Polymorphism of follicule stimulating hormone gene receptor in Albanian male population

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UNIVERSITY OF ZAGREB

SCHOOL OF MEDICINE

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POLYMORPHISM OF FOLLICULE STIMULATING HORMONE GENE RECEPTOR IN ALBANIAN MALE POPULATION

DISSERTATION

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POLIMORFIZAM GENA ZA RECEPTOR FSH U MUŠKARACA ALBANSKE ETNIČKE PRIPADNOSTI

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Zagreb, 2019

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LIST OF ABBREVIATIONS

AC Adenylate cyclase

Akt RAC-alpha serine/threonine-protein kinase

AMH Anti-Mullerian Hormone

AR Androgen receptors

ARKO AR knockout

ART Assisted reproductive technology

ATP Adenosine triphosphate,

AURKC Aurora kinase C

AZF Azoospermia factor

AZO Azoospermia

BMI Body mass index

CAG Trinucleotide

CaM Calmodulin

cAMP Cyclic adenosine monophosphate

CBAVD Congenital bilateral absence of the vas deferens

CFTR Cystic fibrosis transmembrane cond. regulator

CNVs Copy-number variation

CYP11A1 Cholesterol side-chain cleavage enzyme

DAX1 Nuclear receptor protein

DAZ1 Deleted in azoospermia 1

DAZL Deleted in Azoospermia like

DDT Dichlorodiphenyltrichloroethane

DHPC Denaturing High Performance Chromatography

DHT Dihydrotestosterone

DMRT1 Double sex and mab-3 related transcription factor 1

DNA Deoxyribonucleic acid

DPY19L2 Developmental pluripotent-associates 19

EAR1 eosinophil-associated, ribonuclease A family m.1

ELISA Enzyme-linked immunosorbent assay

EPAC Exchange protein directly activated

ERK Extracellular signal-regulated kinase

FSH Follicle stimulating hormone

FSHR FSH receptor gene

FSHRKO FSH receptors mice knockout

GDP Guanosine diphosphate

GnIH Gonadotropin-Inhibitory Hormone

GnRH Gonadotropin releasing hormone

GPCRs Glycoprotein hormone receptors

GTP Guanine triphosphate

GWAS Genome-wide association studies

HBsAg Hepatitis B Virus Surface Antigen

hCG Human chorionic gonadotropin

HCV Hepatitis C virus

HIV Human immunodeficiency virus

ICSI Intracytoplasmic sperm injection

IGF1 Insulin-like growth factor 1

IMI Idiopathic male infertility

INSL3 Insulin-like 3 gene

IUI Intrauterine insemination

IVF In vitro fertilization

LD Linkage disequilibrium

LH Luteinizing hormone

LRR Leucine-rich repeat

MAPK Mitogen-activated protein kinase

MTHFR methylenetetrahydrofolate reductase

NAS Normoasthenozoospermia

NGF Nerve growth factor

NOA Non-obstructive azoospermia

NS Normozoospermia

OA Obstructive azoospermia

OAS Oligoasthenozoospermia

PCR Polymerase chain reaction

PDE Phosphodiesterase

PKA Protein kinase

PRL Prolactin

PRM/TNP Sperm nuclear protein genes

PTP Protein phosphatase

RNA Ribonucleic acid

ROS Reactive oxygen species

SCARKO Selective androgen receptor knockouthCG

SDF Sperm DNA fragmentation

SF1 Splicing Factor 1

SHBG Sex hormone-binding globulin

SNPs Single nucleotide polymorphisms

SOX5 SRY-related HMG-box

SOX9 Sex determining region Y box 9

SRY Sex-determining region Y

STDs Sexually transmitted diseases

TR Tandem repeats

TDS Testicular dysgenesis syndrome

TEFNA Testicular epididymis fine needle aspiration

TESE Testicular sperm extraction

TEX11 Testis-expressed gene 11

TGFβ Transforming growth factor beta

TH Transmembrane helices

TP Transitional proteins

WHO World Health Organization

1. INTRODUCTION

1.1 Overview

Infertility means diminished ability to produce descendants, with major effects on a couple's life, including marital satisfaction, causing jealousy, disbelief, shame, anger, trauma, stress, and withdrawal from social contacts with a strong emphasis on childbearing (1, 2).

Male infertility is the inability of the body to produce enough male cells for successful fertilization. Male infertility is a heterogeneous group of disorders, which accounts for 30-50% of known causes, while the rest are unknown. (3).

In the daily medical practice, among other diseases and pathologies, clinicians in the field of urology also encounter occasional cases when a patient complains about a problem of infertility, which tends to feel like it will never end and be resolved (3). Before evaluating the causes of infertility, couples' information should be carefully reviewed. The causes of infertility are equally distributed between males and females and most of them have more than one causal factor. As part of routine medical examinations, the clinician must obtain information about the history and the consequences of the problem in collaboration with the gynecologist, to evaluate the fertility ability as a couple, including female and male factors (4, 5).

The World Health Organization (WHO) Human Reproduction Programme 2016 defines infertility as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy in one year or more of regular unprotected sexual intercourse between a couple (5, 7).

Infertility can be:

- Primary – when pregnancy is not achieved; and
- Secondary – when a woman is unable to bear a child either due to the inability to become pregnant or the inability to carry a pregnancy to term (5).

Epidemiology:

Male infertility is a common multifactorial pathological condition which affects approximately 7-8% of the male population (5). There are no reliable statistics for the overall prevalence of infertility, but according to WHO estimates, about 70-80 million couples suffer

from fertility problems (6). Worldwide, infertility affects about 13-15% of couples. The prevalence of infertility varies widely, with the lowest percentage in developed countries and the highest percentage in developing countries, where limited resources for investigation and treatment are available (7).

Unfortunately, this number does not represent all regions of the world because we were unable to calculate some statistics due to the lack of reports from regions like Asia and the Pacific (7, 8). The male factors are responsible for 30% of infertility cases; 40-50% of all cases of infertility are due to female factors alone, and the remaining 20-30% is due to a combination of male and female factors (7, 8). For a large number of patients, genetic factors are implicated in gametogenesis, which accounts for 15–30% of male factor infertility (9, 10). Recent studies show that the number of infertile couples in the general population is growing (10). Most patients are subfertile rather than sterile (infertile), but the degree of subfertility is difficult to predict (11).

Apart from female infertility, male infertility is responsible for around 50% of these couples' inability to conceive and is the result of acquired or congenital abnormalities. According to the National Center for Health Statistics, the number of impaired fecundity increased from 4.5 million in 1982 to 9 million in 2012. Moreover, the infertility rate in young men decreased by 15% (8, 12).

Idiopathic male infertility occurs in ~50% of all cases. Men with idiopathic infertility often have normal findings on physical examination, normal endocrine functions, and no previous history associated with fertility problems, but they exhibit declaimed of sperm count, sperm motility, or poor sperm morphology (11, 13).

The genetic abnormalities of male infertility are highly complex and contribute to a considerable percentage of male infertility (9, 10, 15, 16). The highest known genetic factors of male infertility (15-30%) are in azoospermia or severe oligozoospermia, but the number of identified genetic factors is constantly growing (9, 10, 15, 16). It is estimated that thousands of genes involved in the genetic control (more than 2000), and the most frequent genetic factors implicated in male infertility are chromosomal abnormalities or genetic translations of the Y chromosome microdeletions and monogenic disorders (9, 10, 15, 16). However, a substantial part of unexplained cases also has a genetic etiology. Several lines of evidence indicate that reduced male fertility is not just one disorder, but also a heterogeneous group of

disorders with a multi-factorial etiology, but the functional role of the majority of these genes in male infertility is still poorly understood (9, 10, 14, 15).

For many decades, in societies such as our community in Kosovo, women have almost always been blamed for being infertile if a couple had no children, but nowadays, it has become clear that both male and female infertility are equally responsible for a couple's infertility (17).

The causes of male infertility may be different, ranging from an unhealthy lifestyle, oxidative stress, smoking, alcohol, drugs, inflammatory processes that damage sperm quality, hormonal dysfunction, physical trauma, congenital malformations, malignant diseases, mumps, erectile dysfunction, retrograde ejaculation, and premature ejaculation (1, 3, 5, 7).

1.2 Human male reproductive system

Reproduction is the process through which organisms create offspring. This miracle is a characteristic that all living things have in common and separates them from non-vital things (18, 19).

The male reproductive tract is comprised of two testicles, two epididymitis, ductus deferents, and ductus ejaculatorius, accessory sexual glands known as seminal vesicles, prostate gland, bulbourethral glands (Cowper's gland), penis, and scrotum. The entire reproductive tract plays an essential role in male reproduction. The testis is the key organ in male reproduction, which is responsible for sperm production (Figure1) (18, 20, 44).

The male reproductive anatomy includes internal and external structures and accessory glands that produce, support, transport, and deliver viable sperm for reproduction (18, 19). These organs are located on the external part of the body and the pelvic cavity.

The main reproductive organs of the male body are the testes, which produce sperm and male hormones (testosterone). The male reproductive system also includes other structures, including the epididymites, vas deferens, the spermatic cord, and the urethra. External genitals include the penis and the scrotum, and accessory organs, including the seminal vesicles, prostate gland, and the bulbourethral glands (18, 19. 20) (figure 1).

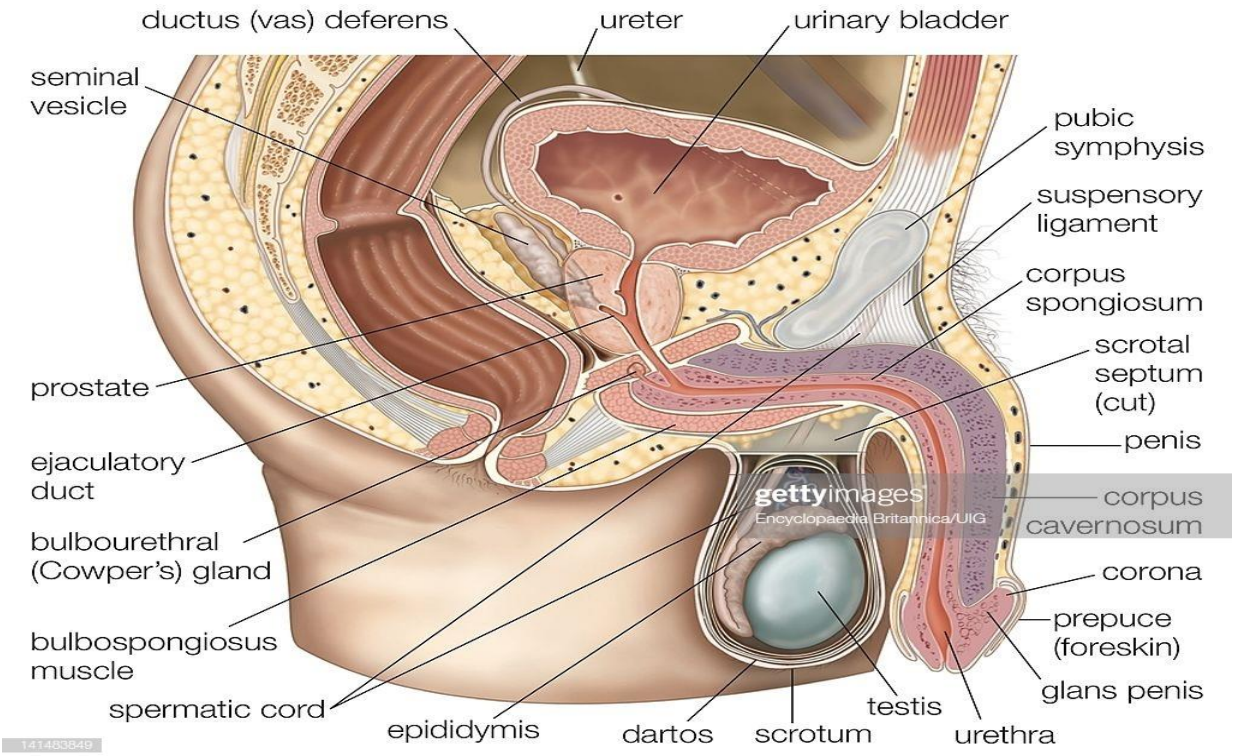


Figure 1. Male reproductive organs (Encyclopaedia Britannica/UIG)

1.2.1 Testes

The pair of important structures in the male reproductive systems, also known as the male gonads, which are enclosed in the scrotal sac, has two primary functions: first, they produce the sperm and the major male hormone, testosterone. Usually, the right testis hangs higher than the left by about 1 cm. The testes hang outside the body because the temperature inside the body is too high to produce sperm, so they are produced in the testes at about 3°C lower than body temperature (figure 2) (18, 20).

1.2.2 Epididymis

At the back of the top and posterior portion of each testis is a cap formed by a single coiled microscopic tube that measures almost 17-20 feet (5-6 meters) in length, called the epididymis. Each epididymis has three regions: head, body, and tail.

When the sperm have developed flagella and are nearly mature, they leave the testicles and enter the epididymis. The function of the epididymis is to collect the immature sperm from

the testis. As the sperm make their long journey through the epididymis, they become mature sperm. This journey takes about 20 days and during its course, the sperm become fertile, and they also become able to move or motile (18, 20).

1.2.3 Vas deferens (sperm duct)

The sperm duct is the continuation of the epididymis, a thick-walled tube that measures about 30 cm, which carries the sperm behind the bladder, and empties in the ejaculatory duct, which then passes through the prostate gland to merge with the urethra (18. 20).

1.2.4 Seminal vesicles

Seminal vesicles are a pair of tubular glands about 5-6 cm in length, located above the prostate that connect with vas deferens to form the ejaculatory ducts that travel through the prostate. The seminal vesicles produce 50-70% of the seminal fluid that nourishes the sperm (18, 20).

1.2.5 Ejaculatory duct

The connection of vas deferens with the seminal vesicle forms the ejaculatory duct (2-3 cm in length), which passes through the prostate gland to merge with the urethra (18, 20).

1.2.6 Prostate

The prostate is a compound tubuloalveolar, walnut-shaped gland that surrounds the urethra, with a weight ranging between 10-20 grams. Along with the seminal vesicles, it produces slightly alkaline fluid secretions that constitute 25-30% of the volume of the semen, which supports and nourishes the sperm. Without this fluid to dilute them, the sperm cannot move easily. The prostate also contains smooth muscles that help expel the semen during ejaculation (20).

1.2.7 Penis

The penis is the male external sexual organ for urination and copulation**. It** is made of three columns of tissue: two corpora cavernosa that lie next to each other on the dorsal side and a corpus spongiosum that lies between the corpora cavernosa on the ventral side. The glans penis is the bulbous end of the penis formed by the corpus spongiosum (18, 20).

1.2.8 Urethra

The urethra is the last part of the urinary tract and traverses the corpus spongiosum. The urethral opening is called the meatus and lies on the tip of the glans penis. The urethra carries urine from the bladder through the penis to the outside, and it also acts as a tube for semen to be ejaculated (18, 20).

1.3 Gonadal differentiation

Gonadal differentiation is related to the genetic makeup at fertilization, starting from the 7^{th} week of gestation (21, 22, 23). Sexual determination and differentiation involve a sequence of related processes that begins with chromosomal sex**,** established at the time of conception (21, 22, 23, 27).

Sexual differentiation includes:
1) Genetic sex: Differentiation of the gonads into testes or ovaries depends on the presence or absence of the Y chromosome in the germ cells (21, 22, 23).
2) Gonadal sex: The differentiation of the gonads into testes depends on the presence of the sex-determining region or gene (SRY) located on the short arm of the Y chromosome. The absence of this gene results in XY female chromosome (21, 22).
a) Internal genital sex: The Mullerian inhibiting substance (MIS) is produced in immature Sertoli cells and is responsible for the regression of the paramesonephric (Mullerian) duct. Testosterone is responsible for the differentiation of the Wolffian duct into the epididymites, vas deferens, and seminal vesicles (21, 23, 25).
b) External Genital Sex: The external genitalia is derived from the genital tubercle (23, 24,

25). The genital folds and swellings, together with the elongation of the genital tubercle, form the shaft and glans of the penis, which develop into male genitalia. The fusion of the urogenital folds encloses the definitive urogenital sinus, forming the penile urethra (23, 24). All these morphological changes are under the influence of androgenic hormones produced by the Leydig cells of the testes (25, 26).

Differentiation of the reproductive tract (figure 2).

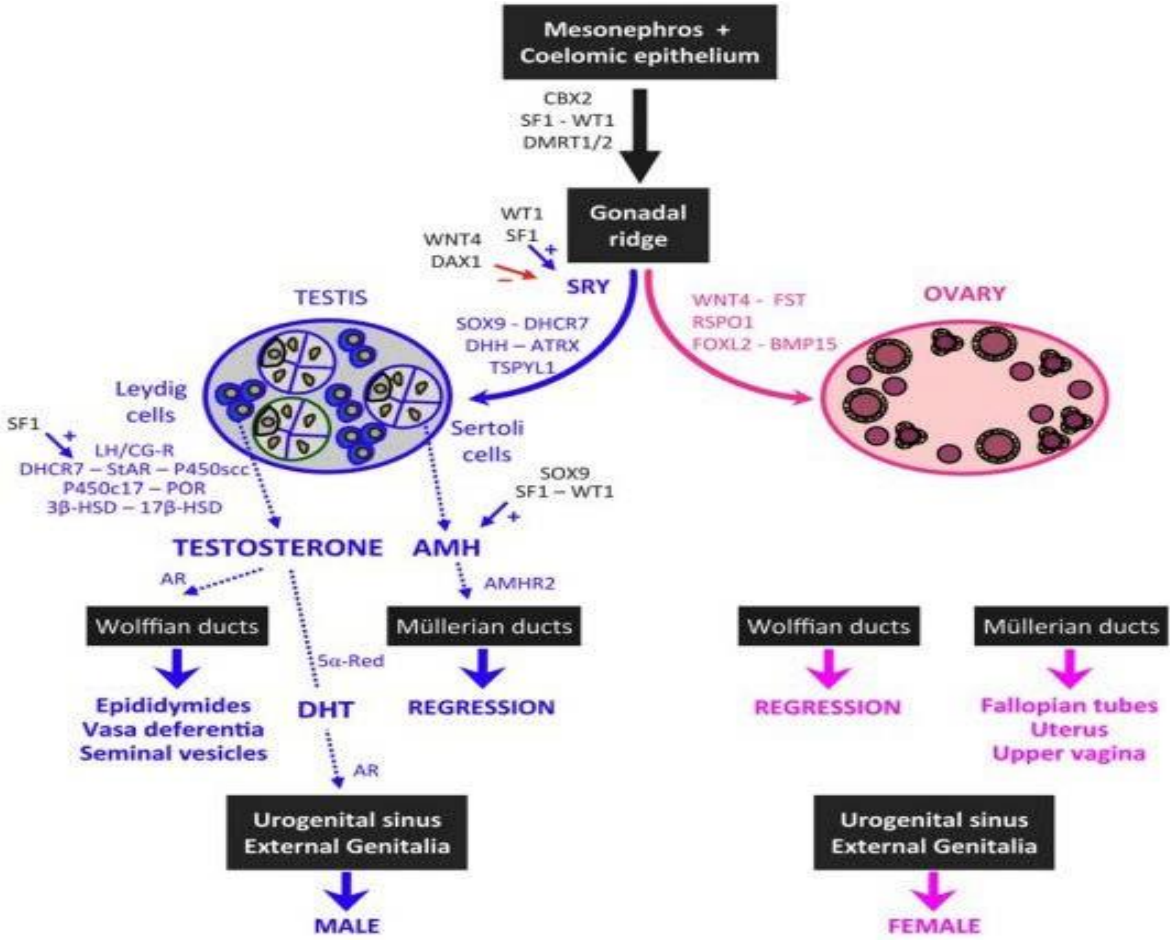


Figure 2. Stages and genetic regulation in normal male sexual differentiation *(adapted from Rey and Grinspon 2011),*

Gonadal Descent- During embryonic and fetal life, the testes are formed in the abdominal cavity and from there descend into the scrotum. The descent depends upon a structure called the gubernaculum. The action of the descent of testes into the scrotum takes place between the 7^{th} week and the 7^{th} month of gestation (Fig. 3). This action occurs under the influence of androgens (testosterone and dihydrotestosterone), which are crucial in the function of the gubernaculum. Failure of one testis or both testes to descend is a condition called cryptorchidism (26).

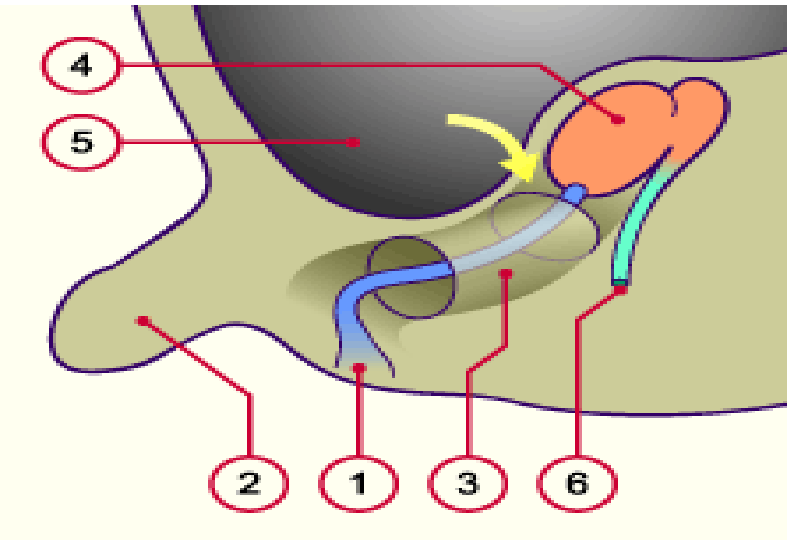


Fig. 3. Between the 3rd and 7th month of pregnancy, the testes remain near the inguinal canal, in order to pass through it. The vaginal process lengthens, while the gubernaculum shortens, thereby drawing the testes. 1. Gubernaculum, 2. Penis, 3. Inguinal canal, 4. Testis, 5. Peritoneal cavity, 6. Deferent ductus.

1.4 Spermatogenesis

Male germ cells contain half of the genetic materials required for life, which start to develop in the seminiferous tubules of the testes throughout life. This whole process of germ cell development is called spermatogenesis (27) (Figure 4).

Spermatogenesis is a continuous and precisely controlled process by which the population of germ cells produces spermatozoa capable of fertilization. Spermatogenesis takes place within the seminiferous tubules, which are ~200 μm in diameter and have a total length of ~600 meters (27, 28).

The whole spermatogenesis process needs detailed information about the organization of the germinal epithelium; the structure and function of germ cells; endocrine and paracrine cells and mechanisms, as well as intratesticular and extratesticular regulation of spermatogenesis (27. 28).

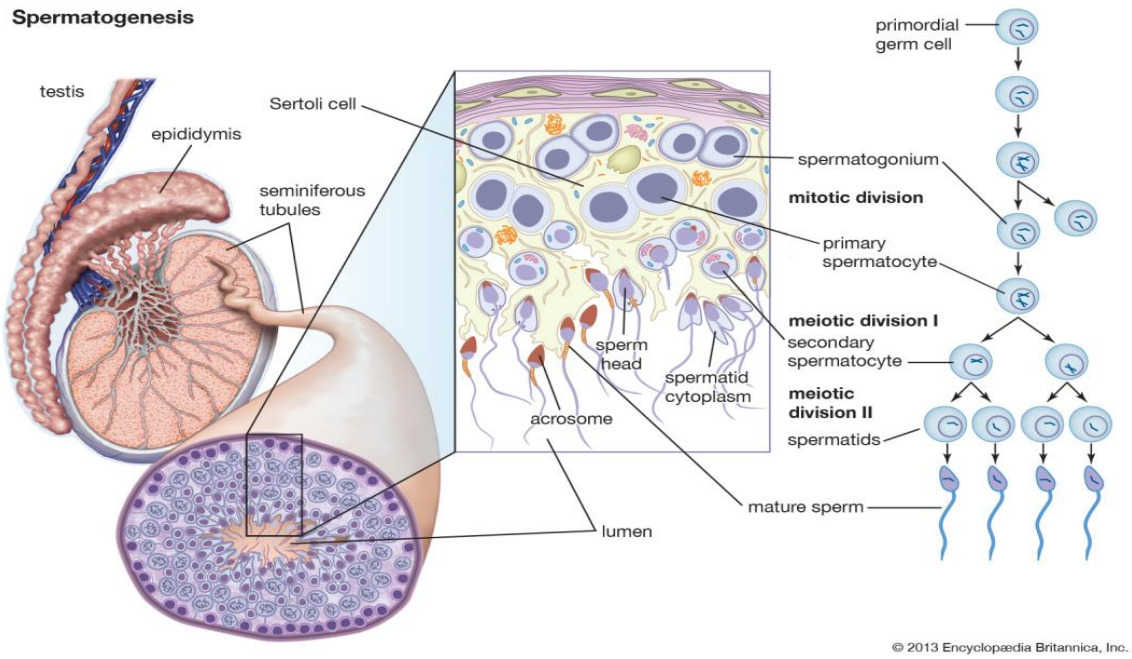


Figure 4. Cross-section through a testis, showing the location of the seminiferous tubules, the vas deferens, and the epididymis (Encyclopaedia Britanica, 2013)

Spermatogenesis begins during puberty and occurs in seminiferous tubules of the testes (29). It starts with the division of stem cells known as basic spermatogonia that lie adjacent to the basement membrane (29, 30), and ends with the formation of the mature gametes, namely spermatozoa.

The spermatogenic process starts with the undifferentiation of stem cells (line type A spermatogonia), which start to divide mitotically to replace themselves and to produce cells for differentiation and maturation. The whole spermatogenic process can be divided into four phases: spermatogoniogenesis, spermatocytogenesis, spermiogenesis, and spermiation (Figure 5) (30, 31).

1.4.1 Spermatogoniogenesis: Spermatogenesis begins at puberty when the diploid spermatogonia (germ cells) undergo mitosis and splitting into two different types of cells, type A and type B spermatogonia. Type A spermatogonia are classified into Ad (dark) and Ap (pale). Ad spermatogonia do not divide or very rarely do so when the spermatogonial population is drastically reduced, such as radiation, chemotherapeutic drugs etc. (32, 68, 70). Ap spermatogonia show typical characteristics of a progenitor; Ap spermatogonia differentiate into two daughter B spermatogonia and Ap spermatogonia. Ap spermatogonia

come back up to the basement membrane, reuse it for the next spermatogonia, and continue to replicate. After differentiation and mitotic division, B spermatogonia evolve into primary spermatocytes (31, 33, 34).

1.4.2 Spermatocytogenesis: During this phase, primary (diploid) spermatocytes continue to move through the tight junctions, commence the process of meiosis, and produce haploid germ cells called secondary spermatocytes or spermatids. At this stage, there is genetic recombination and exchange of genetic information, which is essential for the diversity of life. Meiosis entails chromosomal duplication, genetic recombination, and a reduction division to produce four haploid spermatids in a cycle that lasts around 12 days (31, 33, 34).

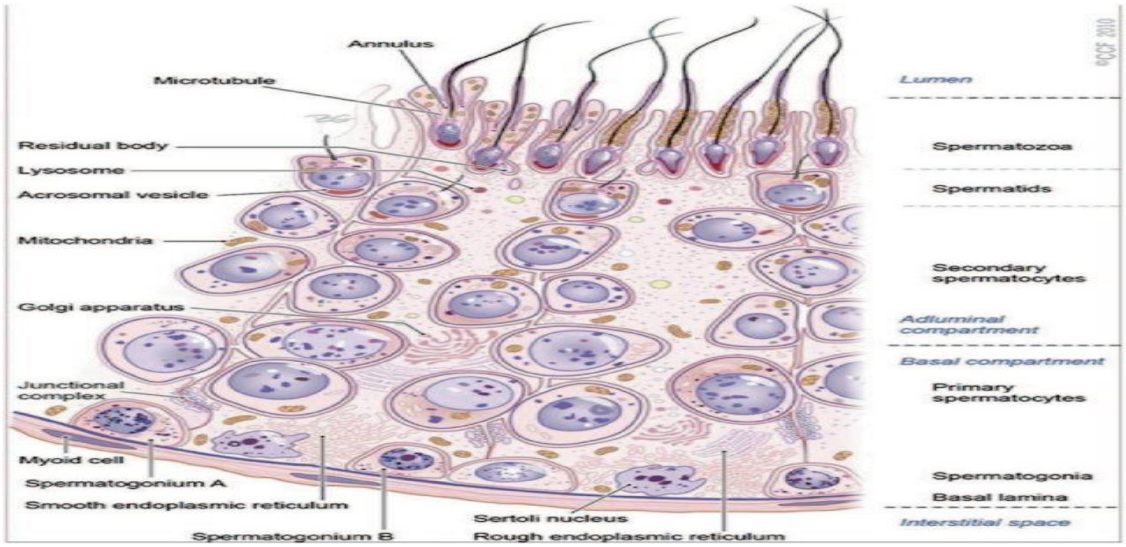


Figure 5. A cross-section of the germinal epithelium in the seminiferous tubule. (Cleveland Clinic Center for Medical Art & Photography © 2010–2013.)

1.4.3 Spermiogenesis: After one more genomic reducing step, secondary spermatocytes divide into haploid cells called spermatids and are mitotically inactive. In this stage, they undergo differentiation, DNA condensation, a decrease of cytoplasm, and transform their shape and content, achieving typically a form of spermatozoa (35, 36). As spermatids elongate, the accessory structures needed for flagella function are assembled around the central axoneme into the sheath of the middle piece, and the accessory structures needed for flagellar function (37).

1.4.4 Spermiation is the final remodeling stage of spermiogenesis when the spermatids line up along the luminal edge, shed their residual cytoplasm, and release the spermatids from the Sertoli cells in the lumen of seminiferous tubules. This is a complex process, which occurs

over several days (~80 hrs) (37, 38). At this stage, spermatozoa continue to move to the epididymis. Transit of spermatozoa through the epididymal duct is important for their final maturation and gains their fertilizing ability (39). The spermatozoa, however, will only complete maturation within the female reproductive tract. To develop into a fully functional and competent sperm for successful fertilization, modifications of the sperm membrane surface during its transit in the reproductive tracts are critical (40). According to Mortimer et al. (2013), this is not an efficient storage process as it is dependent on the individual, spermatozoa become moribund or die after a relatively short period. As a result, without ejaculation occurring every few days, spermatozoa in an ejaculate may well be compromised (41).

1.5 Regulation of spermatogenesis

The spermatogenesis process in seminiferous tubules is maintained by different internal and external factors (Figure 6).

