al.* 2009).

Table 2.1 Endocrine disorders and their impact on male infertility

Endocrine disorder	The main effects	References
Hypogonadotropic hypogonadism Kallman syndrome	Infertility and delayed puberty brought on by the migration of GnRH-secreting neurons ceasing gonadotropin production	(Kim and Schlegel 2008) (Fraietta <i>et al.</i> 2013)
Hypergonadotropic hypogonadism	Increased FSH/LH normal or decrease testis volume infertility	(Yan <i>et al.</i> 2005)
Hyperprolactinemia	Normal or decrease FSH/LH decrease testosterone	(Rama Raju <i>et al.</i> 2012)
Androgen excess	Normal or decreased FSH/LH secretion when GnRH production is inhibited	(Menon 2003)
Estrogen excess	Decrease T:E2, decrease semen parameters (T:E2 :Testosterone to estradiol ratio)	(Amaral <i>et al.</i> 2006)

The axis of male reproductive hormones is referred to as the hypothalamic-pituitary-gonadal axis (Figure 2.3) , it is composed of three principal parts: the hypothalamus, pituitary and testicular glands (Corradi 2016, Kuiri-Hänninen 2014). While this axis normally supplies the right amount of hormones to support men's sexual development and function, infertility may be caused by any medical condition (Xiong *et al.* 2014). The inability of the brain to produce gonadotropic releasing hormone (GnRH) leads to a lack of testosterone and the inability to produce sperm; this disease is known as hypogonadotropic hypogonadism (Monaco *et al.* 2015, Ullah *et al.* 2017). One of them is referred to as "Kallmann syndrome," which is connected to immaturity and a change in sense of smell (Kotan *et al.* 2014).

Increased prolactin may also result in decreased sperm production, libido, and erectile dysfunction (Figure 2.3). Hyperprolactemia causes infertility in 11% of oligospermic individuals (Marrag *et al.* 2015) LH indirectly affects spermatogenesis by stimulating testosterone production (Oduwole *et al.* 2018). Although endocrine abnormalities are linked to male infertility in a small percentage of cases (1% to 2%), individuals may benefit from a tailored approach to preserving and restoring fertility when these illnesses are treated (Kim *et al.* 2008).

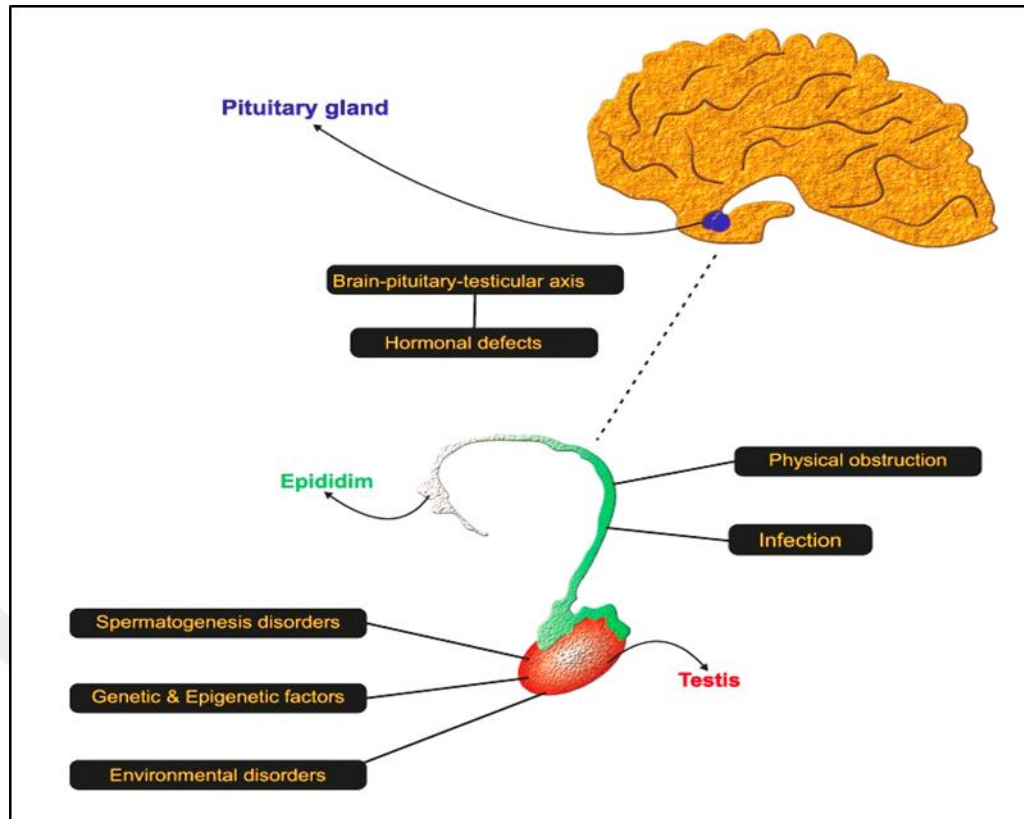


Figure 2.3 Brain-pituitary-testicular axis (Babakhanzadeh *et al.* 2020)

2.2.2 Environmental and life style factors

Given that a multitude of factors might affect male fertility, one reason for the downward trend might be the existence of environmental, occupational, or lifestyle variables that lead to sperm quality decrease (Jurewicz *et al.* 2014).

Multiple environmental conditions, like pollution, influence fertility through epigenetic Routes, there are varying degrees of evidence about the possible harmful influence that different lifestyle factors may have on male fertility (Sharma *et al.* 2013), recent research has focused on the epigenetic signature that is unique to sperm, as well as its transfer to oocytes and the consequences it has on the development of embryos, the epigenome links the genome to the surrounding environment and may facilitate the transmission of epigenetic markers from one generation to the next.(Cescon *et al.* 2020).

Male infertility risk is increased, semen quality is poor, and the number of motile sperm is lowered when dangerous physical and chemical substances are exposed at work (Cescon *et al.* 2020). Other environmental concerns include mobile phone and laptop radiation exposure, tight-fitting undergarments, frequent hot baths and sauna use, and exposure to endocrine-disrupting chemicals, additionally, infertility may be influenced by extended sitting, being in places with high temperatures like bakeries and metal shops, and being under a lot of stress, in addition to psychological, physical, and sexual issues, job strain or workload is positively associated with perimenopause (Leisegang *et al.* 2021).

Most non-communicable diseases have microbial dysbiosis as a risk factor, and there have been some observed correlations between the microbiome and male fertility, However, additional investigation will be required to confirm these associations (Venneri 2022).

The human microbiome plays an important role in regulating health and disease via circadian, endocrine, and molecular interactions for example, obesity brought on by a poor diet may have an influence on male fertility by altering sexual behavior and sleep, as well as hormone profiles, scrotal temperatures, and the quality of sperm. There is a substantial chance of miscarriage in males who are obese (Wang *et al.* 2022).

Lifestyle encompasses all factors that have an impact on health, such as diet, exercise, and drinking or smoking (Campbell *et al.* 2015). Also, men who are underweight or overweight are more likely to have azoospermia than men who are the right weight, people have found that sex hormone-binding globulin concentrations in obese males are lower which has been linked to high insulin levels and high levels of total estradiol, on the other hand, weight-loss programs have been linked to less damage to DNA in cells, more motile sperm, and better shape of sperm (Mir *et al.* 2018).

A diet rich in fat may lead to intestinal dysbiosis and make it more challenging to conceive by elevating blood endotoxin, levels causing inflammation, epididymitis, and uncontrolled gene expression in the testes, intestinal dysbiosis can make it more

difficult to conceive, diet, alcohol and cigarette usage, recreational drug use, stress, and other drug use are all factors that might induce changes in the microbiota that live in the gut, these alterations may have an impact on reproductive potential (Ding *et al.* 2020), while a balanced diet (such as the Mediterranean diet) has been linked to enhanced sperm quality, high-energy, nutritionally deficient processed foods have been linked to an increased incidence of asthenozoospermia, in addition to conventional medications, lifestyle changes, especially regarding the caliber of the food ingested, are advised to treat low semen quality (Skoracka *et al.* 2020).

Smoking and excessive alcohol use have negative impacts on various sperm parameters, lowering fertility and passing epigenetic abnormalities to the progeny (Hart *et al.* 2019). Alcohol seems to prevent testosterone from being produced, GnRH, FSH, and LH, as well as the functioning of Sertoli and Leydig cells at all levels of the hypothalamus-pituitary-gonadal axis (HPG axis), this appears to be where alcohol's effects on the male reproductive system are manifested, alcohol also seems to inhibit the production of sperm, in turn, this might impede spermatozoa's ability to produce, develop morphologically, and mature (Emanuele *et al.* 1998).

Increased alcohol use seems to cause a steady reduction in spermatogenesis (Pajarinen *et al.* 1996). Heavy drinkers had a significantly higher risk compared to nondrinkers of displaying partial or total spermatogenic arrest as well as Sertoli cell-only syndrome. (Pajarinen *et al.* 1995). Chronic alcohol intake has been demonstrated to have a detrimental effect, not only on the quantity but also on quality of the sperm and the hormones that are involved in male reproduction (Muthusami *et al.* 2005). Chronic ethanol use has been demonstrated to lower the antioxidant and steroidogenic enzyme activity in the testicles, leading to an increase in oxidative stress that might alter the manufacture of testosterone and impair fertility (Maneesh *et al.* 2005).

Male fertility is impacted by excessive caffeine use and recreational drug use, including cannabis, opiates, and anabolic steroids (Ricci *et al.* 2017). Tobacco smoke contains reactive oxygen species (ROS) at amounts that may surpass the capacity of the body's natural antioxidant defenses, which has been linked to the condition known as

leucocytospermia, which is a significant endogenous source of ROS, in addition, smokers have greater amounts ROS in their seminal fluid, which puts spermatozoa at risk of oxidative stress and, as a result, reduces male fertility (Harlev *et al.* 2015).

Tobacco use has not only been linked to a decrease in male fertility, but it has also been linked to an increase in DNA damage, aneuploidies, and sperm mutations, the processes that are behind smoking's impact on sperm quality are still a mystery, but it is known that smoking has a negative impact on sperm quality (Beal *et al.* 2017).

As a consequence, increased oxidative stress, DNA and cell death may be factors that contribute to the negative impact smoking has on male fertility damage (Parameswari and Sridharan 2021), this may explain not just the low quality of the sperm produced by smokers, but also the delayed spermatogenesis, maturation, and function of smokers in contrast to non-smokers, male smokers are more prone to have these detrimental consequences than female smokers because to the larger levels of nicotine, its metabolite cotinine, pyrene, benzo a and cadmium that they consume, there are approximately 7,000 different chemicals that can be found in cigarette smoke (Soleimani *et al.* 2021), tobacco smoke contains a variety of chemicals, some of which are known to cause cancer. These include nitrosamines unique to tobacco, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-nitrosonornicotine, as well as polycyclic aromatic hydrocarbons and volatile organic compounds like benzene. Cigarette smokers are also more likely to inhale carbon monoxide and heavy metals like lead and cadmium, as well as tar and other substances (Beal *et al.* 2017). Other crucial lifestyle factors affecting men's ability to reproduce include inactivity, stress, and sleep deprivation (Kumar *et al.* 2021).

2.2.3 Anatomo-pathophysiological factors

The testes, epididymis, seminal ducts, or prostatic utricles may be damaged or blocked by physical malformations in the male reproductive system, which can reduce fertility (Singh *et al.* 2012), and various abnormalities have varying harmful implications on male reproductive function, some physical abnormalities, such as an inguinal hernia,

may not directly cause male infertility; but, they are often connected with other fertility-threatening disorders, and surgical correction of an inguinal hernia may also lead to male infertility, some anatomical defects, such as bilateral lack of the vasa deferentia, may not directly result in male infertility, but they are often connected with other fertility-threatening diseases (Tomiya *et al.* 2005).

Male infertility can be caused by a variety of anatomical and pathophysiological factors. These include, but are not limited to, sperm duct blockage, epididymal or vas deferens obstructions, issues with ejaculation, testicular disease or injury, hormonal imbalances, inherited abnormalities, and other illnesses (iatrogenic factors). Five percent of infertile men have duct blockage, which hinders ejaculation and indicates azoospermia or severe oligozoospermia (Acherma *et al.* 2021). Infertility, hormonal imbalance, and sexual dysfunction may be brought on by testicular injury or malfunction (Saab *et al.* 2018).

A disorder of the bladder sphincter termed as retrograde ejaculation is characterized by semen ejaculating into the bladder, this problem accounts for 2% of all cases of infertility, the reproductive hormones of males are essential for the development and proper functioning of the male genitalia, retrograde blood flow is caused by the abnormal vein growth known as varicocele in the scrotum and may lead to infertility (Lucas 2014, Babakhanzadeh 2020).

When the testis twists within the scrotum, it may compress sperm arteries and interrupt blood supply to the testicles, which can lead to testicular injury. It is also well-known that male infertility may be caused by acute or persistent genital tract infections (Senanayake 2008). Treatment is often only required to regain fertility since it is brought on by a sphincter anotomy (Kamischke and Nieschlag 2002).

Infertility may be caused by some bacteria, the Zika virus, and a number of sexually-transmitted diseases (STDs) which affect the reproductive system and can cause it to malfunction (Farsimadan *et al.* 2020). Furthermore, genitourinary tract infections and other conditions affecting the male reproductive system may lead to oxidative stress (OS), which has been linked to male infertility (Dutta *et al.* 2019). In these conditions,

either the sperm production is slowed or the ejaculatory process is impacted by physical issues, the most prevalent physical condition affecting 40% of infertile men is varicocele, an enlargement of the internal spermatic veins (Nagler *et al.* 1997).

2.3 Fertility and the Hormonal Regulation of Spermatogenesis

Infertility may have a variety of reasons, including atypical puberty and poor hormone control that can happen at any age following puberty, the three primary endocrine centers that make up the hypothalamic-pituitary-gonadal (HPG) axis control both puberty and fertility hormonally, about 15% of couples have decreased fertility, the causes of reduced fertility, however, differ from case to case (Rosati *et al.* 2020).

Several physiological processes are generated, integrated, and regulated by the hypothalamus. These include hormone balance, blood pressure, basal homeostasis, body temperature, and circadian rhythms. The regulation of secretions from the pituitary gland and the gonadal gland is essential for the onset of puberty and the preservation of fertility. Infertility and improper reproductive function can result from changes in hormone levels (Guijarro *et al.* 2006).

The testes, the anterior and posterior pituitary glands, and the brain are all components of the hypothalamic-pituitary-gonadal axis (HPG axis). Reproductive function depends on the HPG axis being under proper control. However, problems with the HPG axis have been found to hinder steroidogenesis and spermatogenesis in the testes, which can lead to infertility and mental health problems (Acevedo-Rodriguez *et al.* 2018).

Pulsatile gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and transported to the anterior pituitary gland via the hypophyseal portal (Sussman *et al.* 2008). According to Selvaraj *et al.* (2020), the hypothalamus has functional relationships with the pituitary gland, adrenal gland, and gonads because of the stress hormones and gonad sex steroids that it releases. When blood levels of sex steroid hormones, especially testosterone in men, drop, the hypothalamus releases

gonadotropin-releasing hormone (GnRH), which controls the HPG axis. In reaction to this, the anterior pituitary releases follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Kandasamy *et al.*2019).

LH works on the Leydig cells in the testes, which makes testosterone, which is needed for spermatogenesis. FSH works on the sertoli cells (Kliesch *et al.*1991).

As part of a feedback loop, the amount of testosterone in the blood causes the hypothalamus to stop releasing GnRH, this tells the pituitary gland how much FSH and LH to make , about 100 times more testosterone is found in the testes than in the rest of the body, Testosterone helps make sperm by acting on the sertoli cells (Clavijo *et al.*2018).

Semen quality is dependent on normal spermatogenesis, sperm maturation, and seminal fluid composition, all of which are governed by uncontested hormonal controls. The hypothalamic-pituitary-testicular axis uses positive and negative feedback loops to regulate testicular activity in response to both internal and external stimuli. Pulsatile GnRH is produced by the hypothalamus, which activates the anterior pituitary gonadotropins. These gonadotropins include lutropin (LH) and follitropin (FSH). The testes are responsible for regulating the key testicular functions of steroidogenesis and spermatogenesis. LH stimulates the production of testosterone and other androgens through receptors on interstitial Leydig cells (Dutta *et al.* 2019).

Testosterone is essential for a variety of male reproductive activities, excluding the testes, such as sexual, libido and anabolic (muscle strength,) processes, FSH is an essential pituitary hormone that stimulates spermatogenesis by acting on Sertoli cells. inhibin ,an inhibitory hormone, and activation a stimulating hormone, in addition to other paracrine substances, are released by Sertoli cells along with testosterone, these hormones are part of the feedback loops that regulate the activities of the HPG axis and influence spermatogenesis, the feedback loops control both the hypothalamic GnRH and the pituitary gonadotropins that are released afterward (Sengupta *et al.* 2019).

A number of additional regulatory variables have been discovered as a result of novel concepts in male reproductive physiology. Kisspeptin, a 54-amino-acid peptide encoded by the KiSS-1 gene, is a regulatory peptide that belongs to the same class as gonadotropin-inhibiting hormone and other short RF-amide peptides with a C-terminal Arg-Phe-NH₂ sequence. G protein-coupled receptor 54 is an important peptide in the onset of puberty, and kisspeptin may impact this process by acting on the hypothalamus. Some research suggests that kisspeptin is also crucial for the start of male puberty (Ilgin *et al.* 2020).

