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Evaluation of Some Hormonal and Molecular Factors in Women with Recurrent Abortion Receiving Dydrogesterone Drug in Karbala Province

A Dissertation

Submitted to the Council of the Collage of Sciences, University of Kerbala in Partial Fulfillment of the Requirements for the Degree of Philosophy Doctorate in Biology

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Dedication

To …

The candles that lighten my way:

My wonderful family

My beloved father

My compassionate mother

My husband (Ammar)

My lovely son and daughter (Al-Hassan and Fatima)

My lovely sister, Beloved brethren

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Summary

Recurrent Pregnancy Loss (RPL) is a condition that is defined as three consecutive pregnancy losses before 20 weeks from the last menstrual period; RPL may be caused, in part, by low progesterone levels during the luteal phase. Dydrogesterone (DYD), a synthetic progestogen, aims to enhance endometrial receptivity and prevent pregnancy loss.

This study included (106) women from the Gynecology and Obstetrics Teaching Hospital, Khadija Al-Kubra Hospital, and Outpatient infertility clinics in Karbala Province. The participants were divided into three groups: the first group involved 35 patients with recurrent pregnancy loss who received dydrogesterone for six months, the second group included 35 patients with recurrent pregnancy loss without treatment, and the third group comprised 36 healthy women as a control group. All participants in the study were matched by age, ranging from 25 to 38 years, and body mass index. The present study was applied to evaluate the concentrations of some hormonal (progesterone, estradiol, prolactin, follicle-stimulating hormone, luteinizing hormone, and testosterone; biochemical factors (progesterone-induced blocking factor and epithelial-cadherin) and relative gene expression of progesterone receptors (nuclear and membrane progesterone receptors for all study subjects.

Progesterone and estradiol levels decreased significantly ($p \leq 0.05$) in RPL patients untreated compared to the control group. No significant difference in progesterone levels between RPL patients receiving treatment and the control group, but a statistically significant increase ($p \leq 0.05$) was noted in progesterone concentration among RPL patients with treatment compared to those without treatment. Also, a positive correlation ($r = 0.522$) between progesterone and estradiol in RPL patients untreated.

The study estimated the concentrations of prolactin, follicle-stimulating hormone, luteinizing hormone and testosterone in RPL women, these parameters are non-significantly different ($p \leq 0.05$) among all groups of this study. The correlation has shown an inverse correlation between progesterone and FSH concentration in RPL patients without treatment and with treatment ($r = - 0.46$, $r = - 0.390$., respectively).

Data on Progesterone Induce Blocking Factor (PIBF) and Epithelial-Cadherin (E-Cad) concentrations indicated a significant decrease ($p \leq 0.05$) in RPL patients without treatment compared to the control group. However, there was a notable rise in PIBF and E-Cad levels between untreated RPL patients and those undergoing treatment. Furthermore, a significant positive correlation between Progesterone and PIBF was observed in both untreated RPL patients and those receiving treatment.

The results of relative gene expression for progesterone membrane receptor (mPRα) have indicated significantly increased gene expression ($p \leq 0.05$) in RPL patients without treatment and control group. Also found the same result between RPL patients with treatment and without treatment. An inverse relationship was found between mPRα gene expression and Progesterone concentration in untreated RPL patients and RPL patients with treatment ($r = - 0.132$, $r = - 0.014$., respectively). The results of patients who had repeated miscarriages without receiving dydrogesterone therapy showed a significantly lower expression of progesterone membrane receptors (mPRß) in their endometrial tissue. Additionally, there was a significant increase in gene expression of mPRß between RPL patients receiving treatment and those without treatment ($p \leq 0.05$). Results of the correlation have shown a significantly positive correlation ($r = 0.380$) between mPRß gene expression and Progesterone in RPL patients without treatment and in RPL patients with treatment ($r = 0.156$).

The analysis of gene expression levels of NPR showed that there was very little variation between untreated patients and the control groups. The same findings were observed when comparing the gene expression of NPR in treated and untreated patients. There was a positive correlation (r = 0.319) between NPR gene expression and Progesterone concentration in RPL patients without treatment and in RPL patients with treatment (r = 0.346).

The findings suggest a reduction of gene expression of membrane progesterone receptor ß (mPRβ) and elevated gene expression of membrane progesterone receptor α (mPRα) in RPL patients may play an important role in the pathogenesis of RPL; and the reduction of mPRα and increased of mPRβ after treatment with the dydrogesterone, that suggest to the dydrogesterone direct effected on endometrial gene expression of progesterone receptors.

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List of Abbreviations

Abbreviation	Term
20a-DHD	20aDi-HydrodyDrogesterone
AJs	Adherens Junctions
AKR	Aldo-Keto Reductases
ASRM	American Society for Reproductive Medicine
AMH	Anti-Müllerian Hormone
aPL	anti-Phospholipid
APS	Anti-Phospholipid Syndrome
anti-TPO	anti-Thyroid Peroxidase
ART	Assisted Reproductive Technology
BMI	Body Mass Index
Ca+2	Calcium ion
CBD	Catenin-Binding Domain
CAM	Cell Adhesion Molecule
CE	Chronic Endometritis
CQ	Cycle Quantification
CT	Cycle Threshold
DNA	Deoxyribonucleic Acid
DHD	Di-Hydro-Dydrogesterone
DYD	Dydrogesterone
ECLIA	Electro ChemiLuminescence ImmunoAssay
EECs	Endometrial Epithelial Cells
ER	Endometrial Receptivity
ELISA	Enzyme-Linked ImmunoSorbent Assay
E-cad	Epithelial Cadherin
EMT	Epithelial-Mesenchymal Transition

E2	**Estradiol**
ESR	**Estrogen Receptors**
ESHRE	**European Society of Human Reproduction and Embryology**
EMBASE	**Excerpta Medical Data BASE**
EVTs	**Extra Villous Trophoblasts**
FSH	**Follicle Stimulating Hormone**
γδT	**Gamma delta T cells**
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**
IFN-γ	**Interferon-γ**
IL	**Interlukin**
JMD	**Juxta Membrane Domain**
kD	**Kilo Dalton**
LPD	**Luteal Phase Deficiency**
LH	**Luteinizing Hormone**
mPR	**membrane Progesterone Receptors**
mPRα	**membrane Progesterone Receptors alpha**
mPRß	**membrane Progesterone Receptors beta**
MVP	**Micronized Vaginal Progesterone**
NC	**No Children**
ND	**Nano Drop**
NK	**Natural Killer**
N-Cad	**Neural Cadherin**
NFAT1	**Nuclear Factor of Activated T-cells 1**
NPRs	**Nuclear Progesterone Receptors**
OD	**Optical Density**
P-Cad	**Placental Cadherin**
PCOS	**Polycystic Ovary Syndrome**

PGT	**Preimplantation Genetic Testing**
P4	**Progesterone**
PGR	**Progesterone Receptor**
PGRMC1	**Progesterone Receptor Membrane Component 1**
PR	**progesterone Receptor**
PIBF	**Progesterone-Induced Blocking Factor**
qRT-PCR	**Quantitative reverse-transcription polymerase chain react**
RPL	**Recurrent Pregnancy Loss**
RSA	**Recurrent Spontaneous Abortion**
RNase	**Ribonuclease**
RNA	**Ribonucleic Acid**
RCOG	**Royal College of Obstetricians and Gynecologists**
RQ	**Relative Quantification**
SPSS	**Statistical Package for Social Sciences**
SD	**Standard Deviation**
Th	**T helper**
TPO-Ab	**Thyroid Peroxidase Antibodies**
TSH	**Thyroid-Stimulating Hormone**
T4	**Thyroxine**
TGF-beta	**Transforming Growth Factor**
TMD	**Trans-Membrane Domain**
T3	**Triiodothyronine**
TNF	**Tumor Necrosis Factor**
URPL	**Unexplained Recurrent Pregnancy Loss**
VE-Cad	**Vascular Endothelial Cadherin**
WOI	**Window Of Implantation**

Chapter One

Introduction

1.1 Introduction

Recurrent pregnancy loss (RPL) is a challenging pregnancy-related condition that impacts 2-3% of couples, to be diagnosed with this syndrome, a woman must experience three consecutive pregnancy losses before reaching 20 weeks from her last menstrual cycle, Multiple factors can lead to a miscarriage, such as genetic factors, uterine anatomical anomalies, thrombophilia, endocrine disorders, environmental factors, infections, and immune system issues (Rahnama *et al*., 2019).

Recurrent Pregnancy Loss can be classified into primary and secondary types; Primary, RPL occurs in women who have never given birth to a live infant, Secondary RPL occurs in women who have given birth to a live infant (Sharba and Alalaf, 2023). However, in around half of the cases, the reason for RPL is still unknown, given that the human endometrium is thought to have a significant role in determining fertility, it has been suggested that an inappropriate endometrium may be a significant contributing cause to RPL (Zhu *et al*., 2023).

Progesterone, a steroid hormone plays important roles in the menstrual cycle, implantation, and maintenance of pregnancy, this hormone is crucial to reproduction (Kolatorova *et al*., 2022). Progesterone plays a critical role in the immune system during pregnancy, protecting the baby from being rejected by the mother by managing her immunological reactions. This is performed by the interaction of progesterone with its receptors, which are referred to as progesterone receptors (PRs). In the decidua, the relationship between progesterone and PRs is particularly important since it controls the mother's immunological responses (Rahnama *et al*., 2019). There are two different mechanisms via which the PRs signal: genomic and non-genomic. The genomic route is associated with nuclear progesterone receptors (NPRs) while the non-

genomic pathway is related to membrane receptors (mPR) such as mPR-α and mPR-β that binds progesterone at the cell surface and rapidly creates intracellular second messengers (Singh *et al*., 2013; Kolatorova *et al*., 2022). Therefore, the therapeutic application of progesterone is targeted to prevent pregnancy complications such as RPL (Coomarasamy *et al*., 2016; Di Renzo *et al*., 2016). Progesterone's reactivity, not its availability, is the reason for its inefficiency. In actuality, progesterone receptor expression or activity is implicated in these situations (Rahnama *et al*., 2019).

Progesterone acts on the immune system through a downstream mediator called progesterone-induced blocking factor (PIBF) (Csabai *et al*., 2020). The protein plays a crucial role in the success of pregnancy. This molecule possesses various immune-modulatory capabilities. Once PIBF binds to PIBF receptors, the receptor forms a heterodimer with the IL-4 receptor and activates the Jak1/Stat6 pathway, which results in an increased production of Th2-type cytokines; that is necessary for the maintenance of normal pregnancy (Raghupathy and Szekeres-Bartho, 2022).

The synchronized growths of the embryo and the endometrium, as well as their interaction, are essential for successful embryo implantation. The term "endometrial receptivity" (ER) describes the endometrium's capacity to receive the growing blastocyst and involves changes in histology and secretory patterns (Bi *et al*., 2022). During this period, the expression levels of some factors or molecules are markedly changed. Epithelial-cadherin (E-Cad) is closely related to the implantation procedure and is a biomarker of ER. During implantation, E-cadherin mediates the early homologous link dialogue between mothers and fetuses and participates in the initial adhesion process of human embryo implantation (Mahdi *et al*., 2021).

In cases where RPL is due to progesterone insufficiency, progesterone or dydrogesterone is usually used in clinical treatment (Saccone *et al*., 2017). It has been used clinically as a natural progestin since the 1960s, but there are side effects, including drowsiness, headache, nausea, and vomiting (Guo and Lu, 2021).

Dydrogesterone, the synthetic form of progesterone, has a similar molecular structure to progesterone but has fewer side effects and superior bioavailability, with 5.6 times the bioavailability of progesterone (Stanczyk *et al*., 2013). Dydrogesterone and its main active metabolites are highly selective for progesterone receptors and cannot bind to androgen receptors. Due to the high bioavailability and receptor selectivity of dydrogesterone, the dose required clinically is also markedly less about 10–20 times lower than progesterone (Guo and Lu, 2021). Dydrogesterone is an effective treatment method for RPL, with an even better clinical efficacy than progesterone (Mirza *et al*., 2016). Because the cellular immune effect is closely related to the development of RPL (Raghupathy and Szekeres-Bartho, 2022).

1.2 The Aim of the study:

The luteal phase of the menstrual cycle is crucial for embryo implantation and early pregnancy development. Dydrogesterone, a synthetic progestogen, aims to enhance endometrial receptivity and prevent pregnancy loss. This research explores its impact on hormone levels (progesterone, estradiol, prolactin, follicle-stimulating hormone, lutenizing hormone and testosterone), biochemical parameters (epithelial–cadherin and progesterone-induced blocking factor), and gene expression of progesterone receptors (mPRα, mPRß, and NPR) in women with RPL receiving dydrogesterone compared to those who do not receive it. Correlations among hormonal levels, gene expression of PRs, and biochemical parameters will also be studied.

Chapter Two

Literatures Review

2.1Recurrent Pregnancy Loss

Recurrent Pregnancy Loss (RPL) is a complicated issue that affects many couples. Yet, there is no universally accepted definition for it. The American Society for Reproductive Medicine (2020) and the European Society of Human Reproduction and Embryology (ESHRE) have defined RPL as two or more failed pregnancies (Bender *et al*., 2018).

RPL has been defined by the Royal College of Obstetricians and Gynecologists (RCOG) as the loss of three or more consecutive pregnancies before viability (Turesheva *et al*., 2023). The term, therefore, includes all pregnancy losses from the time of conception until 24 weeks of gestation as shown in Table 2-1 (Youssef *et al*., 2019).