1.5.1 Intrinsic regulation is dependent on the ability of Leydig cells to secrete testosterone, neuroendocrine substances, and growth factors.

These hormones, transmitters, and growth factors are directed to Leydig cells, blood vessels, to the lamina propria of the seminiferous tubules and Sertoli cells. They are involved in the mending of Sertoli cells and the cells of the peritubular tissue; they influence the contractility of myofibroblasts and regulate the transport of spermatozoa (42).

They also contribute to the regulation of blood flow in the intertubular microvasculature (Fig. 6).

Furthermore, Sertoli cells produce different growth factors (IGF1, TGFβ, and NGF) that participate in the regulation of cell functions and developmental processes of germ cells (43).

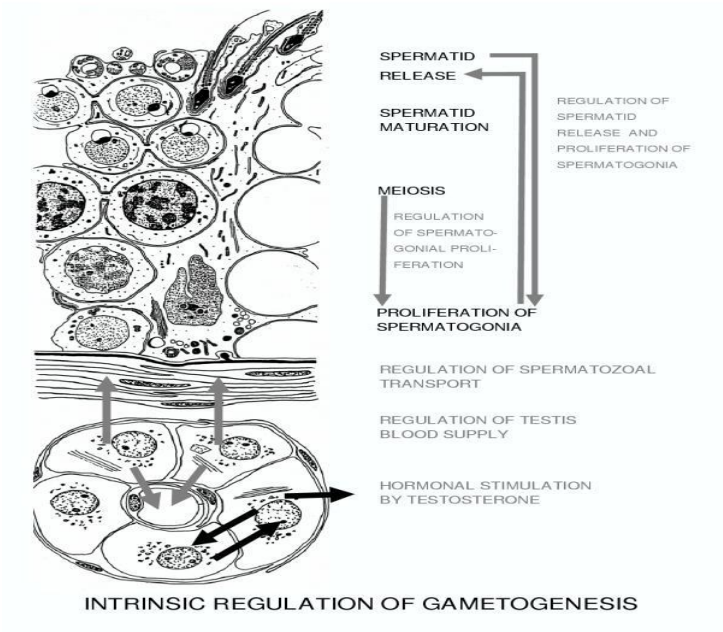


Figure 6. Intrinsic regulation of gametogenesis. On the left, a section of the germinal epithelium with basal lamina propria and below a cluster of Leydig cells surrounding a capillary are outlined. Arrows indicate different influences of secreted hormones and growth factors (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC293421/)

1.5.2 Extrinsic regulation - On the other hand, the local regulation of spermatogenesis requires the extrinsic (endocrine regulation) of spermatogenesis in the testis requires the well-known extratesticular stimuli provided by the hypothalamus and hypophysis. Pulsatile secretion of gonadotropin-releasing hormone (GnRH) of the hypothalamus initiates the release of luteinizing hormone (LH) of the hypophysis. Due to the action of LH with the LH receptor, Leydig cells convert cholesterol to testosterone. Testosterone influences not only spermatogenesis in the seminiferous tubules of the testes but it also provides feedback reaction (after aromatization in estradiol) to the hypothalamus and hypophysis related to the secretory activity of Leydig cells. Stimulation of Sertoli cells by the pituitary follicle-stimulating hormone (FSH) is necessary for the maturation and nutrition of germ cells. In the case of high testosterone levels, Sertoli cells produce the inhibin hormone, which inhibits the feedback mechanism in the production of GnRH and FSH (Fig 7) (43).

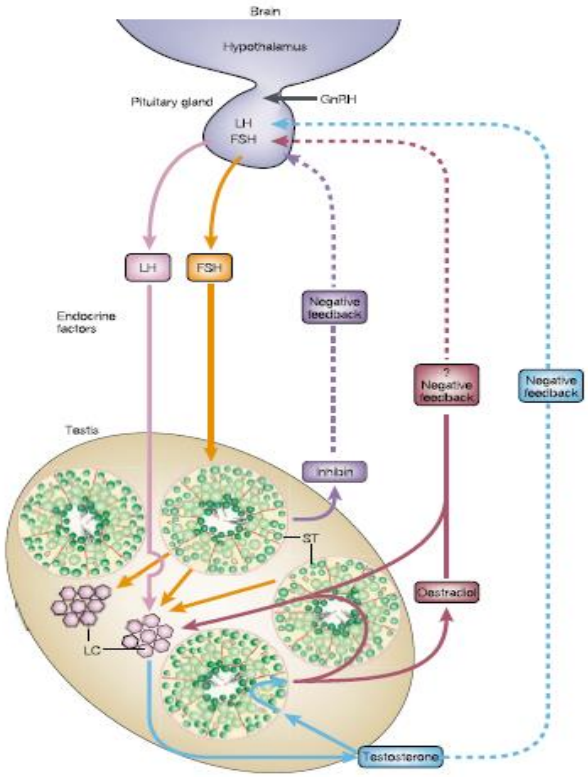


Figure 7. Extrinsic regulations of spermatogenesis (Cooke and Saunders, 2002).

1.5.3 Spermatozoon Organization and Mechanisms of Sperm Production

The mature spermatozoon is a highly specialized haploid cell that is the male gamete (44) and plays a vital role in the continuance of mammalian species with its primary purpose being to deliver an intact complement of the paternal haploid genome to the oocyte at fertilization. Fertilization in mammals is one of the most carefully regulated cell-to-cell interactions in human beings, involving two morphologically disparate cells that must recognize, bind, and fuse with each other in a very specific way. It can be defined as the process of union of two germ cells, whereby the somatic chromosome number is restored, by transferring genetic information from one generation to the next (45). A normal sperm cell is about 50 µm in length and is organized into three distinctive regions: it consists of the head, neck, and tail.

Head – The head of the sperm measures 4.5x3 µm and is the most important part of the male gamete, which should be morphologically oval in shape, with an elongated nucleus (44), which contains highly condensed paternal genetic material (DNA) with 23 chromosomes (45). The head is made up of an acrosome region, which is a product of the Golgi complex and contains acrosin enzymes and polysaccharides. Acrosome has an inner and outer membrane,

which lies between the interior plasma membrane and nuclear membrane, and plays a role in chemotaxis and acrosome reaction when the sperm comes in contact with the oocyte membrane (fig. 8).

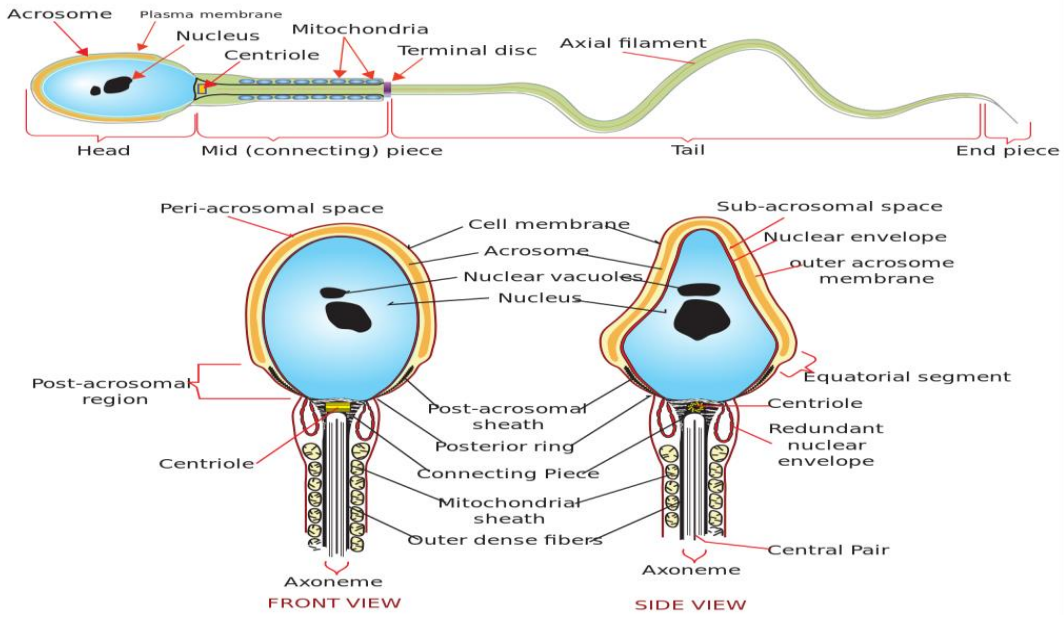


Figure 8. Sperm anatomy (Gray's anatomy 36th edition, Williams & Co.).

The DNA in spermatozoa is stabilized by protamines. Protamines bind to the phosphate of DNA using the arginine-rich domain as grapnel, allowing the DNA to fold and to create a toroid. A sperm cell can contain up to 50,000 toroid structures in its nucleus. A small percentage of DNA is bound to histones; however, the majority (80-90%) of histones are replaced by protamines during the maturation process to provide tighter compaction (47). This change reduces the head size and increases the sperm flow through liquids, preserves genome integrity that must be transferred to the offspring, and helps in various physiological events of the sperm for successful fertilization. Sperm DNA integrity is a fundamental premise for successful fertilization and the quality of embryos (3, 47).

DNA integrity of spermatozoa is often compromised in infertile men and DNA fragmentation represents the most common abnormality under the influence of various factors, which may result in abnormal chromatin structures prior to being ejaculated at any time of sperm's life: in the tests, in the epididymitis, in the ejaculatory duct, and during the ejaculation (41, 47). DNA

damage in spermatozoa can affect both mitochondrial and nuclear DNA and can be induced by six main mechanisms (figure 10) (3, 47, 48)

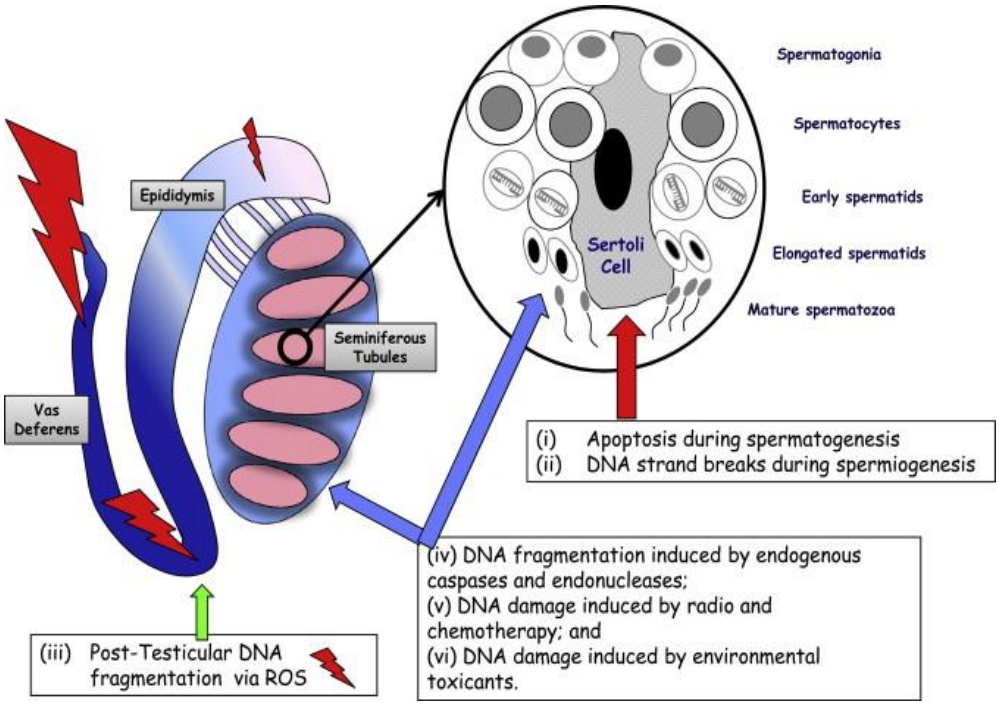


Figure 9. Mechanisms of DNA damages (American Society for Reproductive Medicine, 2010)

Midpiece (neck) takes about 10% of the total sperm length. The midpiece contains mitochondria, which provide the energy required for the sperm movement. Mitochondria create ATP via cellular respiration and take an important role in cell survival and differentiation (apoptosis) (44).

Tail is a thin, elongated structure that makes 80% of the length of the sperm. The tail includes a connecting piece, which connects the flagellum to the sperm head principal piece and the end piece of the flagella, which generates the waveform that allows for movement of the sperm (49).

1.6 Etiology of male factor infertility

Reproduction is a very important event in human life and is strongly linked with happiness, harmony, and family consolidation.

Infertility is a multi-dimensional problem with cultural and socioeconomic consequences. According to the guidelines of the European Association of Urology (EAU), approximately

13-15% of all couples are affected by infertility, and the causes of infertility are equally distributed. The male factor is responsible for approximately 50% of cases with abnormal semen parameters; in half of them, no male infertility factor is found (idiopathic male infertility) (51, 95, 109). Idiopathic infertility presents one of the most frequent types of male infertility, which is characterized by the presence of one or more abnormal semen characteristics with unknown etiology but otherwise normal findings on physical examination and endocrine, genetic, and biochemical laboratory testing (5, 51, 52, 95, 98).

The diagnosis of male infertility is generally based on the results of semen analysis. Semen analysis has been standardized by the publication of the WHO Laboratory Manual for the Examination and Processing of Human Semen (5th ed.). Problems commonly associated with abnormal semen parameters including sperm concentration, motility, morphology, and vitality. A nomenclature related to semen quality was defined in the World Health Organization (WHO) Manual 2010 (52, 111) as:

- Azoospermia (no spermatozoa in the ejaculate);

- Oligozoospermia (sperm concentration < 15 million spermatozoa/mL);

- Asthenozoospermia - < 32% progressive motile spermatozoa;

- Oligoasthenoteratozoospermia (Disturbance of concentration, motility, and morphological parameters);

- Teratozoospermia - < 4% normal forms;

- Cryptozoospermia (spermatozoa absent from fresh preparation but observed in a centrifuged pellet);

- Aspermia (No ejaculate) (52, 111).

Male infertility has many causes, which can be divided into four main areas: Hypothalamic-pituitary disorders (Table1), testicular defects (Table 2), genetic disorders of spermatogenesis (Table 3), and developmental sperm transport disorders (Table 4) (13).

Table 1**.** Hypothalamic-pituitary disorders

Congenital disorders
Congenital GnRH deficiency (Kallmann syndrome)
Hemochromatosis
Multiorgan genetic disorders (Prader-Willi syndrome, Laurence-Moon-Biedl syndrome, familial cerebellar ataxia)
Acquired disorders
Pituitary and hypothalamic tumors (pituitary macroadenoma, craniopharyngioma)
Infiltrative disorders (sarcoidosis, histiocytosis, tuberculosis, fungal infections)
Lymphocytic infundibulitis or hypophysitis
Trauma, post-surgery, postirradiation
Vascular (pituitary infarction, aneurysm)
Hormonal (hyperprolactinemia, androgen excess, estrogen excess, cortisol excess)
Drugs (exogenous androgens, opioids and psychotropic drugs, GnRH agonists or antagonists)
Systemic disorders
Chronic illnesses
Nutritional deficiencies
Obesity

Table 2. Testicular defects in sperm/hormone production.

Congenital disorders
Klinefelter's syndrome (XXY) and its variants (XXY/XY; XXXY)
Cryptorchidism
Myotonic dystrophy
Functional prepubertal castrate syndrome (congenital anorchia)
Androgen insensitivity syndromes
5-alpha-reductase deficiency
Estrogen receptor or synthesis disorders
Acquired disorders
Infections - Viral orchitis (mumps, echovirus, arbovirus); Granulomatous orchitis (leprosy, tuberculosis); Epididymo-orchitis (gonorrhea, chlamydia)
Drugs - Alkylating agents, alcohol, marijuana, antiandrogens, ketoconazole, spironolactone, histamine 2 receptor antagonists, ionizing radiation
Environmental toxins - Dibromochloropropane, carbon disulfide, cadmium, lead, mercury, environmental estrogens and phytoestrogens; smoking; hyperthermia
Immunologic disorders, including polyglandular autoimmune disease and anti-sperm antibodies
Trauma
Testicular torsion
Systemic illness
Renal failure, hepatic cirrhosis, cancer, sickle cell disease, amyloidosis, vasculitis, celiac disease

Table 3. Genetic disorders of spermatogenesis

Y chromosome microdeletions and related disorders
Autosomal and X chromosome defects
Mutations causing severe defects in sperm morphology

Table 4. Developmental sperm transport disorders.

Developmental and sperm transport disorders
Varicocele
Sperm transport disorders
Epididymal dysfunction (drugs, infection)
Abnormalities of the vas deferens (congenital absence, Young's syndrome, infection, vasectomy)
Seminal vesicles and prostate
Ejaculatory ducts disorders
Sexual dysfunction
Ejaculatory dysfunction (spinal cord disease, autonomic dysfunction); premature ejaculation; erectile dysfunction

1.6.1 Endocrine and systemic disorders

The incidence of primary endocrine and systemic disorders in infertile men is less than 3%. Any hypothalamic or pituitary disease can cause gonadotropin-releasing hormone (GnRH) or gonadotropin deficiency (hypogonadotropic hypogonadism) and therefore infertility. These causes can be divided into congenital, acquired**,** or systemic disorders. It is important to diagnose secondary hypogonadism because gonadotropin treatment is often successful (52).

Congenital idiopathic hypogonadotropic hypogonadism – These patients show decreased levels of gonadotropins and testosterone resulting in eunuchoidism (arm span 5 cm greater than height, decreased hair and muscular development, infantile genitalia, anosmia, renal malformations, and cryptorchidism). The underlying cause of hypogonadism is a defect in GnRH secretion (X linked inheritance on the short arm of chromosome X) (53).

Acquired disorders occur during various diseases including pituitary macroadenomas or surgical and radiation therapy of macroadenomas and other sellar masses, infiltrative diseases, vascular lesions, hyperprolactinemia, and excess of certain hormones (estrogen, androgen, glucocorticoids) or use of opioid-like drugs including many psychotropic drugs (54).

Systemic disorders or chronic nutritional deficiencies can cause hypogonadotropic hypogonadism and infertility.

- Renal failure leads to decreased T levels and increased gonadotropins and prolactin (55)

- Cirrhosis of the liver in 50% of the cases increase the level of estradiol (56)

-Sickle cell anemia occurs testicular atrophy and sperm maturation arrest (Hypoxemia and zinc deficiency might play a role) (57)

- Gastrointestinal diseases in coeliac and Crohn's disease, a decreased mobility and teratospermia has been observed as in 35% of patients (*58*)

- Neurological diseases: moderate oligo- and asthenospermia is observed in neurological diseases (retrograde ejaculation, urinary tract infection) (59).

Obesity in men results in hypogonadotropic hypogonadism with total testosterone, free testosterone, and low or inappropriately normal gonadotropin concentrations.

The obesity-associated decrease in serum sex hormone-binding globulin (SHBG) contributes to the low serum total testosterone concentrations. Other factors contributing to the hypogonadotropic hypogonadism seen with obesity include an increase in estrogens through aromatization in adipose tissue, insulin resistance, DNA integrity, metabolic syndrome, and diabetes mellitus. The degree to which obesity attacks the level of hormones, seminal parameters, and DNA integrity is variable and may be the result of other comorbid factors. We advise weight loss to obese men seeking infertility treatment, given the known negative effects of obesity on serum SHBG and testosterone concentrations (61).

1.6.2 Primary testicular defects in sperm production

Primary testicular defects in sperm production can be caused by a congenital trait or acquired from various diseases. Also, environmental factors can vastly influence sperm production and should be taken into account when discussing the quality of sperm production (62).

Congenital disorders are greatly associated with primary testicular defects in sperm production.

Klinefelter syndrome (XXY; XXY/XY; XXXY) is the most common form of hypogonadism in men causes of primary hypogonadism, and therefore male infertility. These patients are present with small testes, gynecomastia, azoospermia, increased height, and elevated levels of gonadotropins. Men with congenital androgen receptor abnormalities and those with 5-alpha-reductase deficiency are nearly always infertile (63).

Cryptorchidism exists in 2-3% of newborns and is present in 6% of infertile patients (65). Men with a history of undescended testes have lower sperm count, poorer sperm quality, and lower fertility rates than men with normally descended testes. Early treatment is therefore recommended to conserve spermatogenesis and hormone production, which may be partially reversible through early surgical intervention, and to decrease the risk for tumors (99, 100).

Acquired defects are often following complications of certain viral infections, among which mumps, is a well-recognized cause of infertility.

Mumps caused infertility is probably due to germinal cell damage, ischemia, or the immune response. Sexually transmitted diseases (STDs) such as gonorrhea, chlamydia, ureaplasma, or mycoplasma can also cause orchitis thus creating an opportunity for infertility development. Serval drugs caused infertility such as alkylating drugs, antiandrogens, spironolactone, ketoconazole, nitrofurantoin, cimetidine etc. These drugs cause testicular dysfunction by inhibiting testicular androgen production or action with impaired spermatogenesis (67).

Radiations and excessive heat to the genitalia have a damaging effect on the testicles. Hence, individuals in direct contact with estrogen-like hormone-disrupting chemicals such as phthalates are of particular concern for infertility in men and for effects on the offspring of women (68, 69).

Environmental toxins may be an underappreciated cause of infertility. Hundreds of synthetic and naturally occurring chemicals can disrupt normal hormone action. Implications of toxins such as physical agents, glues, pesticides, volatile organic solvents, dibromochloropropane, mercury etc. are well-known pollutants linked to infertility.

However, the chemicals with estrogenic or antiandrogenic activity including insecticides and fungicides may lower sperm count. Studies on men exposed to pesticides indicate that changes in semen quality may occur through multiple mechanisms: direct interaction with hormone receptors, indirect enhancement or suppression of a receptor's ability to respond to endogenous hormones (70, 71), or modulation of endogenous hormone levels including effects on spermatogenesis, abnormal sperm morphology, DNA damage, and the above-mentioned estrogenic or anti-androgenic compounds (72).

Tobacco smoking - The testis is considered to be vulnerable to hypoxia because of its high metabolic requirements owing to the continuous spermatogenesis process (73, 77). Therefore,

tobacco smoking compromises the transport of oxygen and is suspected to have a pernicious effect on testicle functions (74, 75, 76, 78).

The effects of tobacco smoking on fertility and reproduction have become apparent but are not generally appreciated. Tobacco smoke contains more than 4000 types of constituents including nicotine, tar, carbonic monoxide, polycyclic aromatic hydrocarbons, radioactive substances, heavy metals, etc.

Data on cigarette smoking and its possible effect on sperm count are inconsistent. At higher concentrations, nicotine reduces the percentage of spermatozoa by inducing the DNA fragmentation and Leydig cells apoptosis (75, 76, 78).

Most studies have reported reduced semen quality, reproductive hormone system dysfunction and impaired spermatogenesis, sperm maturation, and spermatozoa function in smokers compared to nonsmokers.

The toxicological mechanism is complicated. Underlying these effects, impairments in the expression of miRNAs and histone-to-protamine transition, oxidative stress, and cell apoptosis may play important roles in the overall effect of tobacco smoking on male fertility (74, 75, 76).

Tobacco smoking leads to a reduction in all semen parameters: semen volume, spermatozoa motility, sperm concentration and morphology, including reproductive hormone system disorders, sperm maturation, and impaired spermatozoa function.

Despite the various harmful effects of smoking on male fertility, most male smokers are still fertile but have a higher risk of sub-fertility or infertility (77, 78).

Hyperthermia has long been thought to impair spermatogenesis. For optimal spermatogenesis, the testicular temperature is maintained at 2–3°C lower than body temperature (79, 80). The effect of temperature on male fertility is evident. An increase of 1°C causes a 14% drop in spermatogenesis, and consequently poorer sperm production (81).

Immunologic disorders, anti-sperm antibodies - Some infertile men have antisperm antibodies in serum or semen and both presumably could impair spermatogenesis.

The presence of sperm agglutination in the semen should trigger the laboratory to test for antisperm antibodies.

Whether antibodies occur spontaneously or only after some testicular injury is not known. Primary hypogonadism occasionally occurs in men with type 2 autoimmune polyglandular syndrome (82).

Trauma - Testicular trauma is a frequent acquired cause of infertility. There was definite evidence of subfertility as assessed by abnormal semen analyses and atrophic testes following testicular trauma. However, the subfertility did not appear to be immune-mediated nor did the patients present with infertility (83, 84).

Testicular torsion refers to the rotation of a testicle which interferes with the blood supply to the testicle and other nearby structures in the scrotum.

Mechanisms of testicular deterioration in torsion are cellular hypoxia, ischemia, leukocyte infiltration, reactive oxygen species (ROS), germ cell apoptosis, antisperm antibodies, etc.

In all cases, early surgical intervention (mean torsion time < 12 hours) was found to preserve fertility, whereas surgical detorsion in which torsion lasted more than 24 hours, subsequent atrophy of the testis was the rule (86).

Prolonged torsion periods followed by orchiectomy jeopardize fertility (87).

1.6.3 Genetic and epigenetic changes in infertile men

Epigenetic changes encompass molecular modifications of DNA or histones that are intimately associated with DNA.

DNA wraps around histones to form nucleosomes. Nucleosomes are packaged into a higher order of structures called chromatin; modifications in chromatin control gene-expression (5, 7, 47, 95, 96, 97).

Genetic disorders affecting spermatogenesis only can be identified in about 10 to 20 percent of cases of male infertility (9, 10, 15, 16, 95, 96, 147).

With the widespread availability of assisted reproductive technologies (ART), men can have children, but there are genetic risks for the offspring, and genetic testing is necessitated in all azoospermic men (figure 10.) (9, 10, 16, 96, 98, 115).

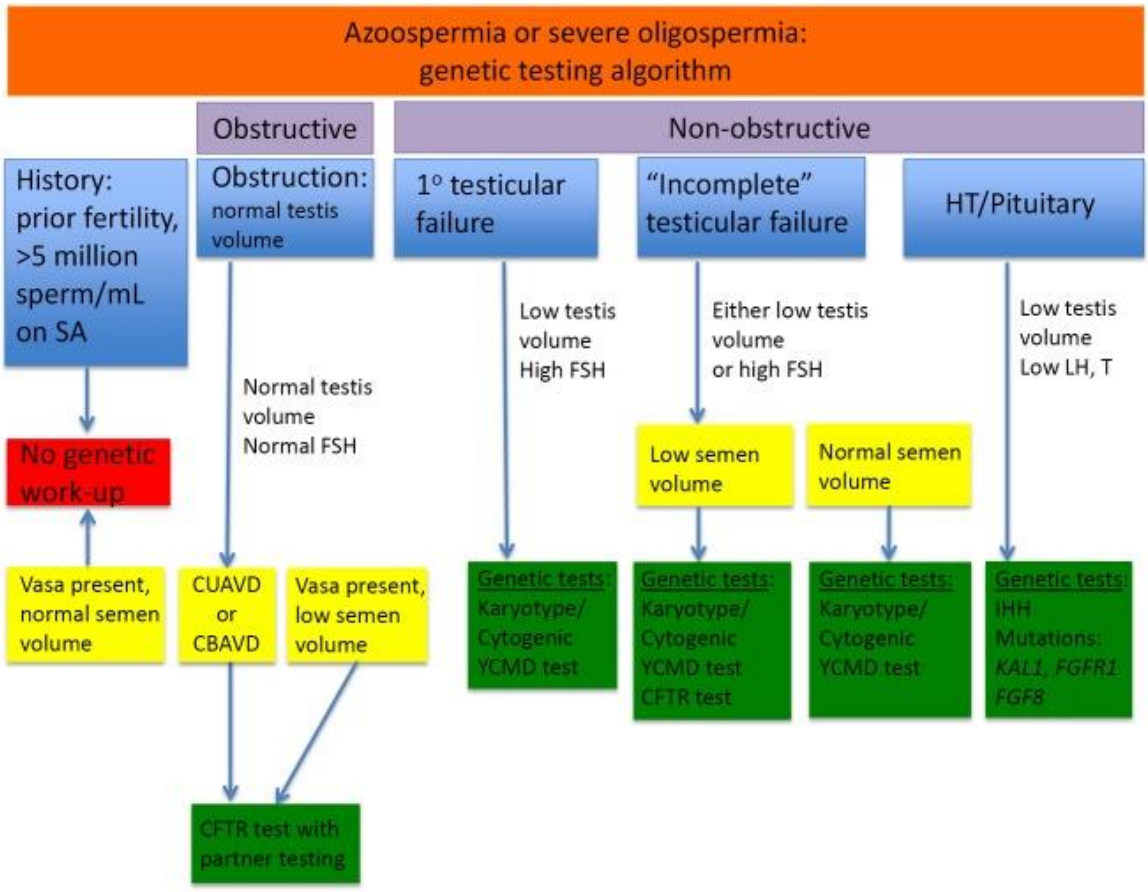


Fig. 10. Genetic testing algorithm. CUAVD, congenital unilateral absence of the vas deferens; CBAVD, congenital bilateral absence of the vas deferens; YCMD, Y chromosome microdeletions; CFTR, cystic fibrosis transmembrane conductance regulator; FSH, follicle-stimulating hormone; SA, semen analysis; HT, hypothalamus; LH, luteinizing hormone; IHH, Indian hedgehog protein; KAL1, FGFR1, FGF8, complex genetics of Kallmann syndrome (Wosnitzer MS.Transl Androl Urol. 2014).

Male infertility can be associated with various genetic factors, including chromosomal aberrations, genetic alterations, and Y chromosome microdeletions (9, 10, 15, 16, 89, 91, 92, 93, 147).

Chromosomal abnormalities may be present in approximately 6% of infertile men, and the prevalence is inversely correlated with sperm count. Azoospermic men can be affected in up to 16% of cases (88). Sex chromosomal aneuploidy (Klinefelter syndrome; 47,XXY) is the most frequent chromosomal disorder present in infertile men and is generally associated with hypotrophic or atrophic testicles, elevated serum FSH levels, and azoospermia. The mutation of the cystic fibrosis gene (CFTR gene), which is located on the long arm of chromosome 7, is

also a relatively common genetic disorder. The long and short arms of the Y chromosome are related to spermatogenesis and testicle development. The Y chromosome region related to infertility is called the azoospermia factor (AZF) locus. The locus can harbor complete or partial microscopic deletions that are isolated or in combination with one another, and in non-overlapping subregions called AZFa, AZFb, AZFc, and AZFd (88, 89, 91, 93, 94).

