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Biochemical Assessment of Endometrial Receptivity and Embryo Evaluation Among Cases of In Vitro Fertilization.

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala, in Partial Fulfilment of the Requirements for the Degree of Master in Clinical Chemistry and Biochemistry

By

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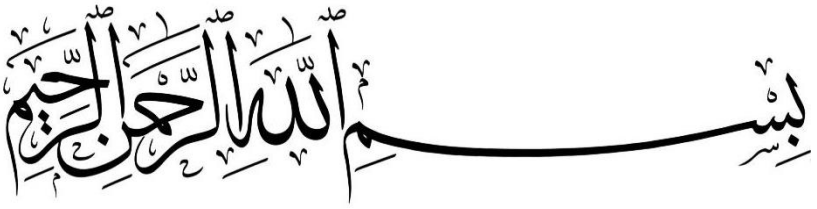

ٱللَّهُ نُورُ ٱلسَّمَٰوَٰتِ وَٱلْأَرْضِ ۚ مَثَلُ نُورِهِۦ كَمِشْكَوٰةٍ فِيهَا
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يُوقَدُ مِن شَجَرَةٍ مُّبَٰرَكَةٍ زَيْتُونَةٍ لَّا شرقية وَلَا غَرْبِيَّةٍ يَكَادُ
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لِنُورِهِۦ مَن يَشَآءُ ۚ وَيَضْرِبُ ٱللَّهُ ٱلْأَمْثَٰلَ لِلنَّاسِ ۗ وَٱللَّهُ بِكُلِّ
شَىْءٍ عَلِيمٌ

صَدَقَ اللهُ العَليّ العَظيمْ

[سورة النور: أية (٣٥)]

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First, and above all, thanks to the Great Merciful Allah who gives me health, strength, patience, and perseverance and facilitated the ways for me to accomplish this work.

My source of strength is my father, my angel and the master of my heart, the man of struggle, the great man, my inspiration and my friend to heaven, my dear mother, and the most precious thing I have, my brothers (Zaid, Zahraa, Zain Mujtaba, Mohammed). My fingers are unable to write any text that would reward you. You were a light on my arduous path.

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I would like to thank all patients for their assistant and participation in this work.

Dedication

To… Allah my Lord,

To…

To the position of my master, the Master of the Age and Time, Imam Mahdi, my soul is sacrificed for the dust of his feet, the absent yet present companion.

To…

My Family (My Eyes Father, My heart Mother, lanterns of light for my way my brothers, and my spiritual companion)

To…

My homeland Iraq, the symbol of civilization and the country to whom I proudly belong, despite the depth of its wounds.

I dedicate this work...

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List of Abbreviations

Abbreviation	Full Form
AFC	Antral follicle count
AMH	Anti-Mullerian Hormone
ART	Assisted reproductive technique
BMI	Body mass index
DICs	Dis-seminated Intravascular Coagulation
E2	Estradiol 2
FF	Follicular Fluid
FSH	Follicle Stimulating Hormone
GDF9	Growth Differentiation Factor 9
GnRH	Gonadotropin Releasing Hormone
GV	Germinal Vesicle
HCG	Human Chorionic Gonadotropin
HLA	Human Leukocyte Antigen
ICSI	Intra Cytoplasmic Sperm Injection
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
MI	Meiosis I
MII	Meiosis II
MUC1	Mucin 1
OSI	Ovarian Sensitivity Index
ROC	Receiver Operating Characteristic
SD	Standard Deviation
SPSS	Statistical Package for the Social Science
U/S	Ultrasound
WHO	World Health Organization

Summary

Background: In Vitro Fertilization (IVF) is a process where an ova is fertilized by sperm outside the body. One of the critical factors influencing the success of IVF is endometrial receptivity, which refers to the ability of the endometrium to allow an embryo to implant and develop. This study was to examine the role of the proposed markers in the IVF process by the following objectives, Estimating the level of (Mucin 1 MUC1, Growth Differentiation Factor-9 GDF-9, and Human Leucocyte Antigen HLA) in IVF cases and investigating the diagnostic preferences of the biomarkers using ROC analysis.

Patients and Methods: A cross-sectional study designed, for a total 120 Females with primary and secondary infertility were collected, blood samples were collected before IVF, and after two weeks of returning the fertilized ova . Study was conducted throughout the period from September / 2023 to August/ 2024. The College of Medicine, University of Karbala, and Karbala Health Directorate validated the study's ethical approval. The approval also taken from the Fertility Center Al-Kafeel super-specialty hospital and from each patient after explaining the nature and purpose of the study

All participants who underwent an IVF treatment with FSH stimulation and had an evaluation of serum AMH levels before treatment were included in this study. level of Mucin, GDF9 and HLA were measured in serum, Statistical analysis was performed with SPSS and Graphical pad prism.

Results showed that Females with secondary infertility have a significantly higher mean level of both mucin (9.26%) and GDF9 (2.7%) compared to those with primary infertility (5.94% and 1.03 %, respectively). While Females with primary infertility have a highly significant higher mean HLA level (33.14%) compared to those with secondary infertility (27.34%).

Results shown an inverse link between AMH levels and Mucin levels. As AMH levels decrease across groups (A to D), the mean levels of Mucin tend to increase. The pattern for GDF9 remains similar. Group A has the lowest mean

GDF9 level, and it increases across groups B and C. a different pattern was found regarding HLA level HLA levels consistently decrease with decreasing AMH levels (A to D). The mean Mucin level was significantly higher in the group with a positive Beta hCG test (7.59 ng/ml) compared to the negative group (6.54 ng/ml), p value was <0.05.

Similar to Mucin, the mean GDF9 level is also higher in the positive Beta hCG group (3.62 ng/ml) compared to the negative group (2.22 ng/ml) p value was <0.001.An interesting difference in biomarker levels were found among the OSI groups: It was shown that infertile women with OSI greater than 4 may have higher levels of Mucin and GDF9, and lower levels of HLA, compared to those with OSI less than 4. The mean Mucin level was higher in the group with OSI > 4 (7.23±1.87) compared to the OSI < 4 group (5.93±2.15). Similar to Mucin, the mean GDF9 level was also higher in the OSI > 4 group (3.46±3.17) compared to the OSI < 4 group (2.25±1.98) Interestingly, the mean HLA level was lower in the OSI > 4 group (18.64±6.58) compared to the OSI < 4 group (33.47±17.99).

Conclusion: The assessment of MUC1, GDF9, and HLA can provide valuable information on endometrial receptivity and embryo viability in IVF. These markers can be used to identify potential issues in implantation and guide personalized treatment strategies to improve the chances of successful pregnancy.

CHAPTER ONE

Introduction And Literature Review

1. INTRODUCTION

1.1. Infertility

Infertility has been established as a significant global health issue, and its incidence rate is rising yearly (1).

Infertility can be defined as the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse or due to an impairment of a person's capacity to reproduce either as an individual or with his/her partner (2) (3).

Infertility may become the 3rd main class of diseases in the 21st century, after tumors, and cardiovascular disorders, Its impact has been observed globally, which has a detrimental impact on family happiness and personal quality of life, but also has an endless negative effect on the level of medical services, reproductive health, social, economic as well as cultural levels in the country and the region (4).

A couple that attempts unsuccessfully to have a baby after a specific period of time is sometimes said to be sub-fertile, that mean a lesser fertile than the typical couples. Infertility and subfertility are defined as the failure to achieve a clinical pregnancy after at least one year or more of regular unprotected sexual intercourse, so often two terms (infertility and sub-fertility) are overlapping (5).

“Infertility” is not synonymous with “sterility” which is define as a permanent state of infertility (6).

Generally, (20-35%) of infertility cases are due to female cause, (20-30%) of cases are due to male cause, (25-40%) of cases due to both of male and female (combined) etiologies and 10% unexplained infertility (7).

For decades the burden of couples' infertility has been often and disproportionately supposed as the women responsibility, as such, for biological and social causes, a couple's infertility has been unequally shared, with the tendency to investigate the female partner over the male (8).

1.1.2. Types of Infertility (9).

1) **Primary Infertility:** It is a failure to get pregnancy after 1 year of regular intercourse without using contraception.
2) **Secondary Infertility:** It is the incapability to conceive after 1 year of regular intercourse without using contraception following a previous pregnancy or miscarriage.

1.1.3. Causes of Infertility

a. Male Factors (10) .

Male infertility may be roughly subdivided into 3 groups:

(1) Hormonal imbalance causing secondary hypogonadism.

(2) Testicular dysfunction (which may be associated with primary hypogonadism).

(3) Obstruction of seminal outflow (usually termed, obstructive azoospermia) and coitus problems.

b. Female Infertility

Can be sub-divided into:

I. Hormonal Disorders

It is an essential reason of anovulation, hormonal disorders that affect ovulation include a hypothalamic\pituitary cause such as hypogonadotropic hypogonadism, or hyperthyroidism, hypothyroidism, and hyperprolactinemia, women with hormonal disproportion impair producing the folliculogenesis to ensure the development of an oocyte (11).

II. Ovarian Disorders

Disorders of ovulation are often present with irregular periods (oligomenorrhoea) or an absence of periods (amenorrhoea), Studies done worldwide prove that polycystic ovarian syndrome is the single most common cause of female factor of infertility (12).

III. Fallopian Tube Damage or Blockage

The most common cause is Genital tract infection, which may result in pelvic inflammatory diseases (Gonorrhea and Chlamydia), endometriosis in most cases leads to pelvic adhesions and then tubal obstruction (13).

IV. Uterine Factors

The uterus has a vital role in allowing for a woman to attain pregnancy and carry it to term successfully, there are many etiologies, but by categorizing the diagnoses into either congenital due to structural defect of uterus or acquired due to fibroids (14).

c. Unexplained Infertility

It is defined as the failure of conception not explained by anovulation, poor sperm quality, tubal pathology or any other causes of infertility, the two most useful treatments for unexplained infertility are intra-uterine insemination and in vitro fertilization (15).

1.1.4. Evaluation and Management of Infertility

Both men and women partners should submit to simultaneous assessment a reproductive history and examination should include in evaluation. The semen analysis (SA) is a significant element in the initial clinical assessment of the men reproductive health. Semen parameters, which are extremely variable biological measurements and can differ dramatically from ejaculate to ejaculate. The importance of getting at least two SAs, ideally obtained at least one month

apart, is highlighted if the first SA shows aberrant values (16), as presented in Figure (1.1)

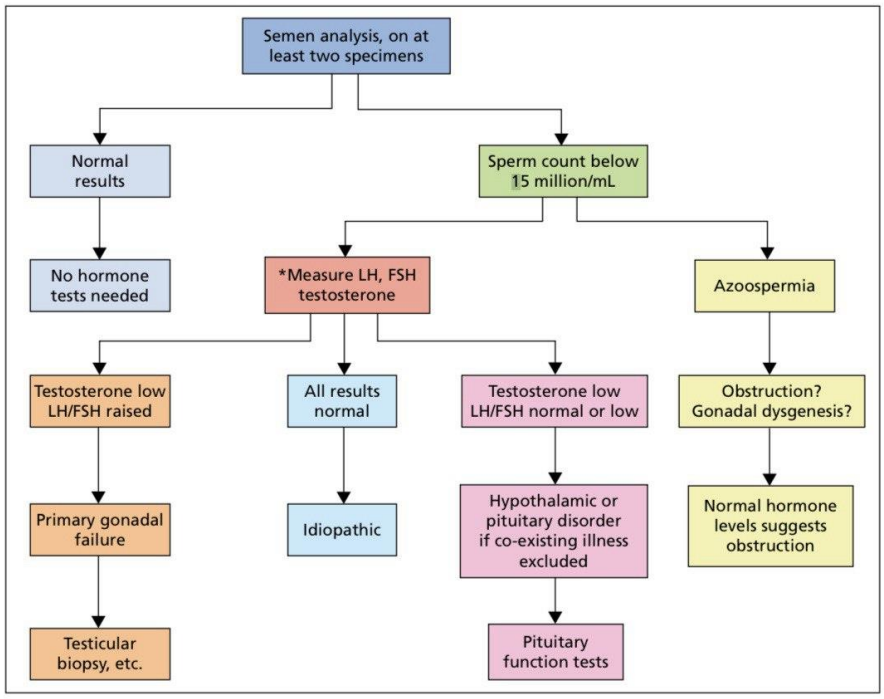


Figure 1.1. Male infertility algorithm (Schlegel *et al.*, 2021)

1.2 Factors Affecting Fertility

1.2.1 Age

A woman's advancing age is one of the most significant non modifiable risk factors for infertility mainly because of the decrease of gamete's quantity and quality with the passage of the years with its peak between the ages of (18 and 24) years while, it begins to drop after age 27 and then drops at a somewhat larger rate after age 35 years (17) .Increased paternal age over 40 years in some studies or over 50 years in other is associated with decrease in sperm quality, increased DNA fragmentation rates with failure in pregnancy, increased risk of autosomal dominant disorders, impaired neurocognitive development, increased risk of adverse postnatal

manifestation of pregnancies with decreased levels of success in IVF, but studies report that when the woman is under 30 years, the increased father's age does not affect fertility rates, fetal quality, and the rate of miscarriages (18).

1.2.2 Weight

Nearly one third of the world's population is categorized as being obese (body mass index (BMI) 30 kg/m^2) or overweight (BMI 25 to 30 kg/m^2). Lower ova quality and quantity, a longer time to conceive, and larger dosages of ovulation-stimulating medicine have all been linked to a higher BMI (19).

Obesity is also related with male infertility, due to hormonal changes secondary to excess adipose tissue, opposite relationship between (BMI) and testosterone, testosterone-to-estradiol ratio, ejaculate volume, sperm concentration, and morphology, the authors also stated higher rates of azoospermia and oligospermia among obese men related with men of normal weight (20).

1.2.3 Smoking

These are modifiable risk factors which are affecting human reproductive function. Smoking is linked to premature menopause in addition to decreased levels of ovarian reserve markers, mediated by a diminishing of antral follicle progress and growth, resultant in cytotoxicity and making of poor-quality oocytes. In men alterations in morphology and decreased concentration, motility and viability of sperm have been observed among smokers (21).

1.2.4 Alcohol

Alcohol consumption is associated with higher levels of estrogens and lower levels of progesterone, as well as decreased luteinizing hormone (LH),

Human Chorionic Gonadotropic (hCG) receptor expression, granulosa cell expression, reduced oviductal smooth muscle cell contractility, irregular menstrual cycles, and ovulatory dysfunction (22).

In men shown a significant decline in testosterone levels, seminal fluid volume and sperm concentration in chronic alcoholics (23).

1.2.5 Environmental Factors

Strong evidence showed that exposure to environmental contaminants could interfere with adult female and male reproductive function, these contaminants include heavy metals, organic solvents, pesticides and endocrine disrupting chemicals that have a potential risk factor for infertility and adverse pregnancy outcomes (24).

1.2.6 Nutrition

Since female reproduction requires a lot more energy than male reproduction does, ovarian activity is repressed in eating disordered women. High-fat diets have an impact on the physical and molecular composition of sperm cells as well as the growing pregnancy and offspring. According to studies, eating meals high in fruits, vegetables, legumes, and seafood consistently is associated with better sperm quality and a lower DNA fragmentation score than people who do not consume such items regularly. Regular consumption of red meat was inversely related to sperm quality (25).

1.3 Female Reproductive Organs and hormonal regulations

The reproductive tract of female is composed of ovaries: in which the follicles develop to fully mature oocytes, the oviducts: where the fertilization occurs and the fertilized ovum(zygote) passes through it to uterus: where implantation occur, cervix and vagina: where, the fetus is passing through them and is born and all these tracts for accommodation and passage of sperms (26).

The hypothalamus is responsible for the pulsatile secretion of gonadotrophin-releasing hormone (GnRH) which stimulate the anterior pituitary

gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH), the ovaries are response to FSH and LH for the production and periodic release of oocytes and for production of estradiol and progesterone, which prepare the endometrium for implantation (27).

The granulosa cells of the growing follicle release estrogen, which has a negative feedback effect on LH production during the first phase of the menstrual cycle. However, as oocytes develop in preparation for ovulation and estrogen levels reach a crucial level, estrogen starts to exert positive feedback on LH production, causing the LH surge through its effect on GnRH pulse rate (28).

As shown in the figure 1.2 demonstrate the regulation of hypothalamic pituitary gonadal axis.