After three losses, it was formerly advised to perform a workup for RPL. On the other hand, current research and recommendations recommend that an assessment for RPL be started following two verified losses. Based on ultrasound results, miscarriages can be further divided into two categories: those that result in the death of a fetus (pregnancy lost after the visualization of the fetal cardiac activity) or those that result in an empty gestation sac (pregnancy lost before 10 weeks gestation) (Kolte *et al*., 2015). There is some evidence from one observational research that whether the pregnancy losses are consecutive or not affects prognosis in unexplained RPL (Egerup *et al*., 2016; Sultana *et al*., 2020).

Despite efforts to standardize terminology, some researchers classify two consecutive miscarriages as recurrent miscarriages since it has been shown that having two miscarriages increases the likelihood that a subsequent pregnancy may result in miscarriage (El Hachem *et al*., 2017).

Table 2.1 The elements of definitions of RPL according to different International guidelines (Youssef *et al*., 2019).

	ESHRE[a]	ASRM[b]	RCOG[c]
Pregnancy	Serum or urine HCG, ectopic and molar pregnancies not to be included in the definition	Clinical pregnancy documented ultrasonography or histo-pathological examination	All pregnancy losses not further defined
Week of gestation	Up to 24 weeks	Only mention that the majority are lost prior to the 10^{th} weeks	Up to 24 weeks
Recurrence	2	2	3
Consecutive	Consecutive or non- Consecutive	Consecutive	Consecutive

a. European Society for Human Reproduction and Embryology,

b. American Society for Reproductive Medicine,

c. The Royal College of Obstetricians and Gynecologists

2. 1. 1 Epidemiology

Recurrent Pregnancy Loss (RPL) is one of the complications during pregnancy which occurs among 2–5% of couples **(**Wang *et al*., 2016**;** Pereza *et al*., 2017**).** This condition is specified as three consecutive pregnancy losses prior to 20 weeks from the last menstrual period (Bender *et al*., 2018). However, the cause of RPL remains unknown in around 50% of the patients. Given that the human endometrium is

thought to play a significant role in determining fertility, it has been suggested that an inappropriate endometrial may be a significant contributing factor to RPL **(**Amirchaghmaghi *et al*., 2015). There could be shared underlying risk factors for miscarriage and other adverse pregnancy outcomes. Several studies have looked at the association between the history of miscarriages and the future risk of other pregnancy complications **(**Dempsey *et al*., 2015**;** Oliver-Williams *et al*., 2015). But less is known about how complications might predict the future risk of miscarriage **(**Magnus *et al*., 2019).

2. 1. 2 Risk Factors

2. 1. 2. 1 Age

Advanced maternal age is considered a risk factor for adverse maternal and perinatal outcomes **(**Dumitrascu *et al*, 2019). The risk of miscarriage is slightly elevated in the youngest mothers and then rises sharply in older mothers **(**Magnus *et al*., 2019). Because of the quantity and quality of the surviving oocytes decrease with increasing maternal age (Youssef *et al*., 2019). Yet, pregnancies of advanced maternal age have become more prevalent over the last few decades. Possible maternal complications of pregnancy at age 35 or older include an increased risk of spontaneous miscarriage, foetal anomalies, preterm labour, gestational diabetes mellitus, pre-eclampsia, stillbirth, chromosomal abnormalities, and cesarean delivery **(**Glick *et al*., 2021). As mothers get older, the frequency of RPL rises, eventually reaching 1 in 4 by the age of 40. This is consistent with meiotic nondisjunction occurring at the oocyte level, which reaches 50% by the age of forty-three **(**Khalife *et al*., 2019). Despite detailed investigations on endocrine imbalances, autoimmune disorders, genetic abnormalities, infections, thrombophilia causes and congenital or structural uterine anomalies, 50% of the RPL cases are still

unexplained **(**Shahine and Lathi**,** 2015). When it comes to male age, the majority of researches assessing male age have found a strong correlation between growing male age and the prevalence of miscarriages **(**Sharma *et al*., 2015). They found associations between advanced paternal age and adverse outcomes in the offspring, particularly with psychiatric disorders like autism spectrum disorders and schizophrenia but also with stillbirth and several birth defects. The age of the father and the mutation rate in the offspring are found to be strongly related, possibly due to the larger number of germline divisions that have occurred in older males (Kong *et al*., 2012). In addition to a higher prevalence of point mutations, data suggests that sperm DNA strand breakage, genetic imprinting mistakes, and chromosomal abnormalities are also linked to miscarriage as paternal age increases (Kobayashi *et al*., 2017).

2. 1. 2. 2 Stress

Research has indicated that maternal stress during pregnancy may be linked to a higher chance of several unfavorable pregnancies and delivery outcomes (Li and Marren, 2018). An association between RPL and stress can be assumed based on many studies, but it is unclear whether stress results from RPL, or whether stress is a causing factor for the next pregnancy loss. Women who experienced RPL showed higher rates of moderate–severe depression, stress and anxiety compared to both controls and men who experienced RPL (Inversetti *et al*., 2023). Stress is associated with RPL, but couples should be informed that there is no evidence that stress is a direct cause of pregnancy loss (Li and Marren, 2018).

2. 1. 2. 3 Environmental or Occupational Exposure

A few studies suggest that exposure to environmental and occupational variables (heavy metals, pesticides, and deficiency in micronutrients) may increase the risk of pregnancy loss in women with recessive partial lipoproteinemia (RPL) (Lopes *et al*, 2019; Akther *et al*., 2020).

2. 1. 2. 4 Endometrial Decidualization

The endometrial alterations during the menstrual cycle and in early pregnancy called endometrial decidualization, endometrial cells appear to function as a quality checkpoint for embryos, causing implantation procedures in the event of normal embryos and the endometrium's quick death (menstruation) in the case of "abnormal" embryos. One of the most important and amazing things that happens to the human endometrium during pregnancy is this process. The impairment of this process leads to a variety of pregnancy disorders, including infertility, recurrent miscarriages, and utero-placental disorders (Garrido-Gomez *et al*., 2017; Wu *et al*., 2018). Previous observations have suggested that in women with RPL, abnormal decidualization may render the endometrium excessively permissive to implantation but unable to sustain the pregnancy, nevertheless, further prospective studies are needed before any firm conclusions or recommendations can be formulated for clinical practice (Lucas *et al*., 2016).

2. 1. 2. 5 Chronic Endometritis

Chronic endometritis (CE) is a poorly investigated pathology that has been related to adverse reproductive outcomes, such as implantation failure and recurrent miscarriage. Chronic endometritis is defined as localized inflammation of the endometrial mucosa characterized by the

presence of edema, increased stromal cell density, dissociated maturation between epithelial cells and stromal fibroblasts, as well as the presence of plasma cell infiltrate in the stroma; These changes at the level of endometrial microenvironment could affect endometrial receptivity (Puente *et al*., 2020). According to other study, women with RPL may have a prevalence of CE ranging from 7% to 58% (McQueen *et al*., 2015). However, there is currently no research that examines the predictive significance of a positive test result for endometritis or compares the incidence of endometritis in women with RPL to control groups or women without RPL **(**Park *et al*., 2016).

2. 1. 3 Health Behavior Modifications

2. 1. 3. 1 Alcohol Intake

The evidence is not consistent, but a large proportion of the studies have shown that alcohol consumption during pregnancy increases the risk of pregnancy loss. Alcohol consumption of 5 or more units per week leads to an increased risk of sporadic miscarriage (Dumitrascu *et al*., 2019). The Mendelian randomization study did not find a link between lifelong regular alcohol intake and pregnancy loss, although it is not impossible that there was a weak link that was missed. Other pregnancy and childhood outcomes, such as small-for-gestational-age and preterm deliveries, were also shown to be associated with moderate alcohol intake (Mamluk *et al*., 2017), but the conclusion remained uncertain (Yuan *et al*., 2021).

2. 1. 3. 2 Caffeine Intake

The association between caffeine intake and female fertility has been studied with inconsistent findings, in other study; a high level of coffee consumption during pregnancy has been associated with an increased risk of fetal death after 20 weeks of gestation and miscarriage (Lyngsø *et al*., 2017). Although other study has shown no association between

preconception caffeine intake and RPL, there are others that have concluded that high daily preconception caffeine consumption increases the risk of spontaneous abortion by 31% (Dumitrascu *et al*., 2019).

2. 1. 3. 3 Smoking

There is a clear correlation between smoking and negative obstetric and neonatal research has also shown links between children's issues such as obesity, psychological issues, cancer, and sudden infant death syndrome with mother smoking during pregnancy (Leung and Davies, 2015). Less is known, though, about how smoking or quitting affects pregnancy loss in women with RPL. It is important to let couples with RPL know that smoking may decrease their chances of a live delivery; hence quitting is advised (Bender *et al*., 2018).

Obstetric problems that have been associated with smoking include miscarriage, premature delivery, ectopic pregnancy and placental abruption. There are many reported effects on the fetus and newborn: prematurity, low birth weight and sudden infant death, among others (Sequí-Canet *et al*., 2022).

Other study has shown that some characteristics of pregnant women influence smoking behavior; these include education levels, maternal parity, partner relationships, and smoking among partners or parents. In addition, Ooka *et al*. (2020) found that certain regional characteristics, including regional socioeconomic status and ethnicity, are associated with maternal smoking behavior. Smoking during pregnancy is associated with spontaneous abortion (Hyland *et al*., 2014).

2. 1. 3. 4 Striving for a Healthy and Body Mass Index

Obesity has a significant impact on female reproductive health; increased body mass index (BMI) is associated with subfertility, poorer outcomes following infertility treatment, and pregnancy loss (Provost *et al*., 2016).

2. 1. 3. 5 Exercise

Exercise during pregnancy is believed to provide various benefits for women's health. A study showed Hegaard *et al*., (2016) that in one systematic review based on 5 studies, the Excerpts Medical data BASE (EMBASE) were conducted with no solid evidence of risks in the first trimester to suggest any change in current guidelines that recommending the regular exercise of moderate intensity during pregnancy.

2. 1. 4 Investigations in Recurrent Pregnancy Loss

2. 1. 4. 1 Family and Medical History

When deciding which investigations are relevant for a patient, their medical and family history, age, fertility, previous investigations, and treatments should be considered (Pillarisetty and Gupta, 2020).

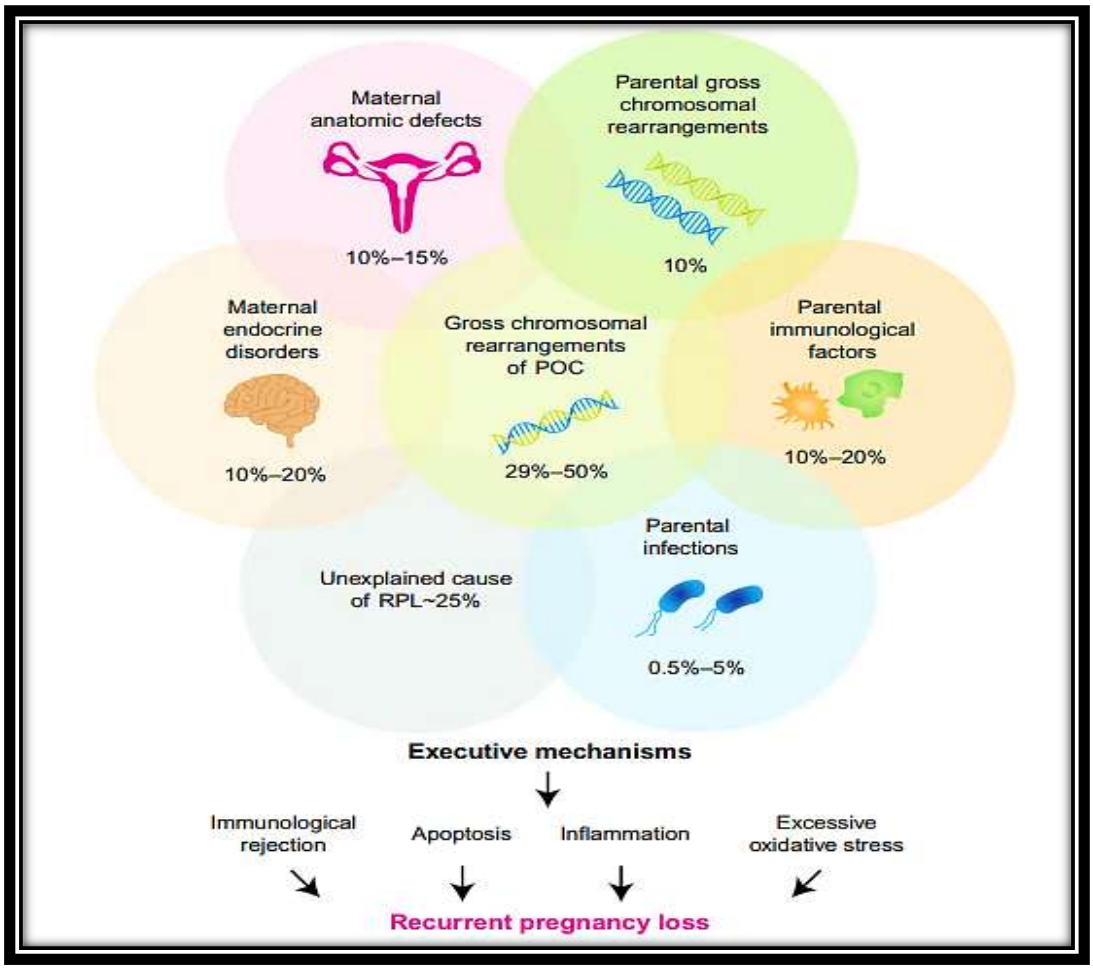


Figure 2.1: Known causes of recurrent pregnancy loss (Kasak *et al*., 2021).

2. 1. 4. 2 Genetic Analysis of Pregnancy Tissue

One possible reason for unexplained Recurrent Pregnancy Loss is genetic abnormalities in the fetus. While it is not always recommended, genetic testing of the pregnancy tissue can help in understanding the cause of RPL. The incidence of aneuploidy, a condition where there is an abnormal number of chromosomes, increases with the age of the mother, and it is a known cause of pregnancy loss. Both women with sporadic and recurrent pregnancy loss have an equal likelihood of having aneuploidies (Vitez *et al*., 2019).