The most common Y chromosome deletion in infertile men is the one affecting the DAZL gene (deleted in azoospermia) located in the AZFc region *SOX5* (Transcription factor SOX-5), and *TEX11* (Testis expressed 11). Mutations in these genes are mostly associated with azoospermia (88, 90, 93). Apart from mutations, also copy number variants (CNVs) in the X chromosome can be a relevant indicator of infertility in men (89, 91). Y chromosome microdeletions are found in 15% of men with azoospermia and 6% of men with oligospermia. The AZF region of Yq11 contains three clinically significant regions: AZFa, AZFb, and AZFc (88, 89). After conception, Yq microdeletions are transmitted to the male offspring, and genetic counseling is mandatory (114, 115).

Epigenetics in male infertility has only recently been studied. Sperm DNA methylation, histone acetylation, and noncoding RNAs (including microRNAs, long noncoding RNAs, and sRNAs) may contribute to defective embryogenesis and idiopathic male infertility (2, 95). Histones are basic proteins of nuclei and are the best candidates for the transmission of epigenetic information and access of transcriptional machinery to genes. DNA wraps around histones to form nucleosomes (96). H2A, H2B, H3 and H4 histones are an integral part of nucleosomes. Histone modifications, such as acetylation, methylation, ubiquitylation, and phosphorylation, have emerged as the main players in epigenetic regulatory mechanisms. Nucleosomes are packaged into a higher order of structures called chromatin (97). The incorporation of protamines into sperm chromatin induces DNA compaction, which is important for the formation of spermatozoa and for providing a safe environment for the genome (50, 70, 81, 98).

1.6.4 Developmental and sperm transport disorders

Infertility is often related to dysfunctional sperm transport and development. The most frequent disorders are cryptorchidism, varicocele, testicular cancer, abnormalities of epididymis or vas deferens and ejaculatory obstruction (5, 13).

Varicocele is a dilatation of the pampiniform plexus of the spermatic veins in the scrotum. The mechanisms by which a varicocele might cause infertility are still not well known. Varicoceles are found in about 10 to 15 percent of normal men and about 40- 45 percent of infertile men (5, 13). The exact association between infertility and varicocele is unknown, but a recent meta-analysis showed that semen improvement is usually observed after surgical correction. Varicocelectomy can reverse sperm DNA damage (101). A Cochrane review from 2013 concluded that varicocelectomy in men with unexplained infertility may improve a couple's chance for spontaneous pregnancies (101, 102). A recent meta-analysis has reported that varicocelectomy may improve outcomes following insert assisted reproductive techniques (ART) in oligozoospermic men (103, 114, 115).

Testicular germ cell tumor (TGCT) is the most common malignancy in Caucasian men aged 15-40 years, and affects 10/100.000 men, and approximately 1% of infertile men, with substantial differences among countries. Several studies have suggested an increased incidence of testicular cancer in men presenting with infertility (104). However, these studies were limited by sample size, and thus, routine screening for testicular cancer in men who present with infertility is not a necessity at this time (4, 5).

Dysfunction of vas deferens is usually caused by obstructions due to infections or genetic mutations of cystic fibrosis transmembrane conductance regulator (CFTR) gene and thioredoxin-nucleoside diphosphate kinase (105).

Ejaculatory duct obstruction is a well-defined, potentially correctable cause of male infertility. It is infrequently diagnosed and is found in only up to 5% of infertile men (106). Patients with ejaculatory duct obstruction present with a low ejaculate volume and seminal fructose with no sperm count and/or very low sperm motility. Ejaculatory duct obstruction is uncommon but can be diagnosed and treated surgically with minimally invasive techniques (106, 107).

Dysfunction of seminal vesicles and prostate - It is not known if the abnormal function of the seminal vesicles and prostate contributes to infertility, but chronic infection of accessory glands may result in leukospermia and might be a possible cause of infertility (108).

1.6.5 Idiopathic male infertility

Male infertility of unknown origin is a condition in which fertility impairment occurs spontaneously or due to obscure or unknown causes and it can present as unexplained male

infertility and idiopathic male infertility. The crucial difference between these two diseases lies in the results of semen analysis. A normal result leads to a diagnosis of unexplained infertility and an abnormal result of idiopathic infertility (51, 95, 98, 109).

1.6.6 Disorders related to sperm function

Sperm dysfunction is one of the most common causes of infertility. Sperm basic parameters such as sperm concentration, sperm motility, and morphology are essential for normal fertilization and play a crucial role in sperm function (110). The diagnostic assessment of sperm function is currently assessed using descriptive semen analysis. According to WHO guidelines, semen analysis includes many parameters like volume (mL), pH, count/concentration (10^6 /mL), motility (%-motile), vitality (eosin) (%), and morphology (%) (111, 112, 113).

These parameters are used to classify male infertility such as decreased spermatozoa motility, (asthenozoospermia), decreased spermatozoa morphology (teratozoospermia), and decreased number of spermatozoa (oligozoospermia) or a combination of each. In case of a complete absence of spermatozoa in the ejaculate in at least two separate centrifuged samples, the abnormality is described as azoospermia.

Any deviations from the reference range of semen variables (Table 5) are considered abnormal.

However, this is a poor method for diagnosing male infertility and it is only considered useful in cases where the concentration of motile sperm is low like in males with azoospermia or severe oligozoospermia (<5 million sperm/mL) (111, 112).

The prognostic value of semen parameters, such as sperm number, motility, and morphology, is influenced by many other factors related to sexual activity and the function of accessory sex glands, making the final diagnosis of infertility very difficult. Routine semen analysis has its limitations and does not account for putative sperm dysfunctions such as DNA fragmentation.

Results from at least two or three separate seminal analyses (recommended intervals between 1-2 weeks) must be obtained before a definitive conclusion is reached (111, 112). This is due to overlap by as much as 60% between the semen parameters of males with proven fertility and males with male factor infertility (113).

Table 5. Lower reference limits (5th centiles and their 95% CIs) for semen characteristics. Adapted from

Jungwirth (Jungwirth et al., 2013)

Parameter	**Lower reference limit (range)**
Semen volume (mL)	1.5 (1.4-1.7)
Total sperm number (106/ejaculate)	39 (33-46)
Sperm concentration (106/mL)	15 (12-16)
Total motility (PR + NP)	40 (38-42)
Progressive motility (PR, %)	32 (31-34)
Vitality (live spermatozoa, %)	58 (55-63)
Sperm morphology (normal forms, %)	4 (3.0-4.0)
Other consensus threshold values	
pH	> 7.2
Peroxidase-positive leukocytes (106/mL)	< 1.0
Optional investigations	
MAR test (motile spermatozoa with bound particles, %)	< 50
Immunobead test (motile spermatozoa with bound beads, %)	< 50
Seminal zinc (μmol/ejaculate)	≥ 2.4
Seminal fructose (μmol/ejaculate)	≥ 13
Seminal neutral glucosidase (mU/ejaculate)	≤ 20

1.6.7 Disorders related to obstructive lesions (azoospermia)

The categories of the etiology of azoospermia are:

1. Pre-testicular azoospermia (2%, hypothalamic or pituitary etiology);
2. Testicular failure or non-obstructive azoospermia (50-90%);
3. Post-testicular obstruction (7-47%, normal spermatogenesis but obstructive azoospermia). (107, 116, 117, 120)

Using ICSI, it is now possible to produce a pregnancy with any live sperm (moving or not), from either the semen or any site within the male reproductive tract. Even men with azoospermia can now be offered sperm retrieval with ICSI. Sperm could be retrieved from any site in the reproductive tract and used for ICSI. These are men who previously had very limited chances to ever have biologically related children (114, 115, 117, 119, 121).

Azoospermia could be due to:

- absence/abnormalities of the vas deferens/seminal vesicles,
- retrograde ejaculation, or
- failure of emission (107, 116, 117)

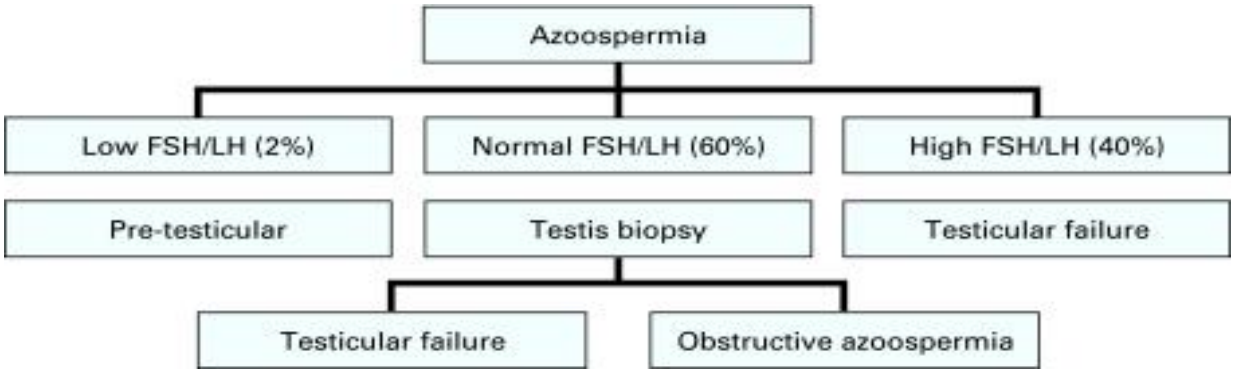


Fig. 11. Algorithm for differentiating the causes of normal semen volume azoospermia. FSH = follicle-stimulating hormone; LH = luteinizing hormone (Canadian Urological Association Journal)

Obstructive azoospermia (OA) is considered a disease with one of the most favorable prognostic conditions for male infertility. OA can result from epididymal, vasal, and/or ejaculatory duct pathology, but it is not linked to a disruption in spermatogenesis (107, 116, 117, 120). Severe genitourinary infections, surgical procedures, and congenital anomalies are other common causes of OA. Approximately 40% of c azoospermia cases result from an

obstruction in the ductal system (107, 116, 117, 120). Azoospermia diagnosis is based on the absence of spermatozoa after centrifugation of complete semen specimens using microscopic analysis according to WHO guidelines (111). Azoospermia affects approximately 1% of the male population and ranges between 10-15% among infertile males. History, physical examination, semen analysis, and hormone profile with serum FSH, LH, and total testosterone are undertaken to define the cause of azoospermia (107, 116, 117).

Males with OA may be managed in one of the following three ways:

1. **Sperm retrieved directly from testis or epididymis** (90% chance of finding sperm) using sperm retrieval techniques like TESE (testicular sperm extraction), TESA (testicular sperm aspiration) or TEFNA (testicular fine needle aspiration) then the sperm is used in IVF or ICSI program. Any of the types of retrievals techniques are acceptable (106, 107, 114, 115, 116, 117).

2. **Surgical correction of the obstruction by bypass/repair** techniques of the obstructed area is possible in less than half of the men with obstructive azoospermia. The most common area of obstruction is within the epididymis. With microsurgical techniques, surgical centers reporting over 85% patency of the anastomosis with over a 50% spontaneous pregnancy rate. Centers with this kind of operation recommend the option to cryo-bank sperm retrieved during the operation in case the surgery is not successful (116,117, 119).

3. **Transurethral resection (TUR) of ejaculatory duct** - This technique is useful in men with ejaculatory duct obstruction. It is important to warn men of the potential complications arising from transurethral resection of the ejaculatory ducts (118).

1.6.8 Disorders related to spermatogenic failure (NOA)

Non-obstructive azoospermia (NOA) is diagnosed in approximately 10% of all infertile males, in which male partners have impaired production of spermatozoa. It represents a failure of spermatogenesis due to either a lack of appropriate stimulation by gonadotropins or an intrinsic testicular impairment (119). These subjects have abnormal spermatogenesis as the cause of azoospermia (107, 116, 117). Approximately 60% of these cases are due to testicular failure (120). Unlike in obstructive azoospermia, the spermatogenesis in males with NOA is disrupted (27).

Therefore, the only way for the affected couples to achieve pregnancy without involving a donor is to improve their spermatogenic function to enable the appearance of sperm in their ejaculate or to retrieve spermatozoa directly from the testes for ICSI (114, 115, 121).

An ideal surgical technique would achieve efficient retrieval of sperm while causing minimal trauma to the testes (121). As presented in Figure 10, the initial diagnostic evaluation is to confirm the cause of azoospermia and to exclude treatable conditions like hypogonadotropic hypogonadism and obstructive azoospermia, and to identify males who are candidates for ART, who are sterile, and who should undergo genetic testing and counseling (107, 114, 117, 120, 121).

1.7 Hormonal regulation of spermatogenesis

In humans, the mechanisms of hormonal regulation of spermatogenesis are not completely understood. However, it is known that the initiation of spermatogenesis occurs at puberty due to the interaction of the hypothalamus, pituitary gland, and Leydig and Sertoli cells. Advances in understanding the hormonal regulation of spermatogenesis (required for the management of male fertility and the potential development of reversible male hormonal contraception) were made possible thanks to several approaches (122).

The reproductive hormonal axis consists of three main components, the hypothalamus, pituitary gland, and the testes. Overall, the hypothalamus secretes the gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland in a pulsatile manner to secrete and release luteinizing hormone (LH) and follicle-stimulating (FSH) hormone. FSH regulates Sertoli cell proliferation and main functions, while LH acts on Leydig cells to stimulate testosterone synthesis. Spermatogenesis initiates at puberty when the adult human testis overcomes the physiological hypogonadotropic hypogonadism to get to a eugonadal state (122, 123). Normal spermatogenesis requires functional somatic cells expressing the androgen receptor (Sertoli, Leydig, and peritubular myoid cells), while germ cells express neither FSH receptor (FSHR) nor androgen receptors (AR). Dysfunction in this axis can result in common disorders such as delayed puberty, ambiguous sexual differentiation, and infertility (122, 123).

1.7.1 Gonadotrophin-releasing hormone (GnRH) is a small neuropeptide called decapeptide, which is composed of 10 amino acids. GnRH secretion is pulsatile and surge modes. Pulsatile mode refers to the episodic release of GnRH, usually every 1-2 hours, and

has a very short half-life (<10 min), where there are distinct pulses of GnRH secretion with undetectable GnRH concentrations during inter-pulse intervals (fig.12) (124, 125).

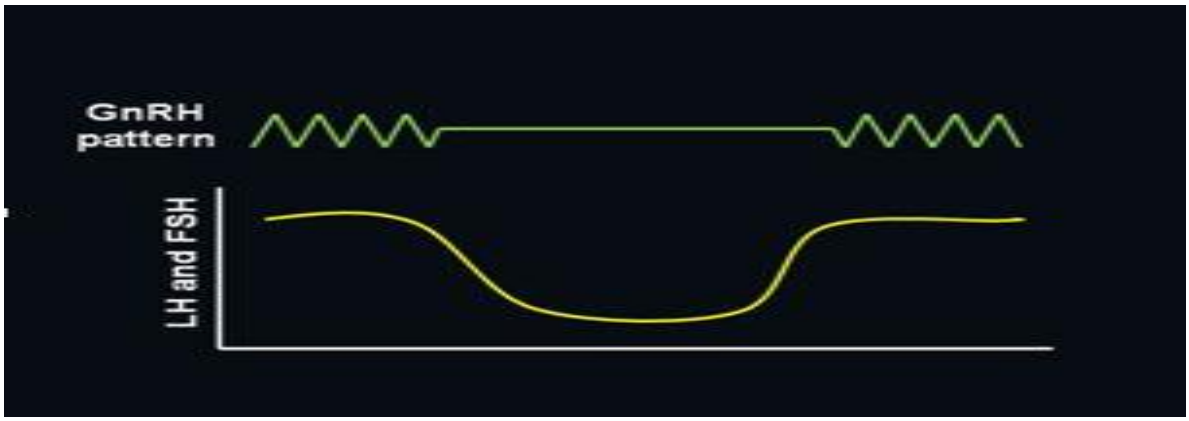


Fig. 12. Importance of pulsatile secretion of GnRH to stimulate the release of LH and FSH. The secretion of both LH and FSH sharply decline as GnRH secretion is changed from pulsatile to continuous (**Global Library of wWomen's Medicine 2013**)

GnRH activity is very low during childhood and increases during puberty. GnRH secretion depends on the activation of the GPR54 receptor located on the surface of the GnRH neurons and stimulated by the kisspeptin secreted in the hypothalamus under the feedback inhibiting mechanism of testosterone and inhibin. Kisspeptin is a product of the KISS1 gene, located on chromosome 1q32.1. Outside the nervous system, the KISS1 gene is expressed in the placenta, testes, pancreas, liver, and intestine (126). The receptor expression is higher when GnRH is given in a pulsatile manner, and the withdrawal of GnRH during the interpulse intervals leads to an increase in GnRH-binding sites just before the next pulse occurs (self-priming). In the absence of GnRH, the gonadotrope cells don't secrete LH or FSH (127).

1.7.2 Gonadotropins LH and FSH are glycoprotein hormones secreted by the pituitary gland that control the development, maturation, and function of the gonads (122, 128).

1.7.3 Luteinizing hormone (LH) acts upon the Leydig cells of the testes and is regulated by GnRH. The Leydig cells produce testosterone under the control of LH, which regulates the expression of the enzyme 17β-hydroxysteroid dehydrogenase that is used to convert androstenedione to testosterone. During fetal development, testosterone causes the formation of the prostate gland, seminal vesicles, and the genital duct; it stimulates the initial phase of testicular migration from the abdominal cavity into the scrotum and inhibits the formation of

female genitalia. The fetal testicle already produces testosterone during the 10th week of gestation under the stimulation of fetal LH and maternal hCG (128).

During infancy, serum testosterone and gonadotropins are very low, and GnRH secretion appears to be extremely limited, even in the presence of negligible steroid production by the gonads. The LH and FSH secretion in serum is detected at the time of puberty first during nighttime, and then progressively also during the day (128).

During puberty, testosterone causes the enlargement of the penis, scrotum, and testes; it influences the secondary male sex characteristics and body hair patterns, stimulates maturation of sperm cells, bone and muscle growth, stimulates the enlargement of the thyroid cartilage and deepening of the voice (129).

The onset of puberty is controlled by two major hormones. FSH initiates spermatogenesis and LH signals the release of testosterone that exerts endocrine and intratesticular activity on spermatogenesis, while LH is released from the pituitary gland, and is controlled by pulses of gonadotropin-releasing hormone.

When testosterone levels are low, it stimulates the pituitary gland to release LH. As the levels of testosterone increase, it will act on the pituitary through a negative feedback loop, leading to increased synthesis and secretion of Gonadotropin-Inhibitory Hormone (GnIH), which consequently inhibits the release of GnRH and LH (128, 129).

1.7.4 Follicle-stimulating hormone (FSH)

FSH and its receptors are essential for normal male and female reproductive function. In males, FSH regulates the function of Sertoli cells where spermatogenesis takes place.

In addition, FSH is necessary for gonadal development and maturation and the initiation and maintenance of spermatogenesis during the fertile period of life (11, 122, 123).

The FSH binds and activates an FSHR in the cell membrane of the Sertoli cells, where it functions as a spermatogonial survival factor.

FSH is 35.5 kDa glycoprotein consisting of an α subunit, which is non-covalently associated with a specific β subunit, secreted by the anterior pituitary that acts directly on the testes, which control the development, maturation, and function of the gonads.

The beta subunit is unique to FSH, which determines the biological receptor specificity consisting of 112-118 aa that accounts for the specificity of the hormone when it binds to its respective receptor, while the α subunit is a member of a family of five glycoprotein hormones that include FSH, LH, TSH, hCG, and inhibins, consisting of 92 aa.

The gene that encodes the α subunit of FSH is located on chromosome 6, while the gene that encodes the β subunit is located on chromosome 11.

FSH is synthesized and secreted from the anterior pituitary gland in response to the binding of hypothalamic gonadotropin-releasing hormone (GnRH) to its receptor in a pulsatile manner (Figure 13).

In addition, research indicates that there are extrapituitary sources of FSH such as the prostate, testes, ovaries, and breasts (43, 130).

FSH stimulates Sertoli cell proliferation, which is the most significant contributor to testicular volume in children. The Sertoli cells produce AMH, which causes the involution of the Mullerian ducts, preventing the formation of female internal genitalia (131).

As other glycoproteins, FSH is subject to intensive posttranslational modifications that lead to the formation of FSH isoforms.

These isoforms differ in their oligosaccharide structure after synthesis; FSH is transported and performs its biological actions to its target site, the Sertoli cells, through specific cell surface receptors FSHR (132).

FSHR is exclusively expressed on Sertoli cells, and global inactivation of FSHR leads to compromised spermatogenesis and subfertility (123, 132).

By quantitatively analyzing testicular development, several studies have documented that FSH signaling through Sertoli cells is required to establish the full capacity of spermatogenesis during prepubertal testicular development, and postpubertal spermatogenic capacity appears to require both FSH and testosterone (123, 132).

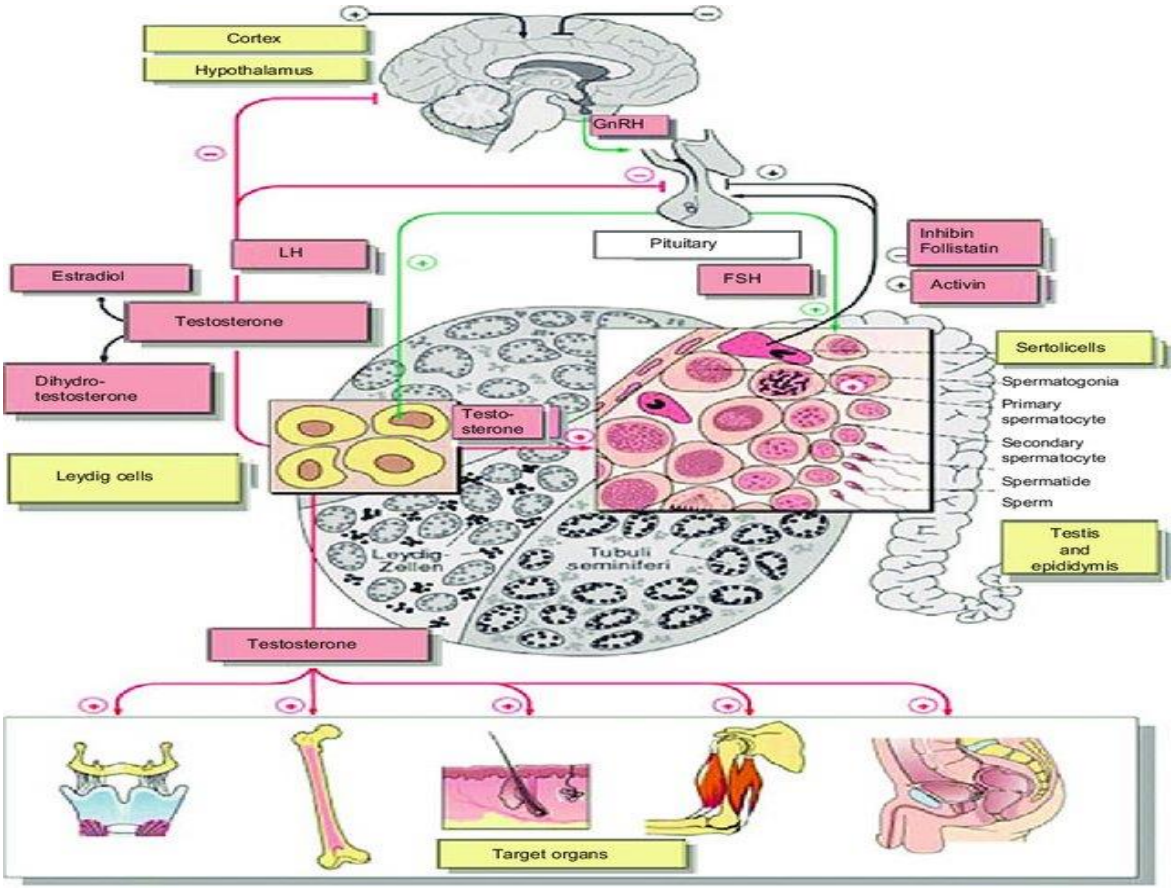


Figure 13. Hormonal regulation of the testicular function and effects of androgens. Secretion and action of FSH; GnRH: Gonadotropic Releasing Hormone, FSH: Follicle-stimulating hormone, LH: Luteinizing Hormone (adapted from Antonio Aversa 2017)

FSH manifests its effects via its FSH receptor (FSHR). After binding to FSHR, conformational changes in the FSHR lead the activation of several extracellular and intracellular signaling pathways depending on heterotrimeric G proteins. The Gαs-subunit interacts with intracellular loops 2 and 3 of the activated FSHR with several intracellular events, i.e. exchange GDP to GTP, dissociate Gαs-subunit-GTP, and stimulate the adenylate cyclase by increasing the intracellular cAMP levels and their propagation into the cell (141). The main signal transduction pathway for the FSHR is to activate its specific receptor the cAMP protein kinase A (cAMP-PKA). Its activation leads to a release of the catalytic subunit of PKA, followed by the phosphorylation of enzymes and proteins like cAMP response element-binding protein (CREB), which activates the transcription of FSH-dependent genes (Figure 14). Moreover, FSH causes an increase in intracellular calcium, mediated by cAMP, which causes the activation of calmodulin (CaM) and CaM kinases that

result in downstream effects including the phosphorylation of CREB. Downstream signaling of PKA includes complex interplay of several cytosolic and nuclear targets such as PLA (phospholipase A), PI3K (phosphatidylinositol 3-kinase), RAS (r*eal* authentic sound), RAF (rapidly accelerated fibrosarcoma), MEK (mitogen protein kinase), ERK (extracellular signal-regulated kinase), and MAPK (mitogen-activated protein kinase). Once activated, the Ras/Raf/MEK/ERK/MAKP signaling cascades to transmit signals from their receptors that result in stimulated spermatogenesis (Fig.14).

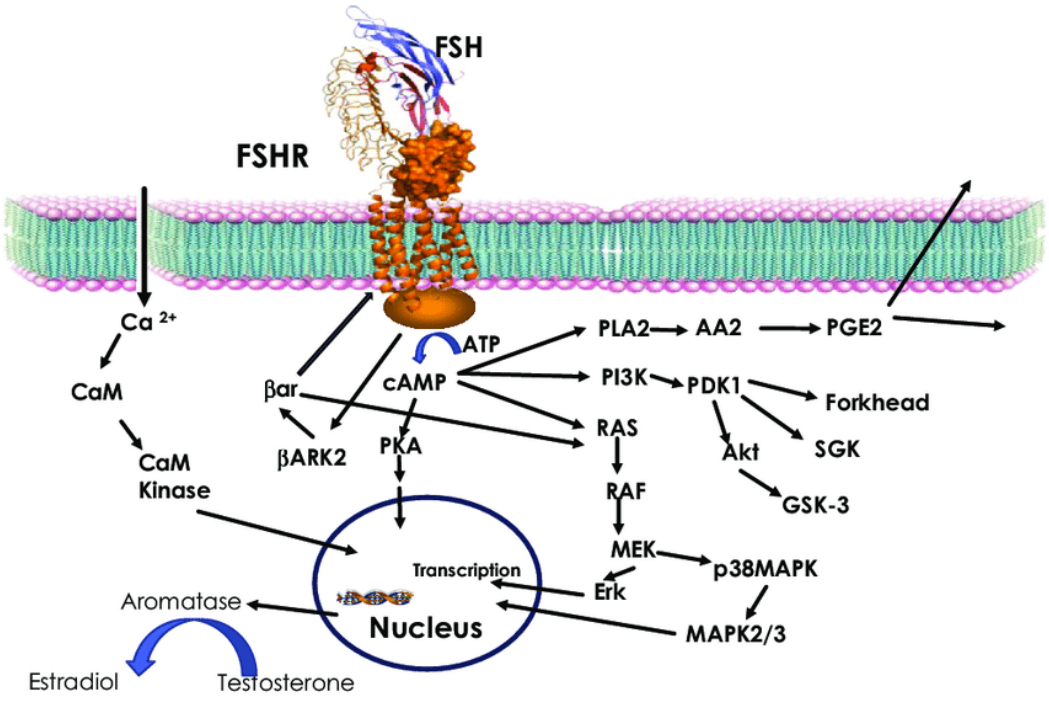


Figure 14. Activation of FSHR by FSH leads to an increase in intracellular cAMP through Gs-adenylate cyclase. Increased cAMP leads to PKA activation, which regulate the expression of several genes through phosphorylation of transcription factors like CREBP. (researchgate.net/publication/282760348 /fendo-06-00142-g001.jpg).

In adult men, FSH plays an important role in maintaining the metabolic functions of Sertoli cells. This contributes to normal spermatogenesis, germ cell survival and male fertility (123, 133). In addition, FSH acts as the main stimulator of seminiferous tubule growth during development. Since the tubules account for 80% of testis volume, it is clear that FSH represents a major determinant of adult testicular size and germ cell number per testis (133).

1.7.5 Roles of FSH in spermatogenesis

Two pituitary gonadotropins, FSH and luteinizing hormone (LH) through the hypothalamic-anterior pituitary-testicular axis (Figure 13) control spermatogenesis (122, 123, 133). LH regulates testosterone biosynthesis at the rate-limiting step of catalysis by the cholesterol side-chain cleavage enzyme (CYP11A1). Testosterone is a major sex hormone in men, influencing several neurotransmitters, and with its levels highly correlated to motivational behavior in status-seeking and maintenance in social groups leading to cooperative and reputation-seeking behavior *(*122, 129, 134*).* The site of testosterone production is the interstitial Leydig cells, and the local intratesticular concentration of testosterone is in the order of 50–100-fold higher than that in the systemic circulation (129, 134, 135). After synthesis, testosterone is liberated from Leydig cells and diffuses to Sertoli cells where it acts together with FSH and stimulates spermatogenesis (129, 132, 133).

The secretion of LH and FSH is regulated by GnRH, a decapeptide released by the hypothalamus. FSH synthesis and secretion depend on slow GnRH pulses (every 2–4 h), while rapid GnRH pulses (every 30 min) lead to the preferential secretion of LH (124, 125, 127). Testosterone inhibits the release of LH (more than FSH), with a minor contribution from peripherally converted estradiol and dihydrotestosterone (DHT) (122, 134, 135). The secretion of FSH is selectively inhibited by the Sertoli cell hormones inhibin A and B (129, 131, 133, 134).

The FSH activates the signal pathway that controls Sertoli cell proliferation during the perinatal and pubertal period, thus determining the testicular volume and the adult spermatogenic capacity (129, 131, 134). FSHR inactivating mutations associate different phenotypes and present variable degrees of spermatogenic failure, but do not show azoospermia or absolute infertility (137, 138, 141, 142). This could also be related to a probable residual activity of FSH in Sertoli cells (129, 136, 138). It is also interesting to mention that hCG administration after having suppressed gonadotropin secretion and decreased sperm production with androgenic treatment increases the number of spermatozoids without restoring normal levels (136, 137, 139). In patients with hypogonadotropic hypogonadism, FSH and androgen administration not associated with hCG fail to initiate or maintain spermatogenesis (136, 139).