Spermatogenesis is an ongoing, intricate biological process, the testis contains sperm at various stages of development, it requires germ cells, which in males are the testicular cells that grow into immature sperm cells. It takes sperm 70 days to grow into a sperm that can fertilize an egg and is detected in seminal fluid, this means that by the time sperm are released from the testis, they have already been developing for weeks (Nieschlag *et al.* 2001).

Germ cells in the linings of seminiferous tubules divide repeatedly to produce sperm, this process, called spermatogenesis, starts in the spermatogonium, and the dividing cells go through several stages of development that lead to a round cell called a spermatid and sperm that look like tadpoles, at the end of the final stage of development, sperm move out of the seminiferous tubules and into the epididymis (Nieschlag *et al.* 2011). When ejaculation starts, waves made by muscle contractions carry sperm and a small amount of liquid from the epididymis out of the body through the vas deferens (Henkel *et al.* 2020).

The neck joins the sperm's head to its tail, in the portion of the tail closest to the neck the mitochondria are located and supply the sperm's movement with energy, the tail's whipping action propels sperm toward the egg, while fluid from the prostate gland is combined with sperm in the prostatic urethra, the urethra receives fluid from the seminal vesicles through the ejaculatory canal, the fatty fluid produced by Cowper's glands aids in the movement of urine through the urethra during sexual activity, fluid from the

testes and other secondary sex glands flows up the penile urethra to the male sex organ, where it is discharged (Agarwal *et al.* 2019).

The epididymis is a long, tightly coiled tube that connects the seminiferous tubules to the vas deferens and extends from each testis, behind the seminiferous tubules, it is positioned at the back of each testis, two to ten days after being discharged from the testes, sperm travel via the epididymis during this phase, sperm develop and become motile, able to move, sperm travel from the epididymis, via the vas deferens, and into the urinary tract (urethra) of the male sex organ at the onset of ejaculation during a woman's fertile days, sperm must enter the vagina for conception to occur, when migratory sperm attach to and enter the egg, fertilization occurs (Ezzati *et al.* 2019).

2.3.1 Gonadotropin-releasing hormone (GnRH)

Since its 1971 discovery in the swine hypothalamus as one of the early hypothalamic releasing hormones by a team headed by Nobel laureate Andrew V., GnRH synthesis has been an essential part of human reproduction. The pituitary gland and the gonadal axis are involved in this process. GnRH is a decapeptide. Schally, GnRH has been a focus of study scientists due to its essential function in reproduction, this is because GnRH was discovered by a team that included Andrew V. Schally (Limonta *et al.* 2012).

The brain secretes GnRH, which travels to the pituitary gland and triggers the synthesis of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones influence the testes to start the process of spermatogenesis and to develop secondary sex traits in males. Both of these mechanisms are carried out by GnRH. Several steps in human spermatogenesis, such as spermatogonia growth and spermatogenesis advancement, are regulated by GnRH, the principal neuroendocrine regulator of reproductive function (Chen *et al.* 2001).

It is believed that humans have between 1,000 and 1,500 neurons dedicated to GnRH, which is transported to the anterior pituitary via the portal circulation after being

secreted into the fenestrated capillaries of the portal circulation by the median elevation. The GnRH cell bodies, which are located in the medial preoptic region (POA) and the arcuate/infundibular nucleus of the hypothalamus, form a neural network with median eminence projections (Stamatiades *et al.* 1997). After its production by neurons in the hypothalamus, specifically in the infundibular nucleus, GnRH controls the secretion of sex hormones by the gonads, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), through its action on the anterior pituitary. This is so due to the fact that the hypothalamus is responsible for producing both FSH and LH (Marques *et al.* 2022).

Research conducted in the last ten years after the identification of the Kisspeptin-Neurokinin B-Dynorphin (KNDy) neural network suggests that it plays a crucial role in regulating the HPG axis (Fink 2000, Tena-Sempere 2005).

Chromosome 8's short arm is where the human GnRH I gene is located, this gene's structure is made up of four exons and three introns. (8p21-p11.2), the medial olfactory placode is the starting point of GnRH's trip, which continues, the two principal gonadotropins, follicle-stimulating hormone and luteinizing hormone, are then stimulated to signal by binding to the gonadotropin-releasing hormone receptor (GnRHR) on pituitary gonadotrophic cells (Stamatiades *et al.* 2018).

GnRH's Biological and Clinical Relevance Reproductive health and pubertal development depend on proper LH pulse frequency regulation, when compared to adulthood, LH pulsatile secretion is elevated in infancy (commonly referred to as "mini-puberty"), likely due to pulsatile GnRH production, but it quickly becomes quiet (Pohl *et al.* 1995). GnRH cannot be measured in peripheral blood since it is produced into the portal circulation and has a short half-life, instead, measures of FSH and LH levels in the blood are used to evaluate the hypothalamic-pituitary function (Ubuka *et al.* 2018).

2.3.2 Follicle stimulating hormone (FSH)

In response to pulsatile stimulation by the hypothalamic-generated gonadotropin-releasing hormone (GnRH), the glycoprotein polypeptide known as FSH is produced and secreted by the gonadotropic cells of the anterior pituitary gland (Stamatiades *et al.* 2018). Following secretion, FSH travels to the gonads, where it is crucial for the maturation of the testicles and the sperm generation process in men (Santi *et al.* 2020). FSH augments the trophic effect of Sertoli cells on tubular spermatogenic cells, Sertoli cells enhance the proliferation and development of spermatogonia into spermatozoa (Mruk *et al.* 2015).

In order for FSH to transduce signals to specific tissues, activate various intracellular effectors, and trigger gene transcription, a specific G protein-coupled receptor (FSHR) must be present (Casarini *et al.* 2019). Molecular weight and isoform specificity are affected by the carbohydrate side chain heterogeneity that the anterior pituitary gland produces in its many FSH isoforms. Gonadotropins and thyrotropins have the same FSH component, which is responsible for their biological specificity; this subunit may contain two asparagine-linked carbohydrate side chains (Bousfield *et al.* 2018).

The ovary controls oocyte selection, folliculogenesis, and the production of sex steroid hormones through the synthesis of follicle stimulating hormone (FSH), which is essential for normal development and reproduction in animals. This sets the stage for fertilisation, implantation, and pregnancy (Messinis *et al.* 2014). This gonadotropin encourages the development and maturation of sperm in male testicles, which contributes to sexual reproduction (Huhtaniemi *et al.* 2015).

The pituitary gonadotrope cells secrete the hormone in response to the pulsatile regulation carried out by the hypothalamus gonadotropin-releasing hormone (GnRH). This is achieved by activating signals on the surface of target cells in the gonads of both sexes that are related to hormone-induced cell proliferation and apoptosis. One part of FSH interacts solely with the G-protein coupled receptor (GPCR) that regulates FSHR. However, in silico and crystallographic structural investigations revealed an interaction

between the subunit and FSHR, suggesting that receptor binding is not limited to the subunit alone (Jiang *et al.*2014).

After receptor conformational changes brought on by hormone binding, the signal is transduced via direct protein interactions at the plasma membrane. This sets in motion a series of biochemical reactions that, when combined, comprise a very complicated signalling network (Jiang *et al.*2012).

FSH increases human spermatogenesis both independently and via a process that partly overlaps with testosterone, spermatogenesis requires autocrine, paracrine and endocrine stimulation, which FSH and testosterone provide (Oduwole *et al.* 2018).

The number of Sertoli cells is determined, spermatogonial proliferation and metabolic and structural support are provided, meiotic development is promoted until the spermatid stage is reached, and nutrient metabolism and transport to the germ cells are facilitated by testosterone in the later stages, while FSH typically acts in the beginning stages, the FSH-dependent phase (Figure 2.4). Through the FSH receptor FSHR, FSH is able to connect with sertoli cells (Kangasniemi *et al.* 1990). In contrast, testosterone is created by the action of luteinizing hormone (LH) on Leydig cells, and there is an amount of testosterone that is between 50 and 100 times more in the testes than there is in the blood (Roth *et al.* 2010). By boosting the transcription of genes involved in DNA replication and cell cycle control in Sertoli cells, FSH modulates the final number of Sertoli cells between infancy and adolescence, this takes place between the ages of 0 and 14 (Zimmermann *et al.* 2015).The circulating levels of FSH are directly related to the number of the adult Sertoli cells and testicular volume (Figure 2.4) (Cheng *et al.* 2002).

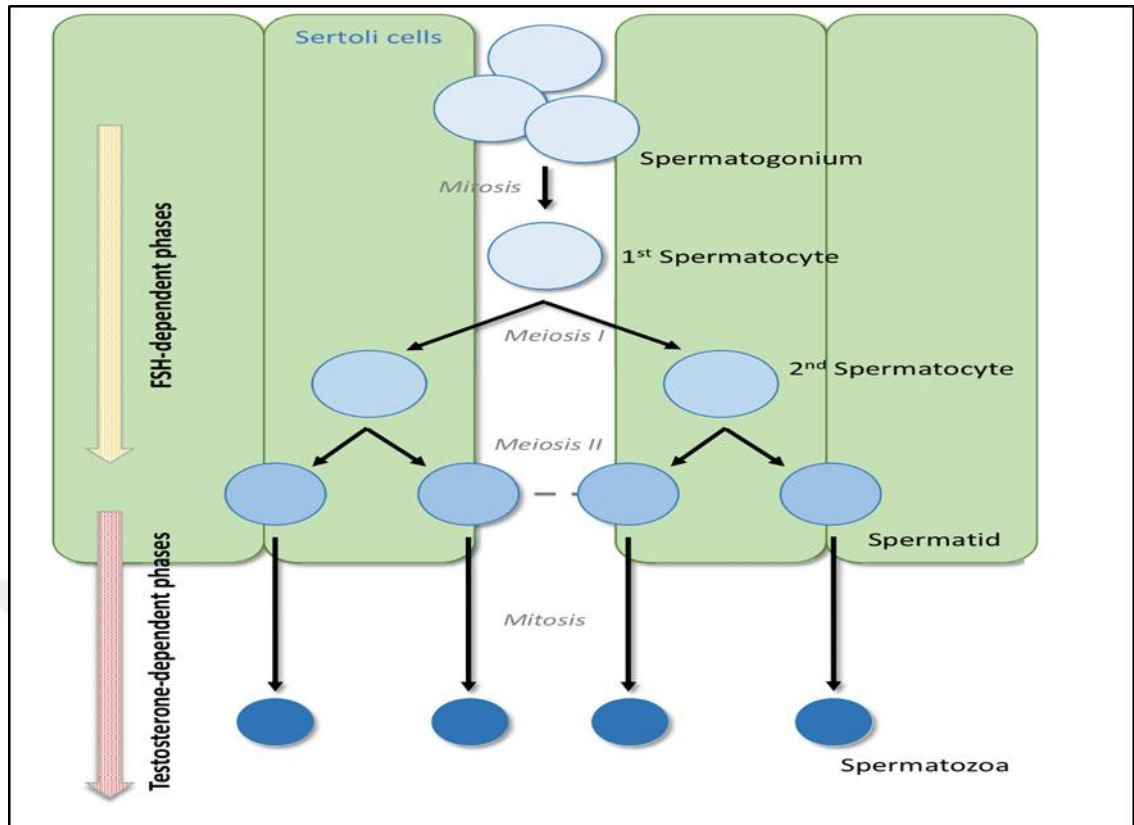


Figure 2.4 The role of FSH and testosterone in spermatogenesis (Simoni and Santi 2020)

2.3.3 Lutropin hormon LH

Because of its action on the interstitial Leydig cell to accelerate steroidogenesis and testosterone production in males, the lutrophin hormone (LH), secreted by the anterior pituitary gonadotropic cells, is also known as the interstitial cell-stimulating hormone (ICSH) (Czieselsky *et al.*2016).

LH activities based on sertoli cells are complementary to those of FSH, hence it indirectly regulates spermatogenesis and maintains sperm quality, leydig cells express LH receptors on their plasma membranes, leydig cells contain around 15,000 LH receptors (Rhoades 2012). It has been shown that a receptor occupancy of 5 % or less is sufficient for LH-mediated effects on the testicles (Casarini *et al.*2018).

Gs-protein coupled LH receptors are responsible for the activation of adenylyl cyclase, Thus, intracellular cAMP production is increased and protein kinase activation, after this, phosphorylation of cholesterol esterase occurs, which frees cholesterol from its storage inside the cell, and/or activation of cytochrome P450 11A1, which begins the process of steroidogenesis ,both of these events are then followed by the release of cholesterol(Strauss *et al.*2013).

Pulses of GnRH at a high frequency in the hypothalamus are believed to increase anterior pituitary LH production, when plasma testosterone levels are inadequate, hypothalamic GnRH increases pituitary LH production, while negative feedback limits GnRH and LH release when testosterone levels are enough, the aromatization of testosterone to estradiol suppresses LH secretion (Pitteloud *et al.*2008).

2.3.4 Testosterone

The stimulation of steroidogenesis in Leydig cells by LH results in the production of androgens, which may regulate their own production by influencing the release of LH through a negative feedback loop; testosterone and its 5-reduced derivative dihydrotestosterone have the highest biological activity, in addition to HPG hormones, androgenic sex steroids are required to maintain regular spermatogenic cycles (Tsai *et al.*1994).

The testis is responsible for producing almost 95% of the testosterone that is circulating in adult males; the adrenal glands are responsible for producing the remaining 5%. The release of testosterone into the bloodstream occurs along a concentration gradient; eventually, the ratio of free hormone to protein-bound testosterone is 2% (Corradi *et al.* 2016).

The significance of testosterone in maintaining the integrity of the spermatogenesis process is critical, Testosterone is produced by converting cholesterol through a

steroidogenesis process initiated by the Steroidogenic Acute Regulatory Protein or StAR protein (Pereira *et al.* 2021).

Research shows that the StAR protein acts as a biomarker of steroidogenesis activity in a number of ways, one of these is that the blocking of this protein can cause the hypothalamus-pituitary-testis axis to break down, which then affects LH secretion (Widhiantara *et al.*2021). Aside from making Leydig cells work better for steroidogenesis, LH and androgens are also important for the growth and differentiation of Leydig cells (Plunk *et al.* 2020). An indirect effect thought to be produced by testosterone aromatization into estradiol is that testosterone suppresses the hypothalamic production of GnRH in the testis via negative feedback (Krajewska *et al.* 2013).

Regardless of the underlying etiology, exogenous androgens generally inhibit gonadotropin release, which reduces intratesticular testosterone levels and decreases spermatogenesis, an accidentally high level of testosterone can be caused by medical treatment, but it can also happen when people use anabolic steroids without a doctor's permission (Dohle *et al.*2003). During spermatogenesis, testosterone is essential for survival and maintenance, especially in the four most crucial phases. About two-thirds of testicular testosterone is either not attached to albumen at all or only weakly attached, while the remaining one-third is strongly associated with either sex hormone-binding globulin or testicular androgen-binding protein (ABP) (Smith *et al.* 2014) through :

1. It keeps the blood-testis barrier from breaking down by helping to put the machinery back together on the bottom of the spermatocyte after the old blood-testis barrier has been broken down.
2. There are several processes that depend on testosterone signalling for meiotic divisions, and when it is inhibited, spermatogenesis stops during meiosis. This means that very few spermatogonia reach the haploid spermatid stage and that elongated spermatid production stops. This impacts sperm quantity and quality, as well as cellular stresses, unfolded protein responses, DNA damage, changes in regulatory proteins required for RNA splicing, post-translational modifications, and oxidative damage.

3. When testosterone signaling isn't working right, the round spermatid are released from the Sertoli cells before they should be because the connection between the Sertoli cell and the long spermatid breaks.

4. Even though the mature sperms are discharged correctly throughout the stage, if there is insufficient testosterone, they are retained and eventually consumed by the Sertoli cells.

2.4 The Molecular Genetics of Male Infertility

It is known that hereditary factors are responsible for 30–50% of instances of male infertility (Rhouma *et al.* 2018). Depending on their frequency and severity, genetic diseases may induce various types of infertility, ranging from small modifications in spermatogenesis to full failure of the gonads, the process of spermatogenesis is regulated by a precise cascade of on/off gene expression (Kasak and Laan 2021).

Male infertility may be brought on by the chromosomal or monogenic defects, mutations in the mitochondrial DNA (mtDNA), deletions of the Y-chromosome, multifactorial diseases, imprinting disorders or genetically based endocrine problems, the spermatogenesis process depends on around 10% of the genome's genes. 15–30% of male infertility instances are caused by genetic abnormalities, which might result in permanent partial or complete spermatogenesis cessation (Zhang *et al.* 2020).

Chromosome abnormalities, or changes to the genetic machinery at the chromosome level, mutations, which may affect a single gene or a set of genes, and X chromosome abnormalities are the most prevalent genetic causes of male infertility. The majority of male infertility cases are caused by chromosomal abnormalities, DNA fragmentation (the breaking of DNA), and other genetic factors (Cariati *et al.* 2019). X and Y chromosome abnormalities account for three quarters of male infertility cases, while other chromosome abnormalities make up the remaining 5 to 15%. Chromosome abnormalities, which are numerical and structural chromosomal aberrations detectable

under the microscope > 4-5 Mb, represent 5 to 10% of oligozoospermia cases, whereas nonobstructive azoospermia patients are 15 to 25% affected (Dada *et al.* 2007).

The majority of azoospermia cases involve sex chromosome abnormalities; however, the most common form of oligozoospermia, which can be inherited, involves imbalances in the organisation of autosomal chromosomes; and neither form of this disorder is associated with the gain nor loss of genetic material (Dada *et al.* 2007).