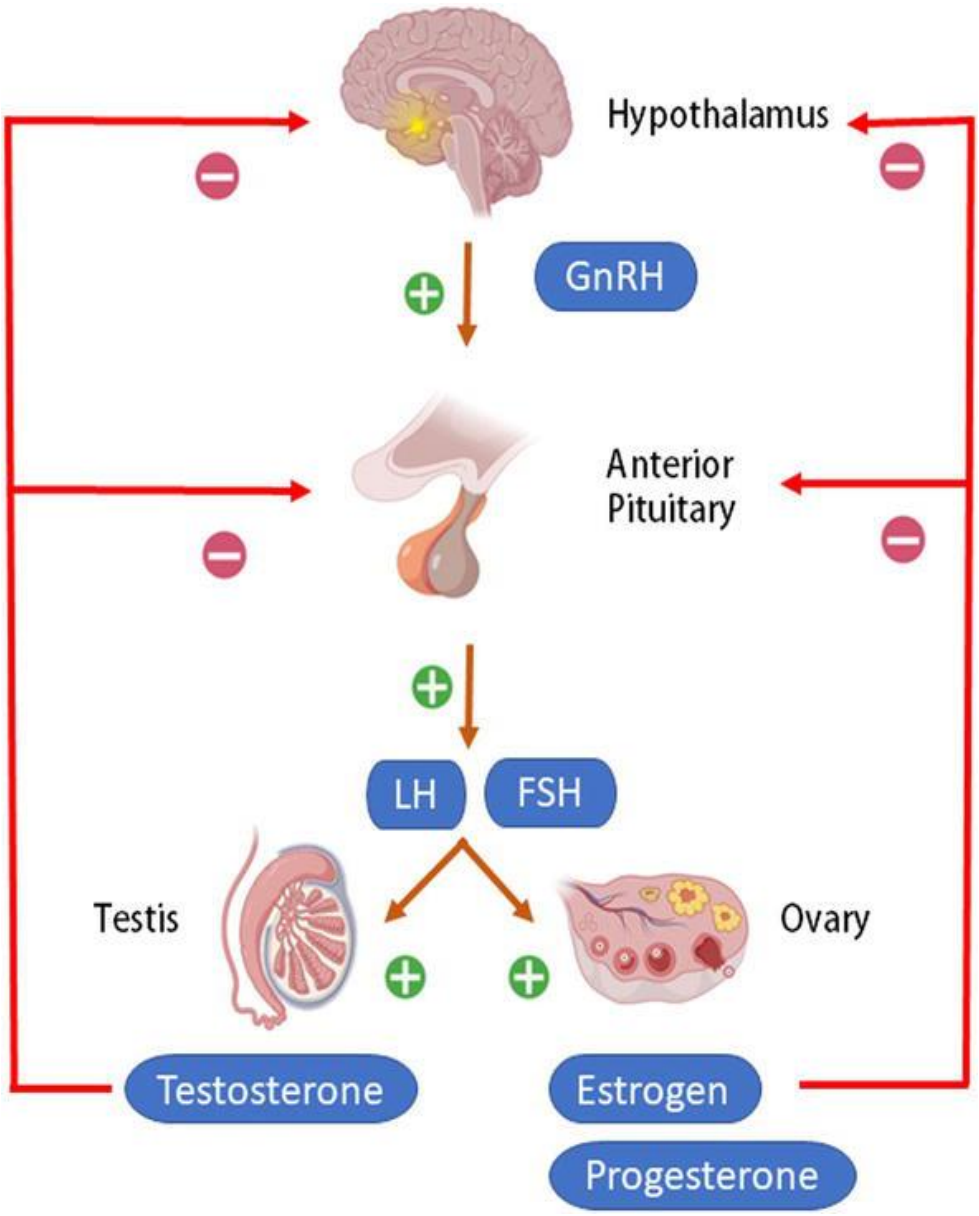


Figure 1.2. Regulation of hypothalamic pituitary gonadal axis (Gupta *et al.*, 2021)

1.4 Ovarian Function

The main ovarian functions are:

1.4.1 Oogenesis

Oogenesis is defined as the process by which the oogonia will differentiate into a mature oocyte, shortly after primordial germ cells arrival to the gonadal ridge, they are differentiated in to oogonia (29) .

Several oogonia have become atretic by the seventh month of prenatal development, and the only remaining oogonia cells identified as primordial oocytes go through mitosis division to produce primary oocytes (30).

After puberty and just before ovulation, the first meiotic division is completed, one of the daughter cells called the secondary oocyte, while the other, is first polar body which degenerate. The secondary oocyte immediately begins the 2nd meiotic division, but it stops at metaphase II(MII) and is completed only if a fertilization occurs, when, the 2nd polar body is discarded and the fertilized ovum started division by mitosis in a process called cleavage to produce blastomers (31) , as presented in Figure 1.3

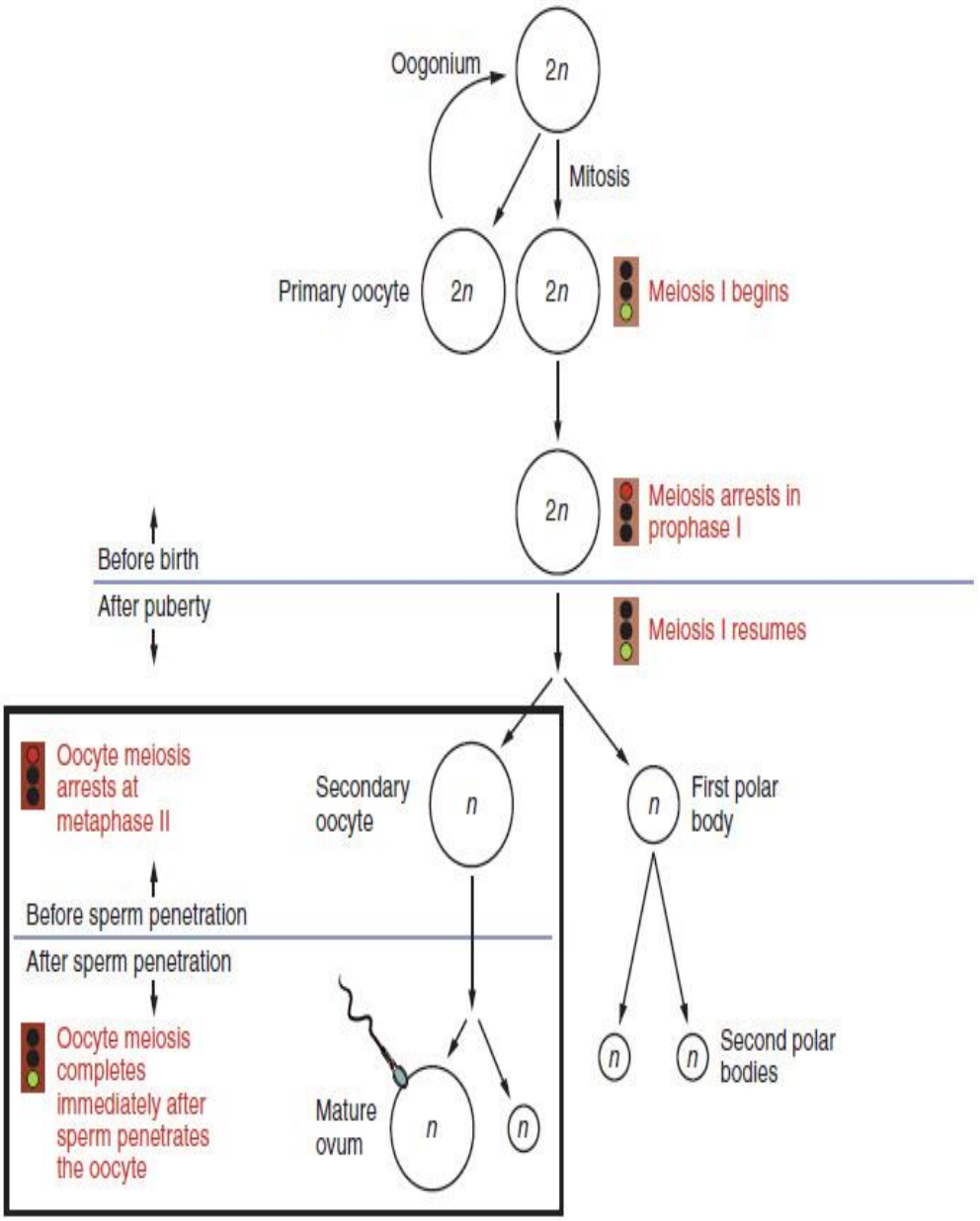

Oogonium
2n
Mitosis
Primary oocyte
2n
2n
Meiosis I begins
2n
Meiosis arrests in prophase I
Before birth
After puberty
Meiosis I resumes
Oocyte meiosis arrests at metaphase II
Secondary oocyte
n
n
First polar body
Before sperm penetration
After sperm penetration
Oocyte meiosis completes immediately after sperm penetrates the oocyte
Mature ovum
n
n
n
n
Second polar bodies

Figure 1.3. Oogenesis process; 2n: diploid nuclear material (46 chromosomes or 23 pairs), n; haploid nuclear material (23 chromosome) (32) (Alfatlawy *et al.*, 2020)

1.4.2 Sex hormones production

Which consist of estrogen, progesterone and androgens, the main estrogenic and most potent form produced by the ovary is b-estradiol also, the ovary is responsible of production of many other peptide hormones, such as Anti-Mullerian hormone (AMH), Inhibin A, Inhibin B, in addition to relaxin (33) .

1.5 Ovarian and Endometrial Cycle

The female ovarian cycle undergoes by 3 phases:

1) Follicular phase: This starts on day 1 of menses until ovulation in which, multiple follicles grow under the effect of FSH, these growing follicles secrete estrogen, which then inhibits FSH secretion in a negative feedback mechanism including the pituitary gland, hypothalamus, in addition to inhibin B (34).

Usually, every twenty-eight days, gonadotropic hormones from the anterior pituitary gland promote eight to twelve new primordial follicles to begin to grow in the ovaries, one of these follicles lastly turn into “mature” and ovulates about the 14th day of the cycle (35).

2) Ovulatory phase: The persistent high estrogen level induces a sudden release of LH from the pituitary gland, LH surge then triggers final maturation and ovulation through increasing collagenase activity and prostaglandin production (36).

3) Luteal phase: The dominant follicle becomes a corpus luteum after ovulation, which secretes estrogen and progesterone. and degenerates after two weeks if pregnancy does not happen to initiate menstruation (37).

The proliferative phase of the endometrial cycle equal to the ovarian "follicular " phase, in which, vascular and endometrial tissues submit to extensive proliferation. After ovulation and formation of a corpus luteum, progesterone is secreted, secretory phase of the endometrial cycle that is equal to (ovarian "luteal" phase), progesterone production is essential for the establishing and maintenance of implantation and pregnancy (38).

If implantation does not happen that lead to regression of the corpus luteum (forming a scar like structure in the ovary known as the corpus albicans) and a consequent sharp decline in circulating progesterone and estradiol concentrations, so menstruation occurs due to fall in progesterone and estrogen levels (39) as presented in Figure 1.4

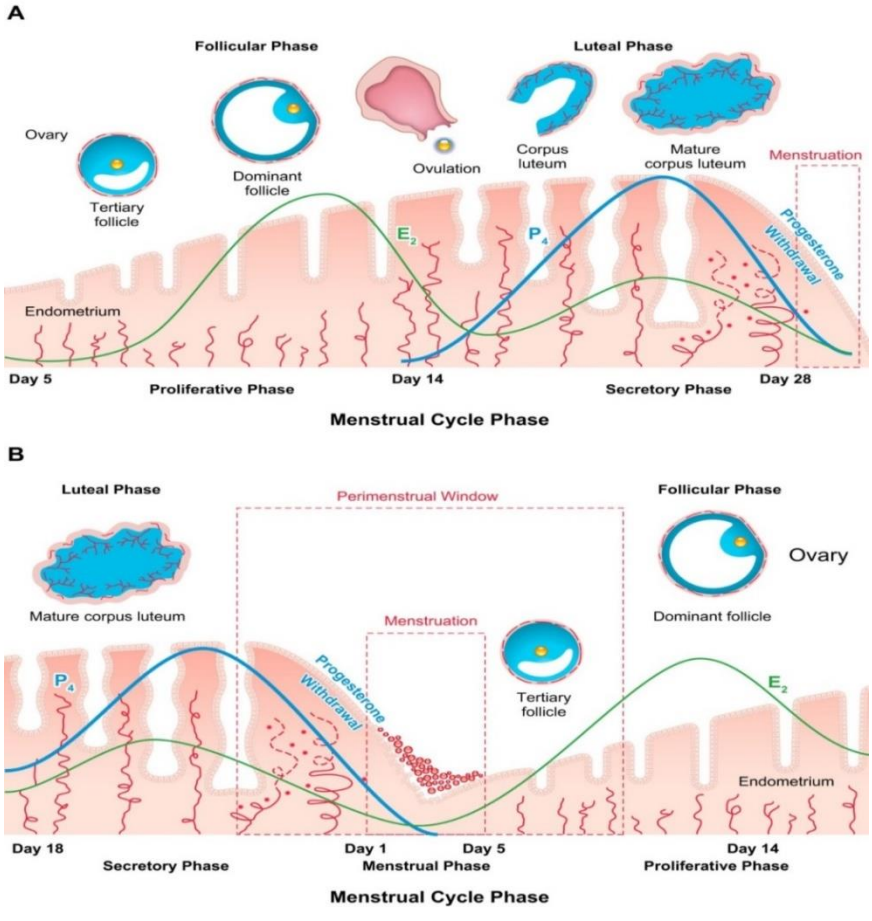


Figure 1.4. menstrual cycle. A: estradiol is the main hormone during the proliferative phase. The secretory phase take place following to ovulation. B: peri-menstrual window: focus on the significant endocrine and endometrial changes that occur through menstrual break and repair (40)**.**

1.6 Ovarian Reserve

Ovarian reserve is determining the capacity of the ovary to provide egg cells which was assessed based on age, the baseline FSH level, and the baseline antral follicle count (AFC), besides baseline anti-Mullerian hormone (AMH) level. AMH is produced by granulosa cells of small, growing follicles in the ovary. Serum levels of AMH are strongly correlate with the number of growing follicles, and consequently AMH has established increasing attention as a marker for ovarian reserve (41).

Beside the age which has an important role influencing egg quality and ovarian reserve. Reduced ovarian reserve describes the diminished quantity and quality of oocytes. Between 6% and 64% of infertile women of various ages have decreased ovarian reserve. These patients also complain of increasing use of ovulation stimulants, ovarian hypo-response, and a high rate of ovulation cancellation, in addition to having fewer and lower-quality surviving oocytes (42).

Following treatment with assisted reproductive technology (ART), these women may still experience a high rate of miscarriage, a decrease in the number of eggs obtained, and a decrease in clinical pregnancy and live births.

The physiological and psychological difficulties on these women are considerably increased by recurrent ovulation cancellation, post-ovulation

fertilization failure, and failures of implantation (43) , as presented in Figure 1.5.

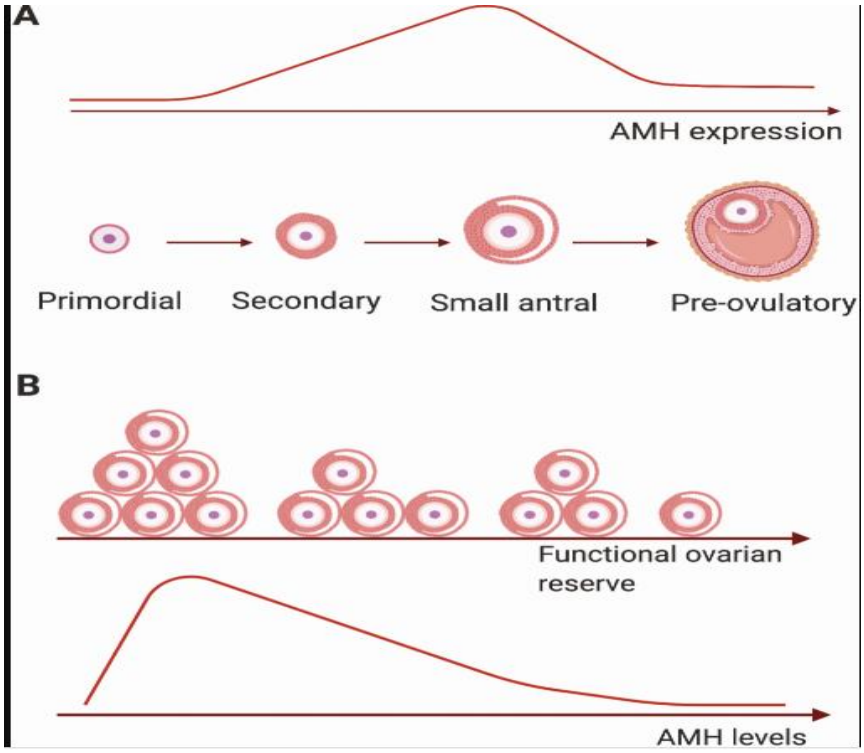


Figure 1.5. Anti-müllerian hormone level in relation to folliculogenesis and ovarian reserve. (A) From the secondary stage onward until the tiny antral follicle stage, AMH expression rises. (B) Because the primordial follicle pool is depleted with age, the functional ovarian reserve decreases.

1.7 Assisted Reproductive Technology (ART)

Assisted Reproductive Technology are a group of medical procedures for treating the infertile human in which both male and female gametes are used outside the body (in vitro) to attain pregnancy. It includes; in vitro fertilization IVF and other techniques (44).

Assisted Reproductive Technique (ART) has been using increasingly over the years as infertility treatment globally between women of different ages since the first baby Louise Brown was born as a result of in vitro fertilization in the UK in 1978 (45).

1.7.1 In Vitro Fertilization (IVF)

It is a type of assisted reproductive tech (ART), and has become the most commonly used technique of in vitro fertilization. the most common form. The term 'in vitro' means outside a living organism as oocytes mature in vivo in the ovary and embryos develop into pregnancy in the uterus, but the oocytes are fertilized in a petri dish. Robert Edwards, Ph.D., and Patrick Steptoe, MD, reported the first live birth from IVF in July 1978 in England. This achievement would later earn Dr. Edwards the Nobel Prize in Medicine in 2010.

Since this major breakthrough in the treatment of infertility, the field of reproductive endocrinology/infertility (REI) has progressed rapidly, and IVF now accounts for 1.6% and 4.5% of all live births in the United States and Europe, respectively. Initially developed as a way to bypass irreparable tubal disease, IVF is now widely applied for the treatment of infertility due to a variety of causes, including endometriosis, male factor, and unexplained infertility. Women who cannot use their own oocytes due to primary ovarian insufficiency (POI) or age-related decline in oocyte number can now become successfully pregnant utilizing donor oocyte IVF.

The cycle of IVF begins with ovarian stimulation. Multiple protocols have been utilized, including no stimulation to various levels of ovarian stimulation using clomiphene citrate, letrozole, and exogenous gonadotropins (FSH and luteinizing hormone (LH). In IVF cycles, gonadotropin-releasing hormone (GnRH) analogs are utilized to eliminate the woman's LH surge allowing the physicians to time oocyte retrieval. Follicular growth is monitored by transvaginal ultrasonography, and blood levels of E2 help determine any indicated changes in the stimulation protocol.