The main causes of spontaneous abortion include chromosomal abnormality, uterine factors (including congenital uterine malformation, intrauterine adhesions, uterine fibroids), endocrine disorders, autoimmune diseases, infections, hyper-coagulation, environmental factors, sperm factors and unexplained factors. Chromosomal abnormality is the most important cause of spontaneous abortion in early pregnancy (Pylyp *et al*., 2018), and 8–10% of intrauterine fetal deaths occurring in the second or third trimester are still caused by fetal chromosomal abnormalities (Reddy *et al*., 2012).

2. 1. 4. 3 Parental Genetic Analysis

In couples with RPL, parental karyotyping is not usually recommended. Genetic history can make it worthwhile to do parental karyotyping (for example, if a previous child has congenital defects, the family has unbalanced chromosomes, or the pregnancy tissue shows a translocation). The test isn't very useful for other couples because there's a really low chance of finding an abnormality: 2% to 4% of all RPL couples have gross chromosomal abnormalities in either or both parents, including asymptomatic carriers who have balanced translocations or inversions (Kasak *et al*., 2021).

As compared to the general population, RPL has a 5 to 10-fold higher rate of balanced parental chromosomal rearrangement, and parental karyotype analysis is still widely used. According to ESHRE guidelines, RPL couples need to know that if a parent has an abnormal karyotype, the chances of a healthy child are good, despite the higher chance of losing a pregnancy (Bender *et al*., 2018). The cost-effectiveness of repetitive parental karyotyping for RPL couples has been proven in recent studies (Bedaiwy *et al*., 2016). Given that, even if a parental chromosomal abnormality is identified, there's still a possibility that the couple could still have a healthy child by natural conception even if a parental chromosomal abnormality has identified. This was validated by two prospective studies and the two most previous systematic reviews on the topic that found no overall difference in live birth rate when Preimplantation Genetic Testing (PGT) was used compared to natural conception in those cases (Ikuma *et al*., 2015; Iews *et al*., 2018).

2. 1. 4. 4 Thrombophilia Screening

Thrombophilia is a hereditary or acquired condition that predisposes women with recurrent pregnancy loss to venous thromboembolism (Nassour-Mokhtari *et al*., 2020).

2. 1. 4. 4. 1 Hereditary Thrombophilia

Patients with a thromboembolic event or their family members are being tested for genetic causes of venous thromboembolism. There is a debate about the usefulness of testing and therapy for venous thromboembolism. However, in cases of recurrent pregnancy loss, genetic thrombophilia factors are believed to be a cause and may be linked to serious obstetric problems. Therefore, women with RPL are assessed for mutations related to Factor V Leiden, Prothrombin, Protein

C, Protein S, and a shortage of Antithrombin (Campello *et al*., 2019; Samfireag *et al*., 2022).

2. 1. 4. 4. 2 Acquired Thrombophilia

Antiphospholipid syndrome (APS) is referred to as acquired thrombophilia. The diagnosis of APS is made on the basis of vascular thrombosis, pregnancy problems, and/or the continuous presence of antiphospholipid antibodies (Kalmanti and Lindhoff-Last, 2020). Moreover, APS is the most common acquired thrombophilia prevalent in 5-20% of females with recurrent pregnancy loss (Pandey and Gupta, 2019). A previous systematic review concluded that testing of APS after two pregnancy losses in couples with RPL is an evidence-based practice since difference in prevalence in APS is unlikely in women with two versus three pregnancy losses (van Dijk *et al*., 2020).

Antiphospholipid syndrome (APS) is an acquired, immune-mediated thrombophilia (Domingues *et al*., 2019). Recurrent miscarriages are a predictable complication in conditions of hypercoagulation. The first reports were in women with APS, of which recurrent miscarriages are a characteristic (Greer, 2011). Other studies described the association between other congenital or acquired thrombophilia and recurrent miscarriages. Previous study showed that women with obstetric APS, who have continuing pregnancies, are at increased risk of placenta-mediated pregnancy complications (Bouvier *et al*., 2014).

2. 1. 5 Hormonal and Metabolic Factors

Hormones play a key role in placentation, and their changes may result in the risk of miscarriage (Bender *et al*., 2018).

2. 1. 5. 1 Polycystic Ovary Syndrome and Insulin Metabolism

The Rotterdam criteria defined polycystic ovary syndrome (PCOS) according to three criteria: chronic anovulation, clinical and/or biochemical evidence of hyperandrogenism, and polycystic ovaries. According to the Rotterdam criteria, two of the three criteria had to be met to meet the definition of PCOS (Yu *et al*., 2016). Almost 10% of women in childbearing age are affected by PCOS (Bozdag *et al*., 2016). Infertility, obesity, amenorrhea, hairiness, infertility, and most importantly, miscarriage are all associated with abnormal endocrine changes. Women with PCOS have a higher miscarriage rate than women without PCOS, according to past meta-analyses (Bahri Khomami *et al*., 2019).

There are lots of pregnancy complications associated with polycystic ovary syndrome (PCOS), including gestational diabetes, pre-eclamptic toxaemia, hypertension caused by pregnancy, and pregnancy loss**.** These complications are different for different people depending on their ethnicity, socioeconomic status, and how well they're cared for (Vanky and Løvvik, 2020). The risk of miscarriage is high with PCOS, according to a large meta-analysis by Yu *et al*. (2016). It's possible that obesity, hyperinsulinemia, hypersecretion of LH, hyperandrogenism, and thrombophilia contribute to the uncertainty around an association between PCOS and pregnancy loss (Apostolos, 2018).

2. 1. 5. 2 Luteinizing Hormone, Follicle Stimulating Hormone and Estrogen

Studies have shown that high levels of luteinizing hormone (LH) in the early to mid-follicular phase have been associated with pregnancy loss, regardless of polycystic ovary syndrome (PCOS). Crosstalk between maternal cells and an embryo is crucial to successful pregnancy. There are

various signals involved in establishing and maintaining pregnancy, including hormones, growth factors, cytokines, microRNAs and proteases (Salamonsen *et al*., 2016). A first trimester abortion is usually caused by an endocrine pathology, mostly an inferior luteal phase caused by hypersecretion of luteinizing hormone, hyposecretion of follicle-stimulating hormone (FSH), hypoestrogenism, hyperandrogenism, damage to endometrial receptors (ex; chronic endometritis), as a rule, normal hormone levels are maintained (Djumaniyazovna, 2022). Elevated follicular phase LH concentration has been associated with detrimental effects on reproductive function—irregular menstrual cycles, reduced rates of ovulation, infertility, and increased rates of recurrent miscarriage (Kumar and Sait, 2011). In the first trimester of pregnancy, estradiol (E2) is crucial for the growth and development of the embryo, and its production is unstable prior to the placenta's full creation (Yang *et al*., 2022). Therefore, estradiol could contribute in some way to enhancing uterine artery blood flow and encouraging fetal development.

2. 1. 5.3 Prolactin disorders

The relationship between RPL and serum prolactin concentration has been debated. Therefore, prolactin testing is not routinely recommended without clinical signs of hyperprolactinemia. Hyperprolactinemia may be treated with dopaminergic agonists to increase live birth rates in women (Bender *et al*., 2018). Most centers routinely test serum prolactin levels because hyperprolactinemia can be easily treated. The hormone prolactin is essential to female reproduction and is responsible for maintaining progesterone secretion and corpus luteum function. Women with unexplained recurrent pregnancy loss were at increased risk of miscarriage in subsequent pregnancy, Prolactin is measured with known ovulatory dysfunction (Atasever *et al*., 2016).

2. 1. 5. 4 Androgens

Recurrent pregnancy losses may be caused by elevated androgen levels and delayed endometrial development during luteal phase. In unexplained recurrent pregnancy loss women (Sultana *et al*., 2020), testosterone levels were higher than those in fertile controls. In unexplained recurrent pregnancy loss women, testosterone levels were higher. In women, deficiency or excess androgens may contribute to pregnancy- and fertility related complications such as PCOS (Huhtinen *et al*., 2014), and recurrent pregnancy loss (Rahman *et al*., 2018).

2. 1. 5. 5 The Corpus Luteum

When the follicle ovulates, it quickly transforms into an adenoid structure which synthesizes estrogen, progesterone, and androgens. The steroid hormone progesterone plays a vital role during pregnancy, preparing the fertilized egg during implantation, facilitating endometrial metamorphosis, and inhibiting uterine contractions (Shah *et al*., 2019). Progesterone also enhances maternal immunity to the foetus and prevents embryo rejection during pregnancy by having anti-inflammatory and immunological effects. Research indicates that placental syncytial trophoblast cells begin secreting progesterone after 8 to 10 weeks of gestation, and that removing the corpus luteum before 7 weeks can cause miscarriage, but that exogenous progesterone can be used to maintain the pregnancy (Li *et al*., 2017).

During early pregnancy, progesterone is vital. In embryo transfer experiments (Gao, 2022 ; Marquardt *et al*., 2019), it has also been found that oestrogen deficiency can prevent implantation or cause infertility or early miscarriage even if the embryo is healthy and hormonally stimulated; oestrogen and progesterone work synergistically to maintain normal pregnancy. A low oestrogen level can also affect progesterone

levels. The corpus luteum produces and secretes steroid hormones along with protein hormones like relaxin and oxytocin. Assisted Reproductive Technology (ART) failure and early pregnancy loss are predicated on underdeveloped corpus luteum and inadequate hormone synthesis (Shah *et al*., 2019).

2. 1. 5. 6 Luteal Phase Insufficiency

A sufficient amount of progesterone is required for a secretory endometrium for embryo implantation and growth. The secretory transformation of the endometrium by progesterone is essential for implantation and early pregnancy maintenance. A number of endocrinopathies can cause luteal phase insufficiency, including PCOS, stress, and prolactin disorders (Agarwal and Kulshrestha, 2018). A corpus luteum deficiency occurs when the secretory activity of the corpus luteum is impaired, but it can also occur when the corpus luteum produces normal hormone levels due to a defective endometrium and immune system response (Ota *et al*., 2015). Asynchronous endometrial and embryonic development is the clinical manifestation, closely associated with infertility and miscarriage, the cause is unknown (Liu, 2021).

In addition to histologic dating of endometrial biopsy, luteal phase progesterone concentration, luteal phase length, and basal body temperature, several diagnostic criteria have been used. A discordant incidence of RPL has been reported (Ke, 2014), ranging from 12% to 28%. Diagnostic tests for LPD are neither reproducible nor reliable, and have not been validated in large studies (El Hachem *et al*., 2017). LPD management is also not well understood. Several treatment regimens have been used, including ovulation induction, supplemental progesterone, estrogen and progesterone, and human chorionic gonadotropin: Due to its

accessibility, ease of administration, and tolerability, progesterone supplementation is widely used as a treatment (Fox *et al*., 2017).

2. 1. 6 Anatomical Abnormalities

The most common congenital uterine abnormality associated with spontaneous miscarriage is septate uterus, which occurs in 19% of women with recurrent pregnancy loss. Recurrent pregnancy loss is associated with congenital uterine malformations. Bicornuate uterus and didelphic uterus are the most common Mullerian tract malformations. It has been reported that women with recurrent pregnancy loss have higher rates of uterine malformations. Women with congenital uterine malformations also have higher rates of miscarriage. Pregnant women with septate uterus and bicornuate uterus had greater chances of losing their pregnancy in first trimester (Venetis *et al*., 2014).

2. 1. 6. 1 Acquired Uterine Anomalies and Recurrent Pregnancy Loss

Depending on the study by Medrano *et al*. (2016), fibroids in RPL range from 0.5% to 1.3%. Fibroids are also associated with infertility. Three types of cells make up leiomyomas: well-differentiated, intermediate-differentiated and fibroid stem cells. . Depending on cell proportions, fibroid stem cells may grow more quickly and impact fertility. Several mechanisms have been suggested that could explain how leiomyomas adversely affect pregnancy development. These include altered uterine contractility, disruptions in endometrial cytokine expression, abnormal vascularization and chronic endometrial inflammation. A number of investigators have also suggested that mechanical stretching of the myometrium and endometrium can alter gene expression (Carbonnel *et al*., 2021).

2. 1. 7 Progesterone Hormone

2. 1. 7. 1 Progesterone Synthesis and Metabolism

The endometrium, the deepest layer of the uterus, is a complex and dynamic tissue made up of luminal and glandular epithelial cells encircled by supportive stromal cells. The endometrium is the layer that is vitally engaged in accepting an embryo, aiding implantation and decidualization, and sustaining embryo growth and development until placentation. The uterus's primary role is to support fertility (Marquardt *et al*., 2019). For a pregnancy to be successfully established, the endometrium must be prepared for decidualization and responsive to blastocyst invasion. This process is regulated by hormones and occurs during a specific time frame known as the window of receptivity, which is a part of the menstrual cycle (Vasquez and DeMayo, 2013). The signaling pathways that lead to successful early pregnancy involve progesterone receptor (PGR) and estrogen receptors (ESR1 and ESR2) (Dinh, 2020). In the endometrium, these pathways are controlled differently in the stromal and epithelial compartments (Wang *et al*., 2017). During the proliferative phase of the menstrual cycle, Estradiol (E2) stimulates epithelial proliferation to increase endometrial thickness. During the secretory phase, P4 suppresses E2-induced proliferation and permits stromal cells to start decidualization **(**Marquardt *et al*., 2019; Patel *et al*., 2017).