Although mosaic individuals with a normal 46XY cell line may be oligozoospermic, males with the chromosomal complement 47,XXY are azoospermic due to seminiferous tubule dysgenesis. Studies on Klinefelter male sperm chromosomes show that the extra chromosome is lost during spermatogenesis, which is a great predictive factor for assisted reproductive technology (ART) and a demonstration of the presence of distinct foci of spermatogenesis. Therefore, identifying the normal cell line is very necessary (Martin *et al.* 2008). Even if the majority of these children have normal chromosomal complements, the danger of producing a kid with chromosomal abnormalities is high (Kailash *et al.* 2021).

Sperm from males with a nonmosaic 47XXY karyotype ranges from 2% to 45% of the time, whereas sperm from Klinefelter mosaics ranges from 1.5% to 7%. (Okutman 2018, Rives 2018). The abnormal translocation of Y material, including the sex-determining region (SRY), to the X-chromosome during paternal meiosis gives rise to the 46XX male chromosomal complement. This happens because the long arm of the Y chromosome is absent, and the presence of the SRY gene causes testicular differentiation rather than spermatogenesis (Dada *et al.* 2002).

There are two forms of well-known chromosomal aberrations: balanced and unbalanced (Artamonov *et al.* 2020). Balanced chromosomal rearrangements, such as inversions, translocations, and marker chromosomes, do not result in the loss or gain of genetic material, but rather in its relocation within the genome; accordingly, carriers of these rearrangements are often phenotypically normal and healthy (Mohammadi *et al.* 2019), in contrast, the emergence of imbalanced rearrangements, deletions and duplications, is

associated with significant issues and is characterized by the replacement of gene dosage ratios, Y-chromosome microdeletions occurring in the locus Azoospermia factor area (AZF) are one of the most prominent genetic causes of male infertility, alongside karyotype disorder, 7 to 10 % of all cases of secretory azoospermia have alterations at this locus (Anifandis *et al.* 2018).

The AZF locus is not the main determinant of spermatogenesis, sterility and suppression of spermatogenesis may be caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, which leads to the severe hereditary disease cystic fibrosis (Pittsley *et al.* 2022).

Rearrangements and point mutations in the Androgen receptor (AR) gene cause testicular feminization or Reifenstein's disease and considerably, >40 % contribute to male infertility, the functional activity of the androgen receptor (AR) is reliant on the number of cytosine–adenine–guanine (CAG) repeats that correspond with the blood levels of free testosterone (Mauvais-Jarvis *et al.* 2020), the reduction in receptor sensitivity is inversely proportional to the number of CAG repeats, the likelihood of developing oligo- and azoospermia rises as the number of CAG-repeats increases, the SRY gene is an example of a gene that contributes to the development of the male reproductive system and sexual differentiation, it is connected with a wide variety of phenotypic and clinical symptoms, ranging from full sex reversal to the underdevelopment of the male gonads (Bosco *et al.* 2018). It consists of double- and single-stranded DNA breaks linked to aberrant chromatin packing or protamine insufficiency during spermatogenesis (Liu *et al.* 2021).

2.4.1 Genes associated with male infertility

Evidence suggests that several genes on the Y-chromosome regulate spermatogenesis, and it has long been known that certain genes on the Y-chromosome's long arm are crucial for normal spermatogenesis (e Silva 2018). Errors can occur in the Y chromosome q11 band because it contains genes that are important for spermatogenesis and because it contains many palindromic sequences, which are genomic sequences that

are similar in polarity and mirror images of each other. These sequences make the band an important tool for studying the genetic causes of male infertility (Rives,2014).

While homologous palindromic sequences prevent chromosomal recombination errors like inversions and gene deletions—which can have a devastating impact on spermatogenesis—intra-chromosomal crossover prevents chromosomal recombination errors like these from occurring (Manotas *et al.* 2020).

2.4.2 Gene mutations and male infertility

Obstructive azoospermia is caused by CFTR gene mutations , which is located on the chromosome 7, these mutations are detected in between 60 and 90 percent of persons who have congenital bilateral azoospermia (Sharma *et al.* 2008).

The acrosin gene is one of several autosomal genes that play a role in germ cell development and sperm production. Another important autosomal gene for fertility is the follicle stimulating hormone receptor gene, which codes for the protein of the same name and is located on chromosome 2. While there is less evidence connecting variants of this gene to male infertility, variations of this gene do affect female FSH receptivity. Several studies have linked alterations to this gene to poor spermatogenesis (Kuijper *et al.* 2011).

2.4.2.1 Mutations of CFTR gene

The gene known as cystic fibrosis transmembrane conductance regulator (CFTR) spans 230 kb and is situated on the long arm of chromosome 7q31.2. The normal allele of this gene produces an mRNA measuring 6.5 kilobases, which in turn encodes a protein with 1480 amino acids that is bound to the cell membrane. its protein acts as a regulated chloride channel in many kinds of epithelial cells; the CFTR gene is in charge of its function (Hwang *et al.* 2010).

The autosomal recessive mode of inheritance is used to transmit CFTR-related diseases from one generation to the next (Yahaya *et al.* 2020). Congenital bilateral absence of the vas deferens (CBAVD), also referred to as obstructive azoospermia, is a type of obstructive azoospermia caused by the separation of the epididymis and the ejaculatory duct, there is a positive correlation between CBAVD and cystic fibrosis (Tahmasbpour *et al.* 2014). According to O'Brien *et al.* (2010), CFTR gene mutations were present in the bodies of 60 to 90 percent of people who were diagnosed with CBAVD.

Men with CBAVD usually have two mild CFTR gene mutations or a mix of a relatively mild mutation and a severe mutation; this is because CFTR mutations are the cause of CBAVD, which makes the genetics of the disease extremely complex (Jamalvandi *et al.* 2018).

The most prevalent form of a severe mutation, a 3-bp deletion (F508del), is seen in 60-70% of individuals with CBAVD and causes the CFTR polypeptide to lack phenylalanine at position 508 (Moskowitz *et al.* 2005). In addition, 21% of the participants had the 5 T polymorphism in intron 8 of the CFTR gene. The level of mutation associated with this polymorphism is likely to be modest (Sertic *et al.* 2001).

G551D, G542X, R553X, N1303K and W1282X were other common mutations that occur at a frequency of 1-2 %, treatments options are available to males with CFTR mutations that produce obstructive azoospermia, including intracytoplasmic sperm injection, testicular sperm extraction, and microsurgical epididymal sperm aspiration (Georgiou *et al.* 2006).

2.4.2.2 Follicle stimulating hormone receptor (FSHR) gene

Follicle stimulating hormone receptor or FSHR, is necessary for reproduction, normal spermatogenesis cell activity, including the synthesis of FSH by spermatogenesis cell, is dependent on the expression of the FSHR gene (Jardon *et al.* 2018). Although FSHR, LHR, and TSHR are all members of the class A glycoprotein hormone receptors

subfamily, the effect of FSHR gene expression on male infertility is not well understood, even though the combination of FSH and FSHR SNPs is critical for predicting patient risk and treatment outcome in infertility cases (Simoni and Casarini 2014).

The link between FSHR polymorphism regions and male infertility has been proven in a large number of case-control studies and meta-analyses. The FSHR is a member of the glycoprotein-coupled receptor family, unique to Sertoli cells, and it goes by the acronym 7TMR because of its seven trans-membrane domains (Gheorghiu 2019, Crosnoe *et al.* 2013).

In males, FSH regulates the development and survival of spermatogonial cells by binding to the architecturally complex FSHR on the surface of testicular Sertoli cells (Siegel *et al.* 2013). Similar to the (TSH HCG and LH) glycoprotein hormone receptors, FSHR proteins are 695 amino acids long and belong to the family of G-protein-coupled transmembrane protein receptors (GPHRs). The first 17 amino acids of these proteins encode the signal sequence (Wopara *et al.* 2021).

The FSHR is unique because it contains a very long NH₂-terminal extracellular domain with possible glycosylation sites that bind to various gonadotropins in various ways, on the surface of Sertoli cells is a protein called an FSH receptor, which has the potential to bind FSH and play a role in the activation and deactivation of the receptor (Asatiani *et al.* 2002).

A large N-terminal region and three loops make up the extracellular hydrophilic domain (ECD) of the FSHR structure (Figure 2.5). This domain connects to a hydrophobic region with seven transmembrane domains (TMDs), and there are three intracellular loops and a C-terminal region of the intracellular hydrophilic domain (Lindgren 2017). On the short arm of chromosome 2, in the 2p21-p16.3 region, between base pairs 48,953,160 and 49,154,526, is a single-copy gene that encodes the human FSHR. The study conducted by (Wang *et al.* 2022). With 10 exons varying in size from 69 to 1,234 base pairs, the gene spans around 190 kb (Sperduti *et al.* 2021).

Exons 1–9 encode most of the extracellular domain, including the hinge region; exon - 10 encodes the C-terminal extracellular domain hinge region, the seven transmembrane domain (a domain with three intracellular and three extracellular loops), and the C-tail of the intracellular domain (Haldar *et al.* 2022).

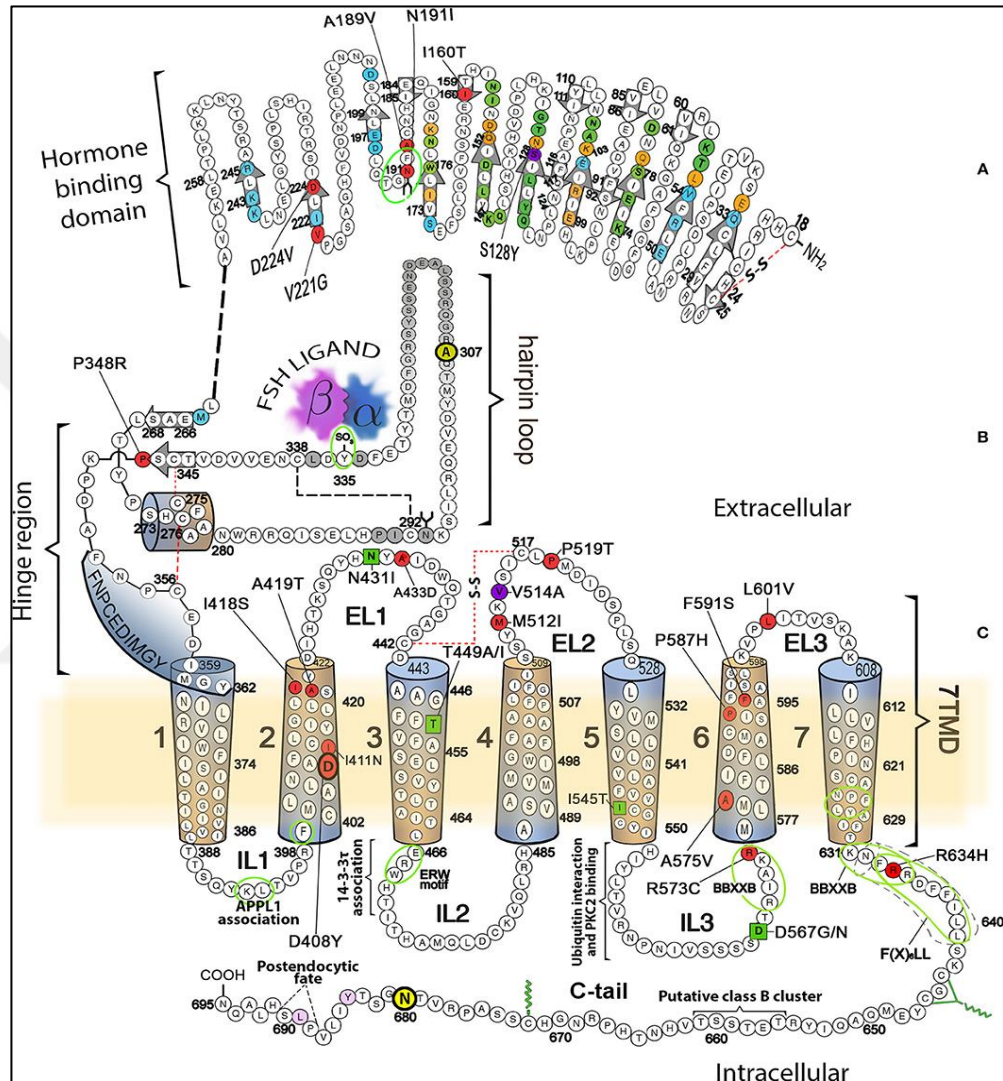


Figure 2.5 Shows the FSHR domains and amino acid sequences involved in agonist binding, activation, and signal transmission

A: HBD. The high affinity-binding location is hidden in the FSH/FSHR interface (orange indicates residues that interact with both FSH subunits; green indicates binding to the FSH α -subunit alone). The colorless arrows show beta strands on the concave or convex surfaces of ECD. Magenta (S128Y) denotes promiscuous ligand binding,

whereas red represents function loss. Many mutations impact the process of receptor trafficking. B: The hinge region, where the green oval is located, includes a sulfated tyrosine (at position 335) that is crucial for ligand-induced binding to FSH subunits, C:7TMD. Squares in green represent mutations that gain-of-function, whereas circles in red represent mutations that lose-of-function (Ulloa-Aguirre *et al.* 2018).



3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

As shown in Table 3.1, the following pieces of equipment were used in the present investigation.

Table 3.1 The study's instruments and where they were made

Equipment	Origin
Centrifuge	Universal/ Germany
Cobas e 411 analyzer	Roche/ Germany
Digital camera	Samsung/ china
Exispin vortex centrifuge	Bioneer/ Korea
Gel electrophoresis	Bioneer/ Korea
High Speed Cold centrifuge	Eppendorf /Germany
Incubator	Memmert/Germany
Light Microscope	Olympus/ Japan
Microcentrifuge tubes	Biobasic/ Canada
Micropipettes 5-50, 0.5-10,100-1000 μ L	CYAN/ Belgium
Microwave	Argose/Germany
Nano drop UV- Spectrophotometer	THERMO / USA
Refrigerator	Concord /Lebanon
Sensitive Balance	Sartorius/Germany
Thermocycler PCR	BioRad/ USA
UV Transilluminator	ATTA/ Korea
Vortex	CYAN/ Belgium
Water Bath	Memmert/Germany

3.1.2 Tools

Table 3.2 lists the instruments that were used in this investigation.

Table 3.2 The tools

The Tool	The Origin
Glass staining jars	China
Graduated classic cylinder	Marienfeld / Germany)
Plain tube	Barcpharma / Jordan
Vacuum collection tube (Gel clot activator tube)	China
EDTA tube	China
Sample cup micro	Germany
Micropippite	China
Syringe 5cc	Denmark

3.1.3 Kits

The kits which have been used in current study are mentioned in Table 3.3.

Table 3.3 The kits used in current study with their origin.

Kit	Origin	Lot No.
Elecys FSH kit	Roche \ Germany	62576901
Elecys LH kit		57071301
Elecys Testosterone kit		62075901
gSYAN DNA- Extraction Kit	Geneaid \ Taiwan	FE02612
GoTaq® G2 Green Master Mix kit	Promega \USA	0000518115

3.1.4 Primers

As shown in the tables below, the primers for the FSHR and CFTR gene polymorphisms were designed using the NCBI-SNP database and the Primer1 online amplification-refractory mutation system (ARMS-PCR) design. The primers were provided by Scientific Research Co. Ltd. of Iraq:

Table 3.4 The CFTR gene $\Delta F508$ mutant (C/T) ARMS-PCR primers, together with their sequence and amplicon size

Primer	Sequence (5'-3')	Product size
Wildtype .Forward Primer.	GTATCTATATTCATCATAGGAAACACCAC	157bp
Mutant. Forward Primer	GTATCTATATTCATCATAGGAAACACCAT	
Common .Reverse Primer	GACTTCACTTCTAATGATGATTATGGGAG	

Table 3.5 The ARMS-PCR primers for FSHR gene (rs1394205) G/A with their sequence and amplicon size

Primer	Sequence (5'-3')	Product size
Wildtype. G Primer	AGGTTTTTCTCTGCAAATGCAGG	567bp
Mutant .A Forward Primer	AGGTTTTTCTCTGCAAATGCAGA	
Common .Reverse Primer	GCCTCCAATTCTGGGTCTCC	

3.1.5 Chemicals

See Table 3.6 for a list of the compounds utilised in this investigation.

Table 3.6 All the chemicals materials that used in current study

Chemical	Origin
Absolute Ethanol	Scharlau \Spain
Agarose	BioBasic\ Canada
DNA Marker Ladder (100bp)	INtRON \ Korea
Ethidium Bromide	BioBasic \ Canada
PCR Premix	Bonier/ KOREA
Primers	Bonier/ KOREA
TBE buffer 10X	BioBasic \ Canada

3.1.6 Additional materials

Other materials that were used in this study are as described in Table 3.7.

Table 3.7 Additional materials

Materials	Origin
Collection tube (2mL)	Roche \ Germany
dNTPs (dATP, dCTP, dGTP, dTTP)	Geneaid \ Taiwan
Elution buffer	Roche \ Germany
GD column	Roche \ Germany
GSB buffer solution	Roche \ Germany
GST buffer solution	Roche \ Germany
KCl	Geneaid \ Taiwan
Loading dye	Geneaid \ Taiwan
MgCl ₂	Geneaid \ Taiwan
Proteinase K 10 mg/mL	Roche \ Germany
Taq DNA polymerase	Geneaid \ Taiwan
Tris.HCl (pH 9.0)	Geneaid \ Taiwan
W1 buffer	Roche \ Germany

3.2 Methods

3.2.1 Design of the study

The present study includes number of 150 samples , were divided into two groups according to the following arrangement:

Patients group: A number of seventy- five infertile men their age ranging from 20 -45 years old Individuals were selected from Infertility Unit, Al-Nasiriyah Teaching Hospital, who had infertility.