In natural cycle IVF, the oocyte is retrieved before the mid-cycle LH surge occurs, or a GnRH antagonist (GnRHant) is used to prevent the release of LH. When the lead follicle reaches a mature size, hCG is given as a substitute for the LH surge. The pregnancy rate is about 8% per cycle with a 21% cumulative rate

after three cycles, with rates as high as 44% in couples with male factor infertility. Natural cycle IVF is not commonly performed because of the lower clinical pregnancy rate (46).

Ovarian stimulation is done in the vast majority of IVF cycles so that approximately 10 to 20 oocytes are retrieved. There are two main protocols; long luteal GnRH agonist (GnRHa) or a GnRHant cycle. The long luteal GnRHa protocol begins with the administration of 0.1 mg GnRHa daily starting on cycle-day 21 in the preceding month. This turns off the pituitary secretion of LH (and FSH) during the ovarian stimulation, and the GnRHa is continued until the hCG injection. Gonadotropins are injected at doses ranging from 75 to 450 IU daily starting on cycle day 2 with dose adjustments based on follicular development and estradiol levels. The hCG injection is administered when at least three follicles reach 18 mm in size.

The GnRHant protocol entails the administration of daily gonadotropins (75 to 450 IU) starting on cycle day 2 or 3. The GnRHant is started to block the endogenous LH surge when the lead follicular diameter reaches 14 mm or on the sixth day of ovarian stimulation. When at least three follicles reach, 18 mm hCG is administered (47).

The minimal stimulation protocol utilizes clomiphene citrate, a selective estrogen receptor modulator (SERM), or letrozole, an aromatase inhibitor, with or without gonadotropins. When decreasing or eliminating gonadotropin stimulation, the cost to the couple is reduced. The minimal stimulation protocol is gaining more support as studies have found that while the live birth rate is slightly decreased compared to the long GnRHa protocol (49% vs. 63%), there are significantly lower rates of ovarian hyperstimulation syndrome and multiple pregnancies (48) .

1.7.2 Oocyte Retrieval

Regardless of the stimulation protocol, mature oocytes are retrieved 34 to 36 hours after hCG administration. Oocyte retrieval is performed using ultrasound-guided transvaginal aspiration and intravenous sedation. The ovaries are visualized using a vaginal ultrasound probe, and an attached needle guide helps the physician direct the needle into each follicle and aspirate the oocyte and follicular fluid.

1.7.3 Embryo Fertilization

Insemination or ICSI is used to fertilize the oocytes. The semen sample is prepared by isolating the sperm by density centrifugation and washing it in media with a high protein concentration to promote capacitation, a process that is necessary for sperm to become fertilizable. Fifty to one hundred thousand sperm are incubated with an oocyte for 12-18 hours. Male factor infertility may require ICSI, where one immobilized sperm is directly injected into the oocyte. This bypasses the need for the sperm to penetrate the zona pellucida, the glycoprotein matrix that surrounds the oocyte.

1.7.4 Embryo Transfer

Fertilized embryos are transferred at the cleavage stage (3 days after fertilization) or the blastocyst stage (5 days after fertilization). The blastocyst stage transfer offers higher live births per cycle and is achieved with fewer embryo numbers and thus lower multiple gestation rates (49). However, the downside of the blastocyst stage transfer is that fewer embryos may be available for transfer due to the loss of embryos that did not survive in culture until day 5.

Embryos are transferred under transabdominal ultrasound guidance into the uterus by a catheter passing through the cervix. The embryo(s) are placed 1 to 2 cm from the uterine fundus. After the transfer, the catheter is checked under the

microscope to ensure no embryos are retained in the catheter and that all embryos were successfully placed in the uterus. The number of embryos transferred will depend on the embryo stage, embryo quality, maternal age, and patient preference. The American Society for Reproductive Medicine recommends no more than two blastocysts to be transferred in women 37 years-old or less, no more than three blastocysts in 38 to 40 year-olds as well as in women 41 to 42 years of age (50). A higher number of cleavage stage embryos can be transferred due to the lower likelihood of successful implantation; no more than two embryos in women < 35 years of age, no more than three embryos in women 35 to 37 years of age, no more than four embryos in women 38 to 40 and in women 41 to 42 years of age, five or fewer embryos. To optimize embryo implantation and a continuing pregnancy, progesterone supplementation is initiated on the day of oocyte retrieval or embryo transfer. Excess good quality embryos are cryopreserved for future use (50).

1.8 Diagnostic Role of serum biomarkers in Females IVF outcome

Biomarkers serve as quantifiable biological indicators, signaling various medical conditions or states within an organism. These molecular beacons are instrumental for the early detection of a dynamic physiologic state or disease, tracking the efficacy of treatment and potentially predicting medical outcomes. They are part of a larger endeavor of precision or personalized medicine, which strives to optimize diagnostic testing and therapy for each individual patient. In assisted reproductive technologies (ART), the discovery of dependable biomarkers related to ovarian and endometrial function is pivotal in elevating ART procedure successes, with the aspiration of ultimately leading to the birth of a healthy child. Predictions of ovarian response and endometrial receptivity are informative to setting realistic patient and physician expectations, tailoring treatment approaches, reducing complications, and elevating the success rates of

ART treatment (51) .The research was aimed at identifying relevant biomarkers for each category—ovarian and endometrial.

1.8.1 Mucin-1 (MUC1):-

MUC1 is an anti-adhesion molecule secreted by human endometrial epithelium, it has been suggested that its expression prevents the adherence of blastocyst to the endometrium (52). MUC1 is a highly polymorphic gene that differs in the size of the region carrying the O-glycosylation sites: the variable number tandem repeat region (VNTR), which can go from 20 to 125 repeats (53).

Mucins, which are O-glycosylated macromolecular proteins, are found on the apical surface of polarized uterine epithelial cells. Mucins are involved in the initial stages of embryo and uterus interactions during implantation (3,4,5), and constitute the main component of the mucus layer. The uterine mucus layer must provide a protective function for the tissues while also allowing embryo adhesion. The process of implantation requires an attachable blastocyst and a receptive uterus (6). During implantation, steroid hormones like estradiol and progesterone modify the mucus layer of the endometrium to make it receptive. These hormones also influence the membrane elements essential for interactions with trophoblast cells (7). It has been shown that high levels of mucins inhibit cell–cell interactions via ligand access to spatial sites on the cell surface and that the uterus must undergo many different transformations to support fertilization and fetal development; a reduction in or loss of function in the mucosal barrier can lead to endometrial inflammation or infection (8).

1.8.1.1 Location of Mucin in the Endometrium

Mucin is a major component of the apical surface of uterine epithelial cells, which are involved in the initial stages of embryo–uterine interactions during implantation. While uterine epithelial cells serve a protective function for the tissue, these cells must also allow the embryo to attach (13). MUC1 (Mucin-1), also known as epithelial mucin, was the first mucin core protein to be cloned from both humans and mice. In a previous study, we observed numerous glycoprotein particles at the apex and base of endometrial epithelial cells. Using immunohistochemistry, we analyzed the distribution of MUC2 (Mucin-2) and detected a positive signal for these glycoprotein particles. This suggests a significant expression of MUC2 in endometrial epithelial cells (14). When inflammatory factors stimulate the uterine tissue, the expression of the mucous layer at the top of the uterine epithelial cells (MUC2 is the main component) is increased, and, in most cases, these mucins appear to protect the mucosal surface from infection and the effects of degrading enzymes as presented in Figure 1.6 [13].

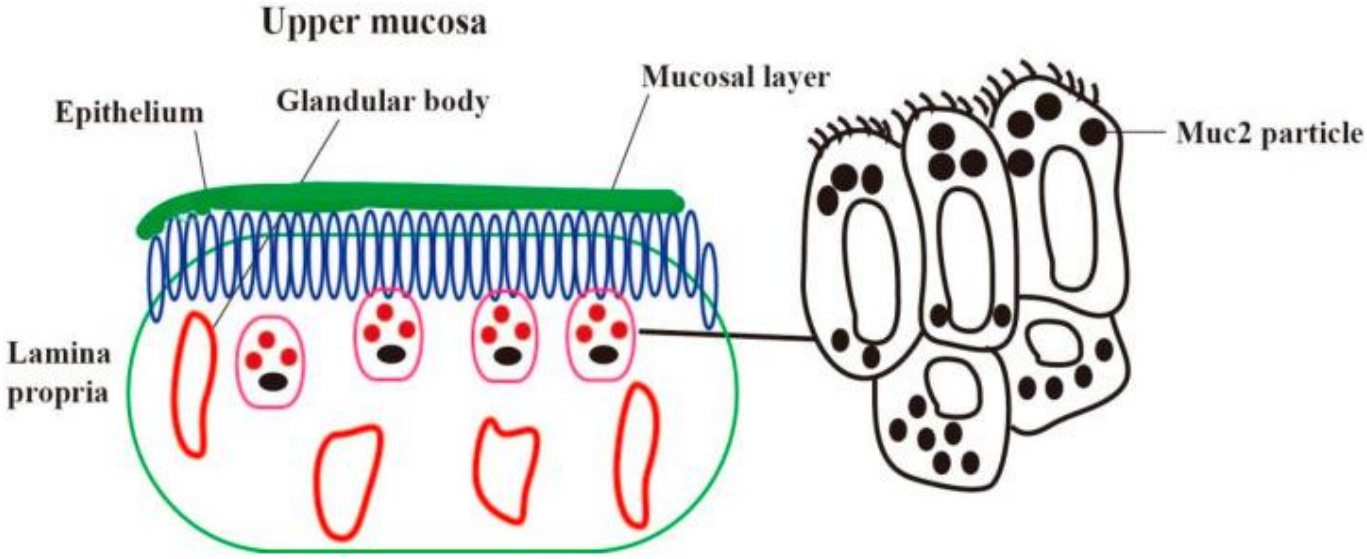


Figure 1.6 Expression and distribution of mucin in endometrial epithelial cells. The mucin distribution of endometrial cells (red dot, left); there are a large number of

glycoprotein particles in the cytoplasm of epithelial cells and basal cells (black square, right).

1.8.1.2 Function of MUC1 in Normal Tissues

In healthy tissues, MUC1 functions as a barrier to protect cells mainly by virtue of its extracellular domain (54). Membrane-bound MUC1 acts as a physical barrier through the action of its extracellular SEA domain, which can help regulate cell shedding and adhesion during metastasis; protect the apical cell membrane of epithelial cells from rupture, harmful environments, and immune attack; provide resistance to stimuli; inhibit immune responses through receptor shielding; and act as a decoy receptor for invading pathogens (55). It is also involved in lubrication, cell surface hydration, and protection from degradative enzymes, as presented in Figure 1.7 (56) . In the normal oral mucosal epithelium, MUC1, together with MUC5B and MUC7, exerts antimicrobial effects by continuously lubricating and stabilizing the mucus on the cell surface and conferring protection against proteolysis, thus preventing dehydration (57). Sherry et al. showed that MUC1*, a transmembrane cleavage product of the MUC1 protein, can help propagate large numbers of pluripotent stem cells for therapeutic interventions (58).

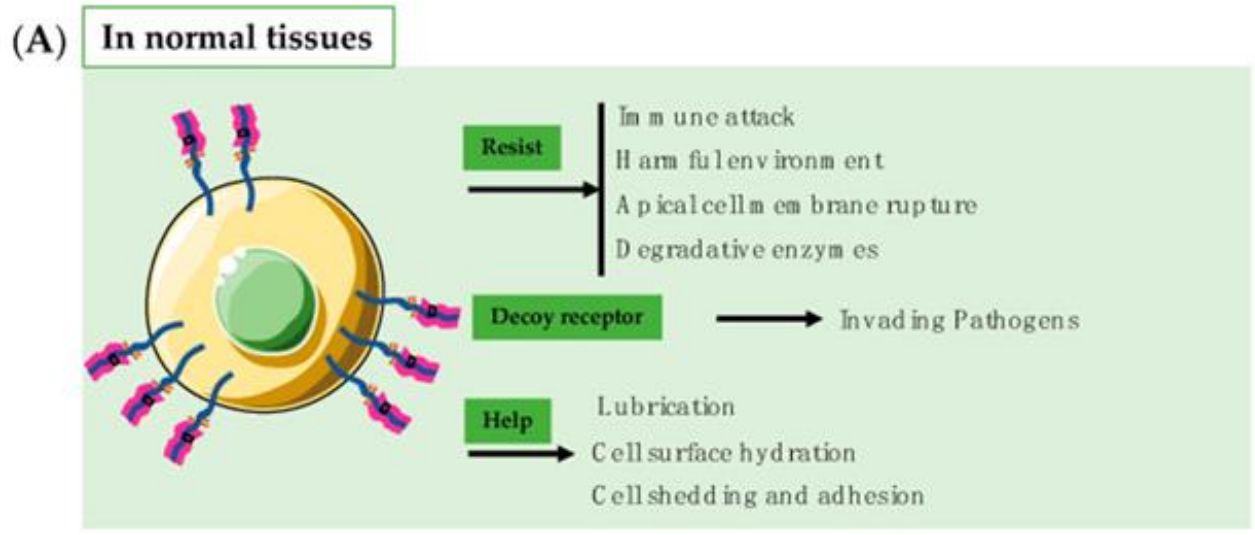


Figure 1.7: Different function of MUC1 in health or cancer tissues.

(A) The function of MUC1 in normal tissues.

1.8.1.3 The Effect of Mucin on the Tight Structure of Uterine Epithelial Cells during Pregnancy:

The integrity of the endometrial barrier is crucial for normal uterine function (47). Mucin plays a pivotal protective role in female reproductive function, shielding the endometrium from enzymatic and microbial attacks. Tight junctions between cells form an epithelial barrier, maintaining cell polarity and inhibiting lipid and protein flow (48).

Mucin is a high-molecular-weight glycoprotein, characterized by a core peptide chain and a glycan chain. Secreted by various epithelial cells, it is primarily found in the mucosal epithelium of the respiratory, urinary, and reproductive tracts [51,52,53]. In the uterus, mucins play roles in anti-adhesion, defense, and blastocyst implantation. Their expression on cell surfaces can inhibit cell-cell and cell–matrix adhesion, as well as prevent receptor–ligand interactions from initiating or mediating cell–cell adhesion on spatial sites [54]. MUC1 can inhibit E-cadherin-mediated cell–cell interactions. While there is evidence of E-cadherin interactions between murine trophectoderm and uterine epithelium, its necessity for embryo implantation remains uncertain [55]. MUC1 might also interfere with other adhesion proteins essential for embryo implantation. It is not clear whether the change in glycocalyx thickness is due to the specific function of MUC1.

1.8.2 Growth Differentiation Factor 9 (GDF-9): -

Growth differentiation factor-9 (GDF9) is an oocyte-secreted members of the transforming growth factor-β (TGF-β) superfamily (59). GDF9 synthesized as precursor proteins, consisting of pro- and mature domains, with the

prodomain templating the folding and dimerization of the mature growth factors (60).

GDF9 play important roles in follicular development including pre-antral follicle recruitment, cumulus expansion, oocyte maturation, and ovulation (61).

Previous studies have shown that mutation, decreased expression, and abnormal structure of the *GDF9* genes may predispose to follicle atresia, multiple ovulation, and early exhaustion of the ovarian reserve (62). These pathological changes are associated with multiple pregnancy, premature ovarian insufficiency (POI), and female infertility (63). Furthermore, the expression of the *GDF9* genes has been associated with oocyte maturation, fertilization, embryo quality, and outcome of IVF treatment. Some researchers have proposed that they may be used as biomarkers for predicting the potential of oocyte development (64).

Growth factors synthesized by ovarian somatic cells directly affect oocyte growth and function (65), but it is unclear whether oocyte-secreted factors play a reciprocal role in modulating somatic cell functions *in vivo* (66) . During the functional analysis of members of the transforming growth factor-β superfamily in mouse development (67).

GDF-9 stimulates the proliferation of theca cells derived from small follicles, inhibiting theca cell IGF-1- and LH-induced progesterone and androstenedione production. Hence, GDF-9 may play a role in increasing proliferation but suppressing differentiation of theca cells from small follicles in bovine ovary.

1.8.3 Human Leukocyte Antigen (HLA)

Human leukocyte antigens (HLAs) are encoded by genes in the major histocompatibility complex (MHC), among the most polymorphic genes in humans. Proteins encoded by the MHC locus mediate antigen presentation and coordination of the immune response. The diversity of HLAs allows the immune system to respond to different pathogens, but certain polymorphisms increase risk for inappropriate responses to self-antigens (68) .

In the maternal-fetal interface, trophoblast cells express HLA class Ib molecules to protect the fetus from maternal immune cells by binding to inhibitory receptors of decidual immune cells (DICs) and shifting Th1/Th2 balance toward Th2 bias. Further studies on the molecular mechanism of HLA class Ib molecules provide a reference for its application in the field of clinical assisted reproduction.

HLA complex is located on the short arm of chromosome 6, with a total of 224 loci. It is divided into three regions according to the structure and function of each point gene and its coding product, namely class I, class II and class III gene region. HLA class I gene region contains non-classical HLA-E, F, G and other loci (69) , the genes on which are called HLA class Ib genes (70). The probability of HLA being identical between two unrelated individuals is extremely small, and this variability leads to allograft rejection. As diploid organisms, humans have two different HLA inherited from both parents (71).

The embryo implantation process can be regarded as a semi-allogeneic transplant process. The embryo with paternal antigen will theoretically cause maternal transplantation rejection, which is contrary to the fact that it is not attacked by the maternal immune system before delivery (72) .Therefore, it can be inferred that there is a special tolerance effect on the maternal-fetal interface to ensure the normal progress of pregnancy.