1.8 Follicle-stimulating hormone receptor

The follicle-stimulating hormone receptor (FSHR) plays a crucial role in reproduction. Normal cells function involved in spermatogenesis, including the production of FSH by these cells, depends on the FSHR gene expression. The human FSHR, together with LHR and TSHR, belongs to the glycoprotein hormone receptors subfamily of class A. Knowledge of combination of SNPs in FSH and FSHR is essential to determine patient risk/treatment outcome in cases of infertility. However, the impact of FSHR gene expression on male infertility is not yet well understood (153, 165).

1.8.1 FSHR structure and function

The FSHR belongs to the seven transmembrane domains receptor (7TMR) family of glycoprotein coupled receptors and is only expressed in Sertoli cells. Up to date, several case-control studies and their meta-analyses have been conducted to evaluate the association between the FSHR polymorphic regions and male infertility (139, 141, 142, 143).
In women, FSHR is located in the plasmalemma of granulosa cells and regulates the menstrual cycle and oocyte maturation through FSH. In men, FSH acts by binding to the FSHR located in the Sertoli cell surface in testes where they function as spermatogonial survival factors and regulate the proliferation of these cells (138, 140).

This structurally complex receptor belongs to the family of G protein-coupled receptors (GPHRs), a superfamily of transmembrane proteins. The FSHR proteins are composed of 695 aa; the first 17 aa encodes the signal sequence, as with the other structurally similar glycoprotein hormone receptors (TSH, HCG, and LH) (140,142). The FSHR is characterized by a very large NH2-terminal extracellular domain known for potential glycosylation sites in the extracellular domain with different binding effect on certain gonadotropins. FSH receptors expressed at the surface of Sertoli cells are available for binding the FSH and participates in the activation/inactivation of the receptor (140, 142, 143). The FSHR structure consists of an extracellular hydrophilic domain (ECD) composed of a long N-terminal part and three loops. This ECD connects to a hydrophobic region comprising seven transmembrane domains (TMDs) and intracellular hydrophilic regions composed of three intracellular loops and the C-terminal part (140, 141, 142, 166).

The extracellular portion is functionally divided into two subdomains, the hormone-binding domain (HBD) consisting of ten consecutive leucine-rich repeats domain (LRR) and the hinge

region located between the HBD and the first TMD. The hinge region contains two additional LRR, followed by a hairpin loop and α-helix flanked on both sides by two cysteine box motifs abled to form cysteine bonds between themselves. The hinge ends with a highly conserved decapeptide (FNPCEDIMGY), located close to the first TMD reported as being necessary for receptor activation. The HBD is composed of 349 aa, and when recognition and binding to FSH occur, it enables the second step of a conformation change in the hinge region. At the COOH-terminal end of the hinge region that links the LRR ECD with the seven-transmembrane α-helical domains (7TMD), plays the main role in receptor activation because this part differs in structure between different FSHR, so it is capable of binding only to the target destination hormone (Figure 15) (141, 166).

The transmembrane portion belongs to the superfamily of G protein-coupled receptors. Each member of this group is characterized by seven hydrophobic cylinders of 20–25 aa predicted to form transmembrane α-helices, spanning the cell membrane and connected by intra- and extracellular loops (141, 142, 166).

The hinge region of all GPHRs is involved not only in affinity binding of the ligand but also in receptor activation, intramolecular signal transduction, and silencing of basal activity in the absence of ligand, whereas the seven-transmembrane domain is associated with receptor activation and transmission of the activation process to intracellular loops comprised of aa sequences, which predicate coupling to effectors, interaction with adapter proteins including agonist-stimulated, deactivation, trafficking, and signal transduction to effectors (141, 142, 143, 166).

The intracellular portion is the signal transduction part, which is responsible for forwarding the message from the hormone-receptor complex into the nucleus.

The FSH receptor coupled to G_s proteins and other interacting proteins involved in signaling, desensitization, and internalization of the receptor, upon receptor activation by the hormonal interaction with the extracellular domain, initiates the cascade of events that ultimately leads to specific biological effects of gonadotropin.

The intracellular domain displays some homology only in the N-terminal portion (aa 631–659 of the FSH receptor), which might be relevant for receptor coupling and regulation of signal transduction by inducing differential gene expression involved in a variety of cellular processes including selectivity, differentiation, and proliferation (141, 142, 153, 166).

The mechanism of FSHR activation goes through a two-step process.

The first stage corresponds to the binding of FSH into the concave surface of the curved HBD domain like a handclasp.

Contact with hormone is secured by ten residues, charged for the most part (L55, E76, D81, R101, K104, Y124, D150, K179). Some of them participate in the FSHR specificity for FSH (L55, E76, R101, K179, I222) (141).

The binding of FSH on the HBD enables the second step of the ligand-induced receptor activation consisting of a structural change in the hinge region.

This structural change has the role of FSHR activation (141, 142, 143).

FSH binds more tightly the HBD, leading to a more rigid FSH conformation, the main reason for the gain in affinity comes from the presence of a sulfated tyrosine (Y335) in the hairpin loop of the hinge region that constitutes a second interaction site between FSH and its receptor (141, 143, 166).

The sulfated tyrosine-binding pocket interacts with the sulfated-Y335 and αresidues of FSHR and hence provokes the tilting of the hairpin loop and the rotation of the helix of the hinge region, consequently causing a conformational change of the TMDs, then the extracellular loops may interact with the charged residues in the hairpin loop and finally stabilize the active state of the receptor.

It is thought that the hinge region may react as a tethered inverse agonist keeping the RFSH in an inactive conformation.

The FSH binding on the HDB could alleviate this inhibitory effect by changing the receptor conformation (141, 142, 143).

As illustrated in figure 15, the extracellular domain consists of several leucine-rich -αstrands (indicated by arrows) and βrepeats (LRRs) that are made up of alternating helices (indicated by a cylinder). The transmembrane domain consists of seven hydrophobic segments spanning the cell membrane and connected by intra- and extracellular loops (141, 142, 143).

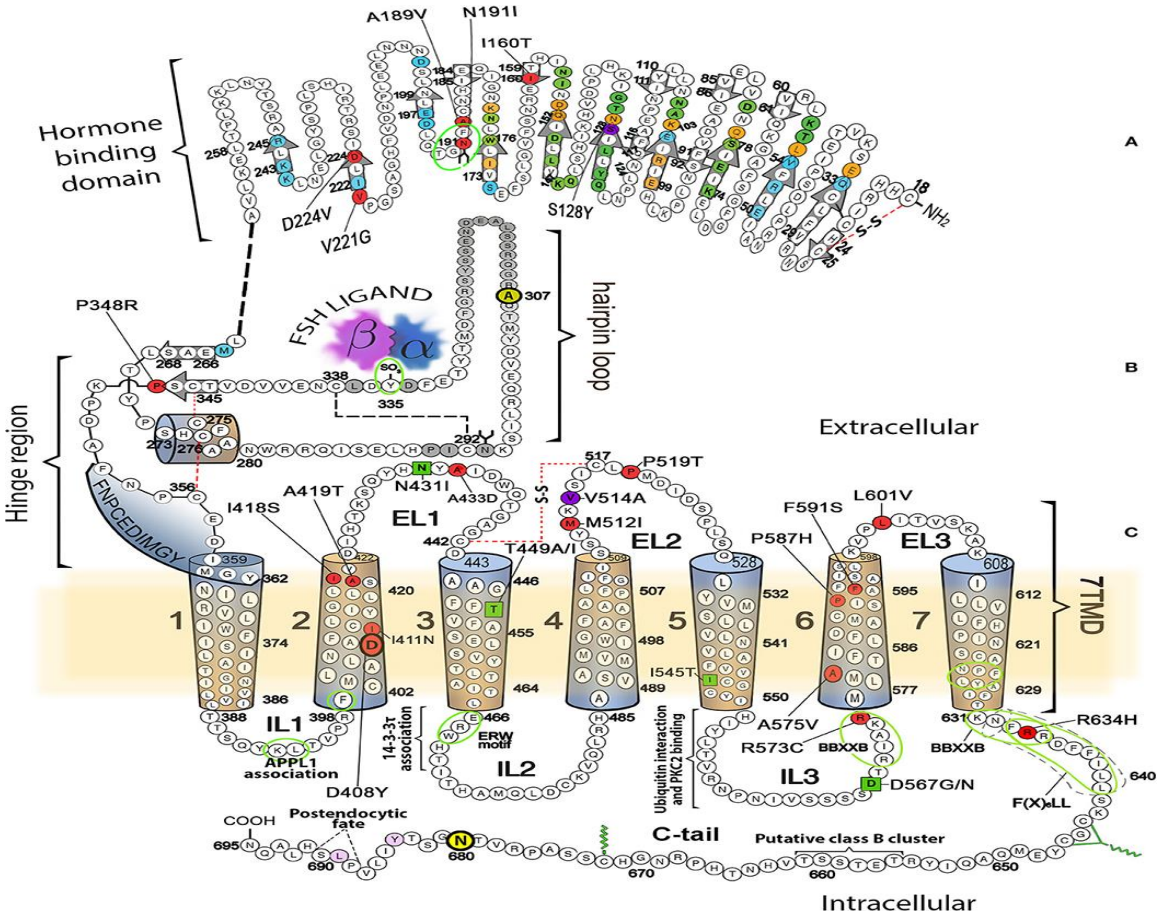


Figure 15. Schematic representation of the FSHR. **(A)** Hormone specific binding domain. Residues buried in the FSH/FSHR interface and located in the high affinity binding site (green, binding to FSH α-subunit; blue, binding to FSH β-subunit; orange, residues that interact with both FSH subunits. **(B)** Hinge region with the sulfated tyrosine involved in ligand-provoked binding to the FSH subunits. **(C)** The transmembrane domain consists of seven hydrophobic cylinders spanning the cell membrane and connected by intra- and extracellular loops. (Copyright© 2016 by The American Society for Pharmacology and Experimental Therapeutics**)**

1.8.2 Location, structure, and organization of the FSH receptor gene

The human FSHR is synthesized by a single-copy gene and spans a region of 54 kb, located on the short arm of chromosome 2 at region 2p21–p16.3 (Figure 16), more precisely, from base-pair 48,953,160 to base-pair 49,154,526 on chromosome 2 (140, 141, 142).

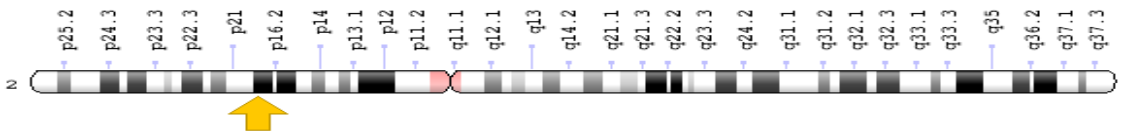


Figure 16. Location of FSHR gene on chromosome 2 (National Institute of Health, 2015)

This gene is about 190 kb in size. Its coding region comprises ten exons, each varying in size from 69 to 1,234 bp, nine introns with sizes 108 to 15 kb (140, 141,142), and promoter region (Figure 17). Most ECD, including the hinge region, are encoded by nine exons (1-9), whereas exon 10 is large and encodes the C-terminal part of the extracellular domain (hinge region), the 7 transmembrane domain (which contains 3 extracellular and 3 intracellular loops), and the C-tail part of the intracellular domain of the receptor (141, 142, 143).

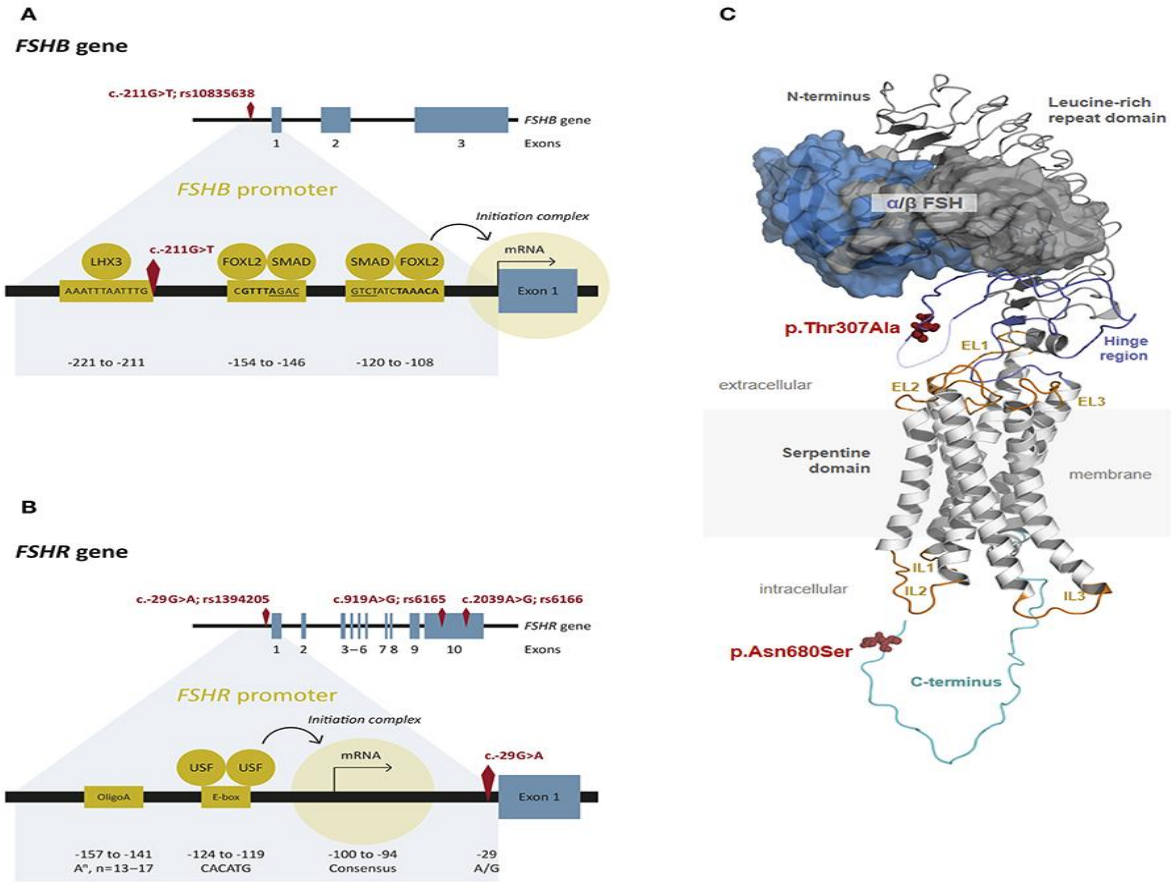


Figure 17. FSHB and FSHR: Gene, promotor and protein structure (A) Structure of the FSHB gene and promotor (B) Structure of the FSHR gene and promotor (C) Protein structure of FSH and FSHR (**Gunnar Kleinau 2018**)

1.9 Genetic Polymorphism

Genetic polymorphisms are defined as the inheritance of a trait controlled by a single genetic locus with two alleles, in which the least common allele has a frequency of about 1% or greater. Genetic polymorphisms may be the result of imperfect proofreading activity of DNA polymerase or may have been induced by external agents (such as viruses, radiation, mutagenic substances) (130).

A Single Nucleotide Polymorphism (SNP) is a single base mutation in DNA, where one nucleotide is substituted to another nucleotide, but there are many gene variations called insertions, deletions, duplications ore translocations. SNPs are the simplest form and most common source of human genetic polymorphism (90% of all human variations that occur in the human genome) and represent genetic differences between people to be used to predict phenotypes and phylogeny (144).

There are two types of nucleotide base replacement resulting in SNPs (144); the transition replacement occurs between purines or between pyrimidines (75% of all SNPs), while transversion replacement occurs between a purine and a pyrimidine (25% of all SNPs). SNPs are not uniformly distributed over the entire human genome, neither overall chromosomes nor within a single chromosome. The sequence variation is much lower for the sex chromosomes (130, 144). An SNP in a coding region may have three different effects on the resulting protein:

- silent mutation (no amino acid change, protein stays exactly the same)
- missense mutation (there is a change in amino acid sequence on the resulting protein)
- nonsense mutation (misplace of termination codon resulting in shorter/longer protein). SNPs may also occur in regulatory regions of genes and can change the amount or timing of a protein's production. These SNPs can change the amount or production timing of a protein (144, 150).

Phenotype, genotype, and haplotype are the most important and basic concepts related to SNPs (144).

1.9.1 Genetic polymorphism as a risk factor for male infertility

For more than three decades, genotyping has been used as a successful concept in a search for male infertility genetic disorders, including both chromosomal and single-gene alterations (146, 147).

To date, a considerable number of genes seem to play an essential role in infertility, and which, when deleted or mutated, can cause pathologic changes in the male reproductive system (146, 147), although some data remain controversial because ethnic or geographical origin appear to play an important role in the expression of certain genetic polymorphisms. When it comes to male infertility, many autosomal genes are currently being investigated for their possible role in male factor infertility/subfertility, while genetic testing has become part of the routine diagnostic procedure in selected groups of patients. Karyotype and Y microdeletion analyses are indicated in patients with severe oligoasthenozoospermia (<5 million spermatozoa/ml) (88, 89, 92, 146, 147).

Congenital bilateral absence of the vas deferens (CBAVD) is an important cause of obstructive azoospermia (147).

Unlike healthy men, the CFTR gene, located on chromosome 7, is mutated in 80–90% of patients with congenital bilateral absence of the vas deferens (CBAVD), suggesting that CFTR gene plays a role in the etiology of infertility in men with obstructive azoospermia (146, 147).

ICSI is a useful method of treatment for men with the CFTR mutation as long as the female does not also carry the CFTR mutation (114, 146, 147). Due to the high incidence of CFTR mutations in patients with obstructive azoospermia, we suggest screening for CFTR mutations before ART.

Partners who both carry the mutation should be advised to have Pre-implantation Genetic Diagnosis (PGD) to avoid passing the abnormality to their offspring (147). Another study of the sex hormone-binding globulin (SHBG) gene located on chromosome 17 has also been studied for a possible role in spermatogenesis.

The gene polymorphism of *SHBG* revealed an association of the effect of polymorphism on semen quality, supporting the significance of these genes in spermatogenesis and semen quality (146, 147, 148).

Other investigated SNPs occur in the estrogen receptor genes (*ESR1 and ESR2*). Estrogens play an important role in spermatogenesis; a deficit or excess of estrogen can alter spermatogenesis as both receptors are highly expressed in human testicular germ cells. The elevated number of *ESR* correlates with lower levels of spermatogenesis (146, 147).

1.9.2 FSH receptor polymorphisms and male infertility

Several studies have investigated the possible impact between Asn680Ser polymorphisms in the FSHR gene and male infertility. Although mostly inconclusive, some of them revealed a significant difference in the distribution of FSHR 2039A>G allelic variants between infertile men and fertile healthy controls (140, 141, 145, 150, 152, 153, 154, 156, 157).

The deterioration of FSH signaling in target cells could be a consequence of its receptor mutations. Recently, a large number of natural mutations in *FSHR* genes have been reported and so far, there are more than 900 SNPs. This high frequency of SNPs in *FSHR* can be explained by the large size of the gene (192 kb) and the SNP distance (282 bp). However, the impact of FSHR gene variants on male infertility is currently not yet well understood (144, 145, 146, 150, 153).

The human FSHR gene spans a region of 54 kb, consists of ten exons and nine introns, and maps to chromosome 2q. The majority of known polymorphisms are located in the introns and have no known effect on receptor activity.

There are three common polymorphisms of the FSH-R gene, one in the promoter region and two in exon 10 (139, 140, 141, 150, 151, 153). The most studied SNP in the promoter region is located at position c.-29 (c.29G>A, rs1394205), while two of them located in exon 10 (c.919A>G, p.Thr307Ala, rs6165, and c.2039A>G, p.Asn680Ser, rs6166).

The first SNP in exon 10 is located in the extracellular domain at codon position 307 (nucleotide 919), which can be occupied either by alanine (Ala) or by threonine (Thr), while the second SNP located in the intracellular domain at codon position 680 (nucleotide 2039) changes an asparagine (Asn) to serine (Ser) (130, 142, 150).

The SNP in amino acid position 680 is in strong linkage disequilibrium with an SNP in amino acid position 307, and those two SNPs hence are inherited together and form two haplotypes. The SNP in amino acid position 307 causes an amino acid change from Thr to Ala. Thr in amino acid position 307, co-inherited with N680, is present in approximately 60% of white populations, while A307, co-inherited with S680, is present in approximately 40% of white populations (130, 140, 150, 151, 153, 166). An exception is the SNP at codon 392 that is silent (Figure 18).

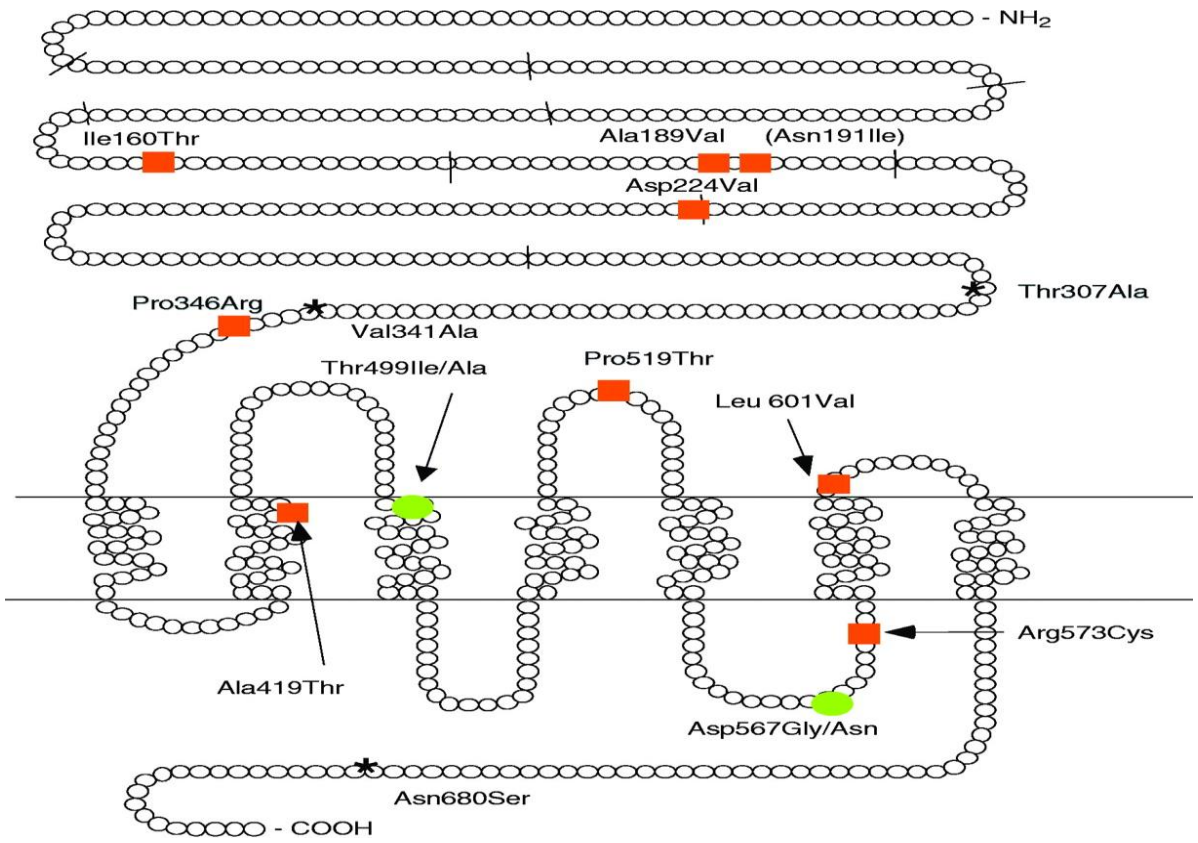


Figure 18. The currently known mutations and aa altering polymorphisms in the human FSH receptor gene. The green circles depict the activating mutations, the red squares inactivation mutations, and the asterisks the polymorphisms (Themmen et al. 2005)

The genomic location of FSHR genes is selected with the position of SNPs because much information in the literature indicates their possible role in the biological response to FSH. As shown in Fig. 19, these SNPs are selected genetic markers among many others belonging to complex genomic regions (153). The HapMap Database shows about 900 SNPs for FSHR organized in distinct linkage disequilibrium (LD) blocks, segments of the genome, in which a given combination of alleles or genetic markers is inherited coordinately. The three-dimensional structure and the mode of FSHR LD blocks differ in their structure between ethnic groups, resulting in various combinations of the different SNPs. This should be considered when assessing association studies obtained from different populations (Figure 19).

Figure 19 shows the genomic location of FSHR genes along with the position of single-nucleotide polymorphisms (SNPs).

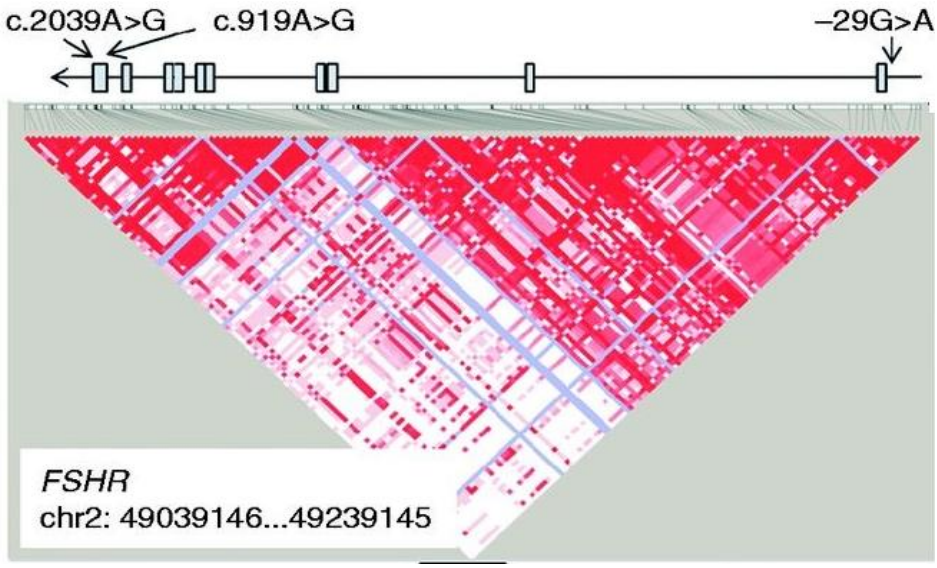


Figure 19. Intron–exon structure, SNP positions, and LD blocks in the human FSHR and FSHB genes

Among mentioned SNPs, Thr307Ala and Asn680Ser attracted widespread attention regarding male infertility. However, the results of previous studies are quite inconclusive (153).

The geographic distribution of the rs6166 (*FSHR* c.2039A>G) allele frequencies is presented in Figure 20.

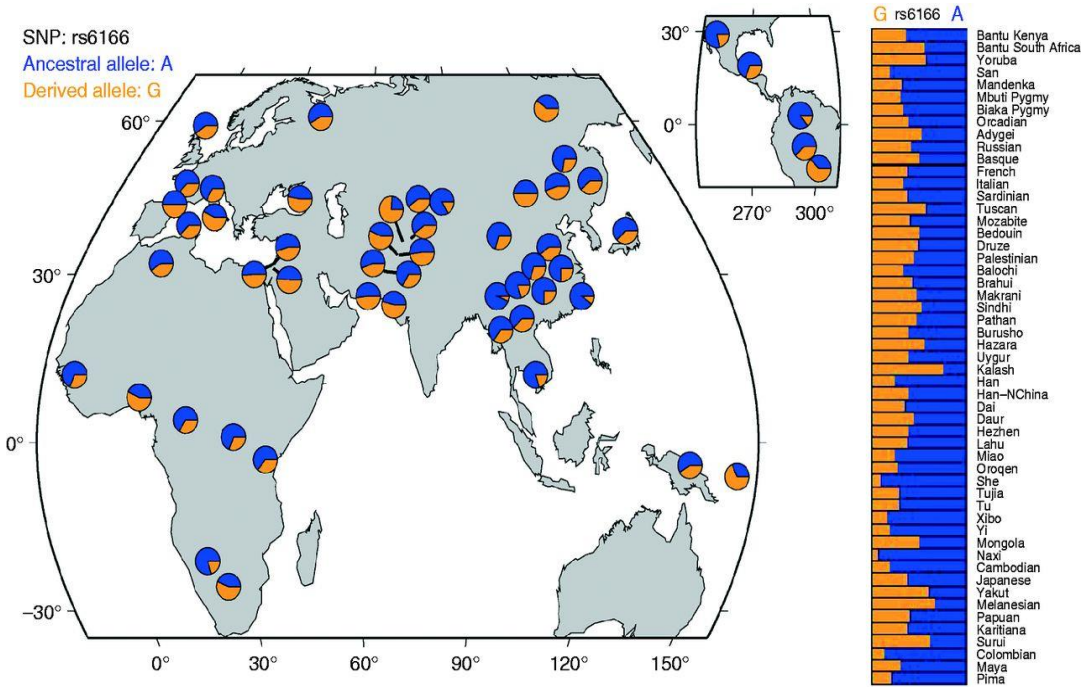


Figure 20. Geographic distribution of rs6166 (*FSHR* c.2039A>G) allele frequencies (Simoni et al. 2014)

FSH with LH and testosterone are the major hormones influencing sperm formation and maturation. FSH does not induce the process of spermatogenesis; however, it is necessary to maintain the metabolic functions of Sertoli cells, normal spermatogenesis, germ cell survival, and male fertility (123, 131, 133).

The interaction between FSH and FSHR plays a crucial role in normal gametes. FSH regulates the function of Sertoli cells through its surface receptor (FSHR). The FSHR gene, located on the surface of cells of ovaries and testicles, defines the receptor response to FSH (123, 132, 133).

The association between FSHR gene polymorphisms and male infertility risk has attracted widespread attention due to the unique biological functions of FSH.

To date, no clear consensus appears to have been reached in the literature on the relationship between FSHR polymorphisms and male infertility risk. Reported results have been contradictory; some studies did not find significant differences in the FSH SNP in infertile patients and controls (153, 154, 155, 157, 170).

Other investigations, however, found significant variations of SNP distributions and suggested that ethnic differences could be involved in polymorphism-related infertility, concluding that abnormalities of the FSHR gene, as well as FSH gene, would affect sperm production in males (153, 156, 157, 158, 166).