Healthy group: Include a number of 75 healthy men in age arranging between 20 to 45 years old .

Male patients were drawn from Infertility Unit, Al-Nasiriyah Teaching Hospital, Thi-Qar, Iraq. To examine masculinity and marital infertility , the clinical cases of the patients were collected after being nominated by the urologist , on other hand some cases were exclusion biased on following criteria were:

1. After comprehensive physical examinations, any detectable reason of infertility determined by genetic disorder laboratory testing.
2. Any immune-related disorders, infectious infections, or significant systemic illnesses that have been identified.
3. abnormalities of the adrenal glands, thyroid issues, or overt hyperprolactinemia.
4. Abnormal psychological stress .
5. Vasectomy, testicular surgery, or other iatrogenic mass infections of the male reproductive system.

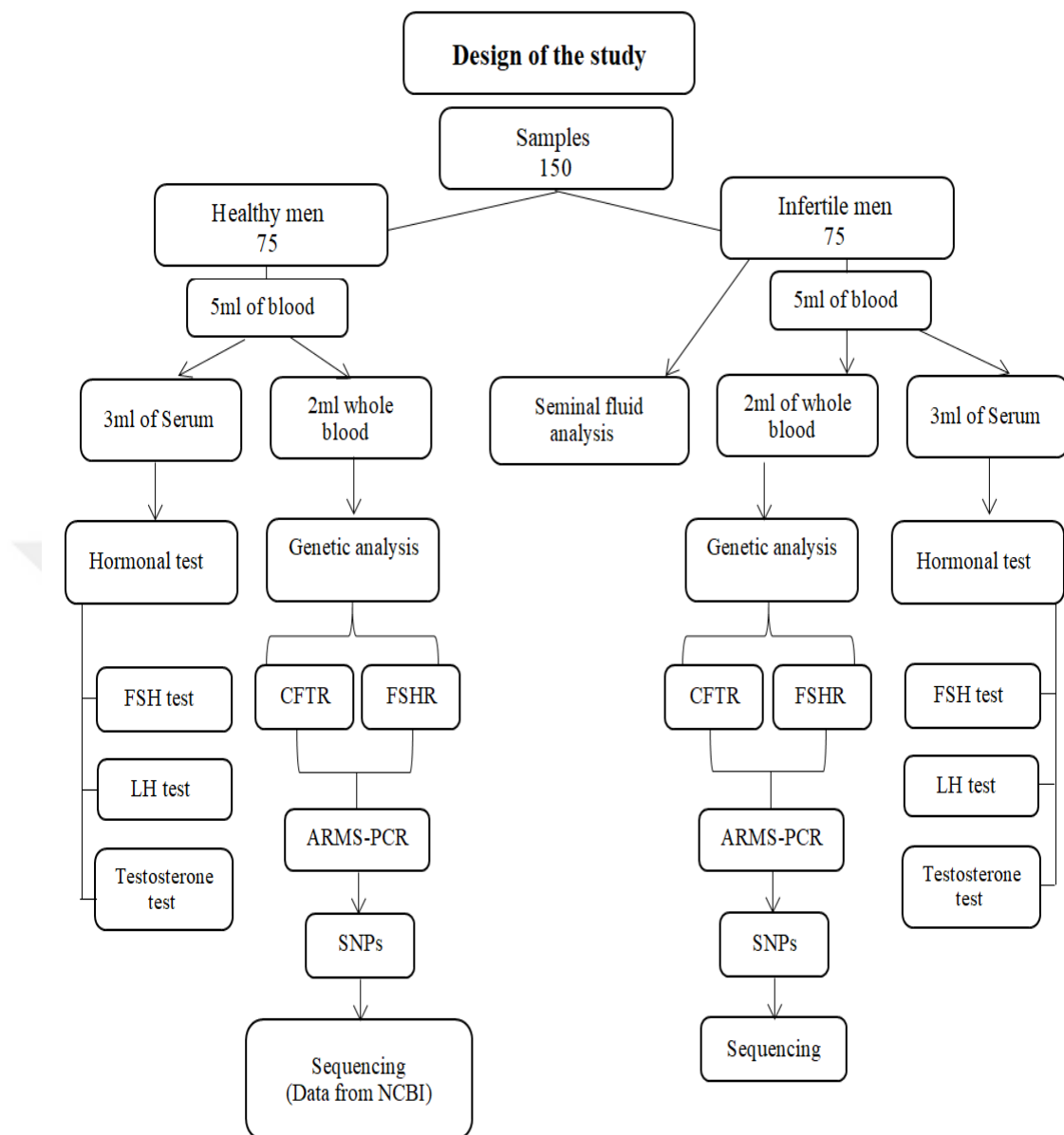


Figure 3.1 Design of the study. The amplification-refractory mutation system ARMS - PCR Allele specific-PCR, (FSH) Follicle-stimulating hormone, (LH) Luteinizing hormone, (SNPs) Single nucleotide polymorphisms

3.2.2 Permissions and ethical consideration

The approvals were obtained from the relevant authorities from Thi Qar Health Office - researches committee (Thi-Qar \ 2022049), In order to conduct the interview and collect the samples, agreement from the patients was obtained. Before any samples were collected or interviews were conducted, patients verbally agreed to participate in the

study after already being informed of its aim, as illustrated in sample of questionnaire (Appendix 1).

3.2.3 Semen collection and analysis

Masturbation in sterile polypropylene containers near the lab was used to collect semen samples from men, both fertile and infertile, after they had abstained from sexual activity for three to five days. Because of this, the time it took to go from collecting semen to analysing it was much shorter. We measured the volume, pH, colour of the seminal fluid, time it took for the semen to liquefy, and viscosity of each sample. The morphology, concentration, motility, and viability of the sperm were assessed by microscopically examining the samples. Next, the value that was obtained is compared to the reference values from the WHO handbook (Paasch *et al.* 2008).

3.2.4 Sample collection

Both healthy and infertile males had their intravenous blood samples separated into two parts. In the first portion, 2 mL of blood was deposited in tubes with Ethylene Diamine Tetra Acetic Acid (EDTA), an anticoagulant. Genomic DNA was extracted from the sample. Concerning the second part, 3 mL of the blood sample was transferred to anticoagulant-free tubes, also called gel tubes, and allowed to coagulate at room temperature in order to extract the serum. Following separation, the serum samples were promptly frozen at -20 C to facilitate future chemical testing, barring immediate usage.

3.2.5 Biochemical tests

3.2.5.1 Follicle-stimulating hormone test

Follicle-stimulating hormone in human serum was quantitatively determined using the FSH hormone test kit. Using the Cobas e411 immunoassay analyzers with the

Electrochemiluminescence Immunoassay ("ECLIA") technique in accordance with the manufacturer's instructions.

3.2.5.1.1 The principle of the assay

This test is based on the sandwich principle. This testing will last around 18 minutes.

- Starting with 40µL of the sample—a biotinylated monoclonal FSH-specific antibody—and a monoclonal FSH-specific AB tagged with a ruthenium complex (Tris (2,2'-bipyridyl)—makes up the first phase in the procedure, which is generating a sandwich complex. Beastall *et al.* (1987) described ruthenium(II)-complexes as Ru(bpy).
- A second round of incubation is required to bind the complex to the solid phase once the streptavidin-coated microparticles have been added.

Aspiration of the mixture inside the measurement cell wherever the magnetically attracted microparticles are on top of the electrode surface, followed by ProCell/ProCell M use to eliminate any unbound materials, by utilizing a photomultiplier to detect the release of chemiluminescent after applying voltage to the electrode.

- The final results were determined using a calibration curve.

3.2.5.2 Luteinizing hormone test

Luteinizing hormone in human serum was quantified using an immunoassay test kit by using Cobas e411 immunoassay analyzers which utilize "ECLIA." the electrochemiluminescence immunoassay.

3.2.5.2.1 The principle of the assay

Based on the sandwich principle. It took approximately 18 minutes to complete this test.

- During the first incubation, a sandwich complex is formed by reacting 20µL of the sample with a monoclonal LH-specific antibody that has been biotinylated and tagged with a ruthenium complex.
- During the second incubation step, the complex is linked to the solid phase as a result of the interactions between streptavidin, biotin, and the streptavidin-coated microparticles.
- After the microparticles were magnetically attached to the surface of the electrodes in the measuring cell, the mixture was removed from the cell. following which removing unattached substances using ProCell/ProCell M. Electrode voltage generation produces chemiluminescent radiation that the photomultiplier can pick up on.
- The final values were determined using a calibration curve.

3.2.5.3 Testosterone test

Proposed use testosterone in human serum and plasma can be determined quantitatively in vitro using an immunoassay. The Elecsys and Cobas e immunoassay analyzers are designed for use with the electrochemiluminescence immunoassay "ECLIA" (Wheeler 1995).

Elecsys Testosterone II is a competitive test assay that uses a sheep high affinity monoclonal antibody that is specifically targeted against testosterone. The biotinylated antibody's binding sites are competed for by endogenous testosterone produced from the sample by 2 bromoestradiol and a testosterone derivative labelled with a ruthenium complex.

3.2.5.3.1 The principle of the assay

Based on the Competition principle. It took approximately 18 minutes to complete this test.

- The first step is to incubate 20 mL of material with a testosterone-specific biotinylated monoclonal antibody. Depending on the concentration of the sample analyte, it binds to the binding sites of the labelled antibody.
- In the second incubation step, biotin and streptavidin interact to bind the complex to the solid phase. This occurs after adding streptavidin-coated microparticles and a testosterone derivative labelled with a ruthenium complex.
- The microparticles are magnetically attracted to the surface of the electrode after the measuring cell aspirates the reaction mixture. After that, all unbound materials are removed using ProCell/ProCell M. Next, a photomultiplier picks up the chemiluminescent emission that results from applying voltage to the electrode (Bablok *et al.* 1988).
- With the use of a master curve supplied by the reagent's barcode or e-barcode and a calibration curve developed for the instrument using the two-point calibration method, the results were achieved.

3.2.6 Genotyping

3.2.6.1 Genomic DNA extraction

A rapid and easy method is provided by extraction kit (Frozen Blood) Geneaid \ USA for the extraction of intact and purified DNA from blood. A micro-centrifuge tube and a binding column are used to process the samples. Per purification, 200µL of blood may be processed. High-quality DNA from the extracted genome can be employed in routine procedures such agarose gel examination, restriction enzyme processing, and PCR analysis.

Cell and RBC Lysis Step, RNase Treatment Step (Optional), DNA Rehydration Step Protein Precipitation (PPT) Step, DNA Precipitation Step, and are the 5 steps that constitute DNA Extraction , genomic DNA from blood sample were extracted by utilized extraction kit, and done according to company instructions as the following steps:

- A sterile 1.5 mL microcentrifuge tube was used to transfer 200µL of frozen blood and 30µL of proteinase K. The mixture was mixed using a vortex and then incubated at 60 °C for 5 minutes.
- Next, each tube was supplemented with 200µL of the lysis buffer GSB, and the contents were vigorously mixed using a vortex. After that, the tubes were placed in an incubator set at 70 °C for 10 minutes. Every three minutes, they were turned over to ensure even incubation.
- The lysate was stirred vigorously immediately after the addition of 200µL of 100% ethanol.
- A 2 mL collection tube was used to transport the whole mixture, including any precipitate, to the DNA filter column. After centrifuging the column at 10,000 rpm for 5 minutes and removing the 2 mL collecting tube with the flow through, it was inserted in a fresh tube.
- After 400µL of W1 buffer was added to the DNA filter column, it was centrifuged at 10,000 rpm for 30 seconds. The collected liquid was discarded while the column was returned to the 2 mL collecting tube.
- Before centrifuging the columns at 10,000 rpm for 30 seconds, 600µL of Washing Buffer (Ethanol) was added to each column. The flow through was then withdrawn, and the columns were returned to the 2 mL collecting tube.
- To remove any remaining moisture from the column matrix, each tube was spun in a centrifuge at 10,000 rpm for three more minutes.
- Next, 50µL of pre-heated elution buffer was added to the column matrix after transferring the dried DNA filter column to a clean 1.5 mL microcentrifuge tube.
- Utilising a Nanodrop spectrophotometer, the genomic DNA isolated from the blood sample was examined for DNA concentration (ng/L) and DNA purity (260 /280 nm) to ensure accurate results.
- We left the tubes in for at least 5 minutes to make sure the matrix absorbed all of the elution buffer. Centrifuging the samples at 10,000 rpm for 30 seconds eluted the pure DNA.

3.2.6.2 The genomic DNA estimation

A Nanodrop spectrophotometer (THERMO / USA) was used to assess the genomic DNA isolated from blood. The instrument assessed the content of DNA in nanograms per millilitre (ng/L) and verified the DNA's purity by measuring absorbance at 260 / 280 nm. The following processes were followed for the evaluation:

- After launching the Nanodrop application, the correct programme was chosen (DNA, nucleic acid).
- The measurement pedestals were dry-wiped many times for cleaning. The bottom measuring pedestals were meticulously filled with 2 μ L of free nuclease water to blank the system.
- The pedestals were cleaned and 1 μ L of blood genomic DNA was introduced to the measurement after lowering the sample arm and initialising the Nanodrop by pressing OK.

3.2.6.3 The amplification-refractory mutation system (ARMS) Assay

The NCBI-SNP database and Primer1 ARMS-PCR primer design website were used in current study to create ARMS-PCR primers for the CFTR gene F508 mutant (C/T) gene polymorphism, on the other hand, was created by (Jafari *et al.* 2022). FSHR (rs1394205) G/A gene polymorphism, these primers were supplied by Scientific Researcher Co. Ltd. in Iraq as the Table 3.3 and Table 3.4.

3.2.6.3.1 The amplification-refractory mutation system (ARMS) -PCR master mix preparation

The GoTaq®.G2 Green Master Mix kit ,was utilized to prepare the ARMS-PCR master mix, and this master mix was used to perform two reactions for each sample in accordance with the Table 3.8.

Table 3.8 The components of the ARMS-PCR reaction Mix preparation

Wild type allele. ARMS-PCR. reaction Mix	
ARMS-PCR Master mix	Volume
Common Primer. (10pmol)	2 μ L
DNA template	5 μ L
G2. .Green Master.Mix	12.5 μ L
PCR water	3.5 μ L
Wild type .primers (10pmol)	2 μ L
Total volume	25 μ L
Mutant type allele ARMS- PCR reaction Mix:	
ARMS-PCR Master mix	Volume
Common Primer (10pmol)	2 μ L
DNA template	5 μ L
G2 Green Master Mix	12.5 μ L
Mutant type primers (10pmol)	2 μ L
PCR water	3.5 μ L
Total .volume	25 μ L

After that, the PCR thermocycler was loaded with the ingredients from Table 3.8's PCR master mix, and the mixture was centrifuged at 3,000 rpm for three minutes using an Exispin vortex centrifuge.

3.2.6.3.2 ARMS-PCR thermocycler conditions

The conditions stated in Table 3.9 for the ARMS-PCR thermocycler.

Table 3.9 PCR thermocycler conditions

PCR step	Temp.	Time	repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30 sec.	35cycle
Annealing	58°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

3.2.6.3.3 ARMS-PCR product analysis

Using Agarose gel electrophoresis, the following procedures were followed to examine the ARMS-PCR products:

1X TBE was used to create a 2% Agarose gel, which was then dissolved in a water bath at 100 °C for 15 minutes before being allowed to cool to 50 °C.

Following that, 3µL of ethidium bromide dye was added to the agarose gel solution.

Following correct alignment of the comb, a solution of agarose gel was gently poured into the tray. The comb was then left to harden at room temperature for fifteen minutes before being delicately removed.

1X TBE buffer was added to the electrophoresis chamber's gel tray, which was fixed therein.

Ten microliters of PCR product was added to each comb well, while three microliters of 100 bp ladder was applied to the first well.

For one hour, the electric current was operated at 80 AM and 100 V.

The ARMS-PCR products were visualised using UV transilluminators.

4. RESULTS AND DISCUSSION

4.1 Demographic Characteristics of Male Patients with Infertility and Control Subjects

The present investigation included 75 guys who were unable to conceive as well as 75 men who seemed to be in good health. Table 4.1 provides a concise summary of the demographic data for both the control and patient groups. With a mean age of 29.28 ± 6.38 years for patients and 29.97 ± 6.33 years for control participants, there were no statistically significant variations in mean age between the two groups ($P = 0.506$). Table 4.1 also showed the age distribution of the controls and patients. Again, when looking at the age distribution of patients and controls separately, no statistically significant difference was found ($P = 0.709$).

The aforementioned findings have guaranteed statistical parity between the patient group and the control group with regard to age, which is a prerequisite for such a case-control study.

Table 4.1 Patient and control group demographics related to infertility

Characteristic	Patients <i>n</i> =75	Control <i>n</i> = 75	<i>P</i>
Age (years)			
Mean ±SD	29.28 ± 6.38	29.97 ± 6.33	0.506 † NS
Range	20.00-40.0 years	20.0-40.0 years	
20-25, <i>n</i> (%)	20 (26.7 %)	21 (28.0 %)	0.709 ¥ NS
26-30, <i>n</i> (%)	28 (37.3 %)	22 (29.3 %)	
31-35, <i>n</i> (%)	10 (13.3 %)	10 (13.3 %)	
36-40, <i>n</i> (%)	17 (22.7 %)	22 (29.3 %)	
(¥): Chi-square test; (†): independent samples t-test; (<i>n</i>): number of cases; (NS): not significant at <i>P</i> > 0.05 ; (SD): standard deviation.			