Maternal-fetal immune tolerance occurs at the maternal-fetal interface, which is composed of maternal decidua and chorion developed from the trophoblast of blastocyst. According to previous studies, it is generally believed that the immune tolerance of the maternal immune system to the fetus is related to the interaction between the immunotolerant microenvironment of the extravillous trophoblast and the deciduous layer. There are a large number of DICs in the immunotolerant microenvironment of the decidual layer to ensure the normal process of embryo implantation and spiral artery reconstruction in early pregnancy (73). DICs exist throughout pregnancy, and the population frequency varies with the stages of pregnancy. Decidual natural killer cells (dNK) account for the majority of DICs, followed by decidual macrophages and decidual T cells and so on. However, when maternal blood is in direct contact with syncytiotrophoblasts (STBs) in late pregnancy, DICs can enter the maternal-fetal interface and carry out immune rejection to the fetus (74).

In recent years, relevant studies have shown that HLA class Ib play an important role in maternal-fetal immune tolerance (75). In order to prevent the fetus from being attacked by the maternal immune system, extravillous trophoblasts (EVTs) express HLA class Ib molecules such as HLA-E, HLA-F and HLA-G to change the function of DICs and regulate its subtypes (76).Therefore, this review will elaborate on the comprehensive mechanisms and frontier applications in HLA class Ib, in the hope of offering new ideas for the diagnosis and treatment of pregnancy-related diseases.

As immune tolerance molecules expressed on the surface of trophoblast cells, HLA class Ib molecules has made a great contribution in the process of pregnancy, protecting the fetus from immune cells and maintaining normal growth. In addition, a deeper understanding of the mechanism of HLA class Ib molecules, especially HLA-G, and their expression in the extravillous trophoblast at the maternal-fetal interface can be used as a non-invasive

biological index for rapid detection of embryo implantation. It can provide clues for maintaining normal pregnancy and further help to improve the success rate of embryo transplantation (77) .

1.9 knowledge gap

The knowledge gap on this topic might be the needing to explore the existing scope and examining ovarian and endometrial biomarkers in their respective roles in ART outcomes. this study would include both traditional and possible emerging biomarkers with discussion of both serum markers. serum ovarian biomarkers provide readily accessible methods through venipuncture, it may ultimately reveal future noninvasive ovarian biomarkers that are currently not realized (51) .

In tandem with ovarian function, the endometrial environment is vital in completing the fertility puzzle. The endometrium's receptivity, a critical determinant for successful implantation, is being increasingly deciphered by various biomarkers. Together, ovarian and endometrial biomarkers have the potential to refine fertility treatments, ushering in an era of targeted therapy and improved prognostic capabilities in reproductive medicine.

1.10 Aim of the study

Examine the role of the proposed markers in the IVF process by the **following objectives:**

- ➢ Estimating the level of MUC1, GDF-9 and HLA among inertial females undergoing IVF
- ➢ Investigating the diagnostic preferences of the biomarkers using ROC analysis.

CHAPTER TWO

Subjects, Materials and Methods

2. Subjects, Materials and Methods

2.1. Study Design

A cross-sectional study design, for a total 120 subjects were collected, study was conducted throughout the period from September / 2023 to August/ 2024. The College of Medicine, University of Karbala, and Karbala Health Directorate validated the study's ethical approval. Approval also taken from in the Fertility Center Al-Kafeel super-speciality hospital and from each patient after explaining the nature and purpose of study.

2.2. Subjects

2.2.1. Patients

Whole blood samples of 120 samples, samples were collected before IVF, and after two weeks of returning the fertilized ovas and performing a pregnancy test at the IVF center of alkafeel super Specialty Hospital, at different ages for women. An interview to collect personal history, family history, demographic information, ultrasound report and laboratory examinations results, attempt of IVF, duration of marriage without pregnancy was performed.

2.2.2. Exclusion criteria

Included endometrial or uterine pathology such as adenomyosis, fibroids, endometrial polyps and hyperplasia, severe endometriosis, endometritis, as well as anovulation & PCOS.

2.2.3. Inclusion criteria

Women with reproductive age, and had no use of hormonal contraception or intrauterine devices for at least 3 months preceding the study.

Female have primary and secondary infertility.

2.2.4. Approval of the Ethical Committee

The protocol of the study was approved by Ethical Committee of Kerbala Medical College, and committee of Oncology unit in Alkafeel Super Specialty Hospital. Samples from serum were obtained after consent from patients or the patients' relatives,120 samples were collected from them.

2.2.5. Sample Collection

Five milliliters of venous blood were drawn from all subjects by using a sterile disposable syringe. The foremost tube contained 5 milliliters of blood, set in gel tubes. Next, it stayed for 10 to15 minutes at room temperature for clotting. Then centrifuged the blood for 10 to 15 minutes at 4000 x rg. for separation of serum and divided into 4 parts and put in Eppendorf tubes then stored at - 20 ˚C till examination of the biomolecules in this study.

2.3. Materials

2.3.1. Chemicals and Diagnostic Kits

All chemicals and kits that were used in this study are listed in Table 2.1.

Table 2.1: Chemicals and Diagnostic Kits used in the study

No.	Chemicals and Kits	Manufactured by
1.	GDF9 (Growth Differentiation Factor 9)	Elabscience/ USA
2.	Human Major Histocompatibility Comp Class I chain-related gene B	BT LAB/ CHINES
3.	MUC1 (Mucin 1)	Elabscience/ USA

2.3.2. Instruments and Equipment

Table 2.2 shows the instruments and equipment's that were used in this study.

Table 2.2: Instruments and Equipment

No.	Instruments and equipment	Company /origin
1.	Appedrofes	
2.	Centrifuge	Hettich (Germany)
3.	Combi Wash For Immuno Assays	Germany
4.	Deep freezer Fisher Scientific US	Fisher Scientific (USA)
5.	ELISA instrument system	Biotek (USA)
6.	Gel tubes vacutainer	
7.	Micro centrifuge	Hettich (Germany)
8.	Micropipettes	Bioasic(Canada)
9.	Pipettes	Bioasic(Canada)
10.	Safty box	American
11.	Tibes	
12.	Vortex mixer Gemmy Taiwan	Gemmy (Taiwan)
13.	Water bath	Memmert (Germany)

2.4. Methods

2.4.1. Measurement of body mass index

The following equation was used to calculate body mass index (BMI).

BMI=Weight kg/ Height in (meter) ^{2}

It is unit (kg/ m^2) (Haslet and Haslet., 2002).

The patients and control weight status were categorized according to their BMI as following (WHO, 2016)

Table 2.3: category of BMI in adult

BMI ($Kg/m^{2)}$	Weight status
<18.5	Under weight
18.5 to 24.9	Healthy weight
25.0 to 29.9	Over weight
≥ 30.0	Obese

2.4.2. Human MUC1(Mucin 1) ELISA Kit

2.4.2.1 Principle:

ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to Human MUC1. Samples (or Standards) are added to the micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human MUC1 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed

away. The substrate solution is added to each well. Only those wells that contain Human MUC1, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of stop solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human MUC1. You can calculate the concentration of Human MUC1 in the samples by comparing the OD of the samples to the standard curve.

2.4.2.2 Kit Components

Table 2.4: Materials provided with ELISA Kit

	Materials provided with the kit	Storage
1	Micro ELISA Plate (Dismountable)	R.T.
2	Reference Standard	- 20 ºC, 12Month
3	Concentrated Biotinylated Detection Ab (100×)	-20 ºC, 12Month
4	Concentrated HRP Conjugate (100×)	-20ºC (Protect from light)
5	Reference Standard & Sample Diluent	2-8ºC
6	Biotinylated Detection Ab Diluent	2-8ºC
7	HRP Conjugate Diluent	2-8ºC
8	Concentrated Wash Buffer (25×)	2-8ºC
9	Substrate Reagent	2-8ºC
10	Stop Solution	2-8ºC
11	Plate Sealer	2-8ºC

2.4.2.3 Reagent preparation

1. Bring all reagents to room temperature (18-25℃) before use. If the kit will not be used up in one assay, please only take out the necessary strips and reagents for present experiment, and store the remaining strips and reagents at required condition.

2. **Buffer Wash:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40℃-water bath and mix it gently until the crystals have completely dissolved.

3. Standard working solution: Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 10 ng/mL (or add 1 mL of Reference Standard &Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed) Then make serial dilutions as needed. The recommended dilution gradient is as follows: 10, 5, 2.500, 1.250, 0.630, 0.310, 0.16, 0 ng/mL.

4. Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 10 ng/mL working solution to the first tube and mix up to produce a 5 ng/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to this step. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube. Gradient diluted standard working solution should be prepared just before use.

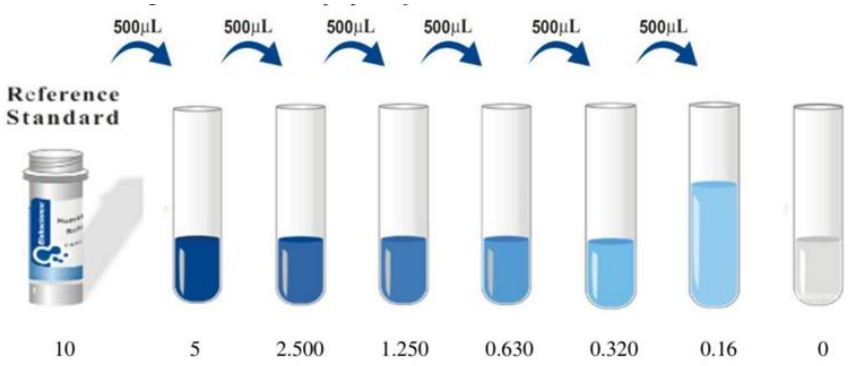


Figure 2.1: Serial Dilution method for MUCIN -1 standard

4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 µL/well). In preparation, slightly more than

calculated should be prepared. Centrifuge the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then dilute the 100× Concentrated biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99). The working solution should be prepared just before use.

5. **Concentrated HRP Conjugate working solution:** HRP Conjugate is HRP conjugated avidin. Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated HRP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99). The working solution should be prepared just before use.

2.4.2.4 Assay procedure

1. Determine wells for **diluted standard, blank** and **sample**.100 μL each dilution of standard, blank and sample were add into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. It is recommended to determine the dilution ratio of samples through preliminary experiments or technical support recommendations). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37℃. Note: solutions should be added to the bottom of the micro-ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Decant the liquid from each well, do not wash. Immediately add 100 μL of **Biotinylated Detection Ab working solution** to each well. Cover the plate with a new sealer. Incubate for 1 hour at 37°C.

3. Decant the solution from each well, add 350 μL of **wash buffer** to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
4. Add 100 μL of **HRP Conjugate working solution** to each well. Cover the plate with a new sealer. Incubate for 30 min at 37°C.
5. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 3.
6. Add 90 μL of **Substrate Reagent** to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
7. Add 50 μL of **Stop Solution** to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

2.4.2.5 Calculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log axis, with standard concentration on the x-axis and OD values on the y-axis. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor. Figure (2.2)

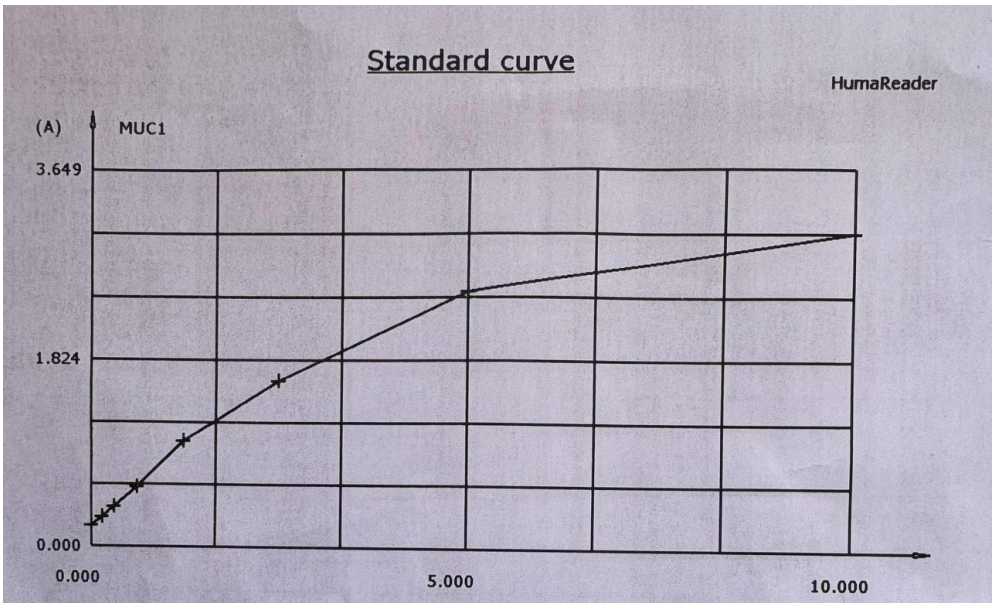


Figure 2.2: standard curve of Human MUCI

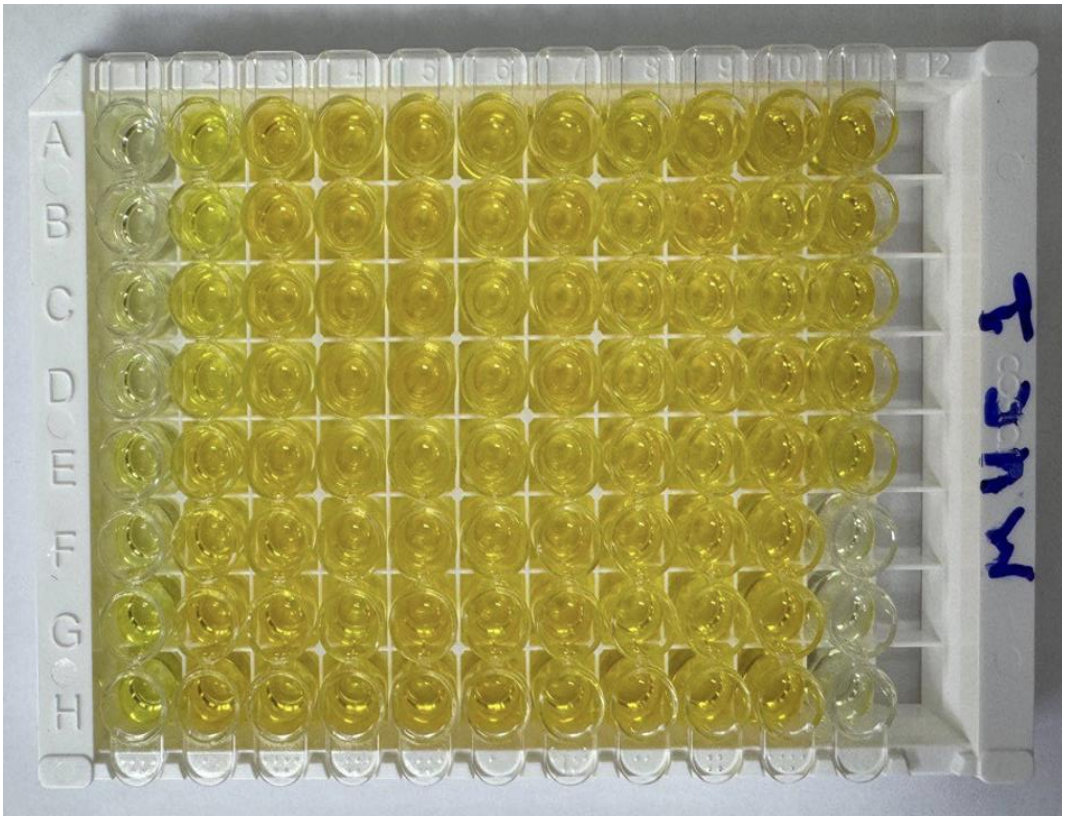


Figure 2.3: Human mucin1 kit.

2.4.3 Human GDF9 (Growth Differentiation Factor 9) ELISA Kit

2.4.3.1 Test principle

This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to Human GDF9. Then a biotinylated detection antibody specific for Human GDF9 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human GDF9, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human GDF9. You can calculate the concentration of Human GDF9 in the samples by comparing the OD of the samples to the standard curve.

Table 2.5: Materials provided with ELISA Kit

	Materials provided with the kit	Storage
1	Micro ELISA Plate (Dismountable)	R.T.
2	Reference Standard	-20 ºC, 12Month
3	Concentrated Biotinylated Detection Ab (100×)	-20ºC, 12Month
4	Concentrated HRP Conjugate (100×)	-20ºC (Protect from light)
5	Reference Standard & Sample Diluent	2-8ºC
6	Biotinylated Detection Ab Diluent	2-8ºC
7	HRP Conjugate Diluent	2-8ºC
8	Concentrated Wash Buffer (25×)	2-8ºC
9	Substrate Reagent	2-8ºC
10	Stop Solution	2-8ºC
11	Plate Sealer	2-8ºC

2.4.3.2 Reagent preparation

1. Bring all reagents to room temperature (18-25℃) before use.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40℃-water bath and mix it gently until the crystals have completely dissolved
3. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 10 ng/mL (or add 1 mL of Reference Standard &Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then make serial dilutions as needed. The recommended dilution gradient is as follows: 10, 5, 2.500, 1.250, 0.630, 0.320 0.16, 0 ng/mL. Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 10 ng/mL working solution to the first tube and mix up to produce a 5 ng/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to this step. The illustration below is for reference.

   Note: the last tube is regarded as a blank. Don’t pipette solution into it from the former tube. Gradient diluted standard working solution should be prepared just before use.