During the late follicular to luteal phase, ovarian follicles produce the largest amount of peripheral progesterone. The corpus luteum, a luteinized ovarian follicle, produces significant amounts of progesterone during the luteal phase and is crucial for establishing uterine receptivity (Wu *et al*., 2018). Progesterone plays a complex physiological role by controlling several different cellular processes. It affects the uterus, breast glands,

and brain, among other organs. Estrogens and progesterone can interact antagonistically or cooperatively. However, in various target organs, the relative concentrations of the two hormones might range dramatically. Progesterone can either encourage or inhibit cell division and proliferation, depending on the surroundings of the cell or tissue (Marquardt *et al*., 2019).

Progesterone generated by the ovaries causes activin A to be produced by endometrial cells, which affects trophoblast implantation. Thus, progesterone is generally acknowledged to be essential in inducing cellular alterations that promote placental decidualization and embryonic implantation (Hantak *et al*., 2014; Shah *et al*., 2019). A possible factor in repeated pregnancy losses could be insufficient progesterone levels and abnormal signaling through progesterone receptors. Progesterone is responsible for triggering changes in the lining of the uterus that are crucial for successful embryo implantation and ensuring a healthy pregnancy (Patel *et al*., 2015). Inadequate progesterone levels and a shortened luteal phase can lead to delayed growth of the uterine lining, which has been linked to recurrent miscarriages (Haas *et al*., 2019).

2. 1. 7. 2 Progesterone Receptor (PR)

The uterus plays a crucial role in women's reproductive wellbeing as it oversees important processes such as menstruation, childbirth, placental growth and embryo implantation. Through its responsiveness to progesterone (P4), the progesterone receptor effectively regulates key aspects of reproductive function in various mammalian species. In particular, the P4/PR signaling pathway is responsible for regulating early pregnancy events such as endometrial receptivity and decidualization, which are critical to ensuring a successful pregnancy outcome (Cope and Monsivais, 2022). PRG's primary role is to bind to progesterone receptors

through both the genomic pathway involves nuclear progesterone receptors (nPR) and the non-genomic pathway involves membrane progesterone receptors (mPR) such as mPRα and mPRß (DiRenzo *et al*., 2016). The most of the existing research on PRG has focused on its classical effects using nPRs in the nucleus as transcription factors, recent evidence suggests that PRG also exerts a broad spectrum of effects through non-classical receptor types located on the cell membrane. These include mPRs/PAQRs and PGRMCs (Czyzyk *et al*., 2017; Di Renzo *et al*., 2016; Aickareth *et al*., 2023).

Human nuclear PRs are produced by a single gene called PGR, located on chromosome 11 (11q22-q23) as show in (Figure 2.2). There are two promoters that regulate the expression of PGR, resulting in the production of two major mRNA transcripts. These transcripts encode two different proteins: full-length PR-B (114 kDa) and while PR-A is slightly smaller at (94 kDa) due to the lack of 164 amino acids in the N-terminal region (Yilmaz and Bulun, 2019). PR-B production is driven by the PR-B distal promoter region and begins at the first AUG translational start codon. On the other hand, PR-A is regulated by the PR-A proximal promoter region and begins at a second AUG translation start codon located 492 bases upstream (Patel *et al*., 2015). The two isoforms of PR (A and B) play different roles as transcriptional regulators during pregnancy and labor. They are also important in the myometrium (Azeez *et al*., 2021).

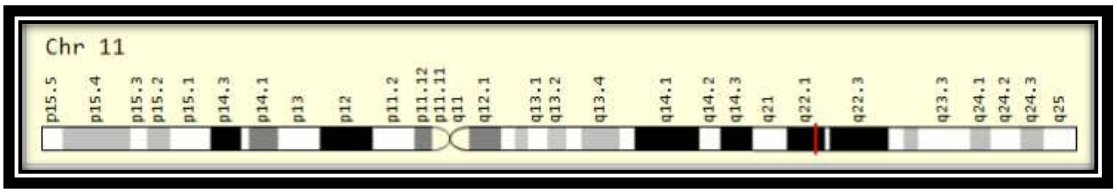


Figure 2.2: The genomic location of PGR Gene (Yilmaz and Bulun, 2019).

During pregnancy, PR-B plays a role in suppressing myometrial contraction by interacting with progesterone. Studies performed in human myometrial cell lines have shown that dominance of PR-B signaling (similar to conditions during pregnancy) leads to a reduction in the expression of pro-inflammatory genes. In particular, progesterone facilitates this response by increasing the expression of inhibitor B, which regulates the activity of the transcription factor nuclear factor B. As a result, both basal and lipopolysaccharide-induced expressions of pro-inflammatory genes are reduced (Tan *et al*., 2012).

In addition, extensive research has been conducted into the use of progestogen therapy in women at risk of preterm labor because of evidence that excessive PR-B expression may result in longer gestational duration. By providing progesterone supplementation, it would bind to PR-B at the molecular level and enhance its ability to suppress labour, thereby preventing premature labour Progestin's are man-made compounds designed to mimic the functions of progesterone, both natural progesterone and synthetic progestin's work through interactions with progesterone receptors. Upon binding ligands, PGRs translocate to the cell nucleus, where they bind to specific DNA promoter/enhancer elements or transcriptional co-regulators to regulate the expression of progesterone-responsive genes. PR-A and PR-B have comparable binding affinities for steroid hormones, but their transcriptional activities differ significantly. Although both variants interact with the same ligands, they exert different effects on gene expression (Zhang and Wang, 2023).

PR-B shows potent transcriptional activity when stimulated by specific ligands, whereas PR-A shows reduced activity but has the ability to repress the transcriptional functions of PR-B. Both PR (A and B) are thought to be antagonistic regulators that play critical roles in modulating

cellular responses to progesterone. The level of response to progesterone is inversely correlated with the ratio between PR-A and PR-B levels in target cells (Haas *et al*., 2019). During implantation, the expression of PR and ER is reduced. Research suggests that this drop in estrogen and progesterone receptor levels during the implantation window significantly affects the receptivity of the endometrium to embryo attachment (Lessey and Young, 2019).

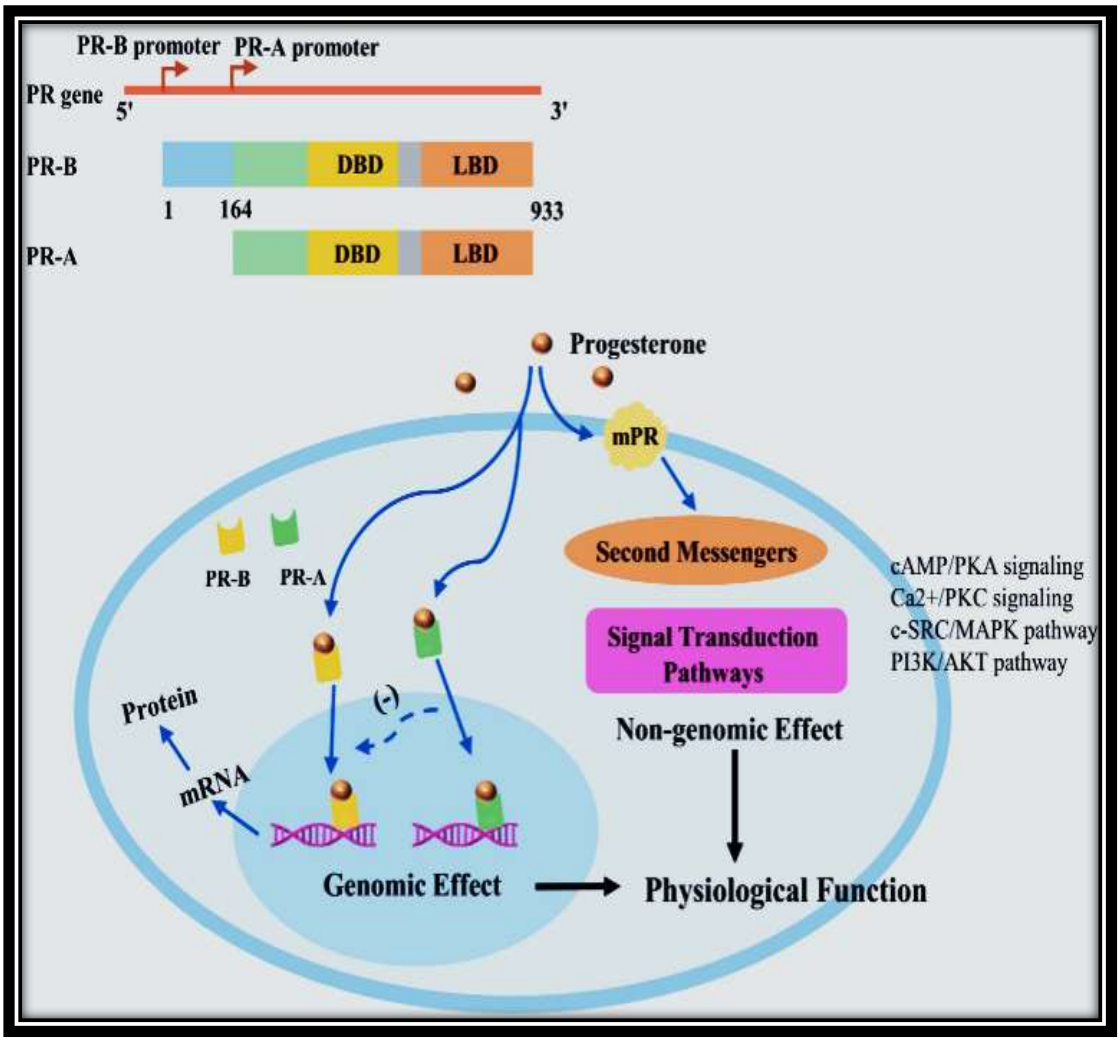


Figure 2.3: Mechanism of progesterone action (Zhang and Wang, 2023).

mPRs are a type of protein-coupled receptors which can be found in five distinct genes (mPRα) (Valadez-Cosmes *et al*., 2016). Proper activation of mPRs is critical to effectively utilizing the full spectrum of action of progesterone in a given tissue or cell. These effects, which

cannot be fully attributed to PR activation alone, can be partially explained by mPR activity (González-Orozco *et al*., 2018). Significantly, various studies have shown that these receptors play a crucial role in numerous diseases, including cancer, due to changes in both their expression levels and activity. The distribution of the mPRs varies by tissue and their activation by progesterone regulates signaling pathways essential to mammary gland development, sexual behavior, ovulation, maintenance of pregnancy and other biological processes (Valadez-Cosmes *et al*., 2016).

mPRs are found in tissues associated with female reproduction and embryonic development, including the endometrium, myometrium, ovary, and placenta. In particular, studies have shown that mPRα, mPRß, mPRγ and mPRε are detected in endometrial tissue. During the secretory phase of the menstrual cycle, α expression is stimulated while expression levels of mPRγ and mPRε decrease (Vázquez-Martínez *et al*., 2020). In addition, it was found that expression levels of mPRα and mPRß along with their respective protein content are lower in endometrial cancer tissues compared to the surrounding, unaffected endometrium. On the contrary, an increase in mPR protein content is observed in endometrial cancer tissue (Sinreih *et al*., 2018).

Progesterone works primarily through the action of the progesterone receptor. When bound to a ligand such as progesterone, this receptor carries signals from outside the cell to regulate gene expression inside the cell. This regulation occurs both through its ability to function as a transcription factor and through other activities that do not involve changes in gene expression (Patel *et al*., 2015). Several levels of regulation can affect the PGRs' ability to transmit progesterone signals. One factor is the transcriptional control of the PGR gene, which

determines its level of expression. In addition, post-translational modifications to the PGR protein play a role in regulating its function. The stoichiometry of different PGR isoforms also affects its signaling abilities and how it interacts with co-regulators on downstream targets (Wu *et al*., 2018).

Previous studies results showed that, in contrast to nuclear progesterone receptors, comparable affinities existed between 3-deoxysteroids and their corresponding 3-keto analogues. These results suggest that mPRs have no preference for binding to the 3-keto group (Polikarpova *et al*., 2017); however, the presence of the 3-keto group is crucial for PRG binding to nPRs. These results provide further evidence that the mechanism by which mPRs and nPRs interact with PRG is different (Aickareth *et al*., 2023).

2. 1. 7. 3 Synthetic Progestogens

Miscarriage or recurring miscarriages during the first trimester worries many women, this risk tends to increase significantly (Quenby *et al*., 2021), and causes significant emotional distress and grief for women and their loved ones. The use of hormone therapy with progestin supplements has been shown to be effective in reducing the risk of miscarriage (Wang *et al*., 2019; Zhao *et al*., 2022) and it is often used in assisted reproductive technologies (ART) to produce a pregnancy by providing luteal support (Griesinger *et al*., 2019).

Synthetic progestins were developed to produce progesterone-like effects and to overcome the rapid metabolism of natural progesterone when taken orally. These compounds are structurally diverse and, depending on their chemical structure, have differential effects on multiple cell types, receptors, and signaling pathways, depending on their exposure dose, the exposure pattern, and, at the target tissue level, the

relative concentrations of the receptors and enzymes involved in and prior to steroid metabolism Tissue exposure to priming factors and factors that activate unligated receptors (Garg *et al*., 2017). To ensure accuracy when studying the effects of early pregnancy medications such as dydrogesterone, it is important to consider and account for potential sources of bias and confounding factors. These may include other interventions or external influences, such as exposure to chemicals, over-the-counter medications, infections, behavioral factors such as smoking or drug abuse, and genomic factors from both parents. When studying the use of progestogens during early pregnancy in women at risk of miscarriage or repeated pregnancy loss (Katalinic *et al*., 2022). Giving extra doses of progestogens could potentially be an option to consider, this can reduce the risk of pregnancy loss (Carp, 2018).