4.2 Subjects Hormonal Analysis Results

The evaluating of serum Follicle-stimulating hormone (FSH) level between male patients with infertility and healthy controls groups has been performed ,Patients with

infertility exhibited median serum FSH levels which were higher compared to the control groups, 3.43 (3.77) versus 2.18 (1.95), the difference was significant at ($P = 0.001$). The comparison of serum Luteinizing Hormone (LH) levels between patients with infertility and healthy controls groups has been performed, Median levels of serum LH in patients with infertility were higher in contrast to the median levels of control groups, 5.64 (3.76) versus 3.35 (1.86), the difference was significant ($P = 0.001$). Median levels of serum Testosterone Hormone in patients with infertility were lower than in comparison the median levels of control groups, 3.82 (2.85) versus 5.42 (1.55), the variations were significant at ($P = 0.001$). The findings are listed in the Table 4.2 and Figure 4.1 , Figure 4.2 and Figure 4.3.

Table 4.2 Median serum Hormones levels of male patients with infertility and healthy controls

	Cases –control comparison		
Hormones	patients with infertility <i>n</i> = 75	Healthy control <i>n</i> = 75	<i>P</i>
FSH Hormone			
Range	1.34- 12.80	0.88-5.34	0.001 † S
Median (IQR)	3.43 (3.77)	2.18 (1.95)	
LH Hormone			
Range	1.58- 11.70	2.14-6.93	0.001 † S
Median (IQR)	5.64 (3.76)	3.35 (1.86)	
Testosterone Hormone			
Range	1.23- 10.20	3.73-8.41	0.001 † S
Median (IQR)	3.82 (2.85)	5.42 (1.55)	
(†): Mann Whitney U test ; (n): number of cases;(S): significant at P < 0.05 ; (SD): standard deviation.			

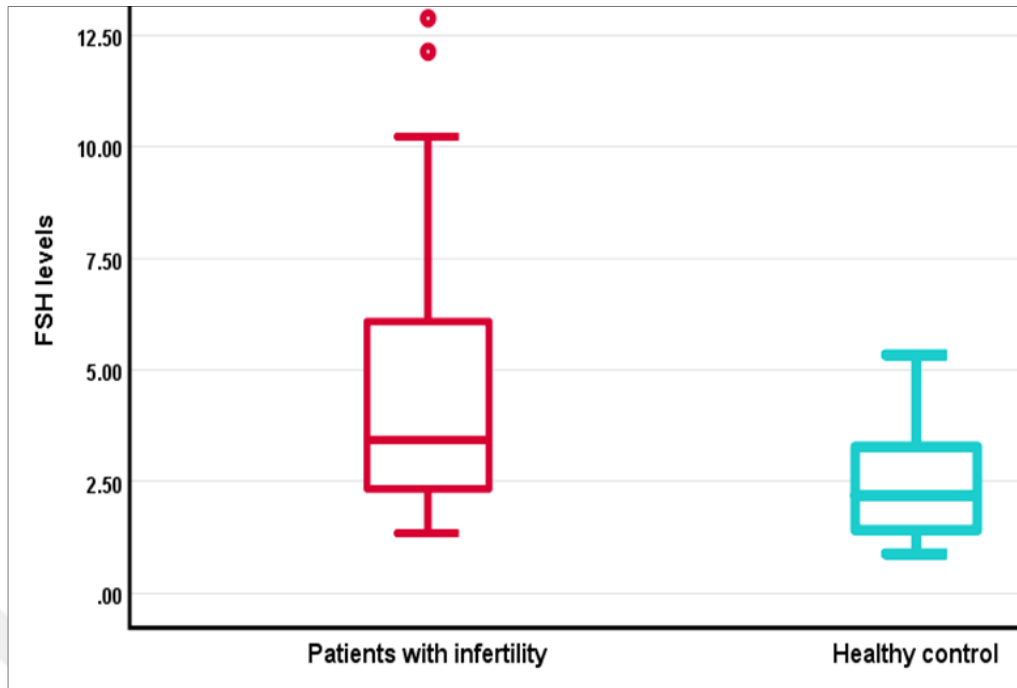


Figure 4.1 Median serum FSH levels in infertile male patients and healthy controls are shown in a box plot

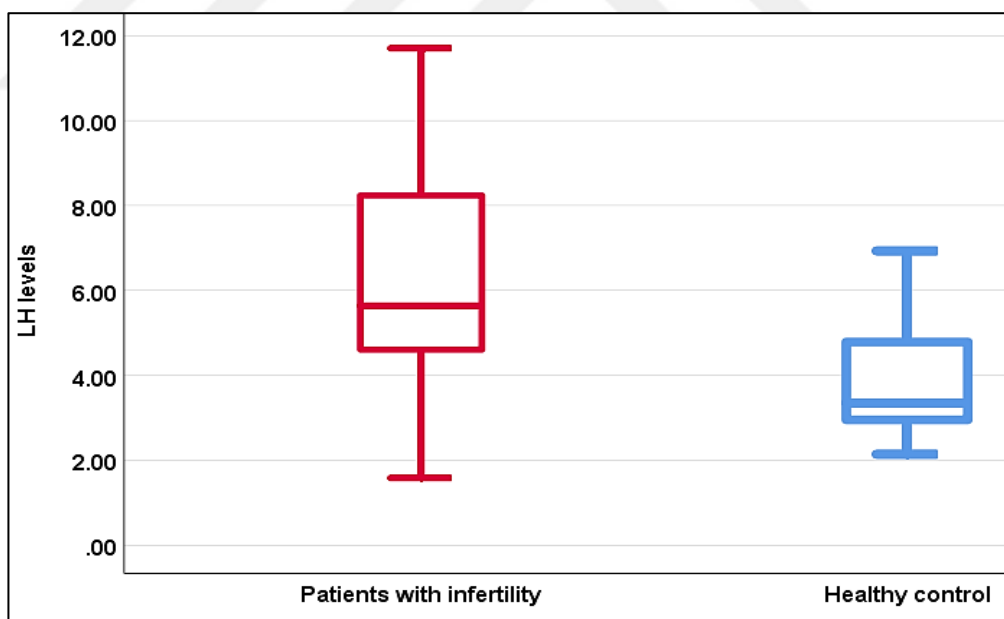


Figure 4.2 Box plot shows the comparison of the Median serum LH levels of male patients with infertility and healthy controls

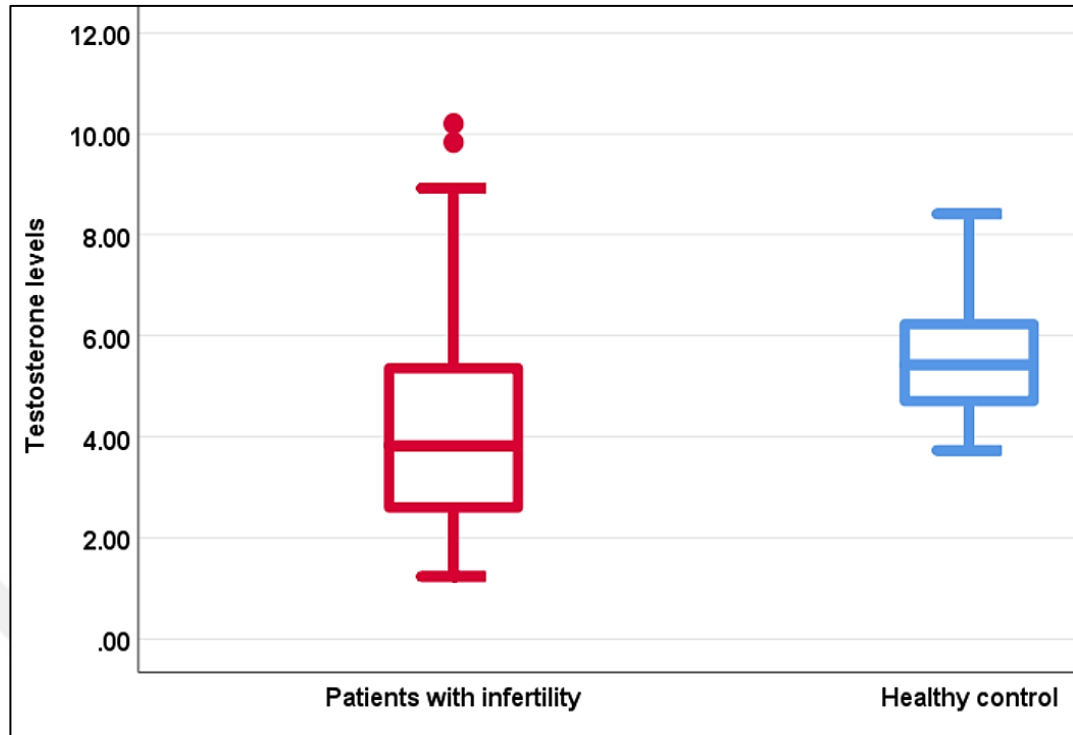


Figure 4.3 Box plot shows the comparison of Median serum Testosterone levels of male patients with infertility and healthy controls

Male infertility has many causes, such as hypothalamic-pituitary disorders , genetic disorders. However, in about 50% of cases, the reason for male infertility is still unknown (Elezaj *et al.* 2020).

The hypothalamus-pituitary-gonad axis controls male infertility as a complicated hormone-regulated disorder, the primary regulators of the development of germ cells are FSH, LH, and testosterone hormones (Nieschlag *et al.* 2011),

Impaired sperm production and function may be caused by several inherited or added adaptive variables that operate at the post-testicular, pretesticular, or directly at the testicular level (Mittal *et al.* 2017).

The three primary hormones that influence the maturation and growth of sperm in adult males are testosterone (T), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Spermatogenesis requires the coordination of follicle-stimulating hormone

(FSH) and endogenous testosterone, which must be maintained in order to ensure the survival of germ cells, proper spermatogenesis, and male fertility (Oduwole *et al.* 2018). Both FSH and LH affect the testes, although in distinct ways. LH helps the interstitial Leydig cells mature so that they can produce FSH, and T stimulates and maintains spermatogenesis by acting on the seminiferous tubules (Prior *et al.* 2018).

Patients with infertility in the current study exhibited median serum FSH levels which were higher compared to the control groups, 3.43 (3.77) versus 2.18 (1.95), the difference was significant ($P = 0.001$), these results were consistent with (Al-Nahi 2015), A reliable biomarker of germinal epithelial injury is thought to be a higher FSH levels (Zhang *et al.* 2003), significantly increased in serum FSH levels correlates with infertility in men that was reported by (Zalata *et al.* 2008).

The comparison of serum Luteinizing Hormone (LH) levels In present study, between patients with infertility and healthy controls groups has been performed. Median levels of serum LH in patients with infertility were higher in contrast to the median levels of control groups, 5.64 (3.76) versus 3.35 (1.86), the difference was significant at ($P = 0.001$), the comparing of serum Testosterone Hormone level in present study, between patients with infertility and healthy controls groups, median levels of serum Testosterone Hormone in patients with infertility were lower than in comparison the median levels of control groups, 3.82 (2.85) versus 5.42 (1.55), the difference was significant ($P = 0.001$), these results agree with what was performed by (Nayyfe *et al.* 2018), another study in the Iranian population, by Safarinejad *et al.* (2010) resulted in a greater FSH level in the infertile males compared to the control group, as well as lower serum LH and T levels; nevertheless, the differences between the two groups were not statistically significant ($P = 0.08$ and $P = 0.06$, respectively).

Infertile men had substantially higher serum FSH levels compared to the control group, significantly lower testosterone levels, and no significant association with serum LH. These findings are consistent with previous research on the Palestinian population (Nahal *et al.* 2017) and the Albanian population (Elezaj 2020). However, serum LH levels were shown to be considerably greater in the current investigation.

Current diagnostic procedures for subfertile couples include monitoring basal FSH in order to assess the prognosis of spermatogenesis. Low testosterone levels may be a sign of hypogonadism that originates in the pituitary or the hypothalamus, whereas elevated blood FSH levels in men indicate infertility due to testicular failure (Babu *et al.* 2004).

Table 4.3 and Figure 4.4 display the findings of the Receiver Operator Characteristic (ROC) curve analysis that was conducted to assess the FSH cutoff value and to forecast male infertility as a diagnostic adjunct. The TSH cutoff value was > 2.49 fold with negative predictive value (NPV), positive predictive value (PPV), sensitivity, specificity, and area under curve of 72.0 %, 72.0 %, 72.0 %, 72.0 % and 0.721 (0.631-0.810).

Table 4.3 Sensitivity and specificity of FSH levels (> 2.49 -fold) in male infertility

FSH level	Male infertility patients <i>n</i> = 75	Healthy control <i>n</i> = 75
> 2.49	54 (%)	21 (%)
< 2.49	21 (%)	54 (%)
Sensitivity %	72.0 %	
Specificity %	72.0 %	
PPV %	72.0 %	
NPV %	72.0 %	
AUC (95% CI)	0.721 (0.631- 0.810)	
(AUC):Area under curve, (CI): Confidence interval, (NPV): negative predictive value, (n): Number of samples, (PPV): positive predictive value.		

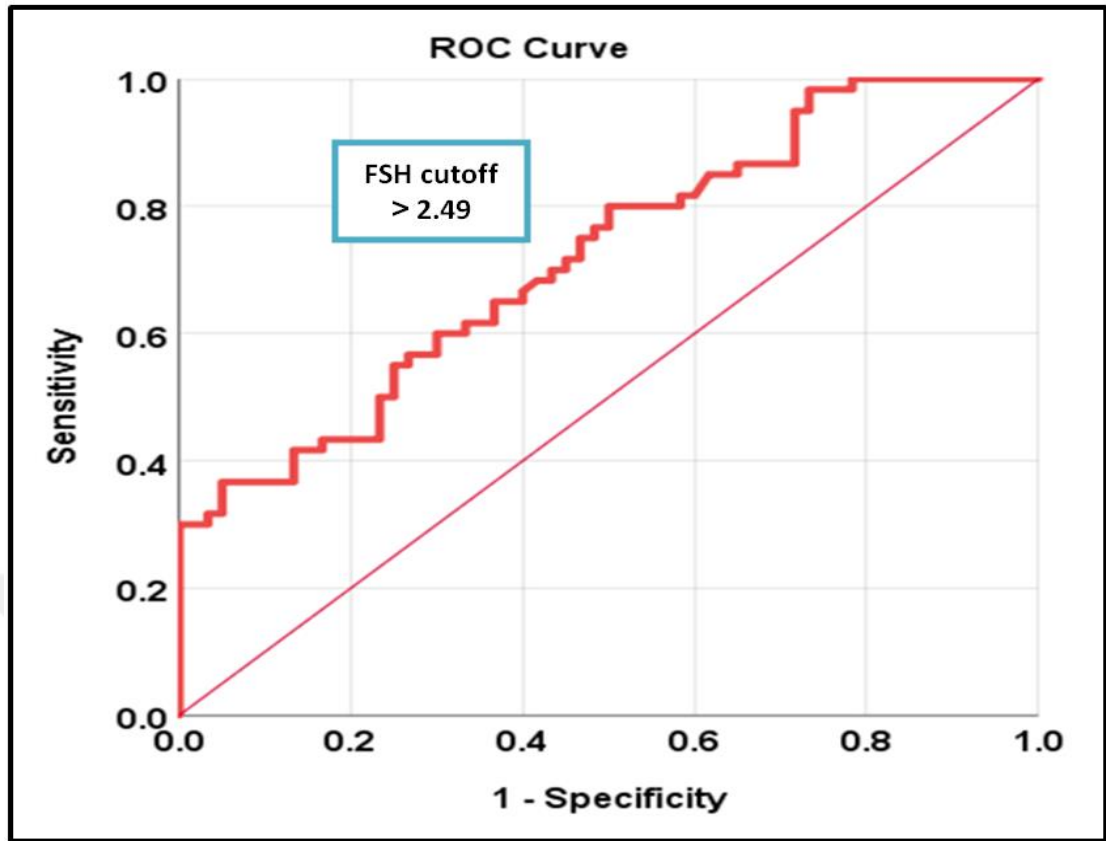


Figure 4.4 Analysis of FSH levels using the receiver operator characteristic curve to determine a potential diagnostic cutoff value

Table 4.4 and Figure 4.5 show the findings of the receiver operator characteristic (ROC) curve analysis, which was used to assess the LH cutoff value and forecast male infertility as adjuvant diagnostic tests or diagnostic tests. The LH cutoff value was > 4.78 -fold with negative predictive value (NPV), positive predictive value (PPV), sensitivity, specificity, and the area under curve of 78.7 %, 78.7 %, 78.7 %, 78.7 % and 0.799 (0.720- 0.878).

Table 4.4 The Sensitivity and specificity of LH levels (> 4.78 -fold) in male infertility

LH level	Male infertility patients <i>n</i> = 75	Healthy control <i>n</i> = 75
> 4.78	59 (%)	16 (%)
< 4.78	16 (%)	59 (%)
Sensitivity %	78.7 %	
Specificity %	78.7 %	
PPV %	78.7 %	
NPV %	78.7 %	
AUC (95% CI)	0.799 (0.720- 0.878)	

(AUC):Area under curve, (CI): Confidence interval, (NPV): negative predictive value, (n): Number of samples , (PPV): positive predictive value.