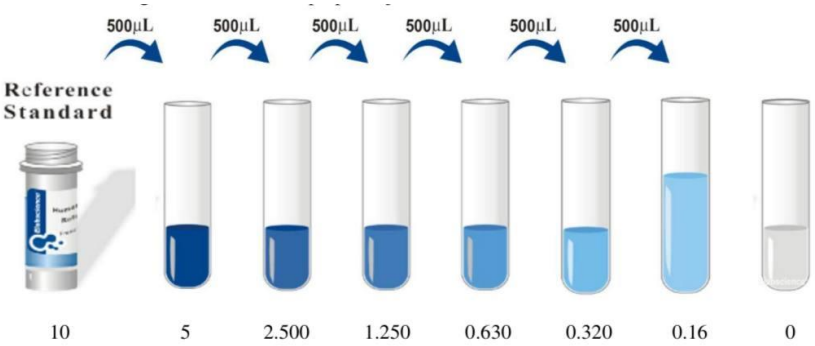


Figure 2.4: Serial Dilution method for GDF-9

4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 µL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the concentrated Biotinylated Detection Ab At 800x g For 1 Min, then diluted the 100x concentrated Biotinylated detection Ab to 1x working solution with Biotinylated detection Ab Diluent (concentrated Biotinylated Detection Ab: Biotinylated detection Ab Diluent =1:99). The working solution should be prepared just before use.

5. Concentrated HRP Conjugate working solution: HRP Conjugate is HRP conjugated avidin. Calculate the required amount before the experiment (100 µL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated HRP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99). The working solution should be prepared just before use.

2.4.3.3 Assay procedure

1. Determine wells for diluted standard, blank and sample. Add 100 µL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. It is recommended to determine the dilution ratio of samples through preliminary experiments or technical support recommendations). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro-ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Decant the liquid from each well, do not wash. Immediately add 100 µL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 hour at 37°C. 3. Decant the solution from each well, add 350 µL of wash buffer to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
3. Add 100 µL of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 30 min at 37°C.
4. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 3.
5. Add 90 µL of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
6. Add 50 µL of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.

7. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

2.4.3.5 Calculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log axis, with standard concentration on the x-axis and OD values on the y-axis. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution.The actual concentration is the calculated concentration multiplied by the dilution factor. Figure (2.5,2.6)

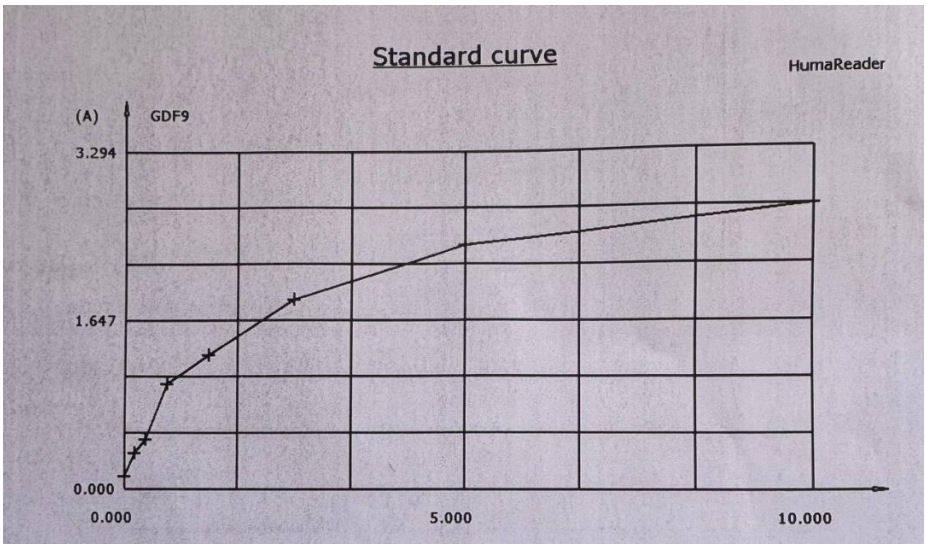


Figure 2.5: standard curve of Human GDF9

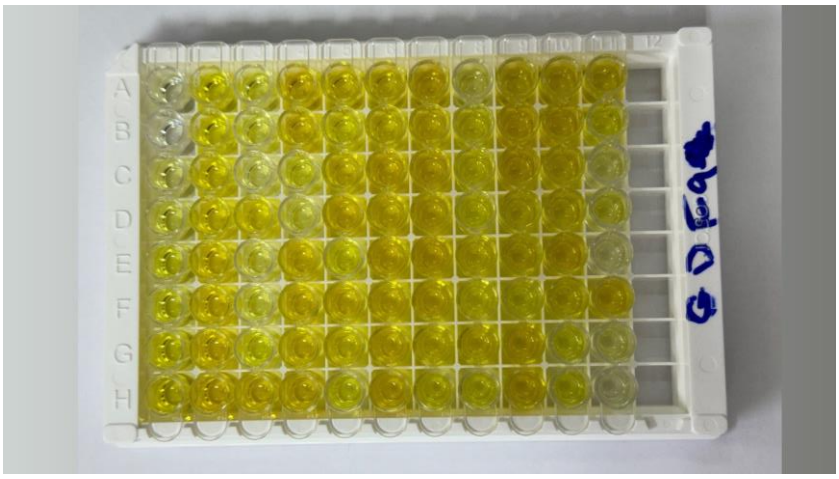


Figure 2.6: Human GDF9 KIT

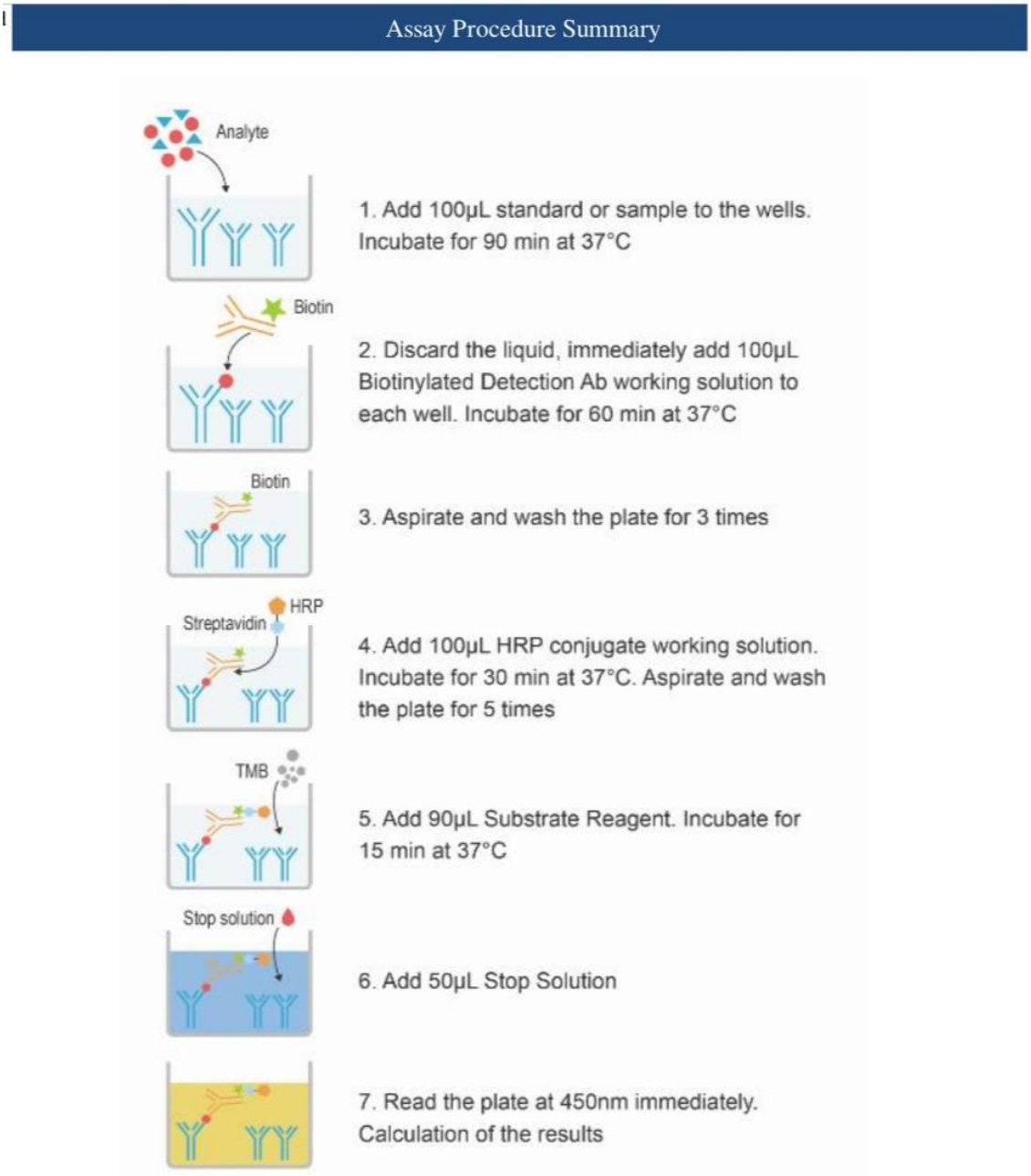


Figure 2.7 Summary of ELISA assay procedure

2.4.4 Human major histocompatibility complex class I chain-related gene B ELISA Kit.

2.4.4.1. Test principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human MICB antibody. MICB present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human MICB Antibody is added and binds to MICB in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated MICB antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and colour develops in proportion to the amount of Human MICB. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm .

2.4.4.2 Kit Component

Table 2.6: Materials provided with ELISA Kit

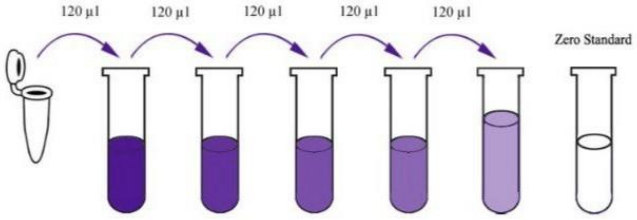
	Materials provided with the kit	**Storage**
1	Standard Solution (80ng/ml)	2-8ºC
2	Pre-coated ELISA Plate	2-8ºC
3	Standard Diluent	2-8ºC
4	Streptavidin-HRP	2-8ºC
5	Stop Solution	2-8ºC
6	Substrate Solution A	2-8ºC
7	Substrate Solution B.	2-8ºC
8	Biotinylated Human MICB Antibody	2-8ºC
9	Wash Buffer Concentrate (25x)	2-8ºC
10	Plate Sealer	2-8ºC

2.4.4.3 Reagent preparation

1. All reagents should be brought to room temperature before use.
2. **Standard** Reconstitute the 120µl of the standard (80ng/ml) with 120µl of standard diluent to generate a 40ng/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (40ng/ml) 1:2 with standard diluent to produce20ng/ml, 10ng/ml, 5ng/ml and2.5ng/ml solutions. Standard diluent serves as the zero standard(0 ng/ml). Any

remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

40ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
20ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
10ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
5ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
2.5ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
80ng/ml	40ng/ml	20ng/ml	10ng/ml	5ng/ml	2.5ng/ml

Figure 2.8: serial dilution of standard stock

Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.4.4.4 Assay procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50µl standard to standard well. **Note**: Don't add biotinylated antibody to standard well because the standard solution contains biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-MICB antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stop Solution to each well, the blue colour will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.4.4.5 Calculation of results

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on
the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

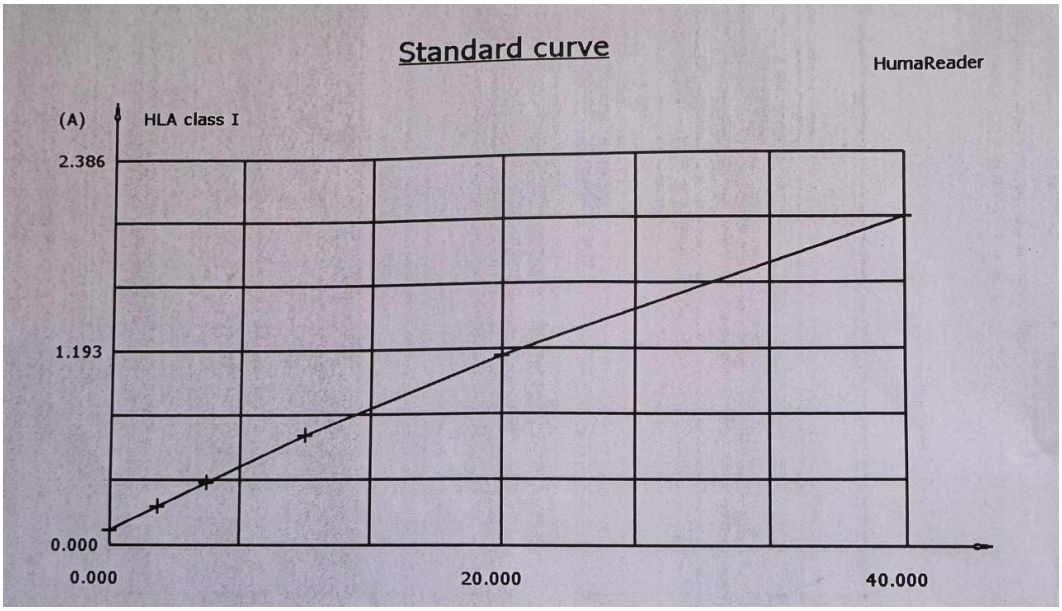


Figure 2.9: Curve of HLA Class 1

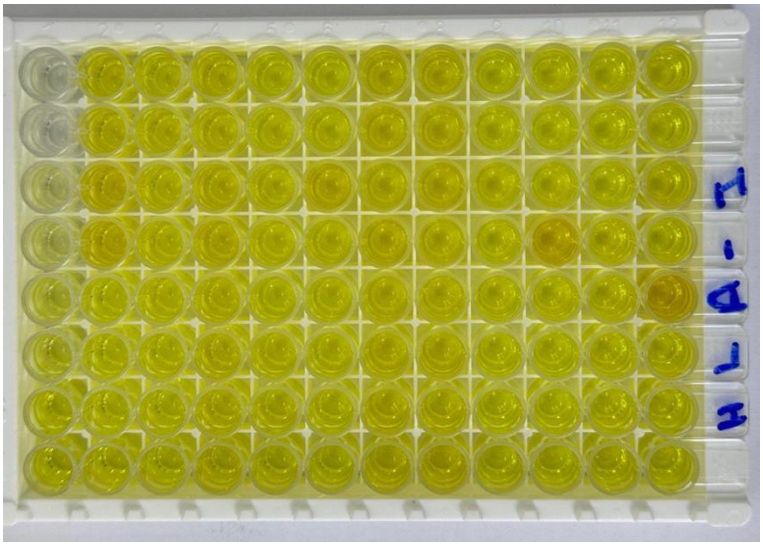


Figure 2.10: HLA-I

2.5. Statistical Analysis

Information from the questionnaire and all test results from study group samples were entered into a data sheet. The data analysis for this work was generated using the graphical Pad prism.

Descriptive statistics were performed on the data of each group. Values were illustrated by n (%) for categorical, Scale variables were presented by mean ± 2standard deviation for normal data while for non-normal data, continuous variables were presented by interquartile range (IQR) and median. The distribution of the data was checked using the Shapiro-Wilk test as a numerical means of assessing normality.

For abnormal distribution, the univariate analysis was performed using an independent Kruskal Wallis Test for continuous variables. Biomarkers were compared using the Spearman rank test to evaluate the relationship within the case study.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p-values <0.05 (two-sided) were considered to be statistically significant.

The simultaneous confidence level for each of the confidence intervals was calculated using Fisher's LSD technique. This simultaneous confidence level is the probability that all confidence intervals contain the true difference.

The optimal threshold with high specificity and sensitivity for study cases was detected using receiver operating characteristic (ROC) analysis.

CHAPTER THREE

Results

3. Results

3.1 The demographics characteristic of the study group

Table (3.1) demonstrates the demographics characteristic of the infertile female participants. The majority of participants fell within the 28–34-year age range (51.9% primary infertility, 42.9% secondary infertility). Younger participants (21-27 years) were less represented (22.2% primary, 7.1% secondary). The proportion of women with secondary infertility increased with age (25.9% at 35-42 years).

For the Body Mass Index, participants were classified as normal weight (57.4% primary, 50.0% secondary). Overweight participants were also well-represented (38.9% primary, 28.6% secondary). And Obesity rates were lower among the study groups (3.7% primary, 21.4% secondary).

Regarding the Pregnancy Outcome (Beta hCG), The success rate (positive Beta hCG) was comparable between primary and secondary infertility groups (35.9% vs 36.4%). **Results were also presented the** distribution of infertility types differed between groups:

Primary infertility: female factor (13.2%) and unexplained infertility (11.3%) compared to secondary infertility. While Secondary infertility were higher prevalence of male factor (42.9%) and combined infertility (21.4%). Most participants (around 87%) had undergone only one IVF attempt in both primary and secondary infertility groups.

This study was also included a detail about the Oocyte Characteristics, The median number of oocytes retrieved was higher in the secondary infertility group (11 vs 8). Minimum and maximum values for all oocyte characteristics (M I, M II, GV, GVL, OSI) were wider in the secondary infertility group, suggesting greater variability in oocyte development and maturity.

Table 3.1: Demographics characteristic among the participants of infertile female

Characteristic		Primary	Secondary
21-27 Years	**Age (years)**	22.2%	7.1%
28-34 Years		51.9%	42.9%
35-42 Years		25.9%	50.0%
Normal Weight	**BMI (kg/m^2)**	57.4%	50.0%
Over Weight		38.9%	28.6%
Obesity		3.7%	21.4%
Positive	***Beta hCG***	35.9%	36.4%
Negative		64.1%	63.6%
Female factor	**Type of infertility**	13.2%	28.6%
Male factor		34.0%	42.9%
Combined		41.5%	21.4%
Unexplained		11.3%	7.1%
One Attempt	**No. of IVF Attempt**	87.0%	85.7%
More than one Attempt		13.0%	14.3%
No Of Oocyte Median (Min-Max)		8(1-35)	11(1-40)
M I Median (Min-Max)		1(0-2)	1(0-6)
M II Median (Min-Max)		7(1-27)	8.5(0-39)
G.V Median (Min-Max)		0.5(0-5)	0.01(0-12)
SOI Median (Min-Max)		2.963(0.40-13.73)	4.1(0.19-47.41)

3.2 Mean differences of Biomarkers based on the types of infertility

Table (3.2) shown the mean levels and standard deviations (SD) of three biomarkers, mucin, GDF9, and HLA, in infertile females categorized by primary and secondary infertility.