Some studies in normal and infertile men and women reported no difference, while other studies in women revealed significant differences in the FSHR SNP are known to influence serum FSH concentrations, length of menstrual cycle, and effective FSH amount during ovarian stimulation for IVF techniques (151, 153, 154, 155, 157, 162).

A recent meta-analysis suggested that although in the single-site association analysis, no significant associations were identified between FSHR Thr307Ala and Asn680Ser polymorphisms, the combined genotypic frequency of Thr/Ala + Asn/Asn was higher in infertile patients than in controls (153, 154, 155).

The SNP in codon 680 was analyzed by Ahda et al. in 742 German men with nonobstructive azoospermia (438 men with nonobstructive azoospermia and 304 controls) (152).

For the first time, Tuttelmann et al. reported that FSHR A2039G affects males as well; the combined effect significantly modulated the more dominant effect of *FSHB* -211G > T on serum FSH and testicular volume, whereas A2039G effects were 2-3 times smaller (145).

The significant role of the FSHR T307/N680 polymorphism in Swedish young men was demonstrated by lower FSH, higher SHBG and E2, and total testosterone concentrations, higher sperm concentration, total sperm count, and larger testicles (166).

The significant role of the FSHR variants and its relationship with seminal AMH among OAT and infertile Egyptian males have been investigated in a study by Zalata et al. (2008).

Their results showed that the frequency of FSHR gene variants among fertile men and OAT men is not associated with significant differences; however, analysis for the Asn680Ser variant was shown to exhibit significantly higher FSH, lower serum testosterone, and lower total testes volume. Seminal AMH was significantly higher in fertile than infertile OAT men.

There was a significant increase in seminal AMH with the Asn/Asn variant of the FSHR gene than those with Asn/Ser or Ser/Ser (158).

A study in the Japanese population concluded that the combination of heterozygous variants of Thr/Ala and Ser/Asn was significantly increased in infertile patients compared to controls and might be responsible for male infertility (156).

Analyzed FSH-R polymorphisms at codons 680 and 307 in Iranian infertile men and age-matched healthy fertile men did not show a significant association of the FSH-R Thr307-Ala and Asn680-Ser polymorphisms with the incidence of male infertility. However, the combination of heterozygous Thr/Ala + Asn/Ser genotypes increases the risk of idiopathic male infertility (157).

The associations of SNP polymorphisms at position c.2039, as well as hormonal and semen parameters, have been studied by D. Tsitlakidis et al.; the data showed no association between FSHR SNPs and male infertility in the Greek population. One SNP combination, the heterozygous SNP (AG) at position 2039 was associated with different sizes of the volume of the right testis (164).

The possible role of the distribution of FSHR Ala307Thr and Asn680Ser polymorphisms in infertile Brazilian men was analyzed and evaluated by Ghirelli-Filho et al. According to the sperm count, relatively similar FSHR polymorphisms genotype and allele frequencies were

found among the groups, and combined genotypes of two polymorphisms did not identify a haplotype associated with sperm count.

When the combined genotypes of the FSHR polymorphisms were compared to FSH serum levels, statistical analysis showed no difference among the groups (171).

The association between three FSHR gene polymorphisms and possible risk for spermatogenetic failure was evaluated in Italian male infertility.

No significant difference was found in allelic variants frequency and genotype distribution between infertile males and normozoospermic controls. The data showed that in the Italian population, FSHR SNP had no influence on FSH concentrations and was not associated with spermatogenetic impairment (170).

Associations between the Thr307Ala and Asn680Ser polymorphisms of the FSHR gene has been well studied by Qiuyue Wu et al. in infertile and fertile men from North China. In the present study, no significant associations were identified between FSHR Thr307Ala and Asn680Ser polymorphisms and male infertility ($p>0.05$).

However, the combined genotypic frequency of Thr/Ala + Asn/Asn was higher in infertile patients than in controls ($p=0.027$).

There was also no evidence of FSHR polymorphism association with male infertility ($P>0.05$) in the meta-analysis; however, the combined genotypes Thr/Thr + Asn/Asn had an increased risk of male infertility ($p=0.049$) (154).

The significant role of the FSHR p.N680S polymorphism in males has been reported by a study involving a very large number of Baltic men (Estonians, Latvians, Lithuanians) and an Estonian male infertility group.

In the meta-analytical approach performed on 1790 men, the data statistically demonstrate the significant association of the FSHR AsnN680Ser polymorphism alone on testis volume, inhibin B, serum FSH, and testosterone levels.

The effect was small, but significant between the FSHR Ser680 variant and higher serum FSH ($p = 0.072$), lower inhibin B ($p = 0.037$), and total testosterone ($p = 0.034$) (155).

In a study conducted by Balkan et al. on the Turkish population, the data demonstrate that FSHR gene polymorphisms seem not to have any statistically significant differences in the FSHR haplotypes.

They concluded that FSHR haplotype has no direct influence on spermatogenesis, but is differently distributed, thus identifying an additional genetic factor that may contribute to the multigenic origin of male infertility (174).

A variety of polymorphisms of genes regulating reproductive functions have been detected during the last decade; however, it should be emphasized that in genetic case-control studies, false positive results can occur (145, 146, 152, 153).

To improve reliability in our study, we recruited an adequate number of subjects (235 in total); the study participants were selected strictly to the purpose.

This way, we removed the bias associated with confounding factors. It is especially important to conduct a case-control study using large and specific ethnic populations; this can lead to data with a higher probability for correct data (153, 165).

These controversial data regarding the significance of *FSHR* gene polymorphisms in male infertility imply that there is a need for further research in this field, particularly in populations that have not been included in previous studies (153, 154, 155, 166).

2. HYPOTHESIS OF THE STUDY

Through this prospective study, it has been proposed to compare and evaluate the impact of FSHR polymorphisms on male infertility in the reproductive age ≥ 24y ≤45y, by measuring hormone values such as FSH, LH, testosterone, prolactin, HIV, HCV and syphilis antibodies, routine microbiological and biochemical tests, spermiogram, and by ultrasound measuring the position, echogenicity, and volume of the testes.

As we have expected, the investigated FSHR polymorphism affects spermatogenesis and it also influences FSH and testosterone levels and volume of the testes in infertile men in the Albanian population.

This study will provide new data that can be used in the field of human fertility over the coming decades that would create an increased need to focus additional research on male infertility factors. As we gain more knowledge on the significance of male factors, it is necessary to present these findings in these areas to provide better treatment for infertility couples. The data obtained from this study are intended to provide useful information to physicians, clinical staff, policymakers, administrators, health care providers, sperm donors, and infertility couples. The findings can be beneficial for the clinical and investigative systems as they adapt their practices to meet the personal needs of individual infertility couples.

Finally, infertile couples can gain meaningful knowledge on lifestyle factors and lifestyle changes that can improve their fertility success rates and enhance their infertility treatment.

3. OBJECTIVES

3.1 General objective

The purpose of this thesis was to genotype Asn680/Ser680 polymorphisms in FSHR gene and to determine the allele variant distribution of Asn680/Asn680, Asn680/Ser680, Ser680/Ser680 in infertile men and fertile healthy controls and to investigate the impact of FSHR gene polymorphisms on human reproductive parameters, thus showing that ethnic differences could be contributory. To our best knowledge, no previous study investigated the effect of FSHR gene polymorphism on male fertility in the Albanian population from the Dukagjini region in Kosovo. Therefore, the present study is the first to assess FSHR polymorphism in infertile Albanian men.

3.2 Specific objectives

- Investigate and identify the possible role of these genetic variants of the FSHR polymorphism influence reproductive parameters in men from the general population, which changes could be a cause of infertility/subfertility;

Find if the occurrence of this genotype in the Albanian male population is influenced by socio-cultural specificities of this population;

- Find if there is a difference in the distribution of genotypes among control group patients, and to compare it with the polymorphism frequency in other population groups;

- Investigate a possible association between these polymorphic variants of the FSH receptor and infertility among the Albanian male population, who are undergoing infertility treatment procedures.

The goal of this study is to improve our knowledge of FSH polymorphism by identifying that when mutated, it causes infertility/subfertility. The ultimate goal is to apply a genetic marker as a routine diagnostic test, which can be used in the evaluation of therapy success rate or the introduction of new procedures for the treatment of male infertility.

4. METHODOLOGY, MATERIALS, AND PARTICIPANTS

4. 1. Study population

We will investigate a group of 123 infertile men (oligoasthenozoospermic, azoospermic, normoasthenozoospermic) and 112 men in the control group with normal spermiogram according to the World Health Organization (111) FSH <10, with at least one healthy child.

Approved fathers were recruited in the General Hospital Department of Urology in Peja. All patients had a fertile female partner as determined by a fertility evaluation. All of them have signed a written consent form. The molecular analysis was conducted in the Laboratory for Epigenetics and Molecular Medicine, Department of Biology and Medical Genetics School of Medicine, Zagreb. The study was approved by the Hospital Ethics Committee, Ethics Committee of the Ministry of Health in the Republic of Kosovo, and Ethics Committee of the Medical School in Zagreb.

4.2 Criteria for the selection of participants

The selection criteria for patients who will be included in this study are as follows:

Inclusion criteria: Infertile men between the ages of 24 to 45 who have been diagnosed with primary or secondary infertility. The infertile group consists of patients who experienced one or more years of history of infertility and clinical examination showed the anatomical presence of both testicles. Only infertile men who spent a minimum period of at least 6 months without taking hormone therapy were included in this study. To strengthen the criteria of the study, only men with non-obstructive azoospermia, oligoasthenozoospermia or normoasthenospermia were selected for the study.

Exclusion criteria: patients with chronic diseases, genital tract inflammation, acute or chronic pelvic inflammatory changes, infertile groups younger than 24 and older than 45 years of age. Patients with genitourinary anomalies or tumors, genetic and autoimmune diseases, patients with a history of hormonal, radio or chemotherapy, patients with general endocrine diseases (pituitary, adrenal, thyroid) or diabetes mellitus type 1.

All patients who presented with history of infertility were classified as follows:

1. Oligoasthenozoospermic (OAS) – sperm count $<15x10^6$spermatozoa per ml of ejaculate, progressive motility PR (A+B) % < 32, n=67;

2. Normoasthenozoospermic (NAS) –sperm count $\geq 15x10^6$ spermatozoa per ml of ejaculate, progressive motility PR (A+B) % < 32, n=28; and
3. Azoospermic (AZO) – no spermatozoa in ejaculate, n=28;

The control group consisted of 112 proven fertile men (fathered at least one child without assisted reproductive technologies) with normal semen parameters according to WHO guidelines (111).

All patients and controls were ethnic Albanians from the Dukagjini region in Kosovo. All participants signed informed consent, while the Institutional Ethics Committee approved the study protocol under the Helsinki declaration.

Selected patients were divided into two age groups:

I. First group ages 24 to 35; and
II. Second group ages 36 to 45.

The following information has been collected from the study participants: age, period of infertility, number of previous medically assisted conception procedures, and the outcomes of those procedures, therapy during the last 6 months, blood type, tests for HIV, HBsAg, HCV, occupation, smoking, alcohol, drugs, and BMI.

4.3 Researcher plan

The research begins after written acceptance for participation in research, which will be formulated as a doctoral dissertation. This means that participants have accepted both diagnostic and therapeutic procedures which may result in improving their hormonal, biochemical, and semen quality (13, 111,165, 170).

All patients filled out a previously designed questionnaire regarding personal, familial, and reproductive history before. We started with performance history, clinical examination of the genitalia, ultrasound of the genitourinary tract, and blood draw to determine FSH receptor polymorphism, hormones, biochemical, hematological, and semen analysis, which will take place once every 3 months (4, 13, 52, 111).

4.4 Medical and hormonal evaluations

After evaluation and according to inclusion criteria, study participants underwent medical examination, including a detailed reproductive history, determination of testicular size by ultrasonography, urine analysis, and complete blood count, biochemical profile, and thyroid function test, measurement of serum FSH (follicle-stimulating hormone), luteinizing hormone (LH), testosterone (T) and prolactin (PRL) serum levels by ELFA method (Enzyme-linked fluorescent assay) in BioMérieux Mini Vidas Automated Immunoassay Analyzer (bioMérieux S.A. 69280 Marcy l'Etoile, France), in "BIOLAB-Zafi" Polyclinic in Peja, Kosovo, following manufacturer's instructions (4, 13, 52, 110, 111, 116).

A blood sample of 15 ml in a test tube with EDTA will be taken for hormonal analysis.

FSH (FSH Vidas 30407-01, test sensitivity 0.1 mIU / mL)

LH (LH Vidas 30406-01, test sensitivity 0.1 mIU / mL)

Testosterone (Total serum testosterone was measured using a radioimmunoassay kit, Biomerieux, S.A. 69280, l'Etoile, France, with a sensitivity of detection of 4 pg/mL)

Estradiol (Estradiol Vidas II 30431-01, sensitivity 9.0 ng / ml)

Prolactin (Vidas Prolactin 30410-01, test sensitivity of 0.5 ng / mL) (111, 122, 128, 129).

4.5 Semen Analysis

After ejaculation, at least two semen samples were provided by each participant after an abstinence period of 3-4 days and one month apart. semen analysis according to WHO recommendations of 2010 (110, 111, 112, 113). The sample was examined immediately for coagulation, color (Brown if RBCs are present, Creamy white indicates high sperm count, clear appearance indicates less sperm concentration). The viscosity of the liquefied sample was evaluated by gentle aspiration into a 5ml pipette and observing the length of the thread formed after allowing the semen to drop by gravity. The ejaculate volume was measured with a 5ml graduated cylinder pH value of the semen was determined by placing a drop of semen on pH paper (14, 111, 112).

Before the procedure, the makler was heated *at 37ºC before using (10 minutes in the incubator or in the heating stage).*

There are several stages of the procedure:

1. Homogenize the sample moving gently the container.
2. Take 5 µl of sample and put it in the center of the makler chamber (in the center of the circle) then cover with the cover round slide of the chamber pressing gently to homogenize the volume of sample on the surface (must have no air bubbles in the chamber) (111, 112, 113).
3. With a 20x-40x objective count the spermatozoa in ten squares (random or on the diagonal).

Organize the counting in:

- motile progressive sperms
- motile no progressive sperms
- motile in situ sperms
- static sperms (14, 110, 111, 112, 113).

The total number of counted sperms represents the total concentration (to obtain the total concentration multiply by 1x10^6). To obtain the percentage of the four categories of sperms divide the total number for the number of each category and multiply by 100.

Total progressive motile= semen volume × count x% rapid motility);

Total nonprogressive motile= semen volume × count x% nonprogressive motility);

Total in situ motile= semen volume × count x% in situ motility);

Total static sperms= semen volume × count x% static sperms);

Total Motile Sperm Count= *semen volume ×total sperm concentration ×% motile (14, 110, 111, 112, 113).*

4.6 Sperm morphology

Sperm morphology was classified according to the WHO guidelines 2010. During morphology evaluation, seminal smears were stained with Giemsa (Gibco) and observed under the microscope for abnormalities of the head, midpiece, and tail (110, 111, 113).

A nomenclature related to semen quality was defined in WHO 2010 as:

- Azoospermia (no spermatozoa in the ejaculate).
- Oligozoospermia (sperm concentration $<15x10^6$/ml)
- Asthenozoospermia (<32% progressively motile spermatozoa).
- Teratozoospermia (<4% morphologically normal spermatozoa).
-Oligoasthenoteratozoospermia (Disturbance of concentration, motility and morphological parameters all together)
- Cryptozoospermia (spermatozoa absent from fresh preparation but observed in a centrifuged pellet).
- Aspermia (No ejaculate) (14, 110, 111, 113).

4.7 Genotyping

Blood samples of 3 ml were taken into a tube containing EDTA and used for the isolation of genomic DNA. Genomic DNA was extracted from peripheral blood leukocytes using the Whole blood PureLink™ Genomic DNA Mini Kit (Invitrogen, Life Technologies Corporation, Carlsbad, California, USA). The FSHR gene polymorphism at codon 680 (N680S, rs6166. 2039A>G) was analyzed by the Taqman allelic discrimination assay (Assay ID: C___2676874_10; cat. number 4351376; Lot Number: P130805-002H05; Assay Mix Concentration; 40X; Forward Primer Concentration 36 µM; Reverse Primer Concentration 36 µM; concentration of forward and reverse primer: 900 nM, probe concentration:200 nM FAM™/VIC® dyes; ContextSequence [VIC/FAM]: AGGGACAAGTATGTAAGTGGAACCA[C/T] TGGTGACTCTGGGACTGAAGAGCA;
Life Technologies Corporation, Carlsbad, California, USA) using the Applied Biosystems Real-Time PCR System 7500 (Applied Biosystems, Foster City, CA, USA). Each polymerase chain reaction (PCR) (25 µl) contained 1.25 µl TaqMan® SNP Genotyping Assay (20X dilution), 12.50 µl TaqMan Universal PCR Master Mix II No AmpErase UNG (2X dilution) (Applied Biosystems, Foster City, CA, SAD), 5 µl of genomic DNA and 6.25 µl of DNase/RNase free water. PCR was carried out in two steps: absolute quantification and allelic discrimination. For absolute quantification the cycles were as follows: AmpliTaq Gold Enzyme Activation at 95^0C for 10 min (1 cycle); 40 cycles: denaturation at 92^0C for 15 s, and annealing/extension at 60^0C for 1 min. The results were verified by Applied Biosystems Real-Time PCR System 7500 allelic discrimination software (Applied Biosystems, Foster City, CA, USA).

5. STATISTICAL ANALYSIS

Normal variables distribution was tested using the Kolmogorov–Smirnov test. Variable with normal distribution (age) is presented as mean and standard deviation, variables with skewed distribution (hormonal and seminal variables, total testis volume) were presents as median and 25^{th} - 75^{th} percentiles (interquartile range). Hardy–Weinberg equilibrium was assessed by chi-squared ($\chi 2$) analysis. Difference in examined variables between control and infertile groups were compared by Student t test for normally distributed variable or by Mann Whitney test for variables with skewed distribution. One-way ANOVA with Tuckey post Hoc test or Kruskal Wallis with Mann Whitney U post hoc test with Bonferroni correction was used for control and infertile subgroups comparison. Categorical variables were analyzed by Chi square test. Association between variables was examined by Spearman analysis.

Logistic regression analysis was used to measure the strength of the association of allelic and genotype frequencies for Asn680Ser FSHR gene polymorphism with disease development. Multiple logistic regression analysis was used to adjust statistical findings for confounding factors (age, BMI, smoking and alcohol consumption). Odds ratio (OR) and 95% CI (Confidence interval) were calculated.

A nominal level of significance $P < 0.05$ was accepted and corrected according to Bonferroni procedure (the corrected level of significance is: $Pc = 0.05/N$; N – number of independent comparisons). All reported P values were two-sided.

All analyses were performed using SPSS software (version 22) (SPSS Inc., Chicago, IL, USA).

6. RESULTS

Following the study inclusion criteria, a work-up was performed on the 123 infertile male patients and 112 control groups who came for their first visit in the General Hospital Department of Urology in Peja and IVF Polyclinic BIOLAB ZAFI in Peja, from the same ethnic group. The research took place over a period of three years, with the same criteria used to collect patient: demographics, anthropometric measurements, clinical characteristics, ultrasound of the testis and biochemical profile. All patients were classified in three groups according to the semen parameters: oligoasthenosospermic, normoasthenozospermic and azoospermic groups. Our primary purpose was to investigate how FSHR polymorphism affect spermatogenesis and influences on FSH, LH, PRL, Testosterone level and volume of the testis. Results are presented as mean ± standard deviation, with the prevalence (%) in each investigated group (% within group, % of total).

6.1 Demographic and clinical characteristics of study participants

Table 6 shows the mean and median values for demographic and clinical parameters in infertile men and control group. As can be seen, no statistically significant differences in age (control: 36.44 ±7.047 years; infertile men group: 35.2±6.25 years), BMI and alcohol consumption were found between the control subjects and studied infertile men groups [one-way ANOVA and Kruskal Wallis test]. However, the number of ever smokers was statistically higher in overall (P <0.001), oligoasthenosospermic (P=0.001), normoasthenozoospermic (P = 0.002) and azoospermic (P = 0.015) infertile men group relative to control participants [Chi square ($\chi 2$) test].

6.1.1Genotype distribution and allele frequencies

The results of statistical analysis of the distribution of Asn680Ser FSHR gene polymorphism genotype and allele frequencies between infertile men (n = 123) and controls (n = 112) are shown in Table 7. The allele frequencies were 54.91 % Ser (S) and 45.09 % Asn (N) for the control group, and 52.6 % Ser and 47.4 % Asn for the overall infertile men group (P = 0.620, $\chi 2$ test). Also, no significant differences in allele distribution relative to control were observed for either of infertility subgroups (i.e., oligoastheno-, normoastheno- and azoospermic) analysed in the study. In addition, no significant difference was observed in genotype distribution between control and overall infertile men group, and the genotypes were in

Hardy-Weinberg equilibrium in both groups [infertile men group: $\chi 2 = 1.47$, df = 1, P = 0.23; control: $\chi 2 = 2.61$, df = 1, P = 0.11]. The significant difference in genotype distribution between oligoazoospermic infertile group and control was detected (P=0.027).

6.1.2Association of N680S FSHR gene polymorphism with infertility

Table 8 shows the results for analysis of Asn680Ser FSHR genotype and allele distribution among the studied groups. As can be seen, OR between the Asn and Ser allele for development of infertility was higher in overall as well as in oligo- and normoasthenozoospermic infertile men group relative to control, thus indicating the Ser allele as protective against infertility. Hovewer the effect did not reach statistical significance after the Bonferroni correction for multiple testing was applied (Table 15, 16, 17, 18).

Crude and adjusted (age, body mass index, smoking and alcohol as covariates) logistic regression analysis revealed increased probability for infertility of heterozygous Asn/Ser genotype carriers in overall [$OR_A = 2.35$; 95% CI: 1.198-4.617)] and oligoasthenozoospermic [$OR_A = 2.75$; 95% CI: 1.24-6.07] infertile men group when compared with Ser/Ser genotype carriers (Table 30). Interestingly, relative to Ser/Ser genotype carriers, the homozygous Asn/Asn genotype carriers in normoasthenozoospermic infertile men group exhibited higher [$OR_A = 3.28$; 95% CI: 0.87-12.30] (Table 32). Nonsignificant probability for infertility compared to Asn/Ser [$OR_A = 2.55$; 95% CI: 0.76-8.56] genotype carriers. Nevertheless, the best-fitting overdominant genetic model (Asn/Ser vs. Ser/Ser + Asn/Asn) further implicates increased probability for male infertility of heterozygous Asn/Ser genotype carriers either in overall infertile men group [ORA = 1.960; 95% CI: 1.114-3.449] (Table 28) or in oligoasthenozoospermic [$OR_A = 2.56$; 95% CI: 1.32-4.96] infertile men group (Table 30).

6.1.3Association of N680S FSHR gene polymorphism and reproductive hormones

The serum FSH levels in the infertile men group [6.12 (3.70-10.5)] was higher relative to control [4.25 (3.19-5.88)], [P <0.001] (table 7). Similar results were obtained in oligoasthenozospermic, normoasthenozoospermic and azoospermic infertile men group compared to control. Contrary, the serum prolactin level was significantly (P <0.001) higher in overall infertile men group compared to control [14.7 (10.50-19.65) and 11.3 (8.61-15.35), respectively]. However, the difference reaches statistical significance when only azoospermic

infertile men subgroup was analyzed (Table 10). The overall infertile men group also showed lower testosterone [4.40 (3.35-5.30)] serum levels relative to control [5.45 (4.07-6.90)]. Again, the testosterone exhibited significantly lower serum level in oligoasthenozoospermic ($P < 0.008$) and azoospermic ($P < 0.008$) infertile men group relative to control (Table 10). LH serum level did not reach statistical significance between two groups (infertile and control) or between any other infertile men subgroups and control.

Likewise, no significant differences were wound in LH, testosterone and prolactin hormone levels between infertile men with different genotypes (Table 7, 21, 23, 25). The effects of N680S FSHR gene polymorphism on semen and testicular parameters

As expected, the semen parameters (normal morphology, concentration, total sperm count and progressive motility grade A and grade A+B) were significantly lower in overall as well as in oligo- and normoasthenozoospermic infertile men group when compared to controls. Notably the progressive sperm motility grade A was also significantly lower in normospermic infertile men group compared to control. In addition, total testis volume showed significantly lower values in overall and oligoasthenozoospermic infertile men group, compared to control (Table 6).

We also analyzed the effect of N680S FSHR polymorphism on major semen characteristics (sperm concentration, total sperm count, ejaculate volume, morphology, total and progressive sperm motility grade A and grade A+B) and total testis volume. Testicular and seminal parameters data for all studied infertile men were analyzed among the different genotype models (Table 22). As it can be seen in the table 22 recessive genetic model once again implicate the increased risk for male infertility among the Asn/Ser and Ser/Ser vs. Asn/Asn genotype carriers, regarding their lower values for major sperm parameters (i.e., total sperm count and concentration).

6.1.4 Demographic, clinical characteristics, biochemistry analysis and hormone concentrations of study participants

The mean and median values along with frequencies of demographic parameters in infertile men comprised of oligoasthenosospermic (OAS), normoasthenozospermic (NAS) and azoospermic (AZO) patients and control group are shown in Table 6. No statistically significant differences in age and BMI, as well as in ejaculate volume were found between control subjects and infertile men. Infertile man had significantly higher prevalence of

smokers. As we expected, total testis volume was significantly lower in infertile men than in control subjects.

Table 6. Demographic characteristics, ejaculate and total testis volume of infertile and control subjects

Variables	**Control** **N = 112**	**Infertile men** **N = 123**	**P**
Age (years)	36.44±7.05	35.2±6.25	0.178
BMI (kg/m^2)	26.3 (24.0-27.8)	26.6 (24.6-29.1)	0.136
Smoking, ever n (%)	24 (21.4)	55 (44.7)	<0.001
Alcohol, ever n (%)	31 (27.7)	47 (38.2)	0.101
Ejaculate volume (ml)	2.70 (2.30-3.40)	2.50 (2.00-3.20)	0.154
Total testis volume (mL)	36.5 (31.0-43.7)	29.0 (21.9-36.4)	<0.001

Normally distributed continuous variables (age) are presented as mean ± SD, variable with skewed distribution (BMI) is presented as median and interquartile range and categorical variable is presented as absolute and relative frequencies. Continuous variables were compared by Student t test for independent population and Mann Whitney U. For comparison of categorical variables chi-square test was used. Hormone concentrations are shown in table 7. There was no difference in LH concentration between examined groups. Infertile man had significantly higher FSH and prolactin hormone concentrations compared to control subjects. Serum testosterone concentration was significantly lower in infertile men.

Table 7. Hormone concentrations in infertile and control subjects included in the study

Variables	**Control** **N = 112**	**Infertile men** **N = 123**	**P**
Serum FSH mIU/ml	4.25 (3.19-5.88)	6.12 (3.70-10.5)	<0.001
Serum LH mIU/ml	3.79 (5.47-2.94)	3.94 (2.70-5.78)	0.740
Serum testosterone ng/ml	5.45 (4.07-6.90)	4.40 (3.35-5.30)	<0.001
Serum prolactin ng/ml	11.3 (8.61-15.35)	14.7 (10.50-19.65)	<0.001

Variables are presented as median and interquartile range and they were compared by Mann Whitney U test.

6.1.5 Genotype distribution and allele frequencies *of study participants*

The results of statistical analysis of the distribution of Asn680Ser FSHR genotype and allele frequencies between infertile men (n = 123) and controls (n = 112) are shown in Table 8 and Figure 21. We didn't find significant difference in Asn680Ser FSHR genotype distribution between infertile group and control subjects (p=0.121). However, there was no difference in number of Ser and Asn allele (p=0.620) and the genotypes were in Hardy-Weinberg equilibrium in both groups [infertile men group: $\chi 2 = 1.47$, df = 1, P = 0.23; control: $\chi 2 = 2.61$, df = 1, P = 0.11].

Table 8. *Allelic and genotype frequencies for Asn^{680}Ser FSHR gene polymorphism in participants included in the study*

Variables		**Control N = 112**	**Infertile men N = 123**	**P**
Genotype	Ser/Ser n (%)	38 (33.9)	31 (25.2)	0.121
	Asn/Ser n (%)	47 (42.0)	68 (55.3)	
	Asn/Asn n (%)	27 (24.1)	24 (19.5)	
Allele	Ser n (%)	123 (54.9)	130 (52.6)	0.620
	Asn n (%)	101 (45.1)	172(47.4)	

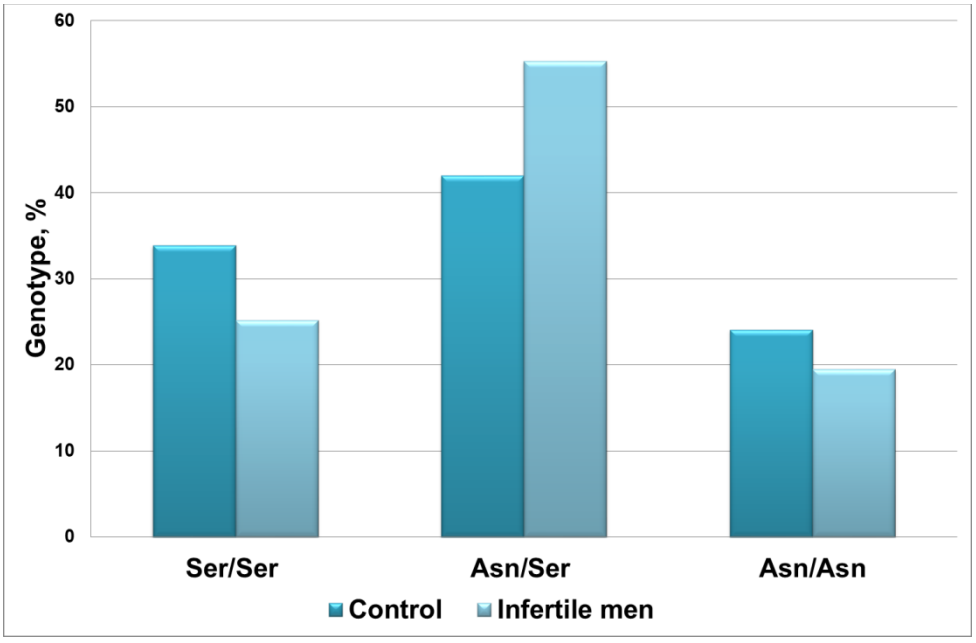


Figure 21. Genotype distribution in control and infertile subjects

6.1.6 Demographic, clinical characteristics and hormone concentrations of study participants stratified according to differential diagnose

After stratification of infertile men group on three subgroups (OAS, NAS and AZO) significant difference between groups (including control group) was not found for age, BMI and prevalence of alcohol consumption. Prevalence of smokers was significantly different between four examined groups. We detected difference in ejaculate and total testis volume. AZO group had significantly lower ejaculate and total testis volume than control group. Total testis volume was also lower in this group compared to NAS group (table 9). In addition, total testis volume was slightly lower in NAS than in control group.