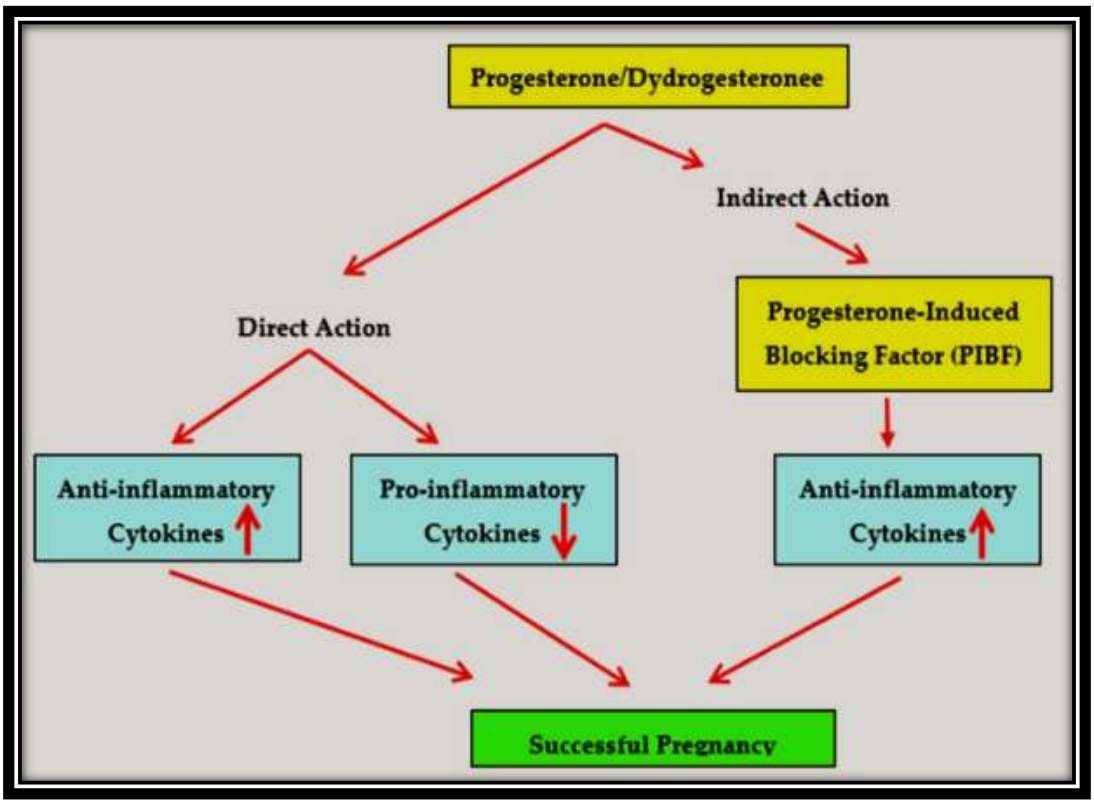


Figure 2.4 Immunomodulatory effects of progestogens (Raghupathy and Szekeres-Bartho, 2022).

2. 1. 7. 4 Oral Dydrogesterone (Duphaston)

One of the main causes of threatened miscarriage is a lack of progestogens, which is due to a malfunction of the corpus luteum. Therefore, progestin supplementation is a widely used treatment approach to manage threatened miscarriage caused by inadequate corpus luteum function. In clinical practice, progesterone and dydrogesterone are among the primary drugs used for this purpose (Greene, 2019).

Progesterone supplementation is a direct method to increase levels of this hormone when secretion from the corpus luteum is insufficient. This treatment option has been shown to be effective in managing threatened miscarriage. Dydrogesterone, an analog of progestogen, closely resembles endogenous progesterone in structure and function. In previous years, it has found wide application in the treatment of threatened miscarriage and assisted reproductive technology support with positive results (Griesinger *et al*., 2018).

Dydrogesterone has been used in over 90 countries worldwide since the 1960s to treat various disorders associated with progesterone deficiency. It is notable for being the only retrosteroid commercially available. While its molecular structure closely resembles natural progesterone (Fig 2.5), it exhibits improved oral bioavailability (Griesinger *et al*., 2019). However, currently, standardized guidelines for proper dosage and duration of treatment of DYD in clinical practice are lacking. In addition, there is no reliable method for accurately assessing luteal function. As such, there is limited high-quality research that supports the effectiveness and safety of using dydrogesterone as a treatment option. In addition, few studies have examined the safety aspects of DYD in the treatment of threatened miscarriage due to corpus luteum insufficiency (Coomarasamy *et al*., 2015; Lou *et al*., 2021).

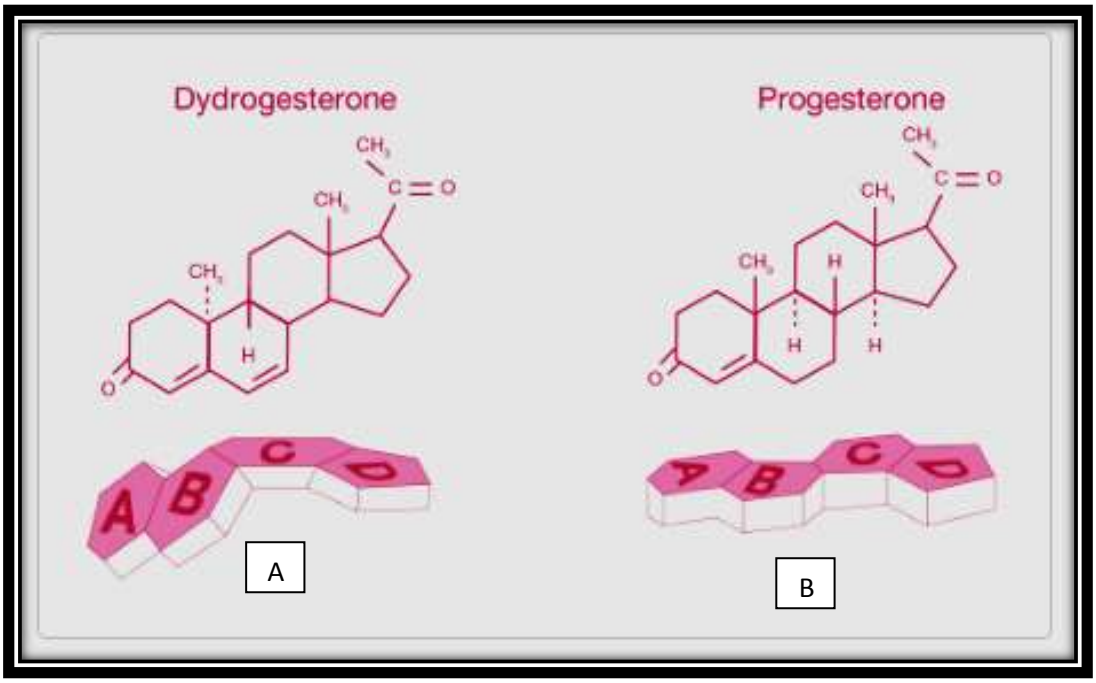


Fig 2.5: Molecular structure of (A) dydrogesterone and (B) progesterone (Schindler. 2009).

Dydrogesterone offers an advantage due to its unique retrostructure and the presence of a C6-C7 double bond. This structural feature results in a rigid conformation that is well suited for binding to the PR. Dydrogesterone's increased rigidity also contributes to its enhanced selectivity, unlike natural progesterone, which can exist in different conformations that are more likely to bind to different receptors; Due to its better bioavailability and the progestational activity of its main metabolites (20-, 21- and 16-hydroxy derivatives) (Guo and Lu, 2021).

Pharmaceutical grade progesterone and dydrogesterone are derived from diosgenin, which is extracted from the dioscorea (wild yam) plant. A particular exposure to ultraviolet light changes the structure of the precursor material, resulting in a distinctive retro-steroid shape that increases the affinity of dydrogesterones for the progesterone receptor. Unlike progesterone, dydrogesterone shows no appreciable binding to nuclear androgen, estrogen, glucocorticoid, or mineralocorticoid receptors. This high level of selectivity over progesterone receptors

minimizes activation of other steroid hormone receptors at the pre-receptor level and reduces adverse effects (Griesinger *et al*., 2019).

Dydrogesterone is developed to using at supporting the luteal phase. Its unique chemical composition improves absorption when taken orally and improves selectivity for progesterone receptors compared to natural progesterone. Other study has shown that oral dydrogesterone is as effective at supporting the luteal phase. However, it is important to note that these studies were not specifically aimed at establishing therapeutic equivalence between the two options (Griesinger *et al*., 2020).

Dydrogesterone is the only oral progestin available for this specific purpose and therefore represents an important treatment option. Progesterone and progestin's are also used to either increase natural progesterone levels or to provide progesterone when the corpus luteum is not present. Although supplementation has been shown to improve the rate of viable pregnancies, it does not address individual progesterone needs. In addition, it may play a role in regulating T-helper lymphocytes and supporting pregnancy as part of the immune process, making it an important factor in the prevention and treatment of recurrent spontaneous abortion (Guo and Lu, 2021).

Progesterone or dydrogesterone are commonly prescribed for the clinical treatment of RPL caused by progesterone deficiency. It can cause certain side effects, such as drowsiness, headache, nausea, and vomiting. Current research suggests that vaginal progesterone treatment may improve RPL outcomes in pregnant women; however, there are still safety concerns about its use (Stewart *et al*., 2021). Dydrogesterone, a progesterone derivative with selective affinity for progesterone receptors and minimal binding to other hormone receptors, is readily absorbed when taken orally. It has a favorable safety profile and low risk of side

effects. Dydrogesterone is considered the optimal choice for luteal support due to its potent potency at relatively low doses, high bioavailability, and minimal impact on liver function. Other study has demonstrated the safety and effectiveness of oral dydrogesterone in the dosage range of 40-80 mg per day (Wan and Lei, 2017).

Dydrogesterone is rapidly absorbed and completely metabolized. When taken orally, peak levels of dydrogesterone and its main metabolite 20-dihydrodydrogesterone are reached within 0.5 to 2.5 hours, with DHD concentrations significantly exceeding those of the original drug. The mean elimination half-lives for dydrogesterone and DHD are about 5-7 hours and 14-17 hours, respectively (Schindler *et al*., 2003). There are no significant interactions in the body's processing of dydrogesterone. Preclinical studies have shown that dydrogesterone has no mutagenic, teratogenic or carcinogenic properties. In addition, data from pharmacovigilance studies do not indicate cases of birth defects associated with the use of dydrogesterone during pregnancy (Schindler, 2009).

Dydrogesterone is efficiently absorbed and, when administered orally, undergoes rapid metabolism leading to the formation of its primary metabolite, 20adihydrodydrogesterone (20a-DHD). This process involves the hydrogenation of the 20-keto group (Olbrich *et al*., 2016). Plasma concentrations of the primary active metabolite are significantly higher than those of the originator drug, with AUC and Cmax ratios for 20a-DHD to dydrogesterone being approximately 45 and 25, respectively. Several other minor metabolites are also formed. A common feature of all identified metabolites is that they retain the dien-3-one configuration found in the parent compound but lack the hydroxylation at position 17a (Schindler *et al*., 2003)

The formation of metabolites after oral ingestion of dydrogesterone is well documented, but there is limited information on the specific enzymes responsible for its metabolic conversion. Previous study has shown that aldo-keto reductases play a role in this process. It has been shown that AKR1C1, 1C2 and 1C3 can convert dydrogesterone to its 20a-hydroxy metabolite when expressed recombinantly. In addition, AKR1D has also been found to be involved in the metabolism of steroid hormones (Rizˇner and Penning, 2014), It is currently unknown whether this particular AKR isoenzyme plays a role in the metabolism of dydrogesterone. In contrast to cytosolic AKR enzymes, no details are available on the contribution of microsomal cytochrome P450 enzymes to dydrogesterone metabolism. However, it should be noted that structurally similar progesterone undergoes metabolic conversion by the enzymes CYP2C9, CYP2C19 and CYP3A4 (Olbrich *et al*., 2016). Dydrogesterone may possess not only genomic effects via the progesterone receptor but also non-genomic effects. The mechanisms mediating these non-genomic effects of it are unclear (Yasuda *et al*., 2018).

2. 1. 8 Progesterone-induced blocking factor (PIBF)

2. 1. 8. 1 Definition

Progesterone-induced blocking factor is a critical protein for the maintenance of human pregnancy and the facilitation of maternal progesterone-dependent immune modulation. PIBF contains 757 amino acid residues and has an estimated molecular mass of 89 kDa (Szekeres-Bartho and Polgar, 2010; Lim *et al*., 2020). PIBF gene has been identified in the chromosomal region 13q21-q22 (Szekeres-Bartho, 2018). Progesterone has been found to suppress uterine muscle contractions and a drop in progesterone levels has been linked to the onset of labour. In

addition, progesterone is thought to play an important role in regulating the maternal immune response and preventing fetal rejection, which is facilitated by PIBF. Lymphocytes expressing receptors for progesterone release PIBF when exposed to progesterone during pregnancy (Lim *et al*., 2020).

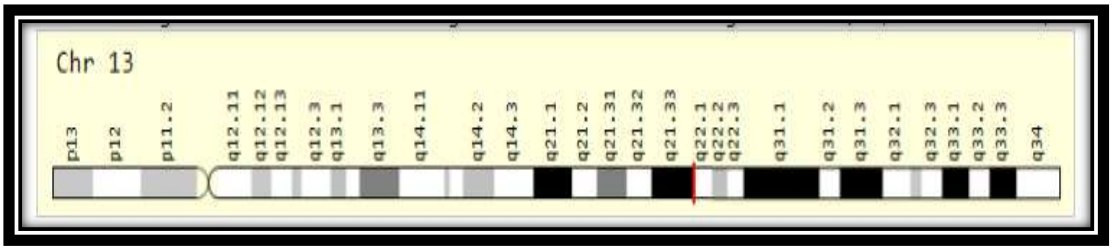


Figure 2.6: The genomic location of PIBF (Szekeres-Bartho, 2021).)