Results were shown that females with secondary infertility have a significant higher mean level of both mucin (9.26) and GDF9 (2.7) compared to those with primary infertility (5.94 and 1.03, respectively). While Females with primary infertility have a highly significant a higher mean HLA level (33.14) compared to those with secondary infertility (27.34), as presented in Figure 3.1

results were indicated the potential differences in biomarker levels between women with primary and secondary infertility. The increasing in mucin and GDF9 levels in secondary infertility might be related to underlying causes. The higher HLA levels in primary infertility could be indicative of an autoimmune response affecting fertility.

Table 3.2: Mean differences of Biomarkers Levels: Mucin, GDF9 and HLA among group of infertile females based on the types of infertility

	Mucin	**GDF9**	**HLA**
Primary Infertility	5.94±1.68	1.03±0.9	33.14±22.33
Secondary Infertility	9.26±3.28	2.7±2.3	27.34±13.5

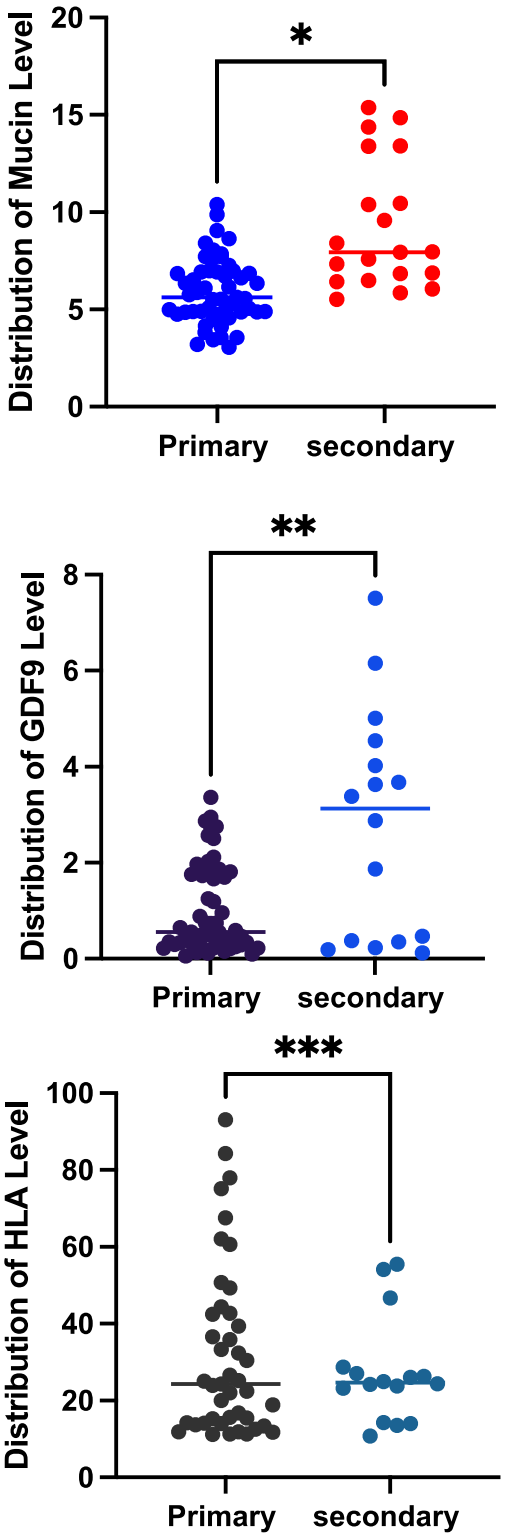


Figure 3.1: Distribution of Biomarkers Levels: Mucin, GDF9 and HLA among group of infertile females based on the types of infertility

3.3 Mean differences of Biomarkers based on circulating AMH level

Circulating anti-Mullerian hormone (AMH) is presently claimed to be the best marker of ovarian reserve and ovarian responsiveness to stimulation. In fact, it has resulted to be superior to patient's age, basal follicle-stimulating hormone (FSH), estradiol, inhibin B [4] in predicting ovarian responsiveness to exogenous gonadotropins. AMH's predictive capacity on oocyte yield is well estabilished; differently, its significance in regard to oocyte quality and pregnancy potential is still under debate, even if recent work shows a good correlation between AMH levels and occurrence of a viable pregnancy [6].

Patients were divided into four subgroups according to the circulating AMH level: below the 25th percentile (AMH 0.25–1.1 *ng/ml*, subgroup A), between 25th and 50th percentiles (AMH 1.2–1.6 *ng/ml*, subgroup B), between the 50th and 75th percentiles (AMH1.7–2.6 *ng/ml*, subgroup C), and above the 75th percentile (AMH 2.7–8.5 *ng/ml*, subgroup D). The $p<0.05$ was considered statistically significant.

This study investigated the mean differences of biomarkers among patients subgroup based on the range on Anti-Müllerian Hormone (AMH) levels. Results were demonstrated a potential link between AMH and these biomarkers

In Table 3.3 results were shown an inverse link between AMH levels and Mucin levels. As AMH levels decrease across groups (A to D), the mean levels of Mucin tend to increase. The pattern for GDF9 remains similar. Group A has the lowest mean GDF9 level, and it increases across groups B and C. a different pattern was found regarding HLA level HLA levels consistently decrease with decreasing AMH levels (A to D).

Results were shown a progressive increase in Mucin levels across the subgroups, with the highest levels in subgroup D (8.01 ng/ml) and the lowest levels in subgroup A (5.63 ng/ml).

Similar to Mucin, GDF9 levels also showed a gradual increase with increasing AMH levels. Subgroup D had the highest GDF9 (7.75 ng/ml) compared to subgroup A (5.08 ng/ml).

Interestingly, HLA levels displayed an opposite trend. The highest levels were found in subgroup A (31.46) and progressively decreased with increasing AMH levels, reaching the lowest point in subgroup D (3.32), as presented in Figure 3.2

Mucin and GDF9: The increasing levels of Mucin and GDF9 with higher AMH could indicate a possible link between these biomarkers and ovarian reserve. AMH is a marker of remaining follicles in the ovary, and these findings might suggest that Mucin and GDF9 play a role in follicular development or function.

While the decreasing trend of HLA levels with higher AMH is intriguing. HLA molecules are involved in immune response, and their lower levels in women with higher AMH could suggest a potential modulation of the immune system related to ovarian function.

3.3: Mean differences of Biomarkers Levels: Mucin, GDF9, and HLA among a group of infertile females according to circulating AMH levels

	Group A	Group B	Group C	Group D
Mucin	5.63±1.98	6.80±2.8	8.08±3.4	8.01±3.7
	1.60±0.45	2.05±0.65	1.65±0.86	1.74±0.32
GDF9	5.082.14	7.14±3.1	7.45±2.5	7.75±2.8
	1.72±0.56	3.25±1.32	2.66±1.21	2.11±0.87
HLA	31.46±12.34	20.73±9.48	16.66±8.2	15.17±6.56
	13.53±6.4	9.25±4.21	4.75±2.1	3.32±1.43

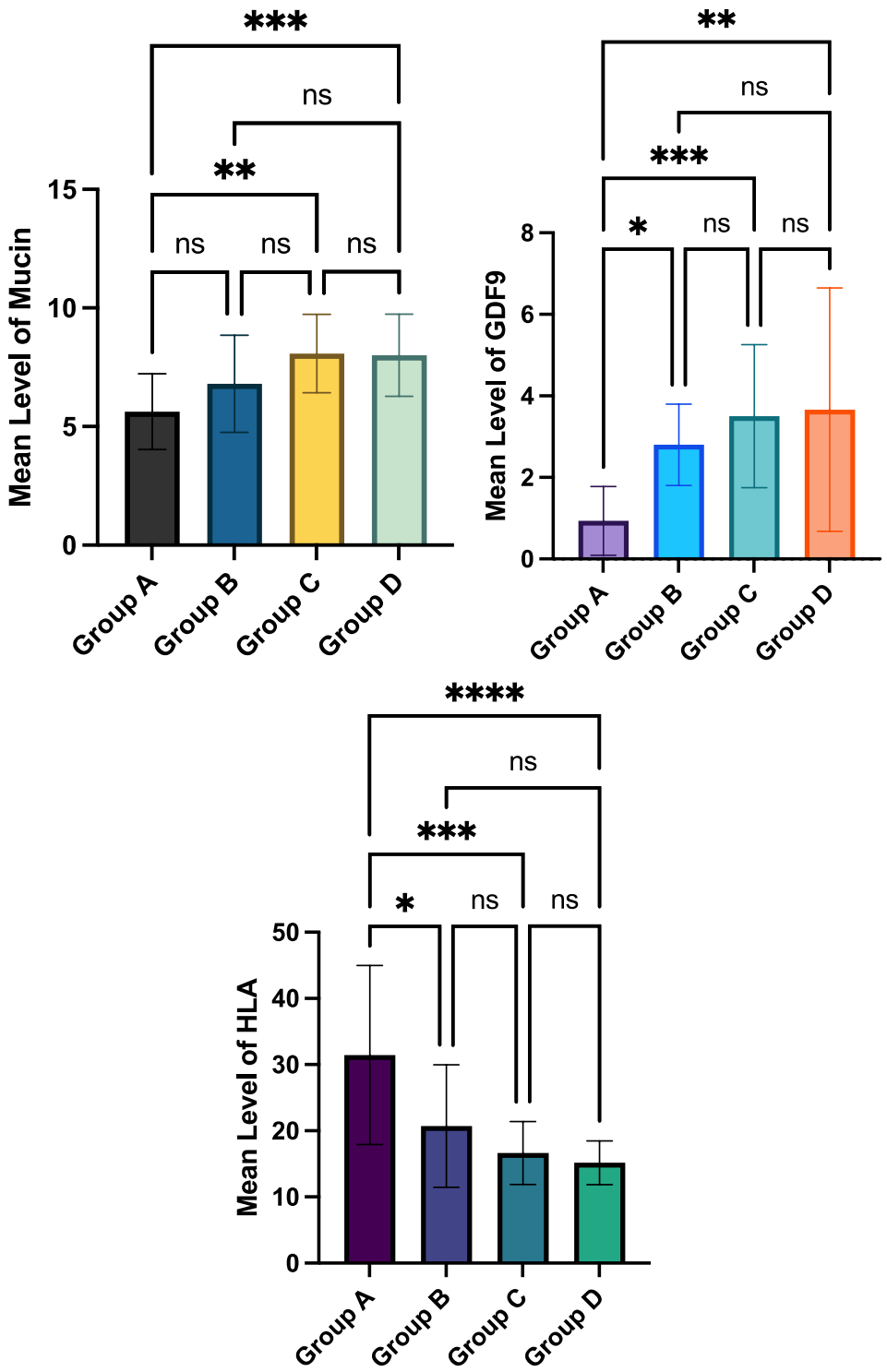


Figure 3.2: Mean differences of Biomarkers Levels: Mucin, GDF9 and HLA among group of infertile females according to circulating AMH levels, patients divided into four groups AMH 0.25–1.1* ng/ml*, subgroup A, AMH 1.2–1.6* ng/ml*, subgroup B, AMH1.7–2.6* ng/ml*, subgroup C, and AMH 2.7–8.5* ng/ml*, subgroup D

3.4 Mean differences of Biomarkers based on final Beta hCG test

Table (3.4) illustrated the mean levels (with standard deviation) of Mucin, GDF9, and HLA in infertile females, categorized by the outcome of the final Beta hCG test (Positive or Negative).

The mean Mucin level was significantly higher in the group with a positive Beta hCG test (7.59 ng/ml) compared to the negative group (6.54 ng/ml), p value was <0.05. it might be due to their role in clinical utility in early pregnancy detection.

Similar to Mucin, the mean GDF9 level is also higher in the positive Beta hCG group (3.62 ng/ml) compared to the negative group (2.22 ng/ml) p value was <0.001, increasing level of GDF9 might reflect a successful implantation or early pregnancy.

The mean HLA level is lower in the positive Beta hCG group (18.31) compared to the negative group (27.19), p value was <0.001, as presented in Figure 3.3. This finding might reflect a possible link between HLA expression and pregnancy success. Lower HLA levels might indicate a more favorable immune environment for implantation.

3.4: Mean differences of Biomarkers Levels: Mucin, GDF9 and HLA among group of infertile females based on the final Beta hCG test

	- Beta hCG	***+ Beta hCG***
Mucin	6.54±2.10	7.59±1.44
GDF9	2.22±2.13	3.62±2.95
HLA	27.19±14.75	18.31±6.26

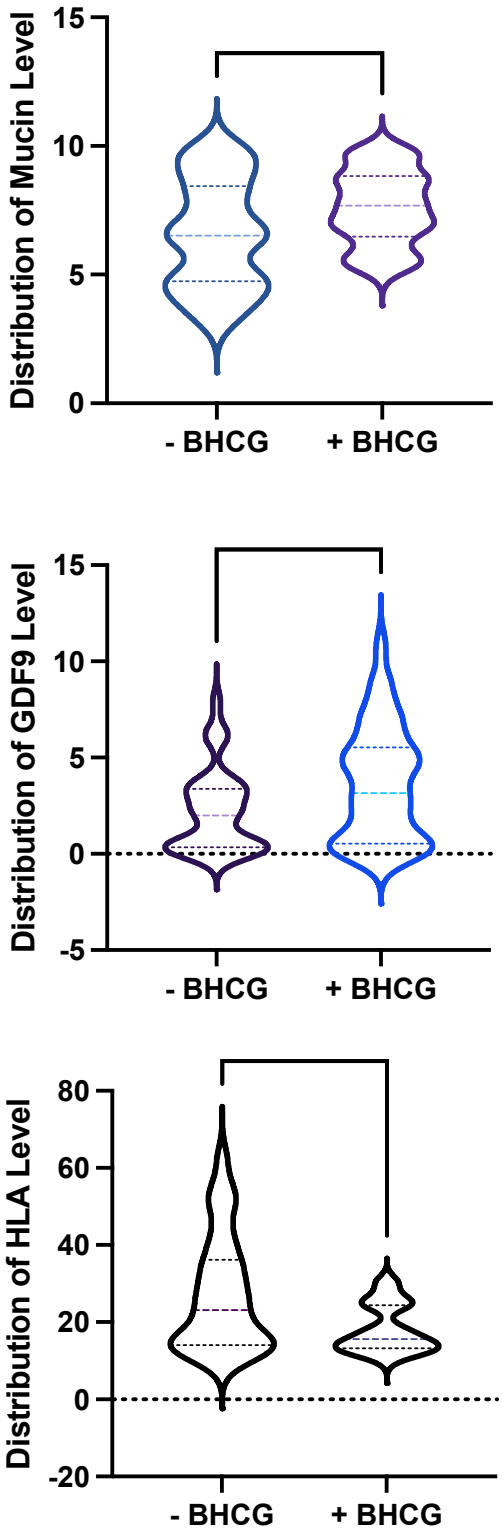


Figure 3.3: Distribution of Biomarkers Levels: Mucin, GDF9 and HLA among group of infertile females based on the final Beta hCG test

3.5 Mean differences of Biomarkers based on Ovarian Sensitivity Index (OSI)

Table 3.5 demonstrated the mean differences of the three biomarkers, Mucin, GDF9, and HLA, and Ovarian Sensitivity Index (OSI) in infertile women. There was no normal value for OSI, a cut-off value was obtained statistically depending upon ROC curve (78) .

Results indicated an interesting difference in biomarker levels between the OSI groups: It was shown that infertile women with OSI greater than 4 may have higher levels of Mucin and GDF9, and lower levels of HLA, compared to those with OSI less than 4.