Table 9. *Demographic variables, ejaculate and total testis volume of control subjects and infertile patients stratified by differential diagnose*

Variables	**Control N = 112**	**OAS N = 67**	**NAS N = 28**	**AZO N= 28**	**P**
Age (years)	36.44±7.05	35.1±6.2	34.5±5.5	36.5±7.02	0.364
BMI (kg/m^2)	26.3 (24.0-27.8)	26.8 (24.6-29.1)	27.5 (24.95-30.13)	25.9 (23.69-27.75)	0.161
Smoking, ever n (%)	24 (21.4)	27 (40.3)	15 (53.6)	13 (46.4)	<0.001
Alcohol, ever n (%)	31 (27.7)	22 (32.8)	12 (42.9)	13 (46.4)	0.195
Ejaculate volume (ml)	2.70 (2.30-3.40)	2.8 (2.0-3.5)	2.5 (2.0-3.2)	2.0 (1.8-2.6) [a]	0.019
Total testis volume (mL)	36.5 (31.0-43.7)	29.6 (22.2-37.5) [a]	33.3 (37.43-40.0)	21.95 (16.55-30.25)[a, c]	<0.001

Normally distributed continuous variables (age) are presented as mean ± SD, variable with skewed distribution (BMI) is presented as median and interquartile range and categorical variable is presented as absolute and relative frequencies. Continuous variables were compared by ANOVA (with Tuckey post hoc test) or by Kruskal Wallis (with Mann Whitney U post hoc test with Bonferroni correction). Significant p values for multiple comparisons

were 0.008 (after Bonferroni correction p=0.05 was divided by 6, taking into account the number of comparisons*).*

After comparison of hormone concentrations between controls, OAS, NAS and AZOS groups, significant difference between groups was not found for LH (table 10). FSH and prolactin concentrations were higher and testosterone concentration was lower in AZO than in control group. FSH concentration was higher and testosterone concentration was lower in OAS patients compared to control subjects. Results are presented in table 10.

Table 10. H*ormonal variables of control subjects and infertile patients stratified by differential diagnose*

Variables	**Control N = 112**	**OAS N = 67**	**NAS N = 28**	**AZO N= 28**	**P**
Serum FSH mIU/ml	4.25 (3.19-5.88)	5.2 (3.6-9.57) [a]	5.29 (3.26-6.99)	11.05 (4.05-18.18) [a]	<0.001
Serum LH mIU/ml	3.79 (5.47-2.94)	3.82 (2.25-5.20)	4.07 (3.16-5.03)	5.48 (2.98-7.78)	0.210
Serum testosterone ng/ml	5.45 (4.07-6.90)	4.30 (3.46-5.10) [a]	4.38 (2.90-5.58)	4.48 (3.27-5.93) [a]	0.001
Serum prolactin ng/ml	11.3 (8.61-15.35)	14.4 (8.78-18.83)	13.70 (10.70-17.90)	17.8 (12.5-22.47) [a]	<0.001

Variables are presented as median and interquartile range. Continuous variables were compared by Kruskal Wallis (with Mann Whitney U post hoc test with Bonferroni correction). Significant p values for multiple comparisons were 0.008 (after Bonferroni correction p=0.05 was divided by 6, considering the number of comparisons). [a] significant difference compared to control group; [b] significant difference compared to OAS group; [c] significant difference compared to NAS group.

6.1.7 Genotype distribution and allele frequencies

The results of statistical analysis of the distribution of Asn680Ser FSHR genotype and allele frequencies between OAS, NAS, AZO and controls are shown in table 11 as well as in Figure 34, 35 and 36. After comparison of subgroups and control group we found more frequent Ser/Ser (33.9%) and Asn/Asn (24.1%) genotype in control group than in OAS group (20.9% of Ser/Ser and 16.4% of Asn/Asn genotype), P=0.027 (Figure 34). Difference in Asn and Ser allele frequencies between NAS and controls was of borderline significance (Figure 35). After comparison of patients' subgroups and control group we did not find statisticaly significant difference between AZO and control group regarding genotype and allele frequencies (Figure 36).

Table 11. *Allelic and genotype frequencies for Asn^{680}Ser FSHR gene polymorphism between OAS, NAS, AZO and control group.*

		Control N = 112	**OAS N = 67**	**P**	**NAS N = 28**	**P**	**AZO N = 28**	**P**
Genotype	Ser/Ser n (%)	38 (33.9)	14 (20.9)	0.027	5 (17.9)	0.209	12 (42.9)	0.289
	Asn/Ser n (%)	47 (42.0)	42 (62.7)		13 (46.4)		13 (46.4)	
	Asn/Asn n (%)	27 (24.1)	11 (16.4)		10 (35.7)		3 (10.7)	
Allele	Ser n (%)	123 (54.9)	70 (52.2)	0.624	23 (41.1)	0.064	37 (66)	0.131
	Asn n (%)	101 (45.1)	64 (47.8)		33 (58.9)		19 (34)	

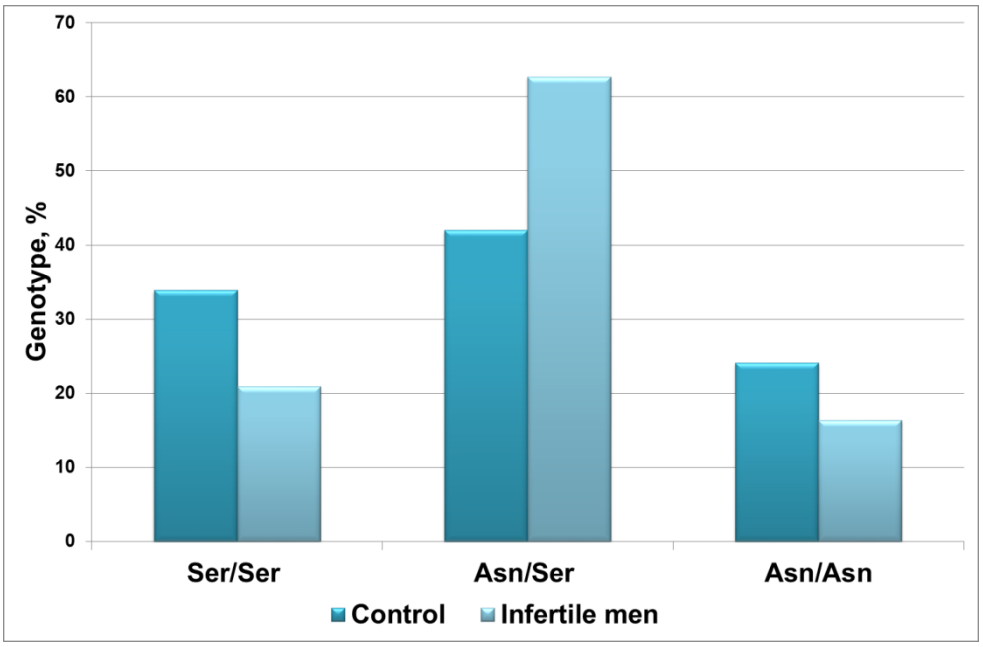


Figure 34. Genotype distribution in control and OAS infertile subjects

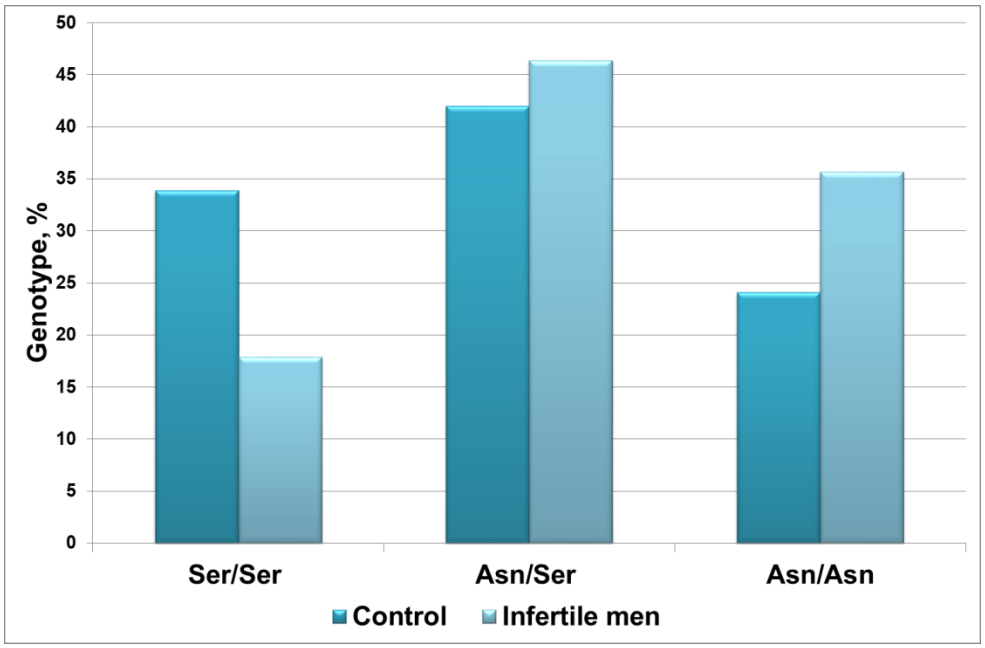


Figure 35. Genotype distribution in control and NAS infertile subjects

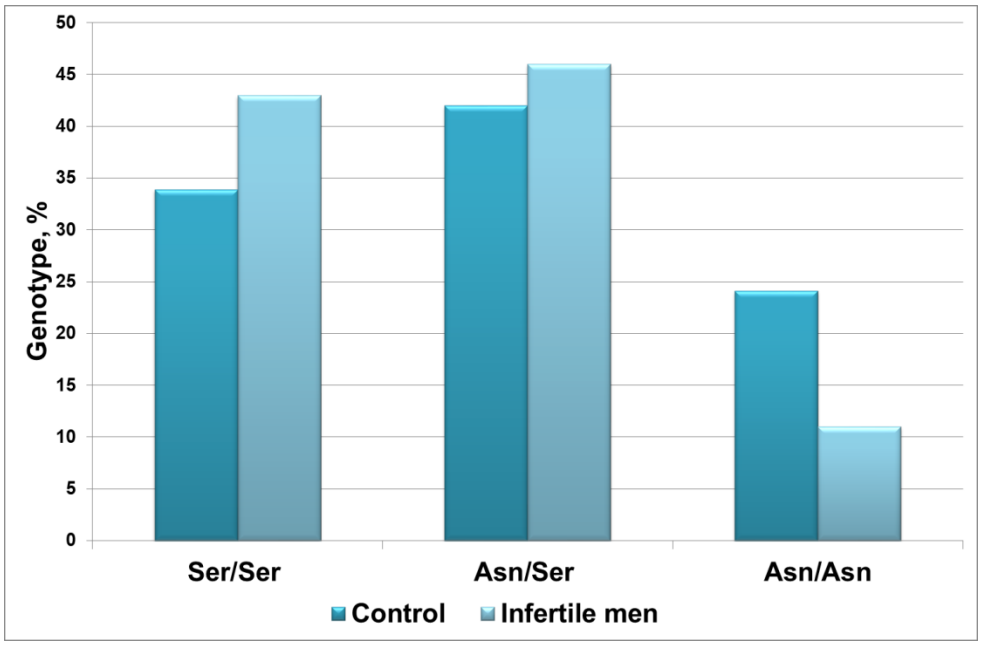


Figure 36. Genotype distribution in control and AZO infertile subjects

6.2. Association of investigated parameters in control and infertile subjects

6.2.1. Association of investigated parameters in control group

Results of association of examined parameters in control group are presented in tables 12 and 13. Age was in negative correlation with ejaculate volume, total sperm count and grade A+B sperm motility (table 12). Age increase was related to decrease in above mentioned three parameters. Similar results were seen for BMI. Increase in BMI is associated with decrease in grade A along with grade A+B sperm motility.

From examined hormones, FSH was in strong positive association with LH. Also, it was in positive correlation with motility parameters and with normal morphology. LH positively correlated with same parameters as FSH. In addition, it correlates with testosterone concentration and with sperm concentration.

Testosterone was in negative correlation with grade A sperm motility and in positive correlation with total testis volume. High concentration of prolactin was associated with low total motility, low normal morphology and low total testis volume.

Table 12. *Association between examined demographic parameters and hormons in control group*

Variables	**Age**	**BMI**	**FSH**	**LH**	**T**	**PRL**
BMI (kg/m^2)	0.089	/	/	/	/	/
Serum FSH mIU/ml	0.055	0.029	/	/	/	/
Serum LH mIU/ml	-0.059	-0.059	**0.576*** **	/	/	/
Serum testosterone ng/ml	-0.014	-0.148	0.120	**0.330***	/	/
Serum prolactin ng/ml	-0.048	-0.086	0.100	-0.064	-0.153	/
Ejaculate volume (ml)	**-0.204***	-0.047	-0.128	-0.125	0.172	0.144
Sperm concentration (106/ml)	-0.086	-0.102	0.175	**0.295****	0.083	-0.104
Total sperm count (106 /ejaculate)	**-0.200***	-0.094	0.076	0.172	0.013	-0.029
Total Motility (%)	-0.126	-0.142	**0.218***	**0.348*** **	0.150	**-0.257****
Grade A sperm motility (%)	-0.141	**-0.189***	**0.253***	**0.369*** **	**-0.236***	-0.148
Grade A + B sperm motility (%)	**-0.188***	**-0.218***	**0.198***	**0.280****	0.139	-0.179
Normal morphology (%)	-0.060	-0.130	**0.196***	**0.437*** **	0.167	**-0.238***
Total testis volume (mL)	-0,008	-0,076	-0,181	0,107	**0,220***	**-0,294****

*In tables are presented R (correlation coefficients). * p calculated by Spearman correlation analyse<0.05; ** p calculated by Spearman correlation analyse<0.01; *** p calculated by Spearman correlation analyse<0.001.*

All parameters of seminal analyse (expect ejaculate volume) presented in table 13 correlated positively to each other. Only higher total sperm count is associated with lower total testis volume and with higher ejaculate volume.

Table 13. *Association between parameters of seminal analyse in control group*

Variables	**Ejaculate volume**	**Sperm conc.**	**Total sp. conc.**	**Total motility**	**Grade A**	**Grade A+B**	**Normal morfology**
Sperm concentration (106/ml)	0.070						
Total sperm count (106 /ejaculate)	**0.479*** **	**0.874*** **	/	/	/	/	/
Total Motility (%)	-0.018	**0.489*** **	**0.397*** **	/	/	/	/
Grade A sperm motility (%)	-0.042	**0.505*** **	**0.419*** **	**0.800*** **	/	/	/
Grade A + B sperm motility (%)	0.009	**0.566*** **	**0.479*** **	**0.883*** **	**0.926*** **	/	/
Normal morphology (%)	-0.034	**0.494*** **	**0.389*** **	**0.778*** **	**0.797*** **	**0.810*** **	/
Total testis volume (mL)	-0.025	**0.196***	0.159	0.071	0.172	0.151	0.158

*In tables are presented R (correlation coefficients). * p calculated by Spearman correlation analyse<0.05; ** p calculated by Spearman correlation analyse<0.01; *** p calculated by Spearman correlation analyse<0.001.*

6.2.2. Association of investigated parameters in infertile subjects

Results of association of examined parameters in infertile men (comprised of OAS, NAS and AZO patients) are presented in table 14. Negative association was observed between total test volume and hormones FSH and LH. On the other hand, total testis volume was positively associated with age. Increase in LH was associated with an increase in FSH. Increase in BMI was related to lower testosterone concentration.

Table 14. *Association between examined parameters in infertile patients*

Variables	**Age**	**BMI**	**FSH**	**LH**	**T**	**PRL**
BMI (kg/m^2)	0.083	/	/	/		
Serum FSH mIU/ml	-0.131	-0.016	/	/		
Serum LH mIU/ml	-0.069	0.010	**0.595*****	/		
Serum testosterone ng/ml	-0.068	**-0.275****	-0.070	-0.003		
Serum prolactin ng/ml	-0.123	0.059	0.100	0.072	-0.118	
Total testis volume (mL)	**0.231***	-0.004	**-0.540*****	**-0.247****	0.028	-0.163

*In tables are presented R (correlation coefficients). * p calculated by Spearman correlation analyse<0.05; ** p calculated by Spearman correlation analyse<0.01; *** p calculated by Spearman correlation analyse<0.001.*

6.3. Associations between Asn^{680}Ser FSHR genotypes and infertility

6.3.1. Predictive ability of Asn680Ser FSHR genotypes for infertility

We investigated predictive ability of Asn680Ser FSHR genotypes on infertility by multiple logistic regressions analyse. After adjustment for age, BMI, smoking status and alcohol consuming significant predictive ability was observed for Asn/Ser and Ser/Ser genotype. Men with Asn/Ser FRSH genotype had almost 2 times higher probability to develop infertility than men with Ser/Ser or Asn/Asn genotype. On the other hand, Ser/Ser genotype decreased probability for infertility development (table 15, Figure 37).

Table 15. Adjusted predictive values of Asn680Ser FSHR genotypes for infertility

Variable	ORa (95% CI)	P
Ser/Ser	0.480 (0.257-0.920)	0.027
Asn/Ser	1.960 (1.114-3.449)	0.020
Asn/Asn	0.862 (0.439-1.692)	0.665

ORa – OR adjusted for age, BMI, smoking status and alcohol consuming

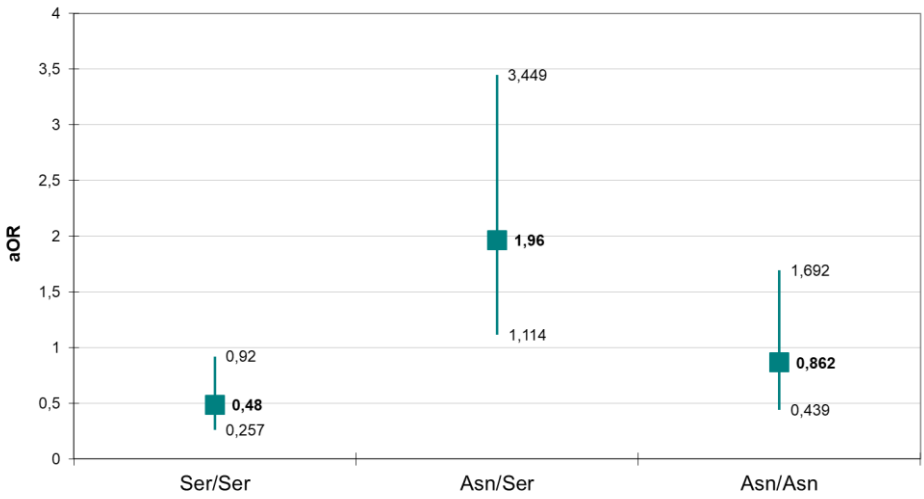


Figure 37. Adjusted predictive values of Asn680Ser FSHR genotypes for infertility development

6.3.2. Predictive ability of Asn680Ser FSHR genotypes for OAS development

After adjustment for age, BMI, smoking status and alcohol consuming significant predictive ability for OAS infertility development was observed for Asn/Ser and Ser/Ser genotype.

Men with Asn/Ser genotype were more likely to develop OAS infertility than men without that genotype (table 16). On the other hand, (Ser/Ser) genotype decreased probability for OAS infertility development (table 16 and Figure 38).

Table 16. Adjusted predictive values of Asn680Ser FSHR genotypes for OAS infertility

Variable	ORa (95% CI)	P
Ser/Ser	0.450 (0.215-0.982)	0.045
Asn/Ser	2.557 (1.318-4.960)	0.006
Asn/Asn	0.601 (0.264-1.366)	0.224

ORa – OR adjusted for age, BMI, smoking status and alcohol consuming

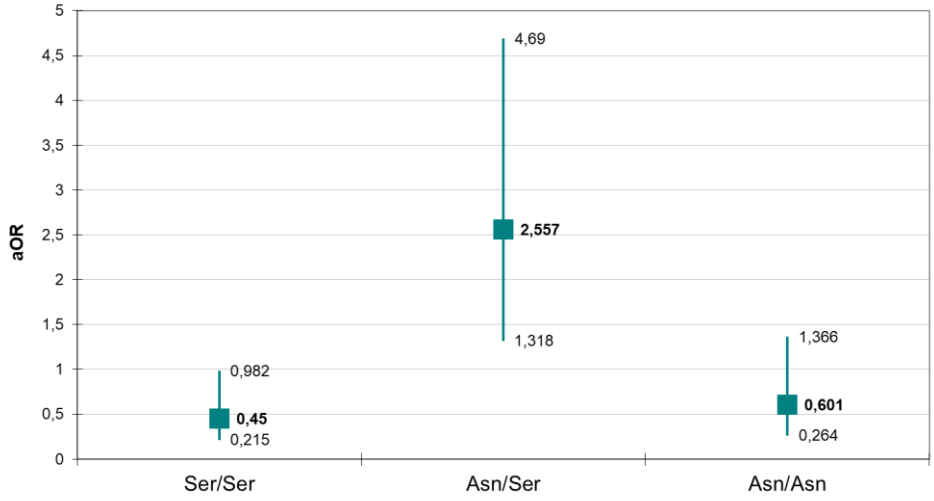


Figure 38. Adjusted predictive values of Asn680Ser FSHR genotypes for OAS infertility

6.3.3. Predictive ability of Asn680Ser FSHR genotypes for NAS development

After adjustment for age, BMI, smoking status and alcohol consumption significant predictive ability of investigated genotypes for NAS development was not found (table 17 and Figure 39).

Table 17. Adjusted predictive values of Asn680Ser FSHR genotypes for NAS infertility

Variable	ORa (95% CI)	P
Ser/Ser	0.358 (0.114-1.119)	0.077
Asn/Ser	1.365 (0.546-3.414)	0.506
Asn/Asn	1.790 (0.655-4.891)	0.257

ORa – OR adjusted for age, BMI, smoking status and alcohol consuming

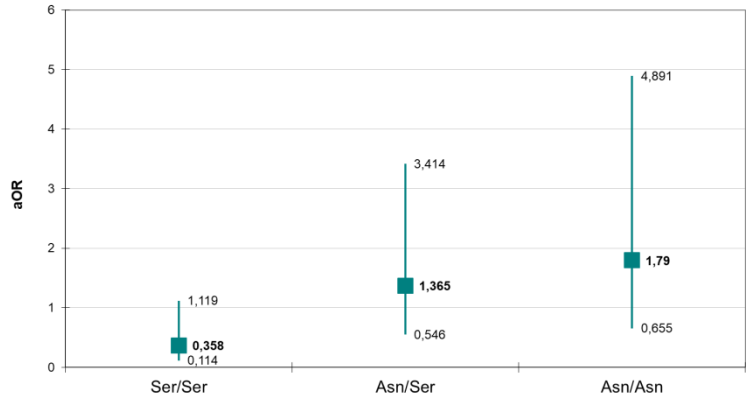


Figure 39. Adjusted predictive values of Asn680Ser FSHR genotypes for NAS infertility

6.3.4. Predictive ability of Asn680Ser FSHR genotypes for AZO development

No statistically significant predictive values of Asn680Ser FSHR genotypes were found after adjustment for age, BMI, smoking status and alcohol consumption (table 18 and Figure 40).

Table 18. Adjusted predictive value of Asn680Ser FSHR genotypes for AZO infertility

Variable	**ORa (95% CI)**	**P**
Ser/Ser	0.737 (0.244-2.227)	0.589
Asn/Ser	1.911 (0.693-5.271)	0.210
Asn/Asn	0.548 (0.143-2.101)	0.381

ORa – OR adjusted for age and BMI, smoking status and alcohol consuming

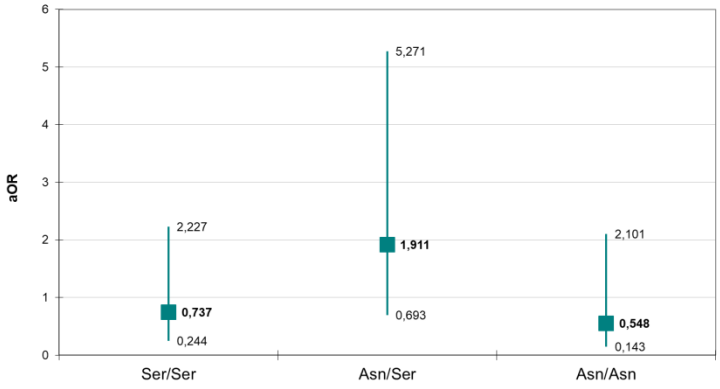


Figure 40. Adjusted predictive values of Asn680Ser FSHR genotypes for AZO infertility

6.4. Examined parameters in three selected SNPs of FSHR gene in different inheritance models

Hormones concentrations and seminal analysis parameters were evaluated in different genetic models (codominant, dominant, recessive and over-dominant).

The major allele was Ser and minor allele was Asn. Dominant model was defined by two groups -minor allele homozygote (Asn/Asn) plus heterozygote (Ser/Asn) and major allele homozygote (Ser/Ser).

Codominant model consisted of three groups, homozygote for the major allele (Ser/Ser), homozygote for the minor allele (Asn/Asn) and heterozygote (Ser/Asn). Recessive model was defined as model with two groups - minor allele homozygote (Asn/Asn) and major allele homozygote (Ser/Ser) plus heterozygote (Ser/Asn). In over-dominant model heterozygote (Ser/Asn) was in one group and (Asn/Asn) + (Ser/Ser) in another group.

6.4.1. Hormone and seminal analyse parameters in codominant model of infertile men

No statistically significant differences in hormone concentrations were found in infertile men in codominant model. Results are presented in table 19.

Table 19. Hormone concentrations in codominant model of infertile men

Variables	**Ser/Ser** **N = 31**	**Asn/Ser** **N = 68**	**Asn/Asn** **N = 24**	**P**
Serum FSH mIU/ml	7.7 (3.50-8.64)	6.64 (3.84-10.73)	5.55 (3.05-11.20)	0.580
Serum LH mIU/ml	3.80 (2.70-5.10)	3.97 (2.59-5.80)	4.27 (2.84-6.44)	0.678
Serum testosterone ng/ml	4.76 (3.7-5.93)	4.16 (3.09-5.10)	4.39 (3.60-6.38)	0.259
Serum prolactin ng/ml	15.33 (9.83-19.55)	13.49 (10.24-19.17)	17.01 (12.58-20.38)	0.450

Variables are presented as median and interquartile range. Continuous variables were compared by Kruskal Wallis test

Similar analyse we conducted for examination of parameters of seminal analyse between men with different Asn^{680}Ser FSHR genotypes. Results in table 20 are shown significant difference

in total testis volume between infertile men with different Asn^{680}Ser FSHR genotypes. After post hoc analyse, significant difference was detected between men with homozygote Ser/Ser genotype and Asn/Asn genotype. Men with Ser/Ser genotype had significantly lower total testis volume then men with Asn/Asn genotype.

Table 20. Semen variables in codominant model of infertile men

Variables	**Ser/Ser N = 31**	**Asn/Ser N = 68**	**Asn/Asn N = 24**	**P**
Ejaculate volume (ml)	2.35 (2.0-3.13)	2.5 (2.0-3.43)	2.8 (2.0-4.0)	0.445
Sperm concentration (106/ml)	8.0 (4.0-15.0)	9.0 (3.0-14.0)	14.0 (7.0-26.0)	0.130
Total sperm count (106 /ejaculate)	27.28 (8.0-41.6)	22.4 (7.0-40.0)	40.0 (15.8-55.3)	0.090
Total Motility (%)	20.0 (11.0-38.0)	20.0 (9.0-33.0)	25.0 (7.0-42.0)	0.772
Grade A sperm motility (%)	6.0 (2.0-11.0)	3.0 (0.0-8.0)	7.0 (0.0-12.0)	0.349
Grade A + B sperm motility (%)	12.0 (6.0-21.0)	10.0 (4.0-19.0)	17.0 (2.5-24.5)	0.673
Normal morphology (%)	7.0 (2.0-16.0)	8.0 (2.0-19.0)	6.5 (3.0-15.0)	0.772
Total testis volume (mL)	26.0 (18.0-32.0)	29.0 (21.8-36.0)	34.5 (29.3-40.8) [a]	0.020

Variables are presented as median and interquartile range. Continuous variables were compared by Kruskal Wallis (with Mann Whitney U post hoc test with Bonferroni correction). Significant p values for multiple comparisons were 0.008 (after Bonferroni correction p=0.05 was divided by 6, considering the number of comparisons). [a] *significant difference compared to Ser/Ser genotype. Sperm concentration, total sperm count, total motility, grade A and grade A+B motility and morphology was evaluated only in OAS and NAS patients. N of patients with Ser/Ser, Asn/Ser and Asn/Asn genotype with those parameters was 19, 55 and 21, respectively.*

6.4.2 Hormone and seminal analyse parameters in recessive model of infertile men

No statistically significant differences in hormone concentrations were found between infertile men with different $Asn^{680}Ser$ FSHR genotypes. Results are presented in table 21.

Table 21. Hormon concentrations in recessive model of infertile men

Variables	Ser/Ser + Asn/Ser N = 99	Asn/Asn N = 24	P
Serum FSH mIU/ml	6.2 (3.79-10.5)	5.54 (3.05-11.20)	0.457
Serum LH mIU/ml	3.94 (2.64-5.53)	4.27 (2.84-6.44)	0.378
Serum testosterone ng/ml	4.40 (3.26-5.20)	4.40 (3.60-6.38)	0.519
Serum prolactin ng/ml	14.1 (10.3-19.1)	17.0 (12.58-20.38)	0.214

Variables are presented as median and interquartile range. Continuous variables were compared by Mann Whitney test

Similar analysewas conducted for examination of parameters of seminal analyse in recessive model. Results in table 22 were shown significant difference in sperm parameters.

Men with Asn/Asn genotype had significantly higher values of total testis volume then men with Ser/Ser and Asn/Ser genotype. Significant difference in sperm concentration, total sperm count, and total testis volume was detected. Men with Asn/Asn genotype had significantly higher values of above-mentioned parameters then men with Ser/Ser or Asn/Ser genotype.