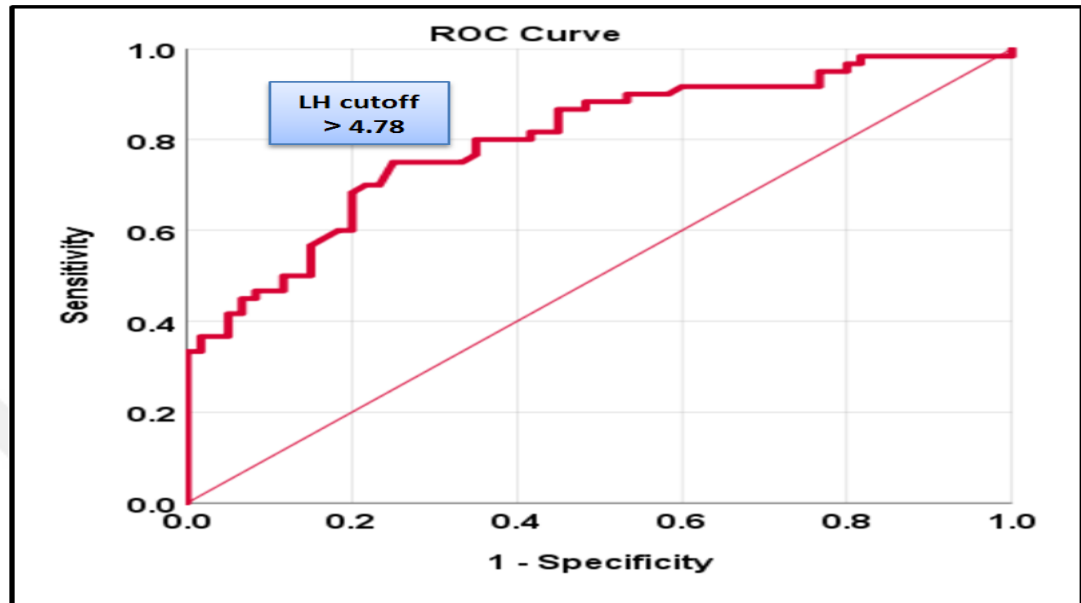


Figure 4.5 The examination of LH levels using the receiver operator characteristic curve allows for the determination of potential

To evaluate the Testosterone cutoff value and forecast male infertility as a diagnostic test or adjuvant diagnostic tool, ROC curve analysis was used. Table 4.5 and Figure 4.6 display the findings. The Testosterone cutoff value was < 4.60 -fold with, positive predictive value (PPV), sensitivity, specificity negative predictive value (NPV), and area under curve of 73.3 %, 81.3 %, 79.7 %, 75.3 % and 0.740 (0.644- 0.837).

Table 4.5 A high testosterone level (>4.6 -fold) is a sensitive and specific marker for male infertility

Testosterone level	Male infertility patients <i>n</i> = 75	Healthy control <i>n</i> = 75
< 4.6	55 (%)	15(%)
> 4.6	20 (%)	60 (%)
Sensitivity %	73.3 %	
Specificity %	81.3 %	
PPV %	79.7 %	
NPV %	75.3 %	
AUC (95% CI)	0.740 (0.644- 0.837)	

(AUC):Area under curve, (CI): Confidence interval, (n): Number of samples , (PPV): positive predictive value, (NPV): negative predictive value.

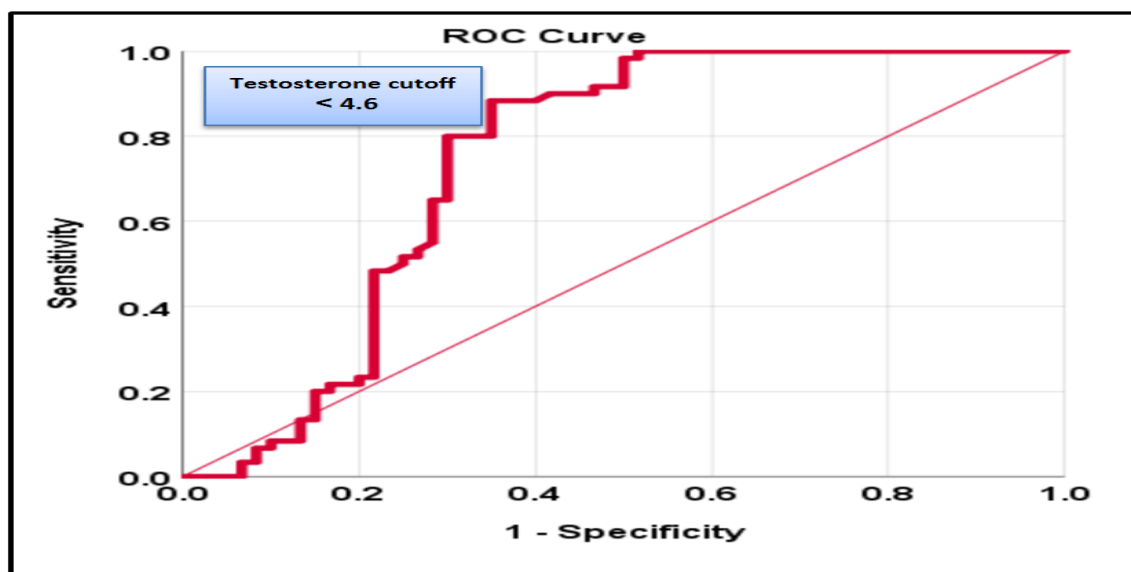


Figure 4.6 Analysing testosterone's receiver operator characteristic curve to determine potential

Current study findings for Testosterone hormone test shows predictive of infertility with a specificity (81.3 %) compared to LH (78.7%) and FSH (72.0%) respectively. In conclusion, determining the levels of the reproductive hormones FSH, LH, and testosterone may identify potential male infertility.

4.3 Molecular Analysis

4.3.1 DNA extraction

The genomic DNA was extracted from blood samples using the (gSYAN DNA) kit extraction method (Frozen Blood) Geneaid. Then, it was measured using a Nano drop spectrophotometer at 260/280 nm. The results showed a range of 1.8-2.2, an average purity of 2, and an average DNA concentration of 20 ng/μl.

4.3.2 Detection of genes polymorphisms

4.3.2.1 Detection of CFTR Δ F508 (C/T) polymorphism

CFTR F508 (C/T) Polymorphism distribution was detected by using ARMS-PCR technique. Three genotypes—CC, CT, and TT—are present at this location. Amplification of the C allele alone The product size in the wild type homozygote genotype was found to be 157 bp. The mutant type homozygote genotype showed just T allele amplification at 157 bp product size. In contrast, the heterozygote genotype showed C and T allele amplification at 157 bp product size, respectively, as shown in the figure (4.7). In all study groups, there was no deviation from Hardy-Weinberg equilibrium in the genotype distribution.

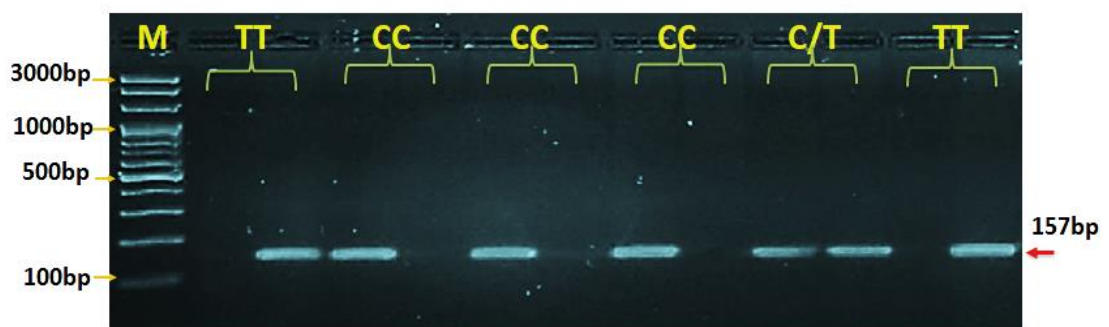


Figure 4.7 The ARMS-PCR product analysis of the CFTR gene Δ F508 (C/T) gene polymorphism in patient samples is shown in the agarose gel electrophoresis picture

(M): marker (3000-100bp). The CC wild type homozygote was solely shown as carrying the C allele. In contrast to the (C/T) heterozygote, who displays both the C and T alleles, the lane (AA) mutant type homozygote displays just the T allele. At the 157-base pair size, the presence of the C or T allele is detected.

Using the Hardy-Weinberg equation, we can see in Table 4.6 how the three CFTR F508 (C/T) genotypes—CC, CT, and TT—were distributed among the control group members. Of the 75 control groups, 32 had the wild CC homozygous genotype, 26 had the CT heterozygous genotype, and 17 had the mutant TT homozygous genotype.

The demonstrated distribution of control subjects by CFTR F508 (C/T) genotypes did not differ significantly from the predicted distribution ($P = 0.055$).

Table 4.6 Hardy Weinberg equation

Genotypes	Observed	Expected	χ^2	<i>P</i>
Homozygote reference CC	32	27.0	5.787	0.055
Heterozygote CT	26	36.0		¥
Homozygote variant TT	17	12.0		NS
¥: Chi-square test; NS: Non-significant at <i>P</i> > 0.05				

Infertile patients and healthy controls were compared with respect to the CFTR F508 (C/T) SNP genotypes and allele frequencies in Table 4.7. In terms of co-dominant mode, the frequency distribution of genotypes did not change significantly ($p = 0.201$) between the control and patient groups. Both the homozygous TT genotype (OR=1.54) and the heterozygous C/T genotype (OR=1.95) were not shown to be significant risk variables according to the risk analysis. This means that compared to those with other genotypes, those with the heterozygous C/T genotype have around a twofold increased risk of having reproductive problems. When comparing the patient and control groups using dominant and recessive modes as well as allele analysis, no statistically significant difference was found ($P > 0.05$).

Table 4.7 CFTR $\Delta F508$ (C/T) POLY genotype frequency in Patients with infertility and healthy control

Mode	CFTR $\Delta F508$	Patients with infertility $n = 75$	Healthy control $n = 75$	P	OR	95% CI
Co-dominant	TT	18 (24.0%)	17 (22.7 %)	0.201	1.54	0.65-3.62
	C/T	35 (46.7%)	26 (34.7 %)	¥	1.95	0.93 -4.11
	CC	22 (29.3 %)	32 (42.6 %)	NS	Reference	
Dominant	TT+C/T	53 (70.7%)	43 (57.4 %)	0.088	Reference	
	CC	22 (29.3 %)	32 (42.6 %)	¥ NS	0.557	0.28-1.09
Recessive	TT	18 (24.0%)	17 (22.7 %)	0.846	1.07	0.55-2.29
	C/T+CC	57 (76.0%)	58 (77.3 %)	¥ NS	Reference	
Alleles	T	71 (47.3%)	60 (40.0 %)	0.200	1.34	0.85-2.13
	C	79 (52.7 %)	90 (60.0 %)	¥ NS	Reference	
¥: Chi-square test; NS: Non-significant at P < 0.001.						

Genetic causes are suggested to be involved in 15-30% of occurrences of male infertility cases (Krausz *et al.* 2015), currently, idiopathic infertility affects 30% of men (Sinclair 2000). Long-term exposure to potentially destructive factors might result in occupational diseases, permanent alterations to the reproductive system (such as diminishing sperm quality or spermatogenesis issues), or even infertility. (Sharpe 2010), Numerous research from various nations have demonstrated that the pathophysiology of male infertility is directly influenced by mutations in the CFTR gene (AlMaghamisi *et al.* 2020), this is, much further as we are knowledge, the first study to investigate at the influence of CFTR gene polymorphism on male infertility metrics in the Iraqi population.

The deletion of three nucleotides, specifically three bases (CTT) at locations 1,653-1,655 in exon 11, causes the most common mutation (F508del), which in about 70% of instances leads to the loss of phenylalanine at protein position 508. (Korf 2003). As a consequence, exocrine gland epithelial cells express much less CFTR protein and aberrant protein folding is caused by the loss of CFTR protein at residue F508. The most common mutation in white individuals, known as p.F508del, occurs in 35–74.6% of cases (Kumar *et al.* 2014).

Researchers have found that the heterozygous C/T genotype is nearly twice as likely to have reproductive problems as other genotypes, but neither the homozygous TT genotype (OR=1.54) nor the heterozygous C/T genotype (OR=1.95) were significant risk factors in the CFTR risk analysis. Consistent with the findings of the research by Stuppia *et al.*, no statistically significant difference was found between the patient and control groups for the dominant mode, recessive mode, or allele analysis ($P > 0.05$). There was no significant difference in the CFTR gene between the control group and patients in a research conducted in 2005 on 1,195 infertile and non-infertile Italian men. This pertains to the CFTR $\Delta F508$ (C/T) gene as it pertains to infertile patients. Despite the fact that the frequency distribution of genotypes does not change significantly between the control and afflicted groups. The results are in line with

previous studies that have looked at CFTR mutations; for example, a study conducted by Chillon *et al.* (1995) on patients from Belgium, France, Spain, and the US, and another study by Dayangac *et al.* (2004) on Turkish patients, both found that genotype TT was associated with a significantly higher risk of infertility than genotype CC. Also there was increased risk of infertility with genotype CT versus CC as Odd ratio was 1.95, and may be considered as etiological fraction for infertility, The occurrence of this mutation is low in Asians, The frequency of mutations within the CFTR gene differs in different ethnicities. which was validated by Leonardi *et al.*(2013).

The CFTR gene is distributed throughout the reproductive system and may contribute to male infertility through interactions with other ion channels and transporters (Chavez *et al.* 2012). Conversely, this channel has been shown to play a crucial part in sperm function via HCO-3 production and its interference with sperm's fertilisation abilities. Human sperm cells have this channel, and it affects both sperm function and male fertility (Jafari *et al.* 2022).

Nevertheless, the gathered evidence strongly establishes the hypothesis that CFTR plays a crucial role in spermatogenesis by controlling sertoli cell as well as germ cell functions at different stages of spermatogenesis, deficiencies of which can as observed in patients with a high prevalence of CFTR mutations, result in impaired sperm production and function (Chen *et al.* 2012).

4.3.2.2 Detection of FSHR (rs1394205) G/A polymorphism

ARMS-PCR was used to identify the distribution of the FSHR (rs1394205) G/A polymorphism. GG, GA, and AA are the three genotypes present at this locus. Only the G allele was amplified in the wild type homozygote genotype at 567 bp product size. The A allele was the only one amplified by the mutant type homozygote genotype at 567 bp product size. In contrast, the heterozygote genotype demonstrated G and A allele amplification respectively, at 567 bp product size, Figure 4.8. In all study groups, the genotype distribution did not deviate from Hardy-Weinberg equilibrium.

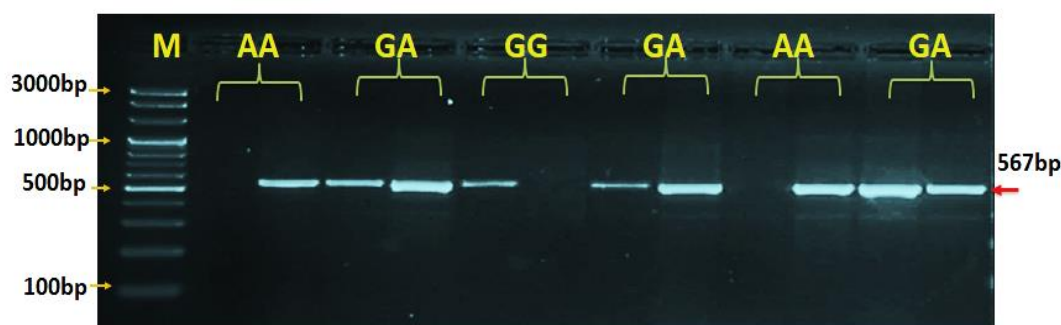


Figure 4.8 The ARMS-PCR product analysis of the FSHR gene (rs1394205) G/A gene polymorphism in patient samples is shown in the agarose gel electrophoresis picture

(M) marker (3000-100bp). The GG wild type homozygote is solely shown as having the G allele. At 567 base pairs, we can see if an A or G allele is present in a mutant homozygote (lane AA), but in a (G/A) heterozygote, we only see the A allele.

Table 4.8 displays the results of the Hardy-Weinberg equation computed to determine the distribution of the FSHR (rs1394205) G/A genotypes GG, GA, and AA within the control group. The control group included 33 individuals with the homozygous wild GG genotype, 27 individuals with the heterozygous GA genotype, and 15 individuals with the homozygous mutant AA genotype.

The observed distribution of control subjects by G/A genotypes for the FSHR gene (rs1394205) was not significantly different from the expected distribution ($P = 0.063$).

Table 4.8 Hardy weinberg equation

Genotypes	Observed	Expected	χ^2	<i>P</i>
Homozygote reference GG	33	28.83	5.540	0.063
Heterozygote GA	27	35.34		¥
Homozygote variant AA	15	10.83		NS
¥: Chi-square test; NS: Non-significant at <i>P</i> > 0.05.				

Table 4.9 shows the results of a comparison between infertile patients and healthy control groups with regard to the FSHR (rs1394205) G/A SNP genotypes and allele

frequencies. The frequency distribution of genotypes with respect to co-dominant mode differed significantly ($P=0.001$) between the control group and the sick group. The results of the risk analysis showed that both the heterozygous G/A genotype and the homozygous AA genotype were significant risk factors; however, the homozygous AA genotype was found to be approximately six times more likely to cause infertility than the other genotypes. There was a very significant difference in the dominant mode, recessive mode, and allele analysis between the patient and control groups at ($P>0.001$).