The mean Mucin level was higher in the group with OSI > 4 (7.23±1.87) compared to the OSI < 4 group (5.93±2.15).
Similar to Mucin, the mean GDF9 level was also higher in the OSI > 4 group (3.46±3.17) compared to the OSI < 4 group (2.25±1.98)
Interestingly, the mean HLA level was lower in the OSI > 4 group (18.64±6.58) compared to the OSI < 4 group (33.47±17.99), as presented in Figure 3.4

3.5: Mean differences of Biomarkers Levels: Mucin, GDF9 and HLA among group of infertile females based on the Ovarian Sensitivity Index (OSI)

Biomarker	OSI < 4	OSI > 4
Mucin	5.93±2.15	7.23±1.87
GDF9	2.25±1.98	3.46±3.17
HLA	33.47±17.99	18.64±6.58

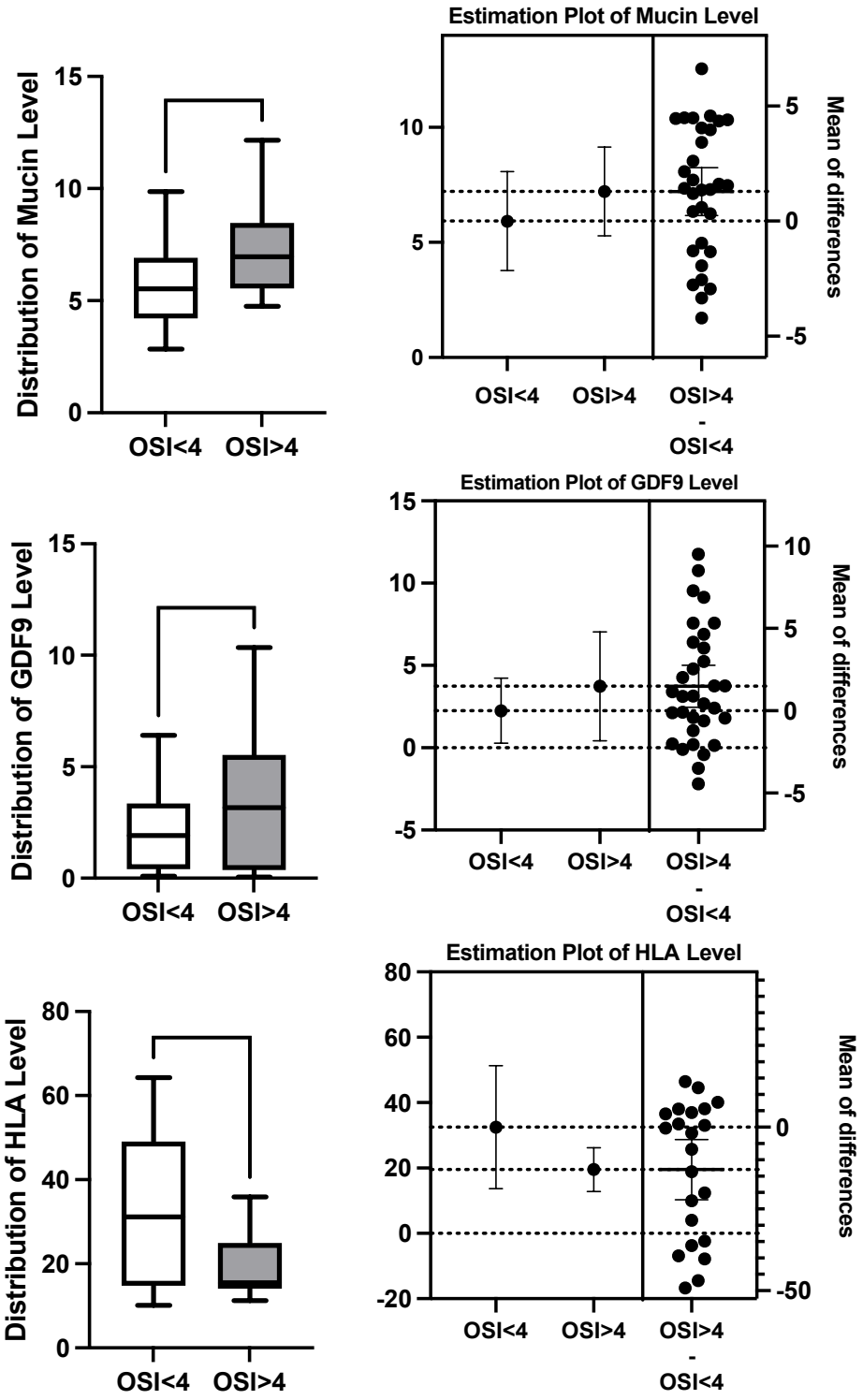


Figure 3.4: Distribution of Biomarkers Levels: Mucin, GDF9 and HLA among group of infertile females based on the Ovarian Sensitivity Index (OSI)

3.6 Receiver operating characteristic curve of Mucin levels among group of infertile females

ROC curve and AUC analysis for the studied biomarkers among among group of infertile females based on the Ovarian Sensitivity Index (OSI) were performed. Results of the receiver operating curve (ROC) was shown AUC, optimal threshold, Sensitivity, and specificity of, data are presented in Table (3.6), Figure (3.5)

the diagnostic performance of Mucin levels in identifying infertile women with OSI less than 4 shown moderate discriminative ability (AUC of 70%). At the optimal cut-off points, Mucin identified 45.2% of participants with OSI less than 4 correctly (sensitivity).

However, it also identified 92.1% of participants without OSI less than 4 correctly (specificity). results of the Sensitivity & Specificity were confirmed using Youden's J statistics to the parameters.

Table 3.6: AUC, optimal threshold, Sensitivity, and specificity of Mucin levels among a group of infertile females based on the Ovarian Sensitivity Index (OSI) to analyze the optimal diagnostic points for predicting participants with OSI<4.

Variable	AUP	Sensitivity %	Specificity %	Youden index	Cut-off points	CI (95%)
Mucin	70%	45.2%	92.1%	0.373	<0.2473	0.558 - 0.822

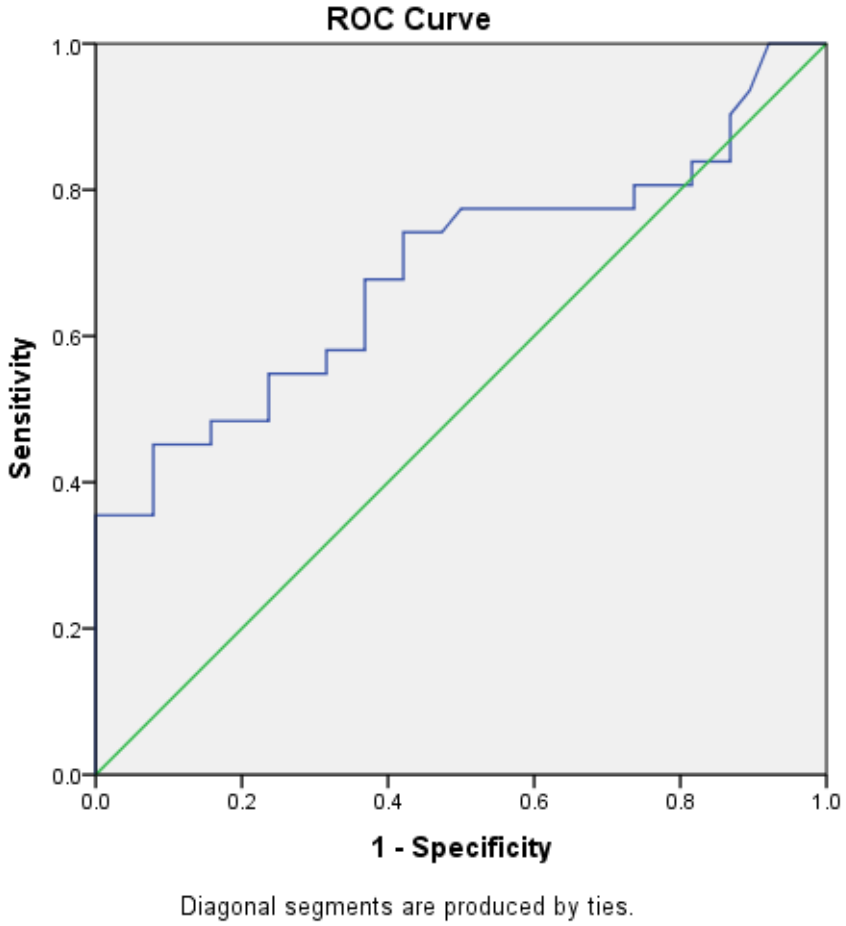


Figure 3.5: Receiver operating characteristic curve of Mucin levels among a group of infertile females based on the Ovarian Sensitivity Index (OSI) to analyze the optimal diagnostic points for predicting participants with OSI<4

3.7Receiver operating characteristic curve of GDF9 levels among group of infertile females

GDF9 levels was also used to identify participants with OSI less than 4, Table (3.7) shown the diagnostic point of GDF9 levels in identifying infertile women with OSI less than 4. GDF9 levels shown a moderate discriminative ability (AUC of 60.5%) for predicting OSI less than 4.

At the optimal cut-off points, GDF9 identified a high proportion of participants with OSI less than 4 correctly (sensitivity of 78.1%). However, it also identified only 50% of participants without OSI less than 4 correctly (specificity), as presented in Figure 3.6

Table 3.7: AUC, optimal threshold, Sensitivity and specificity of GDF9 levels among group of infertile females based on the Ovarian Sensitivity Index (OSI) to analyze the optimal diagnostic points for predicting participant with OSI<4.

Variable	AUP	Sensitivity %	Specificity %	Youden index	Cut-off points	CI (95%)
GDF9	60.5%	78.1%	50%	0.281	<3.371	0.450 - 0.720

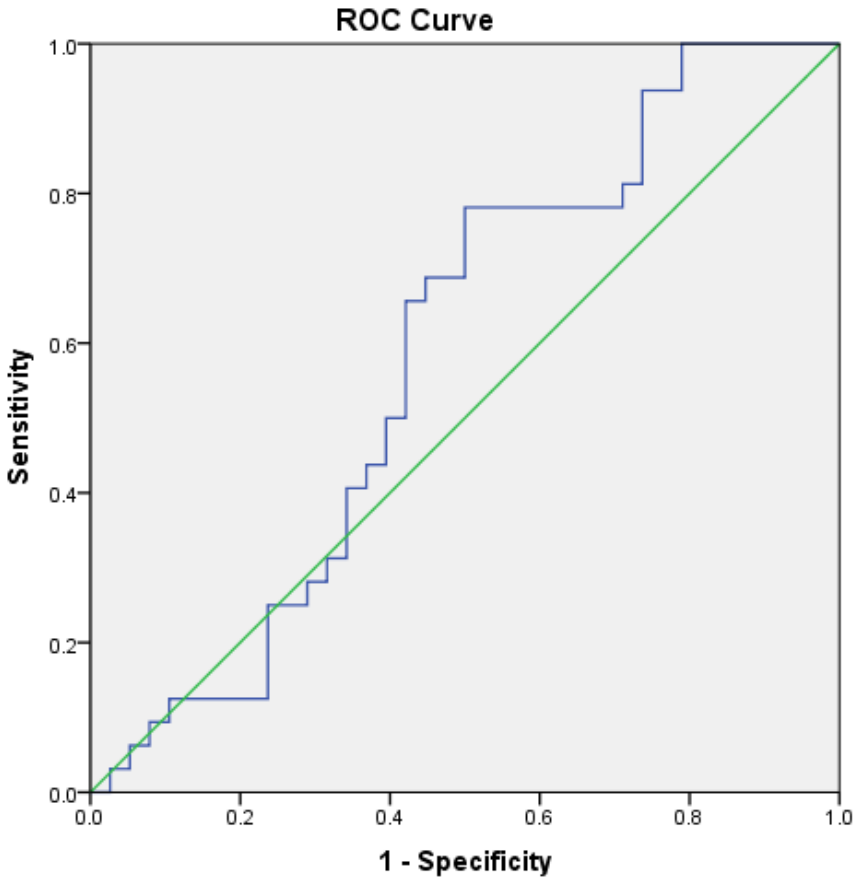


Figure 3.6: Receiver operating characteristic curve of GDF9 levels among group of infertile females based on the Ovarian Sensitivity Index (OSI) to analyze the optimal diagnostic points for predicting participant with OSI<4.

3.8 Receiver operating characteristic curve of HLA levels among group of infertile females

Furthermore, HLA levels was demonstrated good discriminative ability (AUC of 71.3%) for predicting OSI less than 4. At the optimal cut-off points, HLA identified over half (53.6%) of participants with OSI less than 4 correctly (sensitivity). Importantly, it also achieved a high specificity of 93.5%, correctly identifying a large proportion of participants without OSI less than 4.

Compared to Mucin (moderate AUC of 69%) and GDF9 (moderate AUC of 60.5%), HLA levels showed the strongest discriminative ability for predicting OSI less than 4, as presented in Table 3.8 Figure 3.7

While GDF9 had a higher sensitivity for identifying participants with OSI less than 4, HLA offered a balance between sensitivity and specificity, making it a potentially more reliable diagnostic tool in this context.

Table 3.8: AUC, optimal threshold, Sensitivity, and specificity of HLA levels among group of infertile females based on the Ovarian Sensitivity Index (OSI) to analyze the optimal diagnostic points for predicting participant with OSI<4.

Variable	AUP	Sensitivity %	Specificity %	Youden index	Cut-off points	CI (95%)
HLA	71.3%	53.6%	93.5%	0.471	>29.5795	0.570 - 0.850

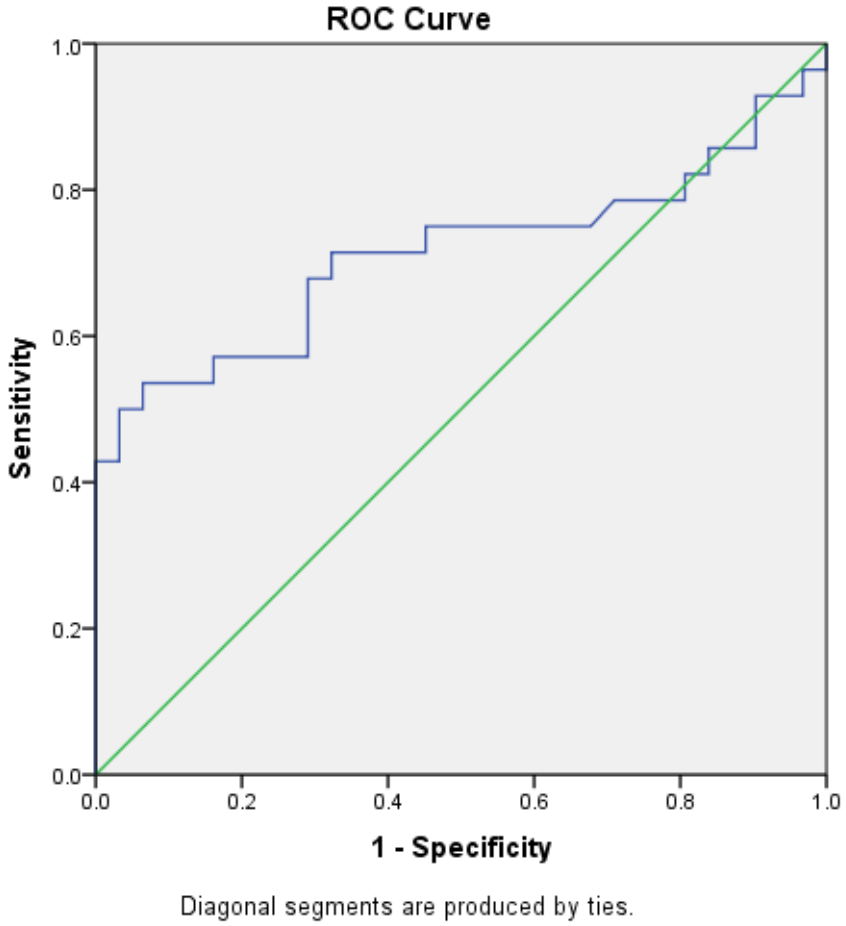


Figure 3.7: Receiver operating characteristic curve of HLA levels among group of infertile females based on the Ovarian Sensitivity Index (OSI) to analyze the optimal diagnostic points for predicting participant with OSI<4.

CHAPTER FOUR

Discussion

4. DISCUSSION

The receptivity of the endometrium is a sign that the endometrium has reached a stage of development where it is prepared to receive the well-formed embryo. It corresponds to find an accurate window of implantation (WOI) (79). Endometrial receptivity is the ability of the endometrium to successfully attach the blastocyst, to nourish it and keep it alive. This can only be achieved after the endometrium underwent a number of histological changes while also increasing in thickness (80).

MUC1 a protein anchored in the apical surface of various epithelial cells. MUC1 belongs to the family of mucins. It is one of the most abundant products of glandular cells in the late secretary endometrium. Change in MUC 1 glycoforms also seem to correlate with the receptive window After glycosylation, it extends 200– 500 nm beyond the surface of the cell (81).

Growth-differentiation factor 9 (GDF9) is oocyte-secreted molecule that play a central role in ovarian function and oocyte quality.

GDF9 is secreted by the oocyte and regulate the quality of the ova by interacting with the surrounding somatic cells. It is thus expected that GDF9 concentration in human serum will vary with differing reproductive pathologies, and recently we demonstrated evidence of an association between serum GDF9 and the number of oocytes retrieved during IVF. Mutations that reduce or disrupt the production of these oocyte-secreted factors have been observed in conditions associated with subfertility including polycystic ovary syndrome (PCOS), primary ovarian insufficiency (POI) and Turners syndrome. An indication that aberrant GDF9 function may be associated with endometriosis arises from reports of lower concentrations of GDF9 protein and mRNA in follicular fluid of patients with severe endometriosis. However, until recently there have not been assay methods available to reliably measure GDF9 in

human sera and thus assess if concentrations are altered in patients with endometriosis (82).

HLA, one of the non-classical HLA classes I molecules, is restricted-expression at extra villous trophoblast. It can concordantly interact with various kinds of receptors mounted on maternally immune cells residing in the uterus for maintaining immune homeostasis of the maternal-fetus interface. HLA is widely regarded as the pivotal protective factor for successful pregnancies. HLA-G expression has been detected in early preimplantation embryos and it has been postulated that a relationship between embryonic expression of this factor and successful pregnancy may exist (83).

The endometrium becomes capable of implantation and then improve the embryo-evaluation, requires the cooperation and timely coordination of various factors and chemical interactions. The knowledge about receptivity markers and the possibility of practical usage of tests designed for their assessment should, in the near future, become routine management in reproductive medicine.

Therefore, **the aims of this study** were to examine the role of the proposed markers in the IVF process by estimating the level of MUC1, DDF-9 and HLA in IVF cases, also to Investigate the diagnostic preferences of the biomarkers using ROC analysis.

This study presented an interesting difference in the levels of three biomarkers, Mucin, GDF9, and HLA, between women with primary and secondary infertility.

Higher in Secondary Infertility: Women with secondary infertility had significantly higher mean levels of both Mucin and GDF9 compared to those with primary infertility.

Elevated Mucin and GDF9 levels in secondary infertility could be a compensatory response to declining ovarian reserve or altered follicular dynamics (84).

On the other hand, Women with primary infertility had a significantly higher mean HLA level compared to those with secondary infertility. HLA molecules are involved in the immune system's response. Lower HLA levels in secondary infertility could indicate a less tolerant immune environment within the ovaries. This might be detrimental to implantation success. Previous pregnancies in women with secondary infertility might have modulated their immune response, leading to lower HLA expression. Alternatively, underlying causes of secondary infertility could be linked to altered immune regulation (85) .

The current study was also illustrated that Both Mucin and GDF9 levels showed a progressive increase with increasing AMH levels. increase in Mucin and GDF9 levels could be associated with a greater number of follicles or enhanced follicular activity in women with higher AMH, which is a marker of remaining ovarian reserve (86).