The binding of hormones leads to changes in the structure of the steroid receptor. These changes allow DNA binding and trigger gene activation, ultimately leading to protein synthesis. An example of a progesterone-regulated gene is the progesterone-induced blocking factor, which produces a protein responsible for modulating the immune system's response to progesterone (Szekeres-Bartho, 2021).

2. 1. 8. 2 Progesterone Induced Blocking Factor structure

Progesterone-induced blocking factor is a gene regulated by PR and has significant immune-modulating effects. This particular form of PIBF has been found to be associated with the centrosome and play a role in cell cycle regulation. However, shorter splice variants of PIBF have also been identified, which show tissue-specific expression both inside and outside of cells. It is believed that these shorter isoforms act as ligands for the PIBF receptor. Cells with high proliferation rates such as trophoblasts, mesenchymal stem cells and tumor cells all show PIBF expression. The expression of PIBF is influenced by hormones and increases during pregnancy. Studies performed on mouse models of hematological cancers

show that PIBF mRNA levels increase or decrease depending on the presence of certain hormones such as progesterone (P4) or RU486 (Cohen *et al*., 2016).

The PIBF receptor is a protein anchored to the cell membrane by glycosylphosphatidylinositol. It transiently binds to the alpha chain of the IL-4 receptor for signal transmission. Activation of the PIBF receptor leads to immediate activation of STAT6. However, incubation with progesterone for STAT6 phosphorylation lasts 24 hours, suggesting that the effect of progesterone on Th2 cytokine production is mediated by PIBF (Szekeres-Bartho. 2018).

A clinical study was conducted on women with recurrent miscarriages found that treatment with dydrogesterone stimulated the production of PIBF, which subsequently regulated Th1 cytokine levels while promoting the synthesis of Th2 cytokines (Cohen *et al*., 2016). During early pregnancy, PIBF induces decidual transformation of mouse endometrial stromal cells, resulting in a significant increase in endometrial expression during the implantation window. This suggests a possible involvement of PIBF in facilitating successful implantation (Check and Check, 2019).

Progesterone-induced blocking factor is a protein that is secreted from two main sources. The major source of PIBF found in serum appears to be the gamma/delta T cell. Interestingly, even in men, serum PBF levels increase significantly in the presence of progesterone. It is worth noting that while exposure to progesterone results in a noticeable increase in serum PIBF levels in the blood, synthetic progestins such as dydrogesterone, as well as naturally occurring 17-hydroxyprogesterone, do not have this effect on increasing serum PIBF levels. In addition, there is an apparent increase in intra-cytoplasmic PIBF levels with exposure to

progesterone, and a significant increase in mRNA levels for PIBFI has also been observed (Check and Check, 2019).

2. 1. 9 Epithelial Cadherin

2. 1. 9. 1 Definition

Transcription of the *CDH1* gene produces the trans-membrane glycoprotein precursor of epithelial cadherin (E-cadherin, cadherin1, or E-cad), a protein with a molecular weight of 135 kDa that serves as a cell adhesion molecule (CAM) that binds to different cell types and is essential for the normal morphogenesis and development of animal tissues (West and Harris, 2016).

The E-cadherin gene (*CDH1*), which is around 100 kb long, is located on chromosome 16q22.1as per (Figure 2.7). The gene region consists of 16 exons that range in size from 115 to 2245 bp. The human CDH1 exon borders were compared to those of other species and cadherin types, and the results showed notable conservation of their splice sites, suggesting gene duplication or conversion throughout the coevolution of cadherin types (Wong *et al*., 2018). A 120 kDa, Ca+2-dependent transmembrane glycoprotein known as mature E-cadherin links polarized and unpolarized epithelial cells at the lateral surface through adhesion junctions (AJs) (Izaguirre and Casco, 2016).

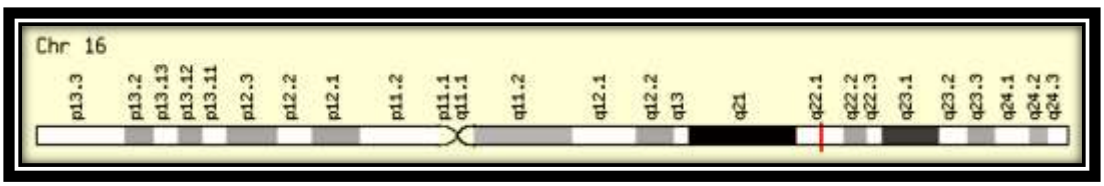


Figure 2.7: The genomic location of *CDH1* Gene (Wong *et al*., 2018).

E-cadherin (Epithelial) is one of the traditional cadherins, combined with the N-Cad (Neural Cadherin), P-Cad (Placental Cadherin), and VE-Cad (Vascular Endothelial Cadherin) (Pal *et al*., 2018). Cells switch to

express different cadherins during cadherin switching, a physiological process. Organ morphogenesis and tissue differentiation are controlled by cadherin switching. A large single-pass transmembrane glycoprotein known as E-Cad is involved in Ca+2-dependent cell-cell adhesion (Christgen *et al*., 2020).

2. 1. 9. 2 Epithelial-cadherin Structure

E-cadherin contains extracellular cadherin repeats with Ca+2 binding sites, which promote homophilic contacts between cadherin molecules expressed on neighboring cells.E-Cad's highly conserved intracellular tail interacts with a variety of cytoplasmic proteins, the majority of which are made up of α, β, and p120-catenins (Mège and Ishiyama, 2017). These various E-Cad binding partners' influence and control E-Cad's function, especially its interaction with the protein of actin-myosin cytoskeleton as shown in (Fig 2.8), its transport and recycling, and its interactions with the different A/B and planar polarity machineries at play in epithelial cells, they represent therefore key players in the remodeling of E-Cad-based adhesion (Sisto *et al*., 2022).

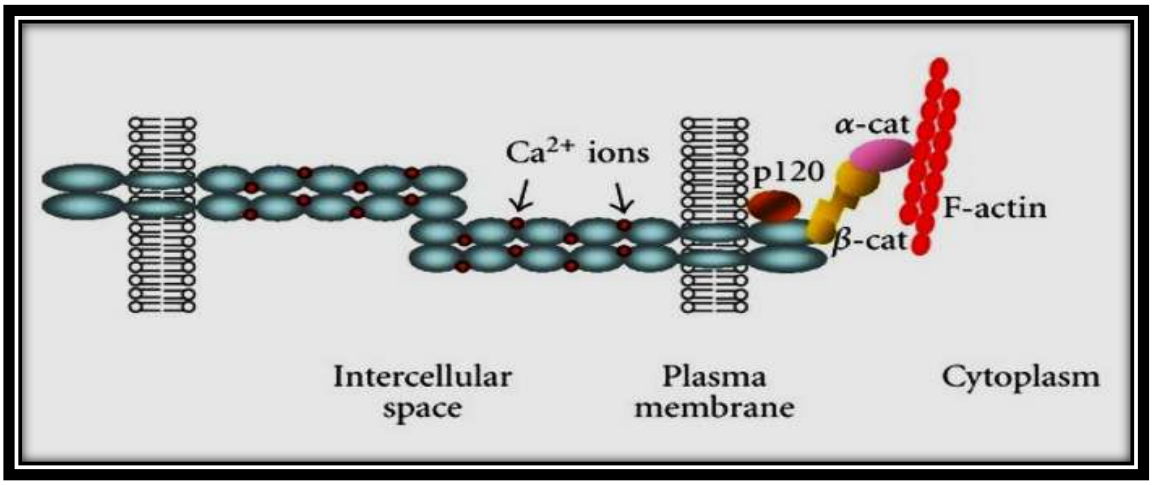


Figure 2.8: Schematic diagram of E-cadherin-catenin complex (Sisto *et al*., 2022).

The connection to the actin cytoskeleton is primarily mediated by β-Cat through its interaction with α-Catenin. It has been postulated that the interaction between E-Cad molecules and the actin/myosin network is mediated by a complex composed of E-Cad, α-Cat, β-Cat, and actin. Indeed, by generating E-Cad/-Cat fusions, direct linkage between -Cat and E-Cad may recover the majority of E-Cad loss-of-function phenotypes, involving remodeling (Desai *et al*., 2013).

The mature E-cadherin protein has three different domains: a cytoplasmic domain that is highly conserved, a single pass transmembrane domain (TMD), and an extracellular domain. E-cadherin's cytoplasmic tail is divided into two sections: the juxtamembrane domain (JMD) and the catenin-binding domain (CBD). β -Catenin and γ - Catenin bind to CBD, while p120ctn binds to E-cadherin's JMD regions. These areas are primarily essential for E-cadherin clustering at cell-cell contacts and provide a significant link to the actin cytoskeleton as shown in Figure 2.9 (Venhuizen *et al*., 2020).

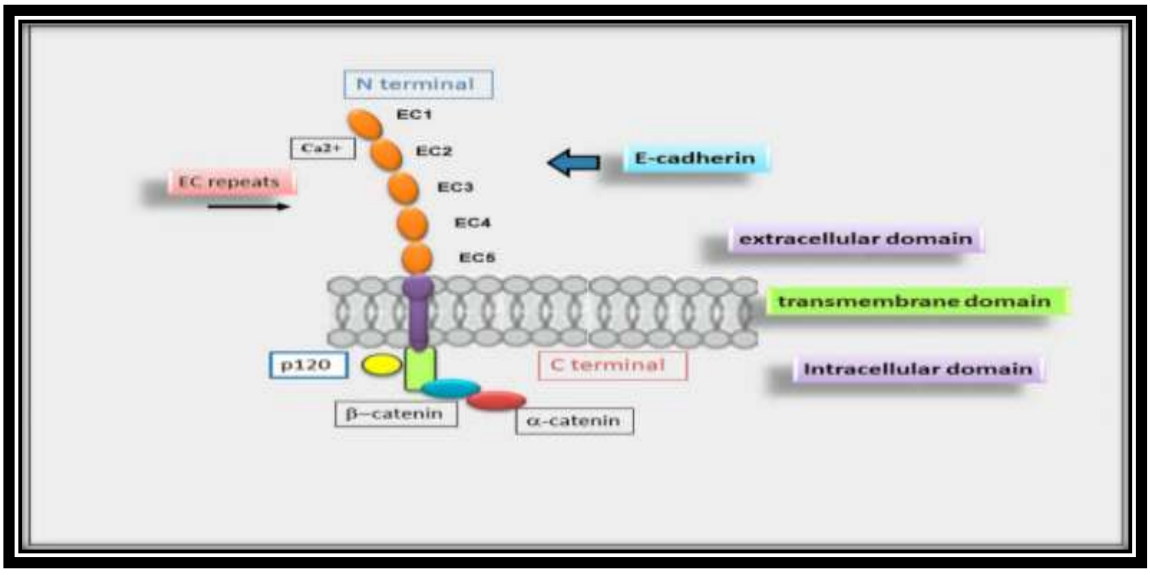


Figure 2.9: E-cadherin domains (Sisto *et al*., 2022).

2. 1. 9. 3 Function and Signaling Pathway

E-cad is a trans-membrane glycoprotein that connects epithelial cells at adherent's junctions. E-cad also forms a complex with four catenin proteins: α-catenin, β-catenin, γ-catenin and p120 catenin (Coopman and Djiane, 2016). Tyrosine phosphorylation of α-Cat increases its translocation to the plasma membrane and association with β-Cat, resulting in increased actin polymerization and AJs stability, whereas Syk deletion inhibits E-cad localization at AJs. Syk tyrosine kinase (also known as spleen tyrosine protein kinase) phosphorylates E-Cad and α-Cat, which acts as a tumor suppressor in epithelial cells. Their phosphorylation by Syk promotes p120-Ctn localization at AJs and the establishment of cell-cell interactions (Kassouf *et al*., 2019). SFKs also directly phosphorylate p120-Ctn, an important regulator of cadherin complexes in mammals that binds and stabilizes E-cad to promote its adhesion and tumor suppressive function (Kourtidis *et al*., 2013; Kassouf *et al*., 2019).

The global morphogenetic processes that result in stereotypical tissues and organs performing their functions during embryonic development as well as the adaptive processes of adult tissues are based on changes in cell size, shape, and relative cell movements. These changes in cell size, shape, and movement are intimately related to the remodeling of the E-Cad complexes and AJs (Röper, 2015).

A highly controlled event, embryo implantation is essential for the beginning of pregnancy. Apposition, adhesion, and invasion are the three steps that follow implantation. The synchronized development of the embryo and the endometrium is necessary for successful embryo implantation. Several regulatory proteins thought to be crucial for cancer cell invasion are also thought to be crucial for implantation. In

trophoblasts from humans, E-cadherin has been discovered, and it is assumed that this protein mediates homophilic contacts between cytotrophoblasts and endometrium. This may imply that E-cadherin was involved in the embryo's implantation process at the attachment stage (Ochoa-Bernal and Fazleabas, 2020). Patients who have spontaneous miscarriage had lower levels of e-cad in the placental villi during the early stages of pregnancy**.** Reduced E-cad expression during villous development was observed in patients with missing and impending miscarriages, as was demonstrated by western blotting in a prior study (Li *et al*., 2017).

In normal cells, E-cad primarily suppresses tumor growth by preventing β-catenin from binding to LEF/TCF, which is responsible for regulating the transcription of genes involved in the proliferative Wnt signaling pathway. Despite the ongoing controversy over whether EMT is caused by or results from the loss of E-cadherin, E-cad functional loss has frequently been linked to a poor prognosis and survival in patients with a variety of malignancies. However, there are instances where genetic changes also contribute to the dys-regulation of E-cad expression that causes carcinogenesis. E-cad expression has been connected to the biological processes of differentiation, cell cycle arrest, growth inhibition, and reduction of invasiveness (Wong *et al*., 2018).