Table 22. Semen variables in recessive model of infertile men

Variables	Ser/Ser + Asn/Ser N = 99	Asn/Asn N = 24	P
Ejaculate volume (ml)	2.5 (2.0-3.2)	2.8 (2.0-4.0)	0.382
Sperm concentration (106/ml)	8.4 (3.15-14.25)	14.0 (7.0-26.0)	0.047
Total sperm count (106 /ejaculate)	23.58 (7.75-40.2)	40.0 (15.75-65.3)	0.028
Total Motility (%)	20.0 (9.75-36.0)	25.0 (7.0-42.0)	0.498
Grade A sperm motility (%)	3.50 (1.0-8.25)	7.0 (0.0-12.0)	0.392
Grade A + B sperm motility (%)	11.0 (4.0-19.25)	17.0 (2.5-24.5)	0.448
Normal morphology (%)	8.0 (2.0-16.0)	6.50 (3.0-15.0)	0.669
Total testis volume (mL)	27.0 (20.8-36.0)	34.5 (29.3-40.8)	0.015

Variables are presented as median and interquartile range. Continuous variables were compared by Mann Whitney test. Sperm concentration, total sperm count, total motility, grade A and grade A+B motility and morphology was evaluated only in OAS and NAS patients. N of patients with Ser/Ser + Asn/Ser and Asn/Asn genotype with those parameters was 74 and 21, respectively

6.4.3. Hormone and seminal analyse parameters in dominant model of infertile men

No statistically significant differences in hormone concentrations were found in dominant model. Results are presented in table 23.

Table 23. Hormon concentrations in dominant model of infertile men

Variables	**Asn/Asn + Asn/Ser** **N = 92**	**Ser/Ser** **N = 31**	**P**
Serum FSH mIU/ml	6.3 (3.8-10.73)	5.71 (3.5-8.64)	0.625
Serum LH mIU/ml	3.97 (2.67-5.88)	3.8 (2.7-5.1)	0.838
Serum testosterone ng/ml	4.18 (3.22-5.24)	4.76 (3.7-5.93)	0.206
Serum prolactin ng/ml	14.8 (10.55-19.95)	14.33 (9.82-19.55)	0.877

Variables are presented as median and interquartile range. Continuous variables were compared by Mann Whitney test.

Results in table 24 are shown significant difference in total testis volume. Carriers of Ser/Ser genotype had significantly lower values of total testis volume then carriers of Asn/AsnorAsn/Ser genotype.

Table 24. Semen variables in dominant model of infertile men

Variables	**Asn/Asn + Asn/Ser** **N = 92**	**Ser/Ser** **N = 31**	**P**
Ejaculate volume (ml)	2.5 (2.0-3.8)	2.35 (2.0-3.13)	0.256
Sperm concentration (106/ml)	9.5 (4.2-17.75)	8.0 (4.0-15.0)	0.675
Total sperm count **(106 ejaculate)**	29.25 (9.7-46.73)	27.28 (8.0-41.6)	0.606
Total Motility (%)	20.0 (9.0-36.75)	20.0 (11.0-38.0)	0.959
Grade A sperm motility (%)	4.0 (0.0-8.75)	6.0 (2.0-11.0)	0.367
Grade A + B sperm motility (%)	11.5 (3.0-20.0)	12.0 (6.0-21.0)	0.805
Normal morphology (%)	8.0 (3.0-17.0)	7.0 (2.0-16.0)	0.503
Total testis volume (mL)	30.0 (22.85-37.0)	26.0 (18.0-32.0)	0.044

Variables are presented as median and interquartile range. Continuous variables were compared by Mann Whitney test. Sperm concentration, total sperm count, total motility, grade A and grade A+B motility and morphology was evaluated only in OAS and NAS patients. N of patients with Ser/Ser and Asn/Ser + Asn/Asn genotype with those parameters was 19 and 76, respectively.

6.4.4. Hormone and seminal analyse parameters in over - dominant model of infertile men

No statistically significant differences in hormone concentrations and semen analysis parameters were found in over-dominant model. Results are presented in table 25 and 26.

Table 25. Hormon concentrations in over-dominant model of infertile men

Variables	**Asn/Asn + Ser/Ser N = 55**	**Asn/Ser N = 68**	**P**
Serum FSH mIU/ml	5.57 (3.40-10.30)	6.64 (3.84-10.73)	0.308
Serum LH mIU/ml	3.86 (2.75-5.53)	3.97 (2.59-5.80)	0.600
Serum testosterone ng/ml	4.75 (3.7-5.93)	4.16 (3.09-5.10)	0.106
Serum prolactin ng/ml	15.7 (10.97-20.33)	13.49(10.24-19.17)	0.384

Variables are presented as median and interquartile range. Continuous variables were compared by Mann Whitney test

Table 26. Semen variables in over - dominant model of infertile men

Variables	**Asn/Asn + Ser/Ser N = 55**	**Asn/Ser N = 68**	**P**
Ejaculate volume (ml)	2.55 (2.0-3.2)	2.5 (2.0-3.43)	0.776
Sperm concentration (106/ml)	9.0 (5.25-20.0)	9.0 (3.0-14.0)	0.183
Total sperm count (106/ejaculate)	34.7 (10.30-50.75)	22.4 (7.0-40.0)	0.154
Total Motility (%)	20.5 (8.75-30.75)	20.0 (9.0-33.0)	0.541
Grade A sperm motility (%)	6.0 (0.25-10.75)	3.0 (0.0-8.0)	0.147
Grade A + B sperm motility (%)	13.50 (3.0-22.75)	10.0 (4.0-19.0)	0.402
Normal morphology (%)	6.50 (2.75-16.0)	8.0 (2.0-19.0)	0.857
Total testis volume (mL)	29.0 (22.5-37.0)	29.0 (21.8-36.0)	0.857

Variables are presented as median and interquartile range. Continuous variables were compared by Mann Whitney test. Sperm concentration, total sperm count, total motility, grade A and grade A+B motility and morphology was evaluated only in OAS and NAS patients. N of patients with Ser/Ser + Asn/Asn and Asn/Ser genotype with those parameters was 40 and 55, respectively

6.5 Predictive values different inheritance models for infertility development

Different genetic models (codominant, dominant, recessive and over-dominant) were used to evaluate the probability for infertility development associated with each SNP. In dominant model minor allele homozygote (Asn/Asn) + heterozygote (Ser/Asn) was compared to major allele homozygote (Ser/Ser). In codominant model homozygote for the major allele (Ser/Ser) serves as the reference). Recessive model was defined as model where minor allele homozygote (Asn/Asn) was compared to major allele homozygote (Ser/Ser) + heterozygote (Ser/Asn). In over-dominant model heterozygote (Ser/Asn) was compared to homozygote (Asn/Asn) + (Ser/Ser), homozygote serve as reference.

6.5.1 Predictive ability of inheritance models for infertility

We investigated predictive ability of inheritance models on infertility by univariate regression analyse. Significant predictor of infertility was associated with presence of heterozygote Asn/Ser allele in codominant model. Men with this allele combination had 1.71 times higher probability to develop infertility than men with Ser/Ser allele (table 27 and Figure 41). In overdominant model similar predictive value (1.7) was seen for Asn/Ser hetorozygote compared to Ser/Ser and Asn/Asn genotype (table 27 and Figure 42).

Table 27. Predictive values of inheritance models for infertility development

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	1.710 (1.020-2.867)	0.042
	Asn/Asn	1.090 (0.527-2.252)	0.817
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	1.524 (0.867-2.680)	0.144
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	0.763 (0.410-1.421)	0.394
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	1.71 (1.02-2.87)	**0.043**
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

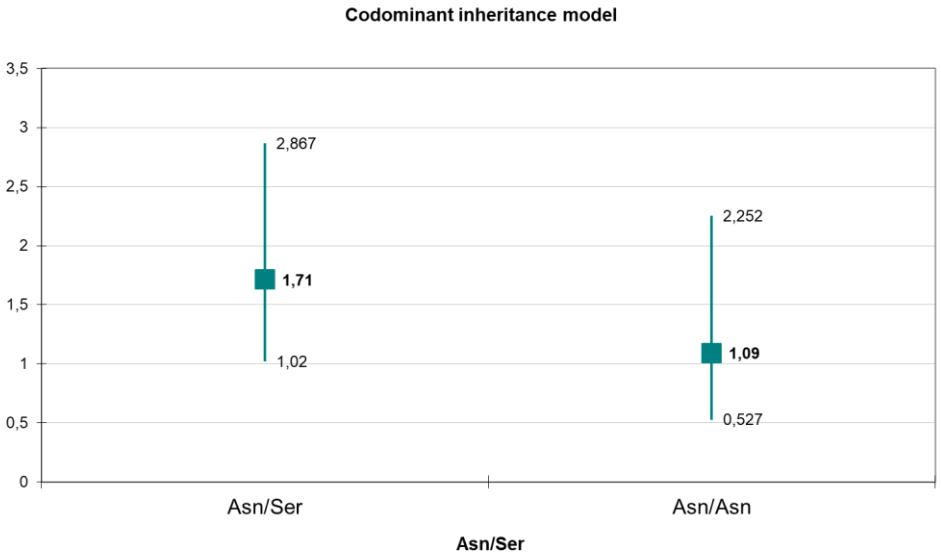


Figure 41. Predictive values of codominant inheritance models for infertility

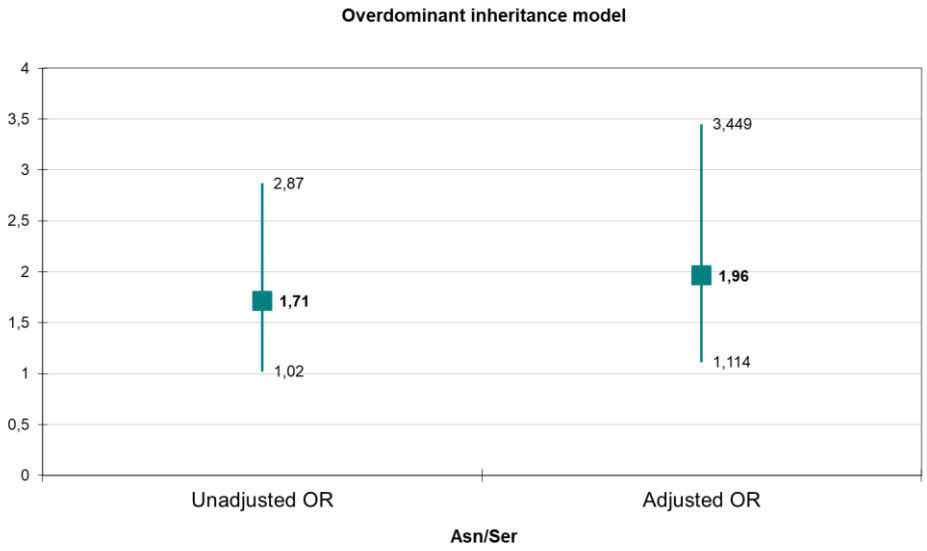


Figure 42. Predictive values of overdominant inheritance models before and after adjustment

After adjustment for age, BMI, smoking status and alcohol consuming heterozygote Asn/Ser allele keep predictive ability for infertility development in overdominant model (table 28 and Figure 42) and codominant model (table 28 and Figure 43). In addition, people with Asn/Ser and Asn/Asn allele combination were also associated with increased probability for infertility development in dominant model (table 28 and Figure 44).

Table 28. Adjusted predictive values of inheritance models for infertility development

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	2.351 (1.198-4.617)	0.013
	Asn/Asn	1.504 (0.668-3.384)	0.324
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	2.057 (1.087-3.893)	0.027
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	0.862 (0.439-1.692)	0.862
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	1.960 (1.114-3.449)	0.020
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

ORa – OR adjusted for age, BMI, smoking status and alcohol consuming

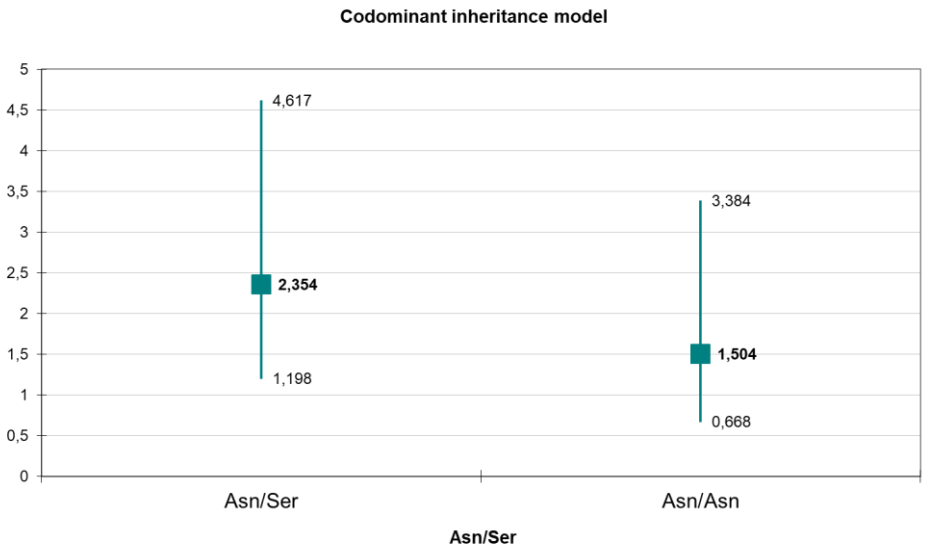


Figure 43. Predictive values of codominant inheritance models after adjustment

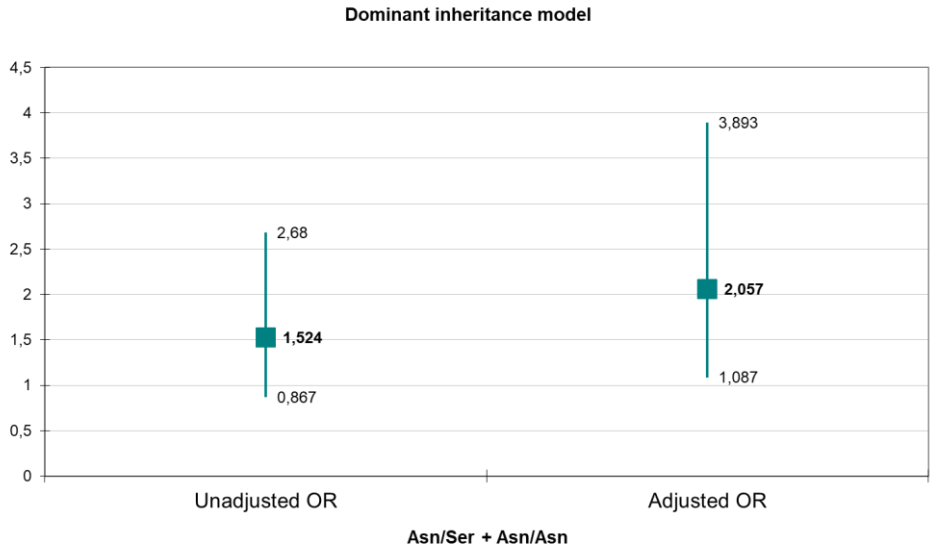


Figure 44. Predictive values of dominant inheritance models before and after adjustment

6.5.2 Predictive ability of inheritance models for OAS development

Significant predictor of OAS infertility was heterozygote Asn/Ser allele. Men with this genotype had more than 2 times higher probability to develop OAS infertility than men with only Ser/Ser homozygote allele (table 29 and Figure 45) or men with either Ser/Ser or Asn/Asn allele in overdominant model (table 29 and figure 46).

Table 29. Predictive values of inheritance model for OAS infertility

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	2.43 (1.16-5.09)	**0.020**
	Asn/Asn	1.11 (0.44-2.81)	0.830
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	1.94 (0.96-3.94)	0.065
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	0.62 (0.28-1.35)	0.226
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	2.32 (1.25-4.32)	**0.008**
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

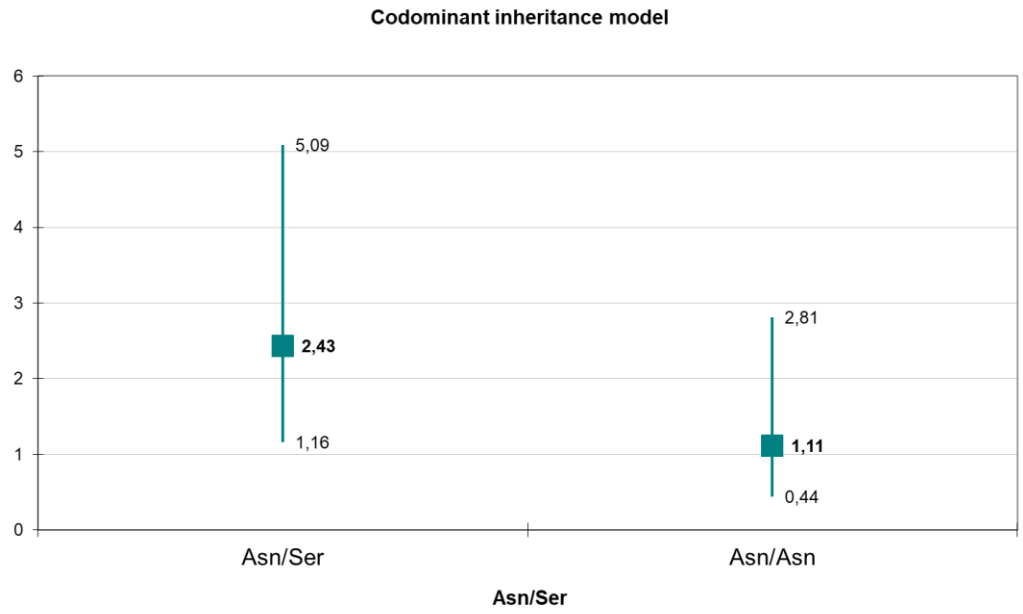


Figure 45. Predictive values of codominant inheritance models in OAS before adjustment

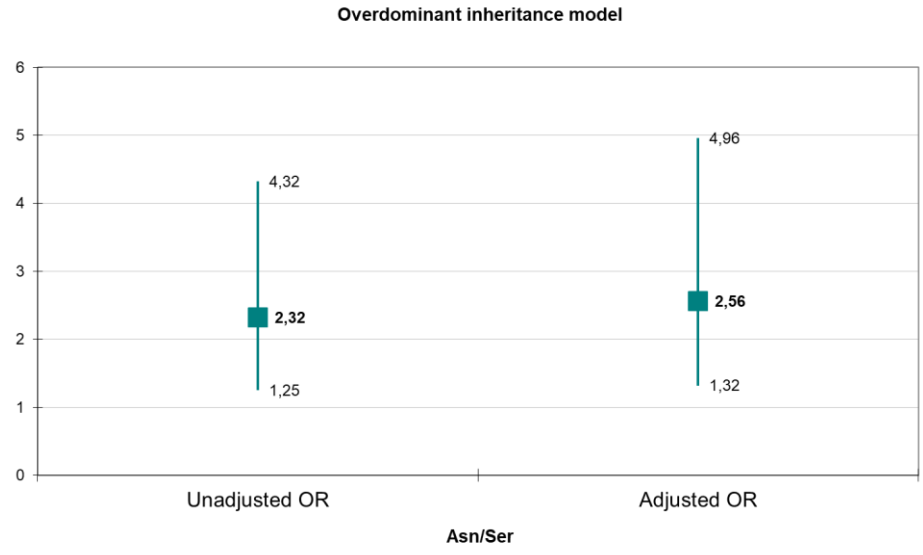


Figure 46. Predictive values of overdominant inheritance models in OAS before and after adjustment

After adjustment for age, BMI, smoking status and alcohol consuming heterozygote Asn/Ser allele kept predictive ability for OAS infertility development in codominant model (table 30 and Figure 47). Similar results were seen for overdominant model. Results are presented in table 30 and Figure 48.

Table 30. Adjusted predictive values of inheritance model for OAS infertility

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	2.75 (1.24-6.07)	**0.010**
	Asn/Asn	1.18 (0.44-3.18)	0.750
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	2.18 (1.02-4.65)	**0.045**
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	0.60 (0.26-1.37)	0.224
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	2.56 (1.32-4.96)	**0.006**
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

ORa – OR adjusted for age, BMI, smoking status and alcohol consuming

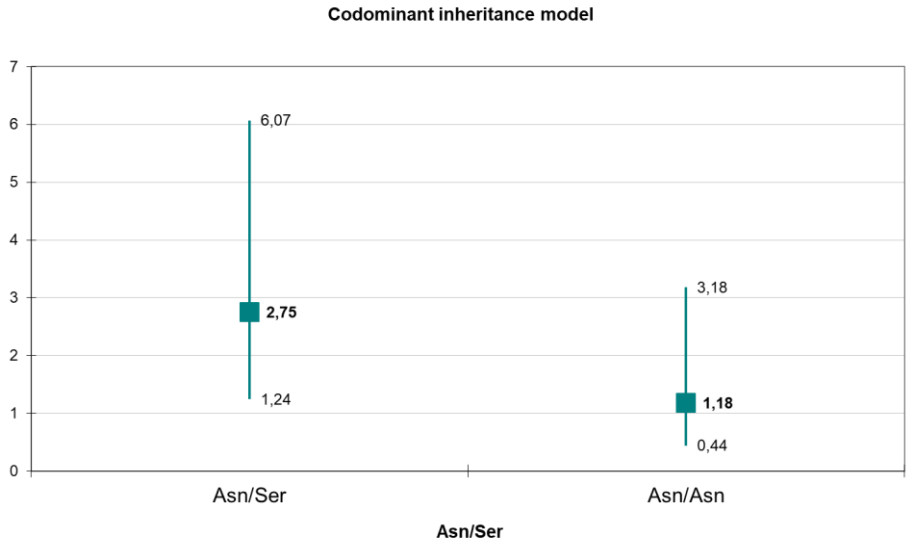


Figure 47. Predictive values of codominant inheritance models in OAS after adjustment

In addition, patients with Asn/Ser and Asn/Asn allele combination were also associated with increased probability for OAS infertility development in dominant model afrer adjustment (table 30 and Figure 48).

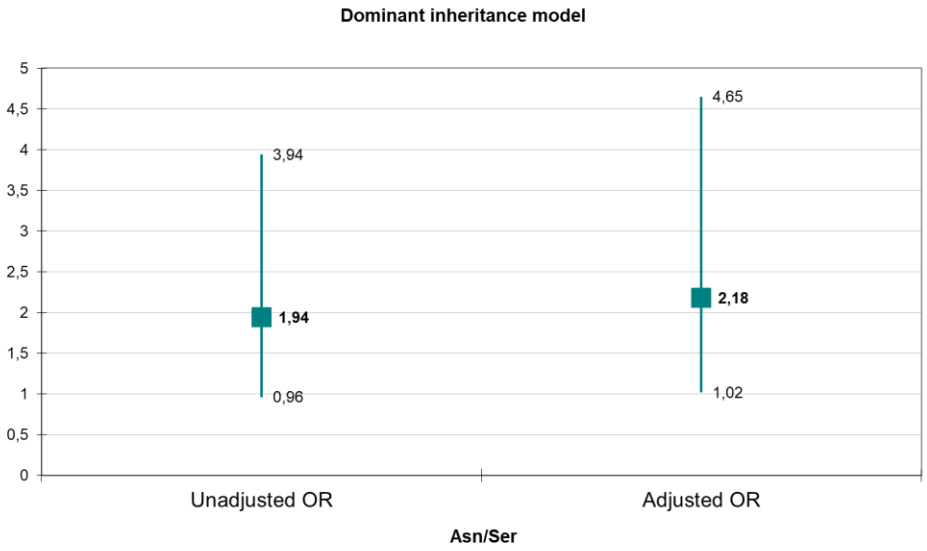


Figure 48. Predictive values of dominant inheritance models in OAS before and after adjustment

6.5.3 Predictive ability of inheritance models for NAS development

No statistically significant association was found between FSHR allele genotype and NAS infertility before and after adjustment for age, BMI, smoking status and alcohol consumption (table 31 and table 32).

Table 31. Predictive values of inheritance model for NAS infertility

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	2.10 (0.69-6.42)	0.190
	Asn/Asn	2.82 (0.86-9.17)	0.090
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	2.36 (0.83-6.71)	0.106
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	1.75 (0.72-4.24)	0.216
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	1.20 (0.52-2.76)	0.670
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

Table 32. Adjusted predictive values of inheritance model for NAS infertility

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	2.55 (0.76-8.56)	0.130
	Asn/Asn	3.28 (0.87-12.30)	0.08
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	2.80 (0.89-8.75)	0.077
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	1.79 (0.66-4.89)	0.257
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	1.37 (0.55-3.41)	0.506
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

ORa – OR adjusted for age, BMI, smoking status and alcohol consuming

6.5.4 Predictive ability of inheritance models for AZO development

No statistically significant association was found between FSHR allele genotype and AZO infertility before and after adjustment for age, BMI, smoking status and alcohol consumption (table 33 and table 34).

Table 33. Predictive values of inheritance model for AZO infertility

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	0.876 (0.358-2.140)	0.771
	Asn/Asn	0.352 (0.090-1.368)	0.352
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	0.685 (0.294-1.593)	0.379
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	0.378 (0.106-0.350)	0.134
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	1.199 (0.522-2.755)	0.670
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

Table 34. Adjusted predictive values of inheritance model for AZO infertility

Inheritance models	**Variable**	**OR (95% CI)**	**P**
Codominat	**Asn/Ser**	1.723 (0.538-5.519)	0.360
	Asn/Asn	0.765 (0.165-3.557)	0.733
Major allele homozygote (Ser/Ser) was defined as reference			
Dominat	**Asn/Ser + Asn/Asn**	1.357 (0.449-4.099)	0.589
Major allele homozygote (Ser/Ser) was defined as reference			
Recessive	**Asn/Asn**	0.548 (0.143-2.101)	0.381
Major allele homozygote (Ser/Ser) plus heterozygote (Asn/Ser) was defined as reference			
Overdominant	**Asn/Ser**	1.911 (0.693-5.271)	0.210
Major allele homozygote (Ser/Ser) and (Asn/Asn) was defined as reference			

ORa – OR adjusted for age, BMI, smoking status and alcohol consuming

7. DISCUSSION

Infertility represents one of the serious health problems that reflects on both the medical and social status of patients. Infertility affects 13–15% of couples. Men with impaired semen quality represent 40–50% of all infertile men. However, the cause of male infertility is still unknown in approximately 50% of cases (4, 5, 8, 9, 10, 16).

Male infertility is a complex disease caused by conditions affecting sperm production and sperm function or disorders related to obstructive lesions.

Impaired sperm production and function can be related to different congenital or acquired factors acting at pretesticular, post-testicular, or directly at testicular level (159). Follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), and Prolactin (PRL) are the major hormones that influence sperm formation/maturation.

FSH does not induce the process of spermatogenesis but it is necessary to form mature spermatozoa with the FSH beta-subunit known as the follicle-stimulating hormone gene beta polypeptide (*FSHB*, 11p13) that determines the reproductive function (133, 138, 140).

In adult men, FSH is important to maintain the metabolic functions of Sertoli cells; it is essential to maintain qualitatively and quantitatively normal spermatogenesis, germ cell survival, and male fertility (133, 138, 140).

The interaction between FSH and FSHR plays a crucial role in normal gametes. FSH regulates the function of Sertoli cells by binding to the FSHR located in the plasma membrane of Sertoli cells of the testes. Through this interaction, they function as spermatogonial survival factors and regulate the proliferation of germ cells. In women, FSHR is located in the plasma membrane of granulosa cells of ovaries and regulates the menstrual cycle and oocyte maturation through FSH (138, 140, 141, 142).

Several polymorphisms have been studied as potential risk factors for spermatogenetic failure, particularly abnormalities of the FSHR gene and the FSH gene, and it would be expected to affect sperm production in males (145, 153).

A variety of mutations and polymorphisms of genes regulating reproductive functions have been detected during the last decade (146, 153); however, it should be emphasized that in genetic case-control studies, false positive results can occur. To improve reliability, we

recruited an adequate number of subjects (235 in total); study participants were selected strictly to the purpose. It is very important to conduct a case-control study using large and specific ethnic populations because this can lead to data with a higher probability of accuracy (145, 149, 153, 154, 155, 165, 166).

In the present study the infertile men and proven fertile control subjects were genotyped to explore the association between the N680S (rs6166 SNP; 2039A>G) FSHR gene polymorphism and male infertility risk in Albanian population from the Dukagjini region in Kosovo. To the best of our knowledge, this is the first study examining the effect of FSHR gene polymorphism on male infertility parameters in the Albanian population.

Our data correspond with those reported in previous similar studies in other ethnicities; however, there are differences in the proportions of genotype and allele distribution between our study and the others (154, 155, 166, 175, 176).

We did not find a significant difference in the Asn680Ser FSHR genotype distribution between the infertile group and control subjects (p=0,121). However, there was no difference in the number of Ser and Asn allele (p=0.620).

Hardy-Weinberg equilibrium in both groups (Table 8) was proven. Our data agree with those reported in previous similar studies in other ethnicities, though there are differences in the proportion of genotype and allele distribution between our study and others (140, 154, 155, 166, 172, 175, 176).

Namely, our study revealed the dominant frequency distribution of the Asn/Ser genotype consistent with its highest rates found in other ethnic groups. However, contrary to other ethnic populations examined so far, the Ser/Ser variant in our population (control and infertile men) was represented in higher frequency compared to Asn/Asn homozygotes (153, 154,155,159, 166, 175, 176).

Similar results were previously reported on the distribution of FSHR ASn680Ser genotype variants in Albanian women from the same region (162, 177).

The sociological and cultural features of the Albanian population, which is manifested by the low incidence of marital union with members of other ethnic groups, thus probably preserving the genetic "pool" originating from the indigenous population, may be responsible for this effect (162, 165).

As a complex hormone-regulated disorder, male infertility is regulated by the hypothalamus-pituitary-gonad axis with FSH, LH, and testosterone as prime regulators of germ cell development (27, 32, 163).

In our study, we found higher FSH and prolactin and lower LH, and serum testosterone levels in infertile men than in controls, but the difference reached statistical significance only for serum testosterone and prolactin levels in overall infertile men and for testosterone levels in oligoasthenozoospermic patient subgroup compared to control group.

In infertile men, a higher concentration of FSH is usually a reliable indicator of severe germinal epithelial damage and it was shown to be associated with azoospermia and severe oligozoospermia (107, 122, 163).

Likewise, low levels of serum LH and testosterone are found in men with oligoasthenozoospermia, while hyperprolactinemia causes infertility in around 11% of oligospermic males (163, 178) (Table 7).