Table 4.9 *FSHR* (rs1394205) G/A poly genotype frequency in Patients with infertility and healthy control

Mode	<i>FSHR</i> (rs1394205)	Patients with infertility <i>n</i> = 75	Healthy control <i>n</i> = 75	<i>P</i>	OR	95% CI
Co-dominant	AA	37(49.3%)	15 (20.0 %)	0.001	6.26	2.6-15.7
	G/A	25 (33.3%)	27 (36.0 %)	¥	2.35	1.01 -5.45
	GG	13 (17.4 %)	33 (44.0 %)	HS	Reference	
Dominant	AA+G/A	62 (82.6 %)	42 (56.0 %)	0.001	Reference	
	GG	13 (17.4 %)	33 (44.0 %)	¥ HS	0.266	0.12-0.56
Recessive	AA	37(49.3%)	15 (20.0 %)	0.001	3.89	1.88-8.03
	G/A+GG	38 (50.7%)	60 (80.0 %)	¥ S	Reference	
Alleles	A	99 (66.0%)	57 (38.0 %)	0.001	3.16	1.97-5.07
	G	51 (34.0 %)	93 (62.0 %)	¥ HS	Reference	

¥: Chi-square test; HS: Highly significant at $P < 0.001$.

A kind of male infertility might entail genetic defects of the *FSHR* because of the importance of FSH signalling in regulating quantitatively adequate sperm production and in adult pubertal beginning of spermatogenesis. There has been little research on the *FSHR* gene variants and polymorphisms in Iraqi males who are unable to conceive. The present research developed a hypothesis suggesting that idiopathic male infertility might be caused by variations in the *FSHR* gene. Results showed that the distribution of *FSHR* genotypes varied significantly between the control group's infertile and fertile males.

The results of the current study with respect to genotypes and allele frequencies for the *FSHR* (rs1394205) G/A SNP, the proportions of homozygous genotype Asn/Asn (AA)

and heterozygous genotype Asn/Ser (G/A) 49.3 and 33.3% respectively in patients group and 20% and 36% in control group. the genotype frequency distribution revealed a highly significant difference with regard to co-dominant mode ($P= 0.001$). The results of the risk analysis showed that both the heterozygous G/A genotype and the homozygous AA genotype were significant risk factors; however, the homozygous AA genotype was found to be approximately six times more likely to cause infertility than the other genotypes. Consistent with the results of the survey conducted by Ghmayes et al. (2020) on the Iraqi population in Baghdad province, with odds ratios ($OR= 4.12$), the present study found a highly significant difference between the patient and control groups in the dominant mode, recessive mode, and allele analysis ($P > 0.001$). This study's findings, however, showed a stronger correlation between the investigated gene SNP and male infertility, as measured by an odds ratio ($OR=6.26$) with a 95% CI.

The present study for FSHR (rs1394205)SNP genotyping frequency of the study individuals indicated that the homozygous genotype (AA) of the FSHR gene was more abundant than (G/A) and (GG) genotypes in infertility, these results agree with what was performed by Abdul-Rahim *et al.* (2021) in Iraq population .

Recent study among Iranian population carried out GharesiFard and his colleagues (2015) found that FSHR gene polymorphism might increase the risks of male infertility which similar to current study findings .

Furthermore, these results contradict those of studies conducted by a number of researchers, including Tuerlings and his colleagues (1998), who found no variation in the frequency distribution of this single nucleotide polymorphism between healthy control groups and infertile groups, and a study by Simoni *et al.* (1999) researchers in the Netherlands and Germany looked at the effects of FSHR gene polymorphism in patient samples, and they came to the same conclusion: FSHR gene mutations don't seem to cause male major consideration idiopathic infertility. Research into the overall effects of the FSHR gene polymorphism on infertility and the identification of the genotype responsible for moderating additional environmental and genetic factors cannot be conducted without considering population stratification, ethnic and

geographic variation, and other potential explanations for the observed differences between their findings and the results of the current study.

The results of this study support previous research suggesting that variations in the Asn680Ser FSHR (rs1394205) polymorphic region could be a major genetic factor influencing the phenotypic manifestation of infertility in Iraqi men. Similar findings have been found in studies conducted in other societies, such as the Albanian population (Elezaj *et al.* 2020) and the United States (Laloti 2011). Several polymorphisms have been studied as potential risk factors for the spermatogenetic process failure; abnormalities of the FSHR gene, in particular, are expected to affect male sperm production (Li *et al.* 2011).

Similar to previous research, this study found that infertile men's genotypic distributions vary. For example, Safarinejad and colleagues (2011) found it in an Iranian population sample, while Shimoda *et al.* (2009) found it in a Japanese population sample. Additionally, Ahda *et al.* (2005) found this form of genetic polymorphism in the German population when they analysed the FSHR gene and found that infertile males had a distinct distribution of genotypes.

The association between genotype and clinical phenotype is still controversial the wide diversity of CFTR and FSHR genes mutations may be due to differences across ethnicities. Besides that, other factors may also be in influence, including environmental factors, bacteria , or viruses.

The influence of CFTR and FSHR genes variants on male infertility It is still unclear. However, the findings of current study have shown that there are no appreciable differences in the distribution of the CFTR gene while there are considerable differences in the distribution of the FSHR gene and its potential functional consequences.

4.4 Association Between CFTR $\Delta F508$ (C/T), FSHR (rs1394205) G/A and Hormones Levels

The association between *CFTR* $\Delta F508$ (C/T) genotype and serum hormones level was summarized in table below Table 4.10. In patients with infertility, the present results show significant increased the serum FSH levels in TT genotype 6.31 ± 3.90 , in compared to CC genotype and CT genotype respectively, 5.42 ± 2.67 and 3.43 ± 3.02 ($P=0.017$). But, the present results show non-significant increased the serum LH levels in TT genotype 7.03 ± 2.34 , in compared to CC genotype and CT genotype respectively, 6.58 ± 2.14 and 5.47 ± 2.40 ($P=0.088$). Whereas, the present results show non-significant decreased the serum Testosterone Hormone levels in TT genotype 3.53 ± 2.37 , in compared to CC genotype and CT genotype respectively, 5.02 ± 2.47 and 4.15 ± 1.90 ($P=0.163$).

Table 4.10 Association between *CFTR* $\Delta F508$ (C/T) genotype and serum hormones level patients groups

Hormones	CFTR $\Delta F508$ (C/T) genotype			P
	CC genotype	CT genotype	TT genotype	
Follicle-Stimulating Hormone (FSH)				P=0.017 † S
Mean± SD	5.42 ± 2.67	3.43± 3.02	6.31± 3.90	
Range	1.56 – 10.23	1.34- 12.80	1.61- 12.73	
Luteinizing Hormone (LH).				P=0.088 † NS
Mean± SD	6.58± 2.14	5.47 ± 2.40	7.03 ± 2.34	
Range	3.64- 9.94	1.58– 11.70	3.80 – 10.58	
Testosterone Hormone				P=0.163 † NS
Mean± SD	5.02 ± 2.47	4.15 ± 1.90	3.53 ± 2.37	
Range	1.89 – 10.20	1.46 – 8.32	1.23– 8.92	

(†) : ANOVA test; (NS): not significant at $P \leq 0.05$;

The association between *FSHR* (rs1394205) G/A genotype and serum hormones level was summarized in table below Table 4.11. In patients with infertility, the present results show non-significant increased the serum FSH levels in AA genotype 5.00 ± 3.28 , in compared to GG genotype and GA genotype respectively, 3.91 ± 2.18 and 4.81 ± 3.70 ($P=0.695$). Also the present results show non-significant increased the serum LH levels in AA genotype 6.41 ± 2.81 , in compared to GG genotype and GA genotype

respectively, 6.35 ± 1.75 and 5.94 ± 2.27 ($P=0.772$). But the present results show non-significant decreased the serum Testosterone Hormone levels in AA genotype 3.73 ± 1.68 , in compared to GG genotype and GA genotype respectively, 5.75 ± 2.62 and 4.12 ± 2.26 ($P=0.056$).

Table 4.11 Association between FSHR (rs1394205) G/A genotype and serum hormones level patients groups

Hormones	FSHR (rs1394205) genotype			P
	GG genotype	GA genotype	AA genotype	
Follicle-Stimulating Hormone (FSH)				P=0.695 † NS
Mean± SD	3.91 ± 2.18	4.81± 3.70	5.00± 3.28	
Range	1.60 – 7.03	1.44- 12.34	1.34- 12.80	
Luteinizing Hormone (LH).				P=0.772 † NS
Mean± SD	6.35± 1.75	5.94 ± 2.27	6.41 ± 2.81	
Range	3.80- 9.12	2.90– 11.70	1.58 – 10.58	
Testosterone Hormone				P=0.056 † NS
Mean± SD	5.75 ± 2.62	4.12 ± 2.26	3.73 ± 1.68	
Range	2.92 – 10.20	1.46 – 8.92	1.23– 7.43	

(†) : ANOVA test; (NS): not significant at $P \leq 0.05$;

The association between CFTR $\Delta F508$ (C/T) genotype serum FSH levels, show significant increasing in FSH levels in TT genotype in compared to CC and CT genotype respectively at ($P = 0.017$), but the present results show non-significant association between CFTR $\Delta F508$ (C/T) genotype serum LH levels at ($P=0.088$). Whereas the present results show non-significant decreased the serum Testosterone Hormone levels in TT genotype in compared to CC genotype and CT genotype respectively at ($P=0.163$).

CFTR is expressed by Sertoli cells, which also secrete different proteins and growth factors to maintain the germ cell , the function of Sertoli cells is tightly regulated by hormones, particularly FSH, a fundamental regulator of spermatogenesis (HILAL et al. 2018). The significant increasing in FSH levels may be attributable to a correlation between the genotype (TT) in patients with infertility of CFTR $\Delta F508$ expression and FSH secretion.

The present results show non-significant association between FSHR (rs1394205) G/A genotype and serum hormones level. Although increased the serum FSH levels and serum LH levels in AA genotype in compared to GG and GA genotype respectively. But the present results show non-significant decreased the serum Testosterone Hormone levels in AA genotype in compared to GG genotype and GA genotype respectively, the observations of the current study relating to the impact the influence of the FSHR polymorphism on hormone concentration are concordant with the findings obtained by Elezaj *et al.*(2020).

According to current investigations, there was no evidence that the FSHR polymorphism caused noticeably different serum FSH levels in Iraqi men that which agreed with findings by Pengo and his colleagues (2006) in the Italian population, similar results were found by Ahda *et al.* (2005) in the German population, Qiuyue Wu *et al.* (2017) in the Chinese population , Safarinejad *et al.* (2011) in the Iranian population, Ghirelli-Filho and his co-worker (2012) on the Brazilian males, Tsitlakidis and his colleagues in the Greek men at (2017), and Balkan *et al.* (2010) in the Turkish population. In contrast, the published articles performed by (Zalata *et al.* 2008) in Egyptian , (Shimoda *et al.* 2009) in Japanese, (Grigorov *et al.* 2013) in Estonian, and by (Lindgren 2017) Swedish young men demonstrated that serum FSH levels correlate to FSHR gene genetic variations, it may be returned to the population frequency of a particular polymorphism may vary by lifestyle , race/ethnicity and environment.

It is the hypothalamus that secretes gonadotrophin-releasing hormone (GnRH) in humans. The gonads are affected by the hormones FSH and LH that are released into the circulation by the anterior pituitary gland in response to GnRH. Similar to the pulsatile secretion of GnRH, the cyclical secretion of FSH and LH occurs in the bloodstream. The expression of the CFTR gene may impact these hidden This is especially true for FSH, however, since its half-life in the bloodstream is two hours, whereas LH's is only twenty minutes (Lindgren 2017).

A better comprehension of factors regulating the expression of the CFTR and FSHR genes is therefore essential, to have a greater understanding of the ways that influence endocrine functions or gene expression.

4.5 DNA Sequence Analysis

The DNA sequencing method was employed for confirmation identification of genetic polymorphism variation analysis in Human cystic fibrosis transmembrane conductance regulator (CFTR) gene and Human follicle stimulating hormone receptor (FSHR) gene.

The phylogenetic tree genetic relationship analysis for Human cystic fibrosis transmembrane conductance regulator (CFTR) gene was showed that local Human CFTR gene ($\Delta F508$) patient No.1 , No.3, and No.4 were showed genetic variant at A mutant type related M28668.1: Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene (4-9). While FSHR patients samples was showed that local Human FSHR gene (rs1394205) patient No.1 , No.2, and No.3 were showed genetic variant at A mutant type related NG_008146.1 Human follicle stimulating hormone receptor (FSHR) RefSeqGene as showed in Figure 4.10.

The homology sequence identity between local CFTR and FSHR patients samples and NCBI BLAST related (CFTR) and (FSHR) RefSeqGene were showed genetic homology sequence identity ranged from (99.73-100%) as showed in Table 4.12 and Table 4.13.

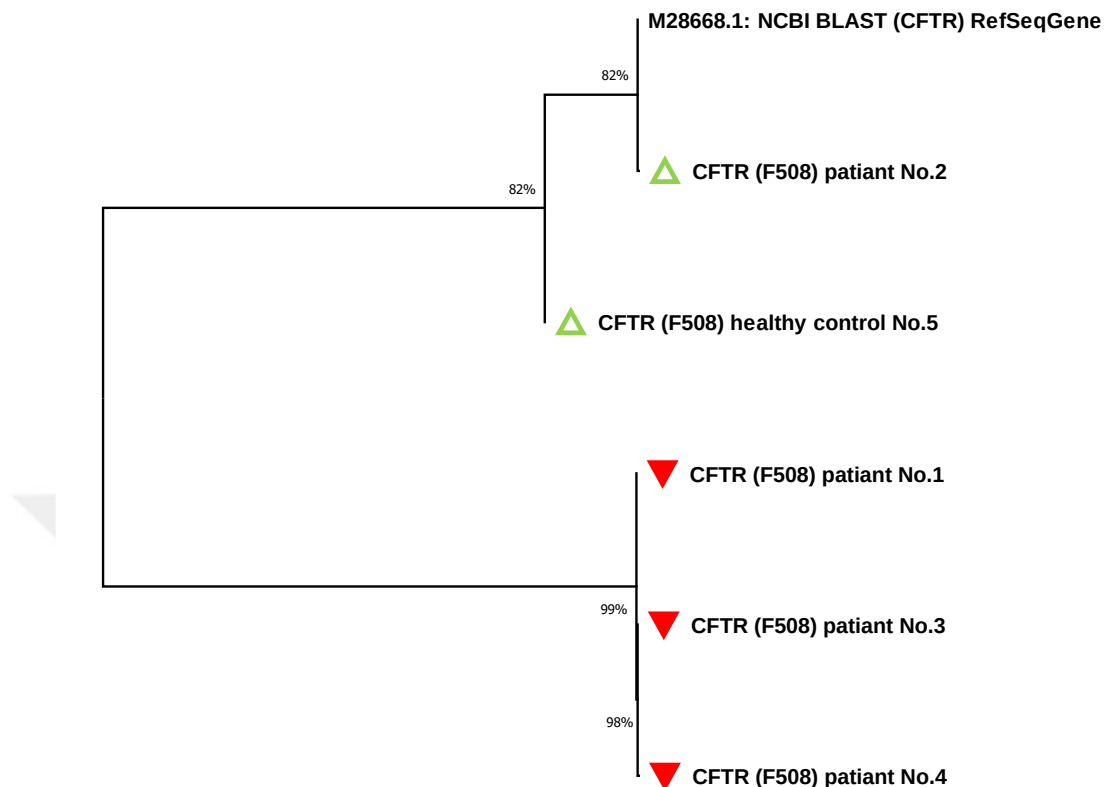


Figure 4.9 Phylogenetic tree analysis based Human cystic fibrosis transmembrane conductance regulator (CFTR) gene

Partial sequence in local CFTR gene (Δ F508) patients that was applied for genetic confirmation analysis. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) was used to construct the phylogenetic tree in (MEGA 6.0 version). The local Human CFTR gene (Δ F508) patient No.1 , No.3, and No.4 are shows genetic variant at A mutant type related M28668.1: Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene.