Chapter Three

Materials

and

Methods

3. Materials and Methods

3.1 Materials

3. 1. 1 Subjects of study

One hundred six female participants, divided into three groups, participated in this clinical study. The first group consisted of 35 women with a history of recurrent miscarriage (Primary RPL). These participants had experienced two or more consecutive spontaneous abortions before reaching 24 weeks gestational age; they received treatment with dydrogesterone, precisely a daily dose of 10 mg twice a day, for six months. The second group also included women with recurrent pregnancy loss (Primary RPL) (n=35) who met the same criteria as the first group but received no medications, including dydrogesterone or other medications, during the six-month observation period. Finally, there was a control group (n=36) composed of women with no history of miscarriage who had previously given birth to at least one child. Data collection occurred from February 2022 to July 2023 at the Gynecology and Obstetrics Teaching Hospital, Khadija Al-Kubra Hospital in Karbala Province, and different infertility clinical in Iraq. All participants were similarly matched in age (25 to 38 years) and body mass index (BMI). The practical part of the study, which included biochemical tests, was carried out at Warith Alanbyaa University.

3. 1. 2 Ethical Issues

The study received approval from the Department of Biology and Scientific Committee of the Science College at the University of Kerbala. Additionally, permission was obtained from the Research and Development Department of the Health Directorate in Karbala province. All participants provided written informed consent after thoroughly explaining the objectives and potential advantages associated with their involvement in this research project.

3. 1. 3 Study Design

Case-Control study:

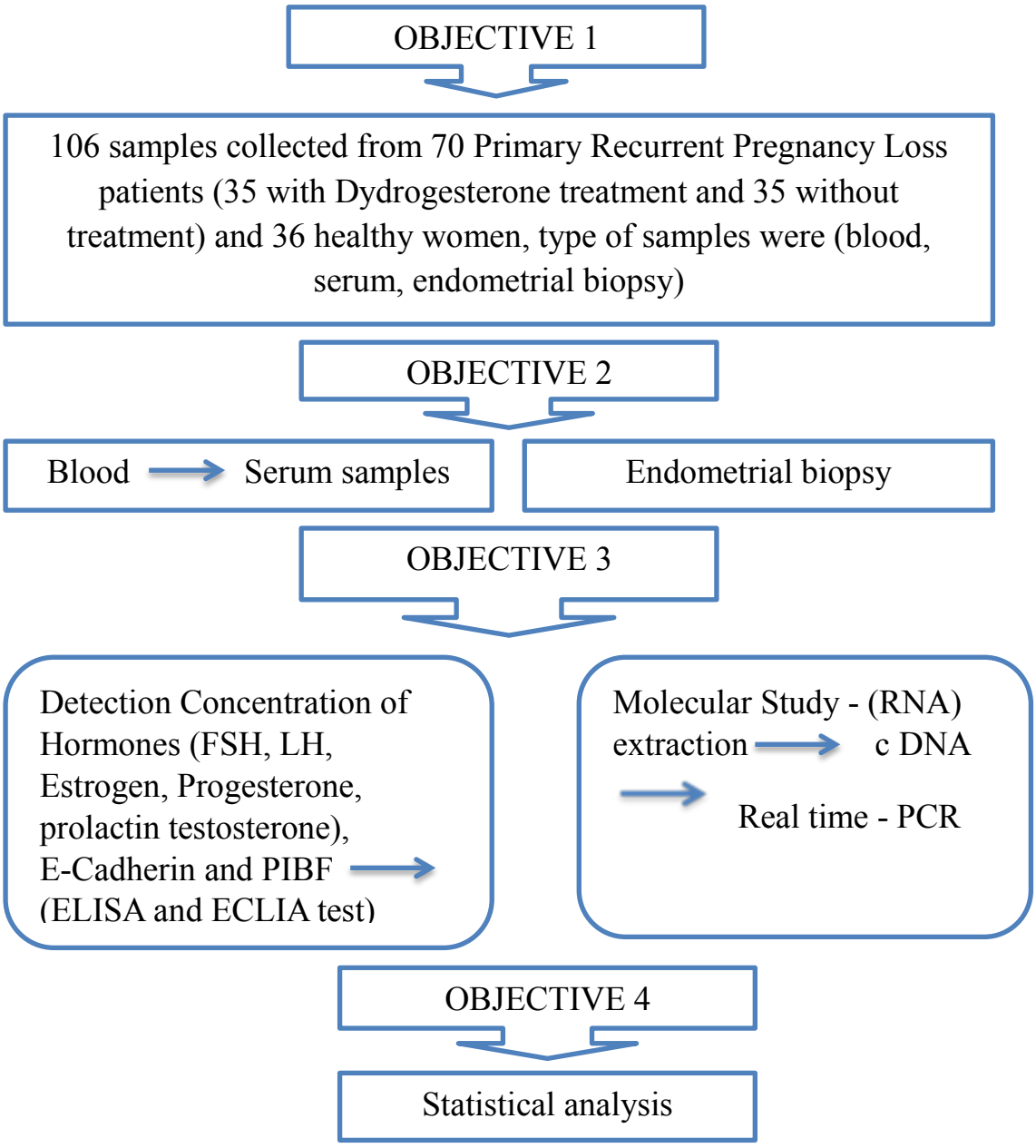


Figure 3-1 Flowchart of the study design

3. 1. 4 Chemicals substances

All the chemicals and the standard kits used in this study are shown in Table 3.1.

Table 3.1 Chemical Substances and Kits Used in the Study

Chemicals	Origin **(country)**
Bright green fluorescence dye	United State
Chloroform	SRL (India)
Ethanol	Holland
Elecsys FSH kit	Roche (Germany)
Elecsys LH kit	Roche (Germany)
Elecsys Estradiol III kit	Roche (Germany)
Elecsys Prolactin II kit	Roche (Germany)
Elecsys Testosterone II kit	Roche (Germany)
Elecsys Progesterone Kit	Elabscience (China)
Human E-Cadherin Level ELISA kit	Elabscience (China)
Isopropanol alcohol	SRL (India)
Liquid Nitrogen	Mosayab Factory for Chemicals (Iraq)
Magnesium chloride MgCl2	High media (India)
PBS- Phosphate Buffer Saline	Sigma-Aldrich (Germany)
PIBF kit	Elabscience (China)
RNAs free water	SRL (India)
RNAzol® RT	Sigma/USA
SYBR Green qPCR Master Mix	Sigma/USA

3. 1. 5 Instruments and Tools

The instruments and tools used in this study are shown in Table 3.2.

Table 3.2 The instruments and tools

Instruments and Tools	**Origin**
0.01 ml pipette tips	China
0.1 ml pipette tips	China
1 ml pipette tips	China
Autoclave	Korea

Cobas e 411 analyzer	Roche (Germany)
Cobas Integra 400 plus	Roche (Germany)
Centrifuge	Hettich /Germany
Cooling Centrifuge	Eppendorf/ Germany
Deep Freeze	GFL / Germany
ELISA reader ELx50	BioTek / USA
ELISA washer ELx800	BioTek / USA
Eppendorf tubes 1.5ml	China
Gel tube (6ml)	Netherlands
Hood	Labtech / Korea
High-speed refrigerated centrifuge	Yingtai/ China
Incubator	Fisher Scient/Germany
Liquid nitrogen containers (7L/45L)	Cryogenic/ USA
Multichannel pipette	USA
Nanodrop Spectroscopy	Biodrop (England)
PCR Thermocycler	Bioneer/ Korea
Powder-free gloves	Jordan
Pipelle Endometrial Suction Curette	USA
Refrigerator	Agur/Turkish
Real-Time PCR	Agilent Technologies/ Germany
Tips	USA
Vortex mixer	Korea
Water bath	GFL / Germany

3. 2 Methods

3. 2. 1 Data Collection

The exclusion criteria of this study were as follows:

Patients were excluded from the study based on medical history and physical and biochemical examination.

1) History of smoking
2) Drinking alcohol
3) Toxoplasmosis
4) Cytomegalovirus
5) Thyroid gland problems
6) Anti-phospholipid syndrome
7) Metabolic disorders
8) Autoimmune diseases
9) Anatomic abnormalities and polycystic ovaries have been excluded by ultrasound examination.

3. 2. 2 Socio-Demographic Data

The survey assessed various aspects as set out in Appendix 3. These included socio-demographic characteristics such as age, duration of infertility, and medical history. In addition, anthropometric measurements were considered, including weight (in kg), height (in meters), and body mass index, which is calculated by dividing weight by height squared (Maqbool *et al*., 2019).

$$\textbf{BMI = Weight (kg) / Square Height } (\textbf{m}^2)$$

3. 2. 3 Blood Collection and Preparation

Venous blood samples were drawn from all subjects using a disposable syringe (10 ml) in the sitting position. The needle was disconnected, and the whole blood was evacuated slowly into a gel tube without anticoagulant. The blood in the gel tube was allowed to clot at 37˚C for 10-15 minutes and then centrifuged at 3500 rpm for 15 minutes.

Separated serum was transferred into plain tubes for hormone concentration measurement (Follicle stimulating hormone, luteinizing hormone, progesterone, estradiol, prolactin and testosterone, Human progesterone-inducing blocking factor and Epithelial-Cadherin).

3. 2. 4 Tissue biopsy collection and preparation

Using a Pipelle Endometrial Suction Curette, endometrial samples were obtained from each group during the luteal phase of the menstrual cycle (Cooper Surgical Medical Devices, USA) for molecular study.

3. 3 Serum analysis

Blood constituent determinations included serum follicle-stimulating hormone, luteinizing hormone, progesterone, Estradiol, prolactin, total testosterone, progesterone-induced blocking factor and E-Cadherin concentration. Collection: The samples were placed in ice-filled containers to maintain them at the proper temperature before being transported to the lab for analysis.

3. 3. 1. Determination of Follicular Stimulating Hormone

The Elecsys FSH test applies a sandwich immunoassay technique to measure the levels of follicle-stimulating hormone in samples (Gudeloglu and Parekattil, 2013) and includes the following steps:

1. During the initial incubation stage, a forty μL sample forms a sandwich complex with a biotinylated monoclonal FSH-specific antibody and monoclonal FSH-specific antibody labelled with ruthenium.
2. During the second incubation stage, the addition of streptavidin-coated microparticles immobilizes the complex to a solid surface through the interaction between biotin and streptavidin.
3. The mixture was expressed into the measuring cell, where particles were magnetically captured onto the electrode surface. Unbound substances were removed with ProCell/ProCell M. A

voltage was applied to the electrode, inducing chemiluminescent emission measured by a photomultiplier.

4. Results: The outcomes were established using an instrument-specific calibration curve created through a two-point calibration process and a primary curve given by the reagent's barcode or e-barcode.

3.3.1.1 Reagents working solutions

i. M Streptavidin-coated microparticles (transparent cap), one bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL.
ii. R1 Anti-FSH-Ab~biotin (gray cap), one bottle, and 10 mL: Biotinylated monoclonal anti-FSH antibody 0.5 mg/L, MES buffer 50 mmol/L.
iii. R2 Anti-FSH-Ab~Ru (bpy) (black cap), one bottle, 10 mL: Monoclonal anti-FSH antibody (mouse) labelled with ruthenium complex 0.8 mg/L, MES buffer 50 mmol/L.

3. 3. 2 Determination of Luteinizing Hormone

The Elecsys LH assay applies the sandwich principle, using antibodies to capture and detect LH molecules in the blood. The working principle of the Elecsys LH kit aligns with the methodology described for the FSH determination kit.

3. 3. 3 Determination of Progesterone

The electrochemiluminescence immunoassay called "ECLIA" is used to quantify progesterone levels in human serum and plasma. It is designed to be operated on Elecsys and Cobas immunoassay analyzers (Anckaert *et al*., 2021). The Elecsys kit for progesterone testing follows the same methodology and standards as the FSH kit.

3. 3. 4 Determination of Estradiol

The Elecsys Estradiol III kit uses ECLIA technology to measure estradiol concentration in human serum. It employs two monoclonal antibodies for competitive testing, where endogenous estradiol competes with added estradiol derivative illustrated with ruthenium complex for binding sites on the biotinylated antibody. Estradiol was measured following manufacturer protocol (Melmed *et al*., 2015).

3. 3. 5 Determination of Prolactin

The Human serum prolactin level was measured with an electrochemiluminescence immunoassay (Elecsys prolactin II test, Roche Diagnostics) (Whyte *et al*., 2015). The process involves binding antibodies to prolactin molecules in the sample. This generates light signals, which are then used to quantify prolactin concentration accurately. The principles of the Elecsys prolactin II test are similar to the methodology described for the FSH determination kit.

3. 3. 6 Determination of Total Testosterone

The total testosterone level in human serum was measured using an electrochemiluminescence immunoassay (Elecsys testosterone II test, Roche Diagnostics) (Rosner *et al*., 2007). This method uses antibodies to bind to testosterone, producing a measurable light signal indicating the sample's testosterone concentration. The Elecsys testosterone II test principles match those of the FSH determination kit methodology.

3. 3. 7 Determination of Human Progesterone Induced Blocking Factor

This Elabscience kit enables the determination of PIBF concentrations in human serum, plasma, and other biological fluids. The test was carried out following the manufacturer's instructions, shown below.

3. 3. 7.1 Assay principle

To measure the human PIBF level in a sample, this kit applies purified antibodies specific to human PIBF that are coated onto microtiter plate wells. Once a solid-phase antibody has formed, PIBF is added to the wells and combined with HRP-labeled PIBF antibodies to create an antibody-antigen-enzyme complex. After thoroughly washing the mixture, the TMB substrate solution turns blue through an HRP enzyme-catalyzed reaction. The color change is measured at a wavelength of 450 nm using spectrophotometry once the sulfuric acid solution terminates the reaction. By comparing the OD of samples against a standard curve, one can determine Human PIBF concentration in their respective samples.