In conclusion, measurement of reproductive hormone levels (FSH, LH, prolactin, testosterone) and sperm parameters serve as an indicator to determine male infertility. From our original work, we can conclude that with the increase of hormone parameters, there is a reduction of sperm parameters and a reduction of male reproductive capacity (165, 178).

A previous study of infertile men in the Egyptian population reported by Zalata et al. also showed significantly higher serum FSH (Ser/Ser vs. Asn/Asn) and lower total serum testosterone (Asn/Ser and Ser/Ser vs. Asn/Asn) in Ser680 variant carriers respectively (158).

They also detected a nonsignificant decrease in sperm concentration, grade A and grade A + B progressive motility, and normal sperm morphology in Ser/Ser and Asn/Ser compared with the Asn/Asn genotype form (158).

Furthermore, Tsitlakidis recently reported a significant association of the heterozygous Asn/Ser genotype form with a different size of the right testis in infertile Greek men but no association of any genotype form with hormonal serum levels (FSH, LH, testosterone, and prolactin) or sperm parameters (semen volume, sperm count, morphology and motility) was detected (164).

Also, a recent meta-analysis reported by Wu et al. detected the association of the Asn680Ser FSHR Ser/Ser genotype form (Ser/Ser vs. Asn/Asn and Ser/Ser vs. Asn/Asn + Asn/Ser) with increased risk of male infertility (154).

One of the main reasons for observed differences in their results and the findings of our study regarding the overall influence of the ASn680Ser FSHR gene polymorphism on infertility status and identification of the effective genotype responsible for the modification of sperm parameters can be found in population stratification and ethnic and geographic variation, but additional genetic and environmental factors cannot be excluded.

Since the phenotypic effects of individual polymorphism may be modulated by overall genetic background, a combined effect of other polymorphic regions and ASn680Ser allele and genotype variants on male infertility should also be examined (153, 154, 155, 157, 176).

For example, Wu et al. reported that a homozygous combination of FSHR gene polymorphism at codon 307 and 680 (i.e., Thr/Thr + Asn/Asn) may be responsible for male infertility in patients from North China (154).

In addition, Lindgren et al. (166) reported that Thr307-Asn680 homozygotes in Swedish young men cohort among others exhibited lower serum FSH and higher total testosterone concentrations, as well as larger testes volume and higher sperm count when compared to heterozygotes and Ala307-Ser680 homozygote carriers. The underlying mechanism was not known when the study was conducted but could be explained by higher activity of the FSH isoform (166).

The discrete codon combination with homo/heterozygous variation of the Thr307Ala and Asn680Ser FSHR gene polymorphisms was also reported in other Asian (156,168, 154) and non-Asian (152, 155, 158, 169, 171, 173, 174) patient groups.

Synergistic effect of FSHR promoter polymorphic region (2039A>G) and other genetic loci (e.g., FSHB-211G>T) with Thr307Ala/Asn680Ser allelic variants were also reported (154, 145,155, 176).

Unfortunately, in this study, we did not evaluate the cumulative effect of common variants found in the FSHR polymorphic regions on the prevalence of Albanian male infertility. Also, the number of patients included in this study was limited; therefore, results should be

interpreted cautiously. Ser/Ser genotype in control and infertile men was 33.9 % and 25.2%, Asn/Ser 42.0 % and 55.3%, respectively and Asn/Asn genotype was 24.1% and 19.5% in control and infertile men, respectively (Table 8).

There was a tendency for Asn/Asn variant FSHR gene to be higher in fertile men, while Ser/Ser variant was higher in infertile men; however, there was no significant difference between these variants among the groups. This finding is consistent with an article published by Zalata AA and others (158).

Previously reported data on the effects of smoking on infertility are not without restriction. However, evidence confirms the negative effect of smoking on several key parameters of the semen, regardless of the study published by (74, 77, 78).

According to the results of our study, the frequency of smokers was significantly higher in the group of infertile men (Table 6, $p < 0.001$). Future studies should be conducted to prospectively analyze the impact of smoking on fertility by including successful rates of pregnancy as the main measure of outcomes (74, 77, 78).

There is significant evidence that male obesity has a negative effect on fertility through changes in hormonal levels as well as direct changes in sperm function and semen composition (60, 61, 167).

In many publications, spermatogenesis and sexual dysfunction have been shown to have a negative effect on other variables in line with poor fertility potential (60, 61, 167). It was found that elevated BMI was significantly associated with sperm concentration, serum testosterone and increased serum estradiol (61, 167).

Our results did not agree with the results of those previously published studies because we did not find a difference in BMI between infertile males and control subjects ($p=0.136$) (Table 6).

Significant differences in total testes and ejaculate volume can lead to a clear diagnosis of fertile and infertile males. In the OAS group, the total testicular volume was lower compared to the control group. In addition, the total testes volume of AZO patients was lower than in NAS patients and control subjects. Also, the ejaculate volume was lower in AZO compared to the control group (table 9). Those results agree with results elaborated in papers (155, 157, 168, 169, 166).

Therefore, this information should be taken into account when assessing the degree of infertility in males. Currently, measuring basal FSH is a routine procedure during the diagnostic work-up of subfertile couples for prognostic evaluation of spermatogenesis. Serum FSH levels in males act as an indicator of male infertility since testicular failure leads to relatively high FSH levels (163).

In our study, we found that serum FSH and prolactin levels were significantly increased in infertile men compared to the normozoospermic group, while serum testosterone levels were significantly decreased. No statistically significant association was identified in serum LH level (table 7) found in Egyptian population results, which were related to our findings (158, 163).

A study in the Iranian population found that the serum FSH level in infertile men was higher than in controls, while serum LH and total testosterone levels did not reach a statistically significant difference between the two groups. There was no difference in the concentration of LH between the examined groups. Elevated FSH levels are a reliable indicator of germinal epithelial damage and are usually associated with azoospermia or oligoasthenospermia, while low testosterone level is an indicator of hypogonadism of hypothalamic or pituitary origin (53, 54, 63, 163).

In our study, a variant analysis of oligoasthenozoospermia, normoasthenozoospermia, azoospermia, and control fertile men was conducted to determine the FSHR, Asn680, and Ser680 variants identified the genotype of FSHR genes at position 680 (Table 8).

After the comparison of subgroups to the control group, we found more frequent Ser/Ser (33.9%) and Asn/Asn (24.1%) genotypes in control group than in the OAS group (20.9% of Ser/Ser and 16.4% of Asn/Asn genotype), P=0,027.

Sperm count was significantly decreased in the mutant (Ser/Ser) FSHR allele compared to wild (Asn/Asn) and heterozygote Asn/Ser FSHR allele (Table 22).

On the other hand, sperm motility showed no significant differences among various groups. Furthermore, there was a tendency for a decrease in semen parameters in FSHR gene variants Asn/Ser and Ser/Ser than Asn/Asn similar result was obtained by Safarinejad (157).

In this context, it was reported that the combination of heterozygous FSHR variants might be responsible for male infertility in the Japanese population (156). In the Iranian population, the

study demonstrated that the genetic polymorphism in the FSHR gene might increase the susceptibility to obstructive azoospermia in men (169).

Other studies showed no statistically significant associations between semen parameters and FSHR gene polymorphisms obtained by different authors (155, 157, 170, 173).

We also examined hormonal parameters across different inheritance models. Like most previous reports, our study also showed no statistically significant association between Asn680Ser FSHR genotype and allelic variants with serum FSH, LH, testosterone, and prolactin levels in infertile male participants. The findings of the present study regarding the influence of the FSHR polymorphism on hormone concentration are not consistent with the findings obtained by (155, 158, 159).

However, this finding contrasts with the finding of Foresta (170). Significantly increased levels of LH were not found in men carrying the Ser/Ser genotypes compared to men carrying Asn/Asn genotypes. That result agreed with the results of Safarinejad (157).

We concluded that the FSHR polymorphism at codon 680 did not result in significant differences in serum FSH levels evaluated by Pengo (173) in the Italian population. Similar results were found by Ahda (152) in the German population, Qiuyue Wu in the Chinese population (154), Safarinejad (157) in the Iranian population, Ghirelli-Filho (171) in the Brazilian population, Tsitlakidis in the Greek men (164), and Balkan M (174) in the Turkish population. Conversely, the published articles (155, 156, 158, 166), found that the genetic variations Asn680Ser of the FSHR gene correlate to serum FSH levels in Estonian, Japanese, Egyptian, and Swedish young men.

The same results were obtained in the meta-analysis of these studies. Similarly, the effect of Asn680Ser FSHR isoforms on clinical reproductive parameters such as FSH level, sperm count, and total testes volume has also failed to show any consistent associations (152, 156, 157, 171, 172, 173, 174).

Some studies failed to prove differences in the prevalence of FSH receptor genotypes in fertile or infertile men and infertile women (157). Most reports suggest that the differences in the genotype and allele frequency of the Asn680Ser FSHR polymorphic region might represent important genetic factors contributing to the phenotypic expression of essential fertility parameters (172). This was clearly demonstrated for influence on basal serum FSH concentrations in women, length and dynamics of the menstrual cycle and effective FSH

concentration during ovarian stimulation for in vitro fertilization techniques (151, 153, 162, 172). Also, the polymorphism at codon 680 is a key determining factor of menstrual cycle length and hormonal dynamics. For example, women with Ser/Ser receptor variant seem to be less sensitive to FSH stimulation and have significantly higher serum FSH levels in the follicular phase of the menstrual cycle than women with the Asn/Asn isoform (151, 153, 162, 177). The common FSHR polymorphisms in exon 10 and the promoter of the FSHR gene have also been extensively studied in male infertility (140, 141, 142, 143, 144, 153). However, so far, there is no clear association of male infertility with the ASn680Ser FSHR polymorphic region. Most reports suggest no significant differences in carrier frequencies of individual FSHR Asn680Ser polymorphic variants between infertile and fertile men from various ethnic groups (140, 150, 153, 155, 154, 155, 166, 172,175, 176). Such different responsiveness to FSH is not apparent in men with or without impaired spermatogenesis (140, 128, 132). This discrepancy is interesting, and it is difficult to explain this functional difference between men and women.

Like most previous reports, our study also showed no statistically significant association of the Asn680Ser FSHR genotype and allelic variants with serum FSH, LH, testosterone, and prolactin levels in control and infertile male participants. However, we did notice an increased, although nonsignificant, risk for male infertility among heterozygous Asn/Ser (vs. Ser/Ser or Ser/Ser + Asn/Asn respectively) genotype carriers regarding their lower values for major sperm parameters (total sperm count, concentration, morphology, and motility) in both the overall infertile men group and infertility subgroups based on various degree of spermatogenic impairment (165).

A slight influence of the ASn680Ser FSHR gene polymorphism on male reproductive parameters was also recently demonstrated by Grigorova et al. (155). They detected a significant association between the FSHR Ser680 allele and lower total testes volume in both the Baltic men cohort and the Estonian idiopathic infertility group (155). A subsequent meta-analysis further supported their findings showing moderate nonsignificant effect between the FSHR Ser680 variant and higher serum FSH level, and significantly lower total testosterone levels. However, no statistically significant associations were identified in serum LH levels or any of the sperm parameters (155). A previous study of infertile men from the Egyptian population reported by Zalata (158) also showed significantly higher serum FSH (Ser/Ser vs. Asn/Asn) and lower total serum testosterone (Asn/Ser and Ser/Ser vs. Asn/Asn) in Ser680

variant carriers respectively (158). They also detected a nonsignificant decrease in sperm concentration, grade A and grade A + B progressive motility, and normal sperm morphology in Ser/Ser and Asn/Ser compared with the Asn/Asn genotype form (158). Furthermore, Tsitlakidis (164) recently reported significant association of heterozygous Asn/Ser genotype form with different size of the right testis in infertile Greek men but no association of any genotype form with hormonal serum levels (FSH, LH, testosterone, and prolactin) or sperm parameters (semen volume, sperm count, morphology, and motility) was detected (164).

In our study, we investigated the predictive abilities of inheritance models on infertility development. Men with heterozygote Asn/Ser allele in the codominant and over-dominant model had an increased probability for inheritance development compared to men with homozygote Ser/Ser allele as well as men with Ser/Ser and Asn/Asn allele. A recent meta-analysis reported by Qiuyue Wu (154) detected the association of the Asn680Ser FSHR Ser/Ser genotype form (Ser/Ser vs. Asn/Asn and Ser/Ser vs. Asn/Asn + Asn/Ser) with increased risk of male infertility (154). One of the main reasons for observed differences in their results and the findings of our study regarding the overall influence of the ASn680Ser FSHR gene polymorphism on infertility status can be found in population stratification and ethnic and geographic variation. However, additional genetic and environmental factors cannot be excluded (165).

We concluded that the FSHR polymorphism at codon 680 did not result in significant differences in serum FSH levels evaluated by Pengo (173) in the Italian population. Similar results were found by authors in articles (152, 154, 157, 164, 171, 174). Conversely, other authors in articles (155, 156, 158, 166), found that genetic variations Asn680Ser of the FSHR gene correlate with serum FSH levels in Estonian, Japanese, Egyptian and Swedish young men.

The same results were obtained in the meta-analysis of these studies. Similarly, the effect of the Asn680Ser FSHR isoforms on clinical reproductive parameters such as FSH level, sperm count, and total testes volume has also failed to show any consistent associations (155, 156, 156, 158, 164, 166).

8. CONCLUSION

- ✓ FSH was significantly increased in the NAS and OAS group compared to control subjects;
- ✓ Testosterone was significantly reduced in NAS and OAS compared to the control group;
- ✓ Prolactin concentration was increased in infertility mostly in AZO compared to the control group;
- ✓ FSHR gene variants did not show any difference in the distribution between NAS, AZO, and the control group;
- ✓ Distribution of FSHR gene variants was statistically significant between OAS and control subjects;
- ✓ Only total testis volume was significantly increased in patients with mutant FSHR (Ser/Ser) compared to those with wild (Asn/Asn) *FSHR* genotype;
- ✓ LH was significantly increased in mutant FSHR (Ser/Ser) compared to wild (Asn/Asn) alleles FSHR. However, there were no significant differences in FSH levels among the different groups;
- ✓ Testosterone was significantly reduced in mutant FSHR (Ser/Ser) alleles compared to wild alleles (Asn/Asn) FSHR;
- ✓ A significant predictor of infertility was Asn/Ser FSHR genotypes after adjustment for age, BMI, smoking, status, and alcohol consumption. Men with this genotype had more than two times higher probability for infertility development compared to men with Ser/Ser or Asn/Asn genotype;
- ✓ A significant predictor of the OAS infertility was Asn/Ser *FSHR* genotypes. Men with this genotype had 2.5 times higher probability to develop OAS infertility than men with Ser/Ser or Asn/Asn genotype (after adjustment for age, BMI, smoking status, and alcohol consumption);
- ✓ A significant predictor of infertility was associated with the presence of the heterozygote Asn/Ser allele in infertility models. Men with this allele combination had a 2.3 and 2 times higher probability to develop infertility than men with Ser/Ser and Ser/Ser together with Asn/Asn allele combination;
- ✓ A significant predictor in the OAS group of infertility models was the heterozygote Asn/Ser allele. Men with this genotype had a more than 2 times higher probability to

develop OAS infertility than men with only Ser/Ser homozygote allele or men with Ser/Ser together to Asn/Asn allele.

The crucial role of FSH for the normal function of cells involved in spermatogenesis is dependent on the expression of the FSHR. A better understanding of factors regulating the transcription of the FSHR gene is therefore important, to better understand the ways they modulate endocrine functions or gene expression.

The clinical importance of the FSHR genotype should be evaluated through future studies, considering differences in ethnicity, genetics, epigenetics, sources of control, methods used, nutritional, and environmental factors influencing male fertility.

In light of the current knowledge, a question arises about the potential of using the pharmacogenetic FSH analogs to improve semen quality in select cases of infertile men. Currently, the most promising approach is to induce reproductive parameters in infertile men by FSH analogs.

The influence of FSHR variants on male fertility is still not well understood. However, the results of our research have revealed significant variations in the distribution of the FSH and FSHR genes and their putative functional effects, thus identifying patients who could benefit from FSH treatment (165, 179).

9. ABSTRACT

Polymorphism of follicle-stimulating hormone gene receptor in Albanian male population

Background: The purpose of this study was to determine the prevalence of allele and genotype variants of follicle-stimulating hormone receptor (FSHR) gene polymorphic region at position Asn680Ser in the Albanian male population and associate them with the clinical parameters of infertility. A total of 123 infertile men (35.04±5.85 years) stratified according to the level of spermatogenetic impairment (oligoasthenozoospermia, asthenozoospermia, and azoospermia), and 112 fertile men (36.44 ±7.05years) with normal semen parameters were enrolled in this study.

Methods: Genotyping of the FSHR gene at position 680 was performed by TaqMan genotyping assay. All participants underwent semen analysis; serum reproductive hormones (FSH, luteinizing hormone, prolactin, and testosterone) were also measured.

Results: The FSHR Asn680Ser genotype frequencies were 42% Asn/Ser, 33.9% Ser/Ser, and 24.1% Asn/Asn for the control group, and 55.3% Asn/Ser, 25.2 % Ser/Ser and 19.5% Asn/Asn for the overall infertile men group ($P = 0.08$,$\chi 2$ test). No statistically significant correlation between serum hormonal levels and semen characteristics or fertility status and FSHR Asn680Ser gene variants was found either in control subjects or the infertile men group. However, adjusted logistic regression analysis (age, body mass index, smoking, and alcohol as covariates) revealed increased odds ratio for male infertility among heterozygous Asn/Ser genotype carriers associated with lower values of semen parameters (normal morphology, concentration, total sperm count, and motility).

Conclusion: In conclusion, our case-control study further confirmed previous reports of no significant association between the FSHR Asn680Ser polymorphisms and male infertility. Nevertheless, the data presented here indicate that Asn/Ser genotype may increase the risk for male infertility in the Albanian population of the Dukagjini Region.

Keywords: FSHR Asn680Ser gene polymorphisms, male infertility, Follicle stimulating hormone, luteinizing hormone, prolactin, testosterone, semen parameters

10. SAŽETAK

Polimorfizam gena za receptor FSH u muskaraca albanske etnicke pripadnosti

Cilj istraživanja bio je odrediti pojavnost alela i varijante genotipa receptora folikularno stimulirajućeg hormona (FSHR) na poziciji Asn680Ser kod muškaraca albanske populacije, u odnosu na kliničke parametre neplodnosti. Istraživanje obuhvaća 123 neplodnih muškaraca (35.04±5.85 godina) svrstanih prema razini oštećenja spermiograma (oligoasthenozoospermia, asthenozoospermia i azoospermia) te 112 plodnih muškaraca (36.44 ±7.05 godina) s urednim nalazom spermiograma.

Methode: Genotipizacija gena FSHR na poziciji 680 učinjena je primjenom TaqMan probe. Kod svih sudionika istraživanja učinjena je analiza sjemena i reprodukcijskih hormona uključujući FSH, luteinizirajući hormon, prolaktin i testosteron.

Rezultati: U kontrolnoj skupini ispitanika kod FSHR Asn680Ser genotipa utvrđena pojavnost Asn/Ser bila je 42%, Ser/Ser 33.9% i Asn/Asn 24.1% , dok se u skupini neplodnih ispitanika incidencija kretala od 55.3% za Asn/Ser, 25.2 % za Ser/Ser i 19.5% za Asn/Asn (P = 0.08, $\chi 2$ test). Nije ustanovljena znakovita statistička povezanost između razine hormona, karakteristika sjemena, stanja plodnosti u varijanti gena FSHR Asn680Ser u kontrolnoj skupini u odnosu na ispitanike u skupini neplodnih muškaraca. Ipak, primjenom prilagođene, logističke i regresijske analize (dob, indeks tjelesne mase, pušenje i alkohol kao kovarijabli) utvrđeno je da postoji veća vjerojatnost javljanja muške neplodnosti kod nositelja heterozigota Asn/Ser koji su povezani sa sniženim vrijednostima parametara sjemena (morfologija, koncentracija, ukupan broj i pokretljivost).

Zaključci: Zaključno možemo utvrditi da ovo istraživanje potvrđuje ranija izvješća da ne postoji znakovita povezanost između polimorfizma FSHR Asn680Ser i muške neplodnosti. Ipak, navedeni podaci upućuju da Asn/Ser genotip može povisiti rizik muške neplodnosti u Albanskoj populaciji Dukagjini Regije.

Ključne reči: *FSHR Asn680Ser polimorfizam gena, muski infertilitet, Follikulo stimulirajuči hormon, Luteinizirajuči hormon, prolaktin, testosteron, Sjemeni parameter*

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12. CURRICULUM VITAE

PERSONAL INFORMATION

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Fax	
E-mail	**shkelzenelezaj@yahoo.com**
Nationality	Kosovo Albanian
Date of birth	20 January,1967

WORK EXPERIENCE

Dates (from –to)	**2002**
Name and address of employer	**Regional Hospital, Peje**
Type of business or sector	**Urologist**
occupation or position held	Chief of Urology& Andrology cabinet
Main activities and responsibilities	Management of helath services which are providing by professional staff in cabinet Diagnosis and treatment of couples infertility
Dates (from –to)	**SEPTEMBER 2017**
Name and address of of employer	**Collegue AAB, Prishtina,**
Type of business or sector	Department of Nursing
Occupation or position held	Professor for two modules
Main activities and responsibilities	Module: Reproductive Health and Ethic 1"- first year of graduated programe. Module: Mental Health second year of graduated programe

EDUCATION AND TRAINING

Dates (from –to)	1990 -1996
Name and type of organization providing education and training	University of Sofia, Faculty of Medicine.
Principal subjects/occupational skills covered	General Medicine
Title of qualification awarded	Medical Doctor
Level in national classification (if appropriate)	University degree

Dates (from –to)	1998 -2002
Name and type of organization providing education and training	University of Ljubljana, Faculty of Medicine.
Principal subjects/occupational skills covered	Specialisation in the field of urology
Title of qualification awarded	Urologist
Level in national classification	Vocational degree

Dates (from –to)	October 2014
Name and type of organization providing education and training	University of Zagreb Faculty of Medicine PhD Program
Principal subjects/occupational skills covered	Biomedicine and Health Sciences
Title of qualification awarded	Student
Level in national classification	PhD cand.

Workshop and training:

1. Histology of the testis and testicular sperm extraction- a practical approach 2019. Croatian Congress on Gynaecologic Human Reproduction and Menopause, Opatija.

2. Application of US in the pathologies of the urogenital tract, KAU, 2018, Prishtina,

3. Treatment of the renal stone with the PCNL, Coock medical, 2015, Germany.

4. CO2 laser application in the treatment of incontinence and chronic vaginal infection. Alma laser, 2014, Leipcig.

5. Treatment of stress incontinence with the method of TVO. Bard GE, 2013, Ljubljana

Participation-presentation in the national and international scientific conferences:

1. Removal of ureteral stones with the URS lithotrypsi procedures in the Regional hospital of Peja, Elezaj Sh. The 10th Symposium of the Kosova urologist association (KUA). April, 2019, Prishtina, Kosovo (KS).
2. The frequency of follicle stimulating hormone receptor 2039A>G gene polymorphism and the risk of male infertility in Albanian population", Elezaj Sh. IV. Kosovo Congress of Obstetirc and Gynecology (KOGA), September 2018, presentation", Prishtina, KS.
3. Pervojat tona ne trajtimin e tumoreve te fshikes urinare me resekcion transurethral-TUR, Elezaj Sh. Simpoziumi I X I urologeve te Kosoves. May, 2015, Prishtina, KS.
4. II Kosovo Congress of Obstetirc and Gynecology, Gashi Z, Elezaj Sh. "Standardisation of the laboratory analysis of sperm in Kosovo. Influence of analytical and biological variation on the clinical interpretation of sperm parameters". September, 2014, KS.
5. Varicocoelae and male infertility, Elezaj Sh. The I congress of KOGA. April, 2012, Prishtina, KS.
6. Pathophysiology and natural history of mail infertility, Elezaj Sh. Symposium of the KUA. May, 2011, Prishtina, KS.
7. Results of intratubar insemination applications at Polyclinic "BIOLAB-Zafi", Elezaj Sh. Mediterranean Symposium in Reproductive Medicine. September, 2010, Budva, MNE.
8. Renal trauma urology ward in the period 2000-2009, Krasniqi Xh, Elezaj Sh. The 7th Symposium of the KUA. Juny, 2010, Prishtina, KS.
9. Treatment of ureter stones with URS method in urology ward in the period 2003-2007, Elezaj Sh. The 6th Symposium of the KUA. May, 2008, Prishtina, KS.
10. The incidence of the urolitiasis regional hospital in Peja in the period 2000-2007, Salihaj R, Elezaj Sh. The 6th Symposium of the KUA. May, 2008, Prishtina,KS.
11. Our first experience in the treatment of localized prostate carcinoma with radical

Prostatectomy, Elezaj Sh. The 4th Symposium of the KUA. May, 2006, Prishtina, KS.

12. Diagnosis of prostate cancer with TRUS biopsy with more samples in comparison with standard sampling, Hoxha A, Elezaj Sh. The 4th Symposium of KUA. May, 2006, KS.

13. Incindental detection of Testis tumor after trauma of scrotal region, Krasniqi Xh, Elezaj Sh. The 4th Symposium of the KUA. May, 2006, KS.

14. The first experience of treatment of stones in the ureter with URS methods, Elezaj Sh. The 3rd Symposium of the KUA, May, 2005, KS.

15. Renal Angiomyolipoma, Salihaj R, Elezaj Sh.. The 3rd Symposium of the KUA. May, 2005, KS.

16. Treatment of bilateral uretheral thumors, Salihaj R, Elezaj Sh. The 3rd Symposium of the KUA. May, 2005, KS.

Scientific work:

1. Vukovic P., **Shkelzen Elezaj** et al, Importance of ovarian tissue cryopreservation in fertility preservation and anti-aging treatment. Journal: *Gynecological Endocrinology* (IGYE). Article ID: IGYE 1611763.doi: 10.1080/09513590.2019.1611763.
2. **Shkelzen Elezaj** et al. The frequency of Follicle-Stimulating Hormone Receptor 2039A>G Gene and Polymorphism and the Risk of Male Infertility in Albanian Population. Acta ClinicaCroatica, Volume 58/2019
3. Zeqiraj A., **Shkelzen Elezaj** et al. Biochemical marker (Creatine kinase) and Sperm DNA Fragmentation in Male Infertility. International Journal of Advanced Biotechnology and Research (IJABR), Volume 10 Issue 1, 2019.
4. Zeqiraj A, **Shkelzen Elezaj** et al. Male infertility. Indo American Journal of Pharmaceutical Science (Indo Am. J. P. Sci), 2019; 06 (01). http://doi.org/10.5281/zenodo.2539325.
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ANNEX: Consent forms

FLETËPËLQIMI
Pëlqimi për të marrë pjesë në studimin e pacientëve të rritur në projektin:

"**POLYMORPHISM OF FOLLICULE STIMULATING HORMONE GENE RECEPTOR IN**
ALBANIAN MALE POPULATION "
Autorë Dr.Shkelzen Elezaj, Programi I studimeve për Doktoraturë-PhD

1. Unë vërtetoj se me datën --------- muaji --------viti -------vendi ----------
e kam lexuar njoftimin për hulumtimin shkencorë të përmendur më lartë, dhe kam pasur mundësi të parashtroj pyetje;

2. Unë e kuptoj se pjesmarrja ime është vullnetare dhe se pjesmarrja ime në këtë studim mund të tërhiqet në çdo kohë, pa dhënë asnjë arsye dhe pa pasoja për gjëndjen time shëndetësore apo statusin ligjorë;

3. Unë e kuptoj se në dokumentacionin tim mjekësor kanë qasje vetëm personeli përgjegjës, që është udhëheqësi I hulumtimit dhe bashkpuntorët e tij, si anëtarët e komisionit etik e të institucionit ku është kryer hulumtimi, si dhe komisioni etik që e ka miratuar këtë studim. Këtyre personave u lejoj qasje në të dhënat e mia mjekësore;

4. Unë pajtohem se mjeku im familjarë (ose anëtari I familjes) të jenë të njoftuar me pjesmarrjen time ne këtë kërkim shkencorë;

5. Pranoj dhe pajtohem që të marrë pjesë në kërkimin shkencorë të sipërshënuar;

Emri dhe mbiemri I të anketuarve: ----------
Nënshkrimi : ----------
Vendi dhe data : ----------

Emri dhe mbiemri I personit I cili ka udhëhequr procesin e informacionit dhe pëlqimit për pjesmarrje : ----------
Nënshkrimi : ----------
Vendi dhe data : ----------

Emri dhe mbiemri I udhëheqësit të projektit : ----------
Nënshkrimi : ----------
Vendi dhe data : ----------

Suglasnost za sudjelovanje odraslog ispitanika u istraživanju:

“ POLYMORPHISM OF FOLLICULE STIMULATING HORMONE GENE RECEPTOR IN ALBANIAN MALE POPULATION “

Autor Dr.Shkelzen Elezaj PhD study programme

1. Potvrdjujem da sam dana ------mjesec -----godinu-------
Pročitao obavijest za ispitanika za gore navedeno znanstveno istraživanje te sam imao priliku postavljati pitanja.

2. Razumijem da je moje sudjelovanje dragovoljno i da se iz sudjelovanja u istraživanju mogu povući u bilo koje vrijeme, bez navodjenja razloga i bez ikakvih posljedica za moje zdravstveno stanje ili pravni status.

3. Razumijem da mojoj medicinskoj dokumentaciji pristup imaju samo odgovorne osebe, to jest voditelj istraživanja I njegovi suradnici te članovi Etičkog povjerenstva ustanove u kojoj se istraživanje obavlja, i Etičkog povjerenstva koje je odobrilo ovo znanstveno istraživanje. Tim osobama dajem dopuštenje za pristup mojoj medicinskoj dokumentaciji.

4. Pristajem da moj obiteljski liječnik (odnosno član obitelji) bude upoznat smojim sudjelovanjem u navedenom znanstvenom istraživanju.

5. Želim i pristajem sudjelovati u navedenom znanstvenom istraživanju.

Ime i prezime ispitanika : ----------
Vlastoručni potpis : ----------
Mjesto i datum : ----------

Ime i prezime osobe koja je vodila postupak obavijesti za
Ispitanika i suglasnosti za sudjelovanje : ----------
Vlastiručni potpis : ----------
Mjesto i datum : ----------

Ime i prezime voditelja projekta : ----------
Vlastiručni potpis : ----------
Mjesto i datum : ----------