Table 4.12 The NCBI-BLAST genetic variation analysis between local Human CFTR gene(Δ F508) patient isolates and M28668.1: Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene

CFTR samples	Homology sequence identity (%)		
	Type of allele	Type of genotype	Identity %
CFTR.No.1	Mutant type allele	TT	99.73%
CFTR.No.2	Wild type allele	CC	100%
CFTR.No.3	Mutant type allele	TT	99.73%
CFTR.No.4	Mutant type allele	TT	99.73%
CFTR.No.5	Wild type allele	CC	100%

1- NCBI BLAST multiple sequence alignment between M28668.1 Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene (Query sequence) and local CFTR (ΔF508) patient No.1 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
678 bits(367)	0.0	369/370(99%)	0/370(0%)	Plus/Plus

Query 1 AGCCTCTTCTTCAGTAATTTCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTC 60

Sbjct 1 60

Query 61 AAGATAGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACTTCA 120

Sbjct 61 120

Query 121 CTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGA 180

Sbjct 121 180

Query 181 AGAATTCATTCTGTTCTCAGTTTTCTGGATTATGCCTGGCACCATTAAAGAAAATATC 240

Sbjct 181 240

Query 241 ATCTTTGGTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCCAACTA 300

Sbjct 241 T..... 300

Query 301 GAAGAGGACATCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGAGAAGGTGGAATC 360

Sbjct 301 360

Query 361 AACTGAGTG 370

Sbjct 361 370

2- NCBI BLAST multiple sequence alignment between M28668.1 Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene (Query sequence) and local CFTR ($\Delta F508$) patient No.2 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
682 bits(369)	0.0	369/369(100%)	0/369(0%)	Plus/Plus

Query 1 AGCCTCTTCTTCAGTAATTTCTCACTTCTTGGTACTCCTGCTGAAAGATATTAATTTC 60

Sbjct 1 60

Query 61 AAGATAGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACTTCA 120

Sbjct 61 120

Query 121 CTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGA 180

Sbjct 121 180

Query 181 AGAATTTTCATTCTGTTCTCAGTTTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATC 240

Sbjct 181 240

Query 241 ATCTTTGGTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCCAACTA 300

Sbjct 241 300

Query 301 GAAGAGGACATCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATC 360

Sbjct 301 360

Query 361 AACTGAGT 369

Sbjct 361 369

3- NCBI BLAST multiple sequence alignment between M28668.1 Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene (Query sequence) and local CFTR (ΔF508) patient No.3 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
678 bits(367)	0.0	369/370(99%)	0/370(0%)	Plus/Plus

Query 1 AGCCTCTTCTCAGTAATTCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTTC 60

Sbjct 1 60

Query 61 AAGATAGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACTTCA 120

Sbjct 61 120

Query 121 CTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGA 180

Sbjct 121 180

Query 181 AGAATTCATTCTGTTCTCAGTTTCTGCTGATTATGCCTGGCACCATTAAAGAAAATATC 240

Sbjct 181 240

Query 241 ATCTTTGGTGTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCCAACTA 300

Sbjct 241 **T** 300

Query 301 GAAGAGGACATCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATC 360

Sbjct 301 360

Query 361 AACTGAGTG 370

Sbjct 361 370

4- NCBI BLAST multiple sequence alignment between M28668.1 Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene (Query sequence) and local CFTR (Δ F508) patient No.4 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
678 bits(367)	0.0	369/370(99%)	0/370(0%)	Plus/Plus

Query 1 AGCCTCTTCTTCAGTAATTTCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTC 60

Sbjct 1 60

Query 61 AAGATAGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACTTCA 120

Sbjct 61 120

Query 121 CTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGA 180

Sbjct 121 180

Query 181 AGAATTTCACTTCTGTTCTCAGTTTTCTGGATTATGCCTGGCACCATTAAAGAAAATATC 240

Sbjct 181 240

Query 241 ATCTTTGGTGTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCCAACTA 300

Sbjct 241**T**..... 300

Query 301 GAAGAGGACATCTCCAAGTTGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATC 360

Sbjct 301 360

Query 361 AACTGAGTG 370

Sbjct 361 370

5- NCBI BLAST multiple sequence alignment between M28668.1 Human cystic fibrosis transmembrane conductance regulator (CFTR) RefSeqGene (Query sequence) and local CFTR (ΔF508) healthy control No.5 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
676 bits(366)	0.0	366/366(100%)	0/366(0%)	Plus/Plus

Query 1 AGCCTCTTCTTCAGTAATTTCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTC 60

Sbjct 1 60

Query 61 AAGATAGAAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACTTCA 120

Sbjct 61 120

Query 121 CTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGA 180

Sbjct 121 180

Query 181 AGAATTTTCATTCTGTTCTCAGTTTTCTGGATTATGCCTGGCACCATTAAAGAAAATATC 240

Sbjct 181 240

Query 241 ATCTTTGGTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCCAACTA 300

Sbjct 241 300

Query 301 GAAGAGGACATCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATC 360

Sbjct 301 360

Query 361 AACTG 366

Sbjct 361 366

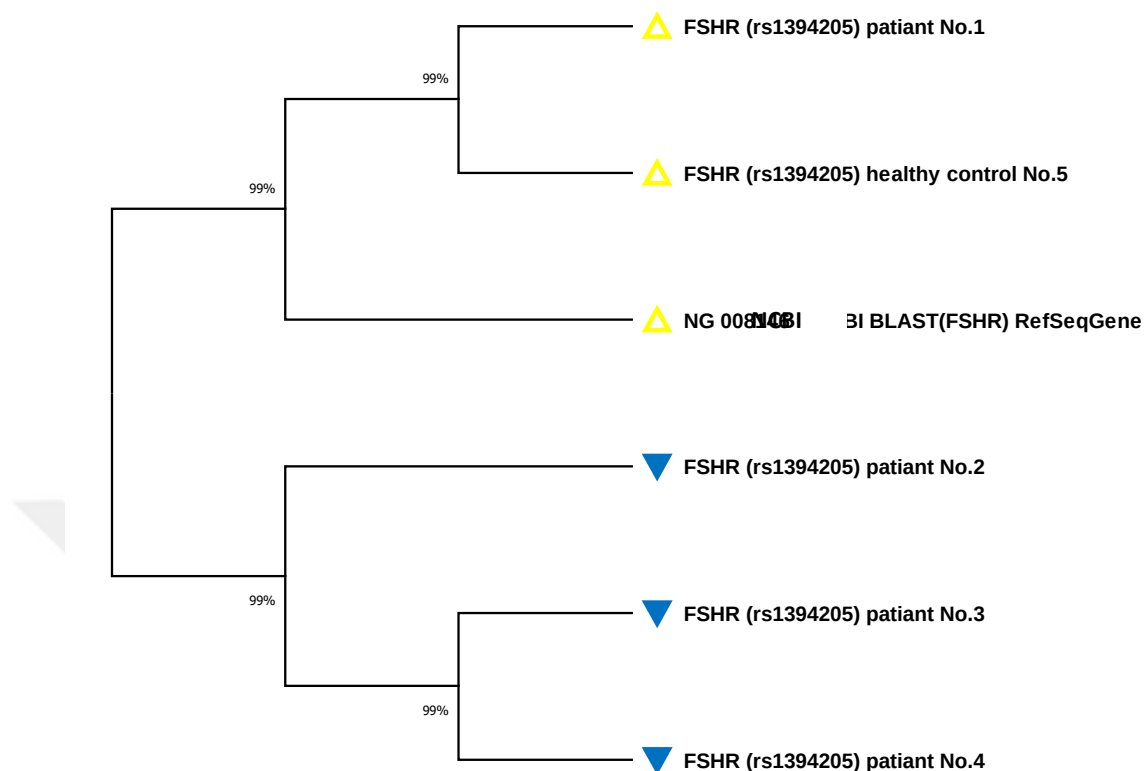


Figure 4.10 Phylogenetic tree analysis based Homo sapiens follicle stimulating hormone receptor (FSHR) gene

Partial sequence in local FSHR gene (rs1394205) patients that performed for genetic confirmation analysis. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) was used to construct the phylogenetic tree in (MEGA 6.0 version). The local Human FSHR gene (rs1394205) patient No.1 , No.2, and No.3 were showed genetic variant at A mutant type related NG_008146.1 Homo sapiens follicle stimulating hormone receptor (FSHR) RefSeqGene.

Table 4.13 The NCBI-BLAST genetic variation analysis between local Human FSHR gene (rs1394205) patient isolates and NG_008146.1 Homo sapiens follicle stimulating hormone receptor (FSHR)RefSeqGene

FSHR samples	Homology sequence identity (%)		
	Type of allele	Type of genotype	Identity %
FSHR.No.1	Wild type allele	GG	100%
FSHR.No.2	Mutant type allele	AA	99.73%
FSHR.No.3	Mutant type allele	AA	99.73%
FSHR.No.4	Mutant type allele	AA	99.73%
FSHR.No.5	Wild type allele	GG	100%

1- NCBI BLAST multiple sequence alignment between NG_008146.1 Homo sapiens follicle stimulating hormone receptor (FSHR) RefSeqGene (Query sequence) and local FSHR (rs1394205) patient No.1 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
673 bits(364)	0.0	364/364(100%)	0/364(0%)	Plus/Plus

Query 1 TCAGGGTGTAAAGAAACCCAATCTTGAAGGAAAACAGAGTAGCTTATCTTGCCTGGAAGTA 60

Sbjct 1 60

Query 61 ACAAAAAAAAAAAAAAAAAAAGCATCCCTTGGTGGGTCACATGACCCTACCAGTTCTCAAGT 120

Sbjct 61 120

Query 121 CAGATCTCTTCTCATAAGGGCACTGTGTGGAGCTTCTGAGATCTGTGGAGGTTTTCTCT 180

Sbjct 121 180

Query 181 GCAAATGCAGGAAGAAATCAGGTGGATGGATGCATAATTATGGCCCTGCTCCTGGTCTCT 240

Sbjct 181 240

Query 241 TTGCTGGCATTCTGAGCTTGGGCTCAGGATGTCATCATCGGATCTGTCACTGCTCTAAC 300

Sbjct 241 300

Query 301 AGGGTTTTCTCTGCCAAGAGAGCAAGGTGACAGAGATTCTTCTGACCTCCCGAGGAAT 360

Sbjct 301 360

Query 361 GCCA 364

Sbjct 361 364

2- NCBI BLAST multiple sequence alignment between NG_008146.1 Homo sapiens follicle stimulating hormone receptor (FSHR) RefSeqGene (Query sequence) and local FSHR (rs1394205) patient No.2 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
667 bits(361)	0.0	363/364(99%)	0/364(0%)	Plus/Plus

Query 1 TCAGGGTGTAAGAAACCCAATCTTGAAGGAAAACAGAGTAGCTTATCTTGCCTGGAAGTA 60

Sbjct 1 60

Query 61 ACAAAAAAAAAAAAAAAAAAAGCATCCCTTGGTGGGTCACATGACCCTACCAGTTCTCAAGT 120

Sbjct 61 120

Query 121 CAGATCTCTTCATAAGGGCACTGTGTGGAGCTTCTGAGATCTGTGGAGGTTTTCTCT 180

Sbjct 121 180

Query 181 GCAAATGCAGGAAGAAATCAGGTGGATGGATGCATAATTATGGCCCTGCTCCTGGTCTCT 240

Sbjct 181**A**..... 240

Query 241 TTGCTGGCATTCTGAGCTTGGGCTCAGGATGTCATCATCGGATCTGTCACTGCTCTAAC 300

Sbjct 241 300

Query 301 AGGGTTTTCTCTGCCAAGAGAGCAAGGTGACAGAGATTCCTTCTGACCTCCCAGGAAT 360

Sbjct 301 360

Query 361 GCCA 364

Sbjct 361 364

3- NCBI BLAST multiple sequence alignment between NG_008146.1 Homo sapiens follicle stimulating hormone receptor (FSHR) RefSeqGene (Query sequence) and local FSHR (rs1394205) patient No.3 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
665 bits(360)	0.0	362/363(99%)	0/363(0%)	Plus/Plus

Query 1 TCAGGGTGTAAGAAACCCAATCTTGAAGGAAAACAGAGTAGCTTATCTTGCCTGGAAGTA 60

Sbjct 1 60

Query 61 ACAAAAAAAAAAAAAAAAAAAGCATCCCTTGGTGGGTCACATGACCCTACCAGTTCTCAAGT 120

Sbjct 61 120

Query 121 CAGATCTCTTCTCATAAGGGCACTGTGTGGAGCTTCTGAGATCTGTGGAGGTTTTCTCT 180

Sbjct 121 180

Query 181 GCAAATGCAGGAAGAAATCAGGTGGATGGATGCATAATTATGGCCCTGCTCCTGGTCTCT 240

Sbjct 181**A**..... 240

Query 241 TTGCTGGCATTCTGAGCTTGGGCTCAGGATGTCATCATCGGATCTGTCACTGCTCTAAC 300

Sbjct 241 300

Query 301 AGGGTTTTCTCTGCCAAGAGAGCAAGGTGACAGAGATTCTTCTGACCTCCCGAGGAAT 360

Sbjct 301 360

Query 361 GCC 363

Sbjct 361 ... 363

4- NCBI BLAST multiple sequence alignment between NG_008146.1 Homo sapiens follicle stimulating hormone receptor (FSHR) RefSeqGene (Query sequence) and local FSHR (rs1394205) patient No.4 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
667 bits(361)	0.0	363/364(99%)	0/364(0%)	Plus/Plus

Query 1 TCAGGGTGTAAGAAACCCAATCTTGAAGGAAAACAGAGTAGCTTATCTTGCCTGGAAGTA 60

Sbjct 1 60

Query 61 ACAAAAAAAAAAAAAAAAAAACATCCCTTGGTGGGTCACATGACCCTACCAGTTCTCAAGT 120

Sbjct 61 120

Query 121 CAGATCTCTTCATAAGGGCACTGTGTGGAGCTTCTGAGATCTGTGGAGGTTTTCTCT 180

Sbjct 121 180

Query 181 GCAAAATGCAGGAAGAAATCAGGTGGATGGATGCATAATTATGGCCCTGCTCCTGGTCTCT 240

Sbjct 181**A**..... 240

Query 241 TTGCTGGCATTCTGAGCTTGGGCTCAGGATGTCATCATCGGATCTGTCACTGCTCTAAC 300

Sbjct 241 300

Query 301 AGGGTTTTTCTCTGCCAAGAGAGCAAGGTGACAGAGATTCTTCTGACCTCCCGAGGAAT 360

Sbjct 301 360

Query 361 GCCA 364

Sbjct 361 364

5- NCBI BLAST multiple sequence alignment between NG_008146.1 Homo sapiens follicle stimulating hormone receptor (FSHR) RefSeqGene (Query sequence) and local FSHR (rs1394205) healthy control No.5 as (Sbjct sequence):

Score	Expect	Identities	Gaps	Strand
673 bits(364)	0.0	364/364(100%)	0/364(0%)	Plus/Plus

Query 1 TCAGGGTGTAAGAAACCCAATCTTGAAGGAAAACAGAGTAGCTTATCTTGCCTGGAAGTA 60

Sbjct 1 60

Query 61 AAAAAAAAAAAAAAAAAAAGCATCCCTTGGTGGGTCACATGACCCTACCAGTTCTCAAGT 120

Sbjct 61 120

Query 121 CAGATCTCTTCTCATAAGGGCACTGTGTGGAGCTTCTGAGATCTGTGGAGGTTTTCTCT 180

Sbjct 121 180

Query 181 GCAAATGCAGGAAGAAATCAGGTGGATGGATGCATAATTATGGCCCTGCTCCTGGTCTCT 240

Sbjct 181 240

Query 241 TTGCTGGCATTCTGAGCTTGGGCTCAGGATGTCATCATCGGATCTGTCACTGCTCTAAC 300

Sbjct 241 300

Query 301 AGGGTTTTCTCTGCCAAGAGAGCAAGGTGACAGAGATTCTTCTGACCTCCCGAGGAAT 360

Sbjct 301 360

Query 361 GCCA 364

Sbjct 361 364

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

The works included in this thesis come to the conclusion that additional investigation is required on polymorphic genes and their effects on male infertility, the investigation described in this thesis contributes to explain how hormones and FSHR/CFTR polymorphisms influence human reproductive function. The data that have been assigned may help in the identification of new targets for the treatment of male infertility.

- LH and FSH were significantly increased in compared to controls and male infertility patient, while testosterone was significantly decreased .
- According to the results of the rs1394205 FSHR genotyping, the homozygous AA genotype has been demonstrated to be a highly significant risk factor and to be more prevalent than GA and GG in male infertility patients suffering infertility.
- There was a tight correlation found between CFTR Polymorphisms and FSH hormone levels.
- The present results show significant increased the serum FSH levels in TT genotype in compared to CC genotype and CT genotype respectively.
- There is no conclusive evidence linking CFTR polymorphisms to male infertility..
- There was no association was found between FSHR variation and serum hormones levels.

5.2 Recommendations

In particular, the role of the polymorphic FSHR and CFTR genes in male infertility outcomes has been better understood because to the research presented in this thesis. The findings offered in this thesis, particularly those pertaining to the effects on male infertility, need, however, more research to validate and expand upon.

- Creating a database for people infected with infertility in Thi Qar Governorate.
- Emphasis on the application gene analysis in blood tests in Iraqi hospitals , to early detection of male infertility, in a more accurate way than other tests currently available.
- Further studies should assess the clinical importance of the FSHR genotype considering into consideration ethnic background, genetics, epigenetics, sources of control, methodologies, dietary, and environmental factors impacting male fertility.rs1394205 FSHR (AA) polymorphism can be used as a biomarker to indicate infertility in male .
- More investigations in detection the number and location of genes by purification of protein (gene) which may be related with male infertility.

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APPENDICES

APPENDIX 1. Information form patient



APPENDIX 1. Information form patient

Case number :				
Full Name:				
Age :				
Address	City		Rural	
Height :		Weight :		
Occupation:				
Education levels	write & read	Primary * Secondary **	College	Higher Education
	Yes	No	Comments	
Do you have diabetes?				
Do / did you smoke cigarettes?				
Do you have a history of genital herpes				
Are you on any medication?				
Did you ever have major trauma or other surgery ?				
Is there a family history of:				
* Birth defects or genetic diseases				
*Infertility				
*Thyroid/endocrine problems				
*High blood pressure				
Semen analysis?	Count (million/cc)	Motility (% moving)	Morphology (% normal shape)	
DIAGNOSTIC FEATURES				
	HORMONAL	TESTICULAR	OBSTRUCTIVE	FUNCTIONAL
Azoospermia (Sperm absent)				
Oligozoospermia Reduced sperm numbers - Severe cases < 5 million per mL				
Follicle-stimulating hormone (FSH)	Low/normal ≤ 8 IU/L	High (> 8 IU/L)	Normal (1–8 IU/L)	write the value
Luteinizing hormone (LH)	Low/normal(≤ 10 IU/L)	High> 10 IU/L	Normal(1–10 IU/L)	write the value
Testosterone	Low <(10 nmol/L)	Often low (< 10 nmol/L)	Normal 910–30 nmol/L)	write the value
Completed by Patient Signature :		Name :		Date : \
\				
Telephone No:				

CURRICULUM VITAE

Personal Information

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Education

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