3. 3. 7. 2 Materials Provided with the Kit

Table 3.3 Material provided

Materials provided with the kit	96 determinations	Storage
User manual	1	
Closure plate membrane	2	
Sealed bags	1	
Micro ELISA strip plate	1	2-8°C
Standard 13.5ng/ml	0.5mlx1 bottle	2-8°C
Standard diluent	1.5ml x1 bottle	2-8°C
HRP-Conjugate reagent	6mlx1 bottle	2-8°C

Sample diluent	6mlx1 bottle	2-8°C
Chromogen Solution A	6mlx1 bottle	2-8°C
Chromogen Solution B	6mlx1 bottle	2-8°C
Stop Solution	6mlx1 bottle	2-8°C
wash solution	(20mlx30 fold) x 1bottle	2-8°C

3. 3. 7. 3 Assay Procedures

1. To prepare the ELISA plates, ten wells were filled and coated with a standard solution. 100 µL of the standard is added to the first and second wells, and then the dilution (50 µL) is added to both wells and mixed thoroughly. Subsequently, 100 l each from the first and second wells are transferred separately to the third and fourth wells. These are also mixed with thinner (50 L). Next, 50 µL each of the contents of the third and fourth wells are discarded before another 50 µL is transferred to the fifth and sixth wells, respectively. An additional 50L is added to these two new wells for proper mixing. Finally, a small amount - specifically a 50 Li aliquot - is withdrawn individually from the contents of the fifth or sixth well to allow further addition to the contents of the seventh or eighth well. Then, 50 L of a diluted standard was added to the seventh and eighth wells and mixed. Then, 50L from these two wells was transferred to the ninth and tenth wells. An additional 50L of the diluted standard was added to these ninth and tenth wells before being combined. After dilution with samples of different densities ranging from 9 ng/mL to as little as 75 ng/mL, a small volume (approximately 50 µL) was removed from each well for further analysis.

2. The empty wells not filled with samples or HRP conjugate reagents were kept separate from the test and sample wells. The process in each well is consistent. Then 40 µL of the sample dilution was added to the

test sample well along with 10 µL of test in the sample (resulting in a final 5-fold dilution).

3. After sealing the plate with a membrane, incubate for 30 minutes at a temperature of 37°C.

4. The liquid was prepared by diluting the wash solution with distilled water in either a 20-fold or 30-fold ratio and kept aside

5. Washing: The closure plate membrane was removed, and the remaining liquid was discarded. The area was dried thoroughly, and a wash buffer was added to each well. It should stand for 30 seconds before deflating. This sequence of steps is repeated five times. HRP-Conjugate reagent 30ul was added to each well except the blank well.

9. Solution A (50ul) and solution B were added to each well and incubated in the dark at 37°C for 15 minutes.

10. Stop Solution50µl was added to each well.

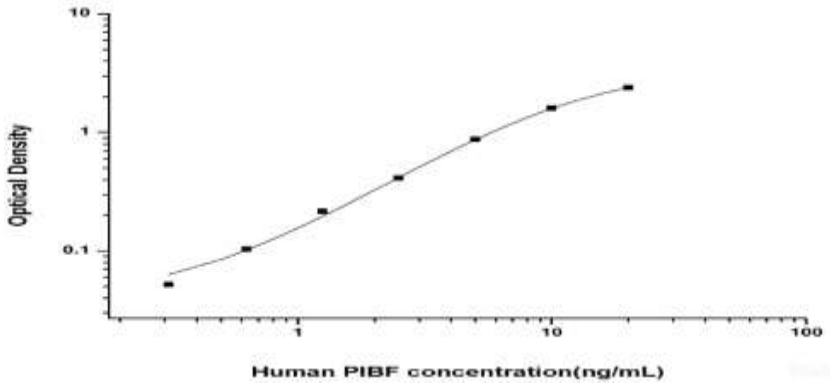
11. The blank value was considered zero, and the absorbance measurement was taken at a wavelength of 450 nm after adding the stop solution within 15 minutes.

3. 3. 7. 4 Calculations

To determine the actual density of the sample, the following procedure was followed:

1. A standard curve was constructed by plotting points on graph paper, with the horizontal axis representing the standard density and the vertical axis representing the OD value.
2. The sample's corresponding density was determined by relating its OD value to the sample curve, which accounts for a dilution factor.

3. Alternatively, a linear regression equation was calculated from the standard density and the OD values of the standard curve. This equation could then be used to calculate the density of a sample by substituting the OD value into this equation.



3. 3. 8 Human E-Cadherin Level ELISA kit

3. 3. 8. 1 Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA) Bioassay Technology Laboratory E0209Hu. The plate has been pre-coated with a Human E-cad antibody. E-cad present in the sample is added and binds to antibodies coated on the wells. Then, a biotinylated human E-cad antibody is added and binds to the E-cad in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated E-cad antibody. After incubation, unbound Streptavidin-HRP is washed away during a washing step. The substrate solution is then added, and colour develops proportionately to the Human E-cad amount. The reaction is terminated by adding an acidic stop solution, and absorbance is measured at 450 nm (According to Elabscience Kit).

3. 3. 8. 2 Reagents Provided

The reagents provided are shown in (Table 3.4).

Table 3.4 Reagents provided.

Components	Quantity
Biotinylated Human E-CAD Antibody	1ml x1
Plate Sealer	Two pics
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Standard Solution (400ng/ml)	0.5ml x1
Stop Solution	6ml x1
Streptavidin-HRP	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1

3. 3. 8. 3 Other Materials

37 0C ± 0.5 0C incubator
Absorbent paper
Precision pipettes and disposable pipettes tips
Clean tubes
Deionized or distilled water
Micro-plate reader with 450 ± 10nm wavelength filter

3. 3. 8. 4 Reagents Preparation

All reagents were allowed to come to room temperature before use.

1. Standard Reconstitute: The standard (400ng/ml) was mixed with standard diluent in a 1:1 ratio to create a 200ng/ml standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation before diluting. Duplicate standard points prepared by serially diluting the standard stock solution (200ng/ml) 1:2 with standard diluent to produce 100ng/ml,

50ng/ml, 25ng/ml and 12.5ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution was frozen at -20°C and used within one month.

2. Wash Buffer: 20 of Wash Buffer Concentrate 25x was diluted into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals formed in the concentrate, they were mixed gently until completely dissolved.

3. 3. 8. 5 Assay Procedure

1. All reagents, standard solutions and samples are prepared as instructed. All reagents are brought to room temperature before use. The assay is performed at room temperature.
2. The number of strips required for the assay was determined. The strips are inserted in the frames for use. The unused strips should be stored at 2-8°C.
3. A volume of 50µl standard was added to the standard well.
4. A 40µl sample was added to the sample wells, followed by 10µl anti-E-CAD antibody and 50µl streptavidin-HRP to the samples and standard wells (not the blank control well). The plate was covered with a sealer and incubated for 60 minutes at 37°C.
5. The sealer was removed, and the plate was washed 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, all wells were aspirated and washed 5 times with wash buffer, overfilling wells with wash buffer. The plate was blotted onto paper towels or other absorbent material.
6. A volume of 50µl substrate solution A was added to each well, and then 50µl substrate solution B was added to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. A volume of 50µl Stop Solution was added to each well, and the blue colour changed into yellow immediately.
8. The optical density (OD value) determined of each well immediately using a micro-plate reader set to 450 nm within 10 minutes after adding the stop solution.

3.4 Molecular study

3. 4. 1 Extraction of RNAs

- **Sample presentation.**

A TRIZOL was used at a ratio of 1 ml per 50 to 100 mg of tissue, and the sample volume was limited to 10% of the total volume used for homogenization (Chomczynski and Mackey, 1995).

- **Phase separation**.

The homogenized sample was incubated for 5 minutes at room temperature to disassociate nucleoprotein complexes fully. Cellular debris was eliminated by centrifugation and transfer of the resulting supernatant into a fresh tube.

1. For each millilitre of TRIZOL Reagent, 0.2 ml of chloroform was included. The samples were vigorously mixed by overtaxing for 15 seconds and incubated at room temperature for roughly two to three minutes before being centrifuged at no more than 12,000 rpm for about 15 minutes at temperatures ranging from 2°C to 8^{o}C.

2. After centrifugation, the mixture separates into lower red phenol chloroform, interphase, and a colorless upper aqueous layer. RNA is found only in the aqueous phase. The volume of this upper layer was measured (approximately 60% of the initial TRIZOL Reagent volume used for homogenization) and then carefully transferred to a new tube without disturbing the interphase.

- **RNA precipitation**

To extract the RNA from the aqueous phase, it is necessary to combine it with isopropyl alcohol. The initial homogenization process requires 0.5 ml of isopropyl alcohol for every 1 ml of TRIZOL Reagent.

1. The specimens were subjected to a temperature range of 15 to 30 degrees Celsius for 10 minutes. Subsequently, they underwent centrifugation at no more than 12,000 times the force of gravity for another period of 10 minutes while maintaining the temperature between 2 and 4^{o}C.
2. The RNA residue that was typically indiscernible before this process now condenses into an amorphous clump adhering to both sides and the base of the container in a gel-like consistency.

- **RNA wash**

1. The RNA pellet underwent one wash using 75% ethanol with at least a volume of 1 ml per ml of TRIZOL Reagent used in the homogenization process after the complete removal of the supernatant.
2. The samples were blended through overtaxing and centrifugation at a maximum of 7,500 rpm for 5 minutes between temperatures of 2 to 8 ^{C}. The washing process was repeated one more time before removing any remaining ethanol.

3. Total RNA quantification and purity were assessed using a Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE).

3. 4. 2 Complimentary DNA (cDNA) Synthesis

The cDNA synthesis was carried out following the instructions provided by the kit manufacturer. The process was performed as follows:

1. Several components were combined and placed on ice to prepare for the reverse transcription reaction. These included an RNA

template, previously dissolved primers, 5 Super RT One Step Buffer, Enzyme Mix, 10x Enhancer solution, and RNase-Free Water.

2. RT-PCR Reaction System Preparation:

Table 3.5 Master Mix Preparation

Reagent	25 µL	Final concentration
5x Super RT One Step Buffer	5 µL	1x
Primer 1	0.5~1 µL	0.2~0.4µM
Primer 2	0.5~1 µL	0.2~0.4µM
Enzyme Mix	1 µL	
10x Enhancer	2.5 µL	1x
RNA Template		
DNase RNase-Free Water	0.5 -2 µL	
Total volume	25 µL	

3. The tubes were shaken and centrifuged to collect a solution at the bottom.

4. The PCR tubes were placed into a thermal cycler and incubated at temperatures up to 45°C. The next step involved executing the RT-PCR procedure.

3. 4. 3 Quantitative Real-Time PCR Analysis

The subsequent steps were followed according to the kit manufacturer's instructions to perform Quantitative Real-Time PCR.

1. Bright Green RNA qPCR Master Mix, template DNA, primers and Nuclease-free water all were thawed on ice. And each solution was mixed well.
2. The master-mix reaction was prepared, as shown in Table (3.5).

3. 4. 3. 1 Reaction Condition:

Reverse transcription	50°C	15min	
Denaturation	95°C	2.5min	
Denaturation	95°C	20 s	40 cycles
Annealing	65°C	25 s	
Extension	72 °C	60s/kb	
Keep temperature	72 °C	10min	
Keep temperature	4°C	Hold	

3. 4. 3. 2 Primers of gene expression

Table 3. 6 The primers of gene expression.

Primers	Sequence	Reference
MPRα	F 5′-CTGAAGTTTGCCTGACACCA-3′ R 5′-AATAGAAGCGCCAGGTCTGA-3′	By Rahnama *et al* (2019)
MPRβ	F 5′-CACGAAGGACCCACAAAACT-3′ R 5′-CAATCCCAAGCACCACCTAT-3′	By Rahnama *et al* (2019)
GAPDH	F5′-GAAATCCCATCACCATCTTCCA-3′ R 5′-CAA ATGAGCCCCAGCCTTC-3′	By Rahnama *et al* (2019)
NPR	F 5′-AGGTCTACCCGCCCTATCTC-3′ R 5′-TCCCACAGGTAAGGACACCA-3′	By Smaglyukova *et al* (2020)

3. 4. 3. 3 Calculating Gene Expression (Fold change)

Two strategies for evaluating qPCR data are absolute and relative quantification. Absolute quantification identifies the input gene amount based on a standard curve.

In contrast, relative quantification determines changes in gene expression relative to a reference gene; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sample accomplished by Errors caused by

standard dilutions when creating a standard curve can also be avoided**.** Groups are of more interest than exact DNA/RNA molecular numbers. Therefore, relative quantification is widely performed. Gene expression, gene fold, or RQ (Relative quantification) value calculated (Pfaffl, 2001).

$$RQ = 2\text{-}(\Delta\Delta CT)$$

Gene fold or RQ is calculated firstly by collecting CT (CT - cycle threshold or CQ - cycle quantification) average value from a real-time PCR device for each triplicated sample, then calculating ΔCT value for both treated and untreated samples as follows:

Δ CT = CT (gene of interest) – CT (reference gene)

To calculate the ΔΔCT value, the following steps were taken:

ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control)

After calculating ΔΔ CT for all samples then, the final equation was used to calculate gene expression or RQ: **Fold change = 2- (ΔΔCT).**

3.5 Statistical Analysis

The data was evaluated by statistical package for social sciences (SPSS 24) software, where data were expressed as the Mean ± standard deviation of the mean independent-sample ANOVA-test, which was used to find the association between the categorical variables, $P \leq 0.05$ was considered statistically significant (Iuliano and Franzese, 2018).

Chapter Four

The