Interestingly, HLA levels displayed an opposite trend. The highest levels were found in subgroup lowest AMH and progressively decreased with increasing AMH. No Previous studies were reported such findings but many studies were confirmed that Anti-Mullerian Hormone was highly related and predicting ovarian response to gonadotropin stimulation due to their role in ovarian primordial follicle recruitment and dominant follicle selection (87) .In line with the study of Hazout et al., greater AMH concentrations were associated with a higher number of mature oocytes, a higher number of embryos, followed by a greater clinical pregnancy (88).

No previous studies were reported such finding but A close explanation might be reflected by other research who link between the studied biomarkers and Endometrial receptivity.

Endometrial "superfertility" is clinically associated with inappropriate uterine receptivity, as it allows defective blastocysts to implant and start pregnancies that cannot be maintained with good health (89).

The glycoprotein MUC-1 is an anti-adhesion molecule that may play a role in endometrial superfertility. Mucin is also a major component of the apical surface of uterine epithelial cells, which are involved in the initial stages of embryo–uterine interactions during implantation. While uterine epithelial cells serve a protective function for the tissue, these cells must also allow the embryo to attach (90) .

MUC1 (Mucin-1), also known as epithelial mucin, was the first mucin core protein to be cloned from both humans and mice. In a previous study, we observed numerous glycoprotein particles at the apex and base of endometrial epithelial cells (91).

Expression of MUC-1 decreases to relatively low levels in healthy women during the implantation "window," to allow the possibility of contact between an embryo and the endometrium. On the other hand, previous studies suggest that MUC-1 expression is extremely low in the endometrium, during the implantation window, in women with recurrent pregnancy loss, compared with fertile women (92). The low levels of MUC-1 may explain why patients seem exceptionally fertile despite having RIF and recurrent pregnancy loss.

Additionally, drastically reduced MUC-1 expression may disturb the immune system during endometrial implantation, thereby adversely affecting the implantation process. In addition, MUC-1 is expressed in T cells as an immunomodulator, acting as a defensive wall against foreign substances and protecting the implanted embryos (93).

Extreme reductions in MUC-1 expression may therefore decrease this protective effect, leaving embryos open to attack. Thus, MUC-1 may play an important role in determining endometrial receptivity. Low levels of MUC-1 could damage the embryo-selection function of the endometrium in infertile patients, thereby increasing rates of miscarriage, or reducing rates of implantation. In a study by Song et al. (94), MUC-1 expression was found to be significantly down-regulated in the endometrium of infertile patients with hydrosalpinx. Similarly, Li et al. (95).

Demonstrated that endometrial MUC-1 expression was significantly lower in patients with hydrosalpinx. These results show that the fluid from hydrosalpinx negatively affects not only the embryo, but also the endometrium, by significantly decreasing the level of MUC-1. The present study reveals that blood and tissue MUC-1 levels of patients with RIF were significantly lower than those of fertile women in a control group. This finding shows that extremely low levels of MUC-1 may have a devastating effect on implantation (96).

The significant differences in the mean serum concentrations of GDF9 among infertile female was in line with previous studies.

GDF9 is secreted by the oocyte and regulate the quality of the ova by interacting with the surrounding somatic cells. It is thus expected that GDF9

concentrations in human serum will vary with differing reproductive pathologies, and recently we demonstrated evidence of an association between serum GDF9 and the number of oocytes retrieved during IVF (97). Mutations that reduce or disrupt the production of these oocyte-secreted factors have been observed in conditions associated with subfertility including polycystic ovary syndrome (PCOS), primary ovarian insufficiency (POI) and Turners syndrome (98).

An indication that aberrant GDF9 function may be associated with endometriosis arises from reports of lower concentrations of GDF9 protein and mRNA in follicular fluid of patients with severe endometriosis (99).

Regarding HLA, the current study was also consistence with the previous research who reported increased level of HLA among infertile female.

During pregnancy, the maternal immune system is in close contact with the cells of the semiallogenic fetus, and a normal immunological homeostasis is needed to avoid rejection of the fetus (100).

The human leukocyte antigen-G (HLA-G) molecules belong to the nonclassical HLA class Ib family and have immunomodulatory, anti-inflammatory, and tolerogenic functions. HLA-G antigens play a crucial role in immune suppression at the maternal-fetal interface; they are expressed mainly in first trimester villous cytotrophoblasts and protect the fetal semiallograft against lysis by maternal natural killer cells. It was reported that HLA expression in the endometrium may play an important role in fertility and embryo implantation, extending their relevance in reproduction beyond the well described expression in extravillous trophoblast at the fetomaternal contact zone during pregnancy (101).

Results obtained in the primary analysis were refined by subgroup analyses. Specifically, when we stratified patients for number of pregnancy losses, a higher frequency of the HLA-G ins/ins genotype was detected both in women with three or more episodes of pregnancy loss compared with healthy controls with successful pregnancy and in those with two or more episodes. There is evidence that pregnancy complications could be associated with abnormal immunologic interactions at the fetal-maternal interface, since the mother's immune system could consider the fetus as nonself and develop antibodies and cytotoxic T lymphocytes against it (102) .

This immunosuppressive role is believed to be mediated, at least partly, by the HLA-G molecule and polymorphisms in the 3′UTR region of HLA-G, and low or undetectable expression of sHLA-G plays a crucial role in the etiology of spontaneous RPL (103) , and failure of IVF (104).

This study was also included assessment of ovarian stimulation of IVF cases, Ovarian Sensitivity Index (OSI) can be easily calculated dividing the total administered gonadotropin dose by the number of oocytes retrieved at OPU. This ratio represents indeed the ovarian resistance to gonadotropins, as the lower is FSH dose, the higher is ovarian sensitivity.

The present study shows that OSI displays a strong, inverse link with AMH levels. This is not surprising as it was shown that neither the total FSH dose administered, nor the absolute number of oocytes accurately reflect ovarian responsiveness to hormonal stimulation; their ratio is much more accurate in describing how the ovary produces oocytes in response to exogenous stimulation (105).

OSI accurately reflects AMH level of women submitted to IVF. According to the present study, OSI appears to be a highly reliable index of ovarian responsiveness

CHAPTER FIVE

Conclusions and recommendations

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions:

- In conclusion, a potential difference in biomarker levels were found among women with primary and secondary infertility. The increasing in mucin and GDF9 levels in secondary infertility might be related to underlying causes. The higher HLA levels in primary infertility could be indicative of an autoimmune response affecting fertility.
- The increasing levels of Mucin and GDF9 with higher AMH could indicate a possible link between these biomarkers and ovarian reserve. AMH is a marker of remaining follicles in the ovary, and these findings might suggest that Mucin and GDF9 play a role in follicular development or function.
- HLA might reflect a possible link with pregnancy success. Lower HLA levels might indicate a more favorable immune environment for implantation.
- Interesting difference was found in biomarker levels when compared the OSI groups: It was shown that infertile women with OSI greater than 4 may have higher levels of Mucin and GDF9, and lower levels of HLA, compared to those with OSI less than 4.

5.2. Future work

The current research project has the following Future work for the future:

- During implantation, the structure of mucin undergoes transformation, preparing the endometrium to bind to the oligosaccharide receptors present on the blastocyst's surface; this process is regulated by many signaling pathways. Since the functional role of mucin O-glycans has not been clearly defined, and future research in this field will be highly valuable, which may offer insights into improving reproductive health or addressing infertility issues in humans.
- It worth to highlight a future research about regulation microRNA of Mucin and GDF9 and their role on protective endometrium receptivity
- Further research is needed on the implication of varying AMH levels within the follicular fluid and other parameters like embryo quality, transfer technique, and endometrial receptivity on pregnancy outcomes needs to be investigated

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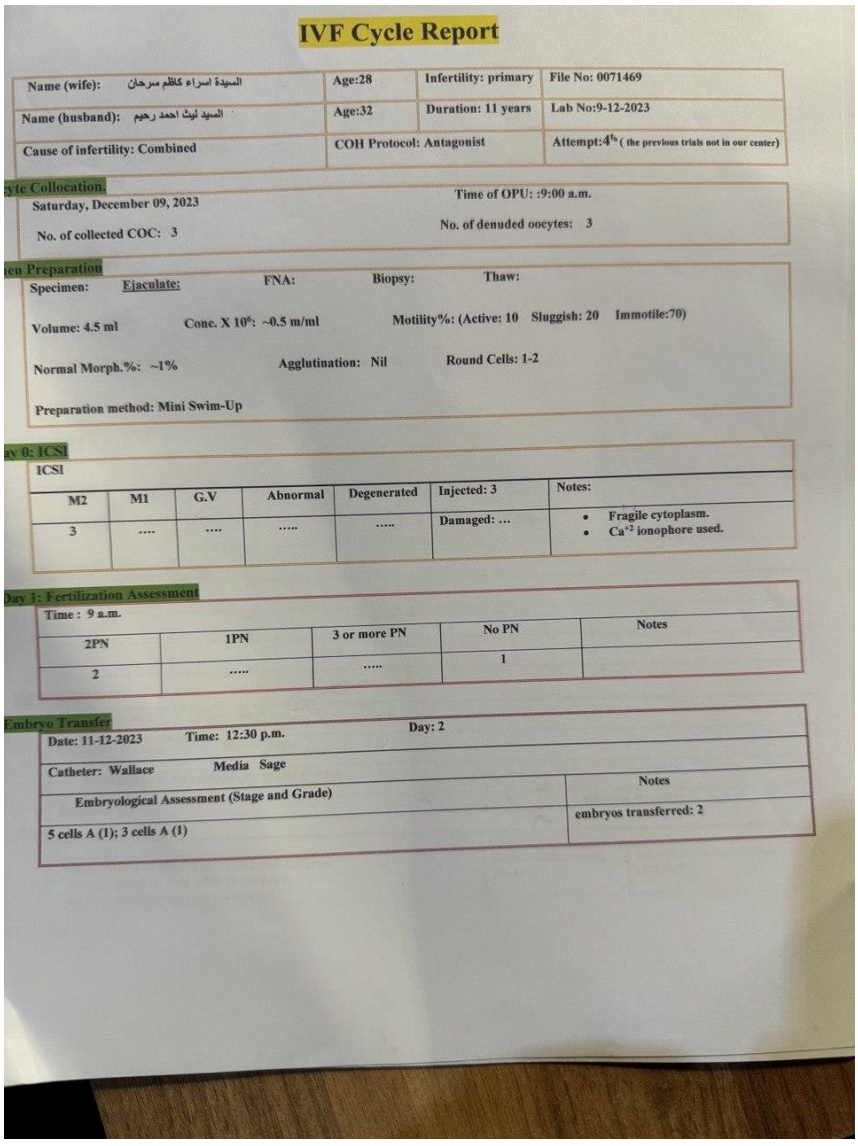
APPENDICES

Appendices

Student Name: Zainab Ahmed Aljarah

Biochemical Assessment of Endometrial Receptivity and Embryo Evaluation among Cases of In Vitro Fertilization.

Type of Study: Cross-section



IVF Cycle Report

Name (wife): السيدة اسراء كاظم سرحان	Age:28	Infertility: primary	File No: 0071469
Name (husband): السيد ثيث احمد رحيم	Age:32	Duration: 11 years	Lab No:9-12-2023
Cause of infertility: Combined	COH Protocol: Antagonist		Attempt:4th (the previous trials not in our center)

yte Collocation:
Saturday, December 09, 2023 — Time of OPU: :9:00 a.m.
No. of collected COC: 3 — No. of denuded oocytes: 3

en Preparation
Specimen: Ejaculate: FNA: Biopsy: Thaw:
Volume: 4.5 ml Conc. X 10^6: ~0.5 m/ml Motility%: (Active: 10 Sluggish: 20 Immotile:70)
Normal Morph.%: ~1% Agglutination: Nil Round Cells: 1-2
Preparation method: Mini Swim-Up

y 0: ICSI
ICSI

M2	M1	G.V	Abnormal	Degenerated	Injected: 3	Notes:
3	....	....	.....	.....	Damaged: ...	• Fragile cytoplasm. • Ca^{+2} ionophore used.

ay 1: Fertilization Assessment
Time : 9 a.m.

2PN	1PN	3 or more PN	No PN	Notes
2	.....	.....	1	

Embryo Transfer
Date: 11-12-2023 Time: 12:30 p.m. Day: 2
Catheter: Wallace Media Sage

Embryological Assessment (Stage and Grade)	Notes
5 cells A (1); 3 cells A (1)	embryos transferred: 2

الخلاصـة

الخلفية: التخصيب خارج الجسم (IVF) هو عملية يتم فيها تخصيب البويضة بواسطة الحيوانات المنوية خارج الجسم. أحد العوامل الحاسمة التي تؤثر على نجاح التلقيح الاصطناعي هو تقبل بطانة الرحم، والذي يشير إلى قدرة بطانة الرحم على السماح للجنين بالزرع والتطور. تهدف هذه الدراسة إلى فحص دور العلامات المقترحة في عملية التلقيح الاصطناعي من خلال الأهداف التالية: تقدير مستوى MUC1 و GDF-9وHLAفي حالات التلقيح الاصطناعي والتحقيق في التفضيلات التشخيصية للواصمات الحيوية باستخدام تحليل.ROC

المرضى والطرق: تم تصميم دراسة مقطعية لعدد 120 أنثى تعاني من العقم الأولي والثانوي، وأجريت الدراسة طوال الفترة من ايلول/2023 إلى اب/2024. كلية الطب جامعة كربلاء وصحة كربلاء التحقق من صحة المديرية الموافقة الأخلاقية للدراسة. كما تؤخذ الموافقة من مركز الخصوبة في مستشفى الكفيل التخصصي ومن كل مريضة بعد توضيح طبيعة الدراسة والغرض منها .

تم تضمين جميع المشاركين الذين خضعوا لعلاج التلقيح الصناعي مع تحفيز FSH وكان لديهم تقييم لمستويات AMH في الدم قبل العلاج في هذه الدراسة. تم قياس مستوى Mucin و GDF9وHLAفي المصل، وتم إجراء التحليل الإحصائي باستخدام SPSS ومنشور اللوحة الرسومية.

أظهرت النتائج أن الإناث المصابات بالعقم الثانوي لديهن مستوى متوسط أعلى بكثير لكل من MUC1 (9.26%) و GDF9 (2.7%) مقارنة بأولئك الذين يعانون من العقم الأولي (5.94 و1.03 على التوالي). في حين أن الإناث المصابات بالعقم الأولي لديهن متوسط أعلى بكثير لمستوى HLA (33.14%)مقارنة بالنساء المصابات بالعقم الثانوي (27.34%).

أظهرت النتائج وجود علاقة عكسية بين مستويات AMH ومستويات .MUC1 مع انخفاض مستويات AMHعبر المجموعات (من A إلىD)، تميل المستويات المتوسطة MUC1 إلى الزيادة. يظل نمط GDF9مشابهًا. المجموعةA لديها أدنى متوسط لمستوىGDF9 ، ويزداد عبر المجموعتين B و .Cتم العثور على نمط مختلف فيما يتعلق بمستوىHLA ، حيث تنخفض مستويات HLA باستمرار مع انخفاض مستويات AMH من (A إلى D) كان متوسط مستوى MUC1 أعلى بكثير في المجموعة ذات اختبار Beta hCG الإيجابي (7.59 نانوغرام/مل) مقارنة بالمجموعة السلبية (6.54 نانوغرام/مل)، وكانت قيمة .$p < 0.05$ قد يكون ذلك بسبب دورها في الفائدة السريرية في الكشف المبكر عن الحمل.

على غرارMUC1 ، يكون متوسط مستوى GDF9 أعلى أيضًا في مجموعة Beta hCG الإيجابية (3.62 نانوغرام/مل) بالمقارنة عندما يكون Beta hCG سلبيا (2.22 نانوغرام/مل) وكانت قيمة p <0.001، وقد يعكس مستوى GDF9 المتزايد عملية زرع ناجحة أو الحمل المبكر. تم العثور على

اختلاف مثير للاهتمام في مستويات المؤشرات الحيوية بين مجموعات :OSI فقد تبين أن النساء المصابات بالعقم مع OSI أكبر من 4 قد يكون لديهن مستويات أعلى من MUC1 وGDF9، ومستويات أقل منHLA ، مقارنة مع أولئك الذين لديهم OSI أقل من 4. المتوسط كان مستوىMUC1 أعلى في المجموعة ذات (1.87 ± 7.23) 4 <OSI مقارنة بمجموعة .(2.15 ± 5.93) 4> OSI على غرار MUC1، كان متوسط مستوى GDF9 أعلى أيضًا في مجموعة (3.17 ± 3.46) 4 <OSI مقارنة بمجموعة .(1.98 ± 2.25) 4 > OSI ومن المثير للاهتمام، أن متوسط مستوى HLA كان أقل في مجموعة (6.58± .(18.64) 4 <OSI مقارنة بمجموعة (17.99±33.47) 4> OSI .

الخلاصة: يمكن أن يوفر تقييم MUC1 و GDF9 و HLAمعلومات قيمة عن تقبل بطانة الرحم وقابلية الجنين للحياة في التلقيح الصناعي. يمكن استخدام هذه العلامات لتحديد المشكلات المحتملة في عملية الزرع وتوجيه استراتيجيات العلاج الشخصية لتحسين فرص الحمل الناجح.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء/كلية الطب قسم الكيمياء والكيمياء الحيوية

التقييم الكيموحيوي لاستقبال بطانة الرحم وتقييم الجنين بين حالات التلقيح الصناعي.

رسالة

دراسة مقدمة إلى مجلس كلية الطب /جامعة كربلاء كجزء من متطلبات نيل شهادة الماجستير في الكيمياء الحياتية

من قبل
زينب احمد الجراح
بكالوريوس تحليلات مرضية – كلية العلوم الطبية التطبيقية , 2015

بأشراف
الاستاذ المساعد الدكتورة رنا مجيد حميد
كلية الطب - جامعة كربلاء

والاستاذة الدكتورة وسن غازي الصافي
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1445هـ 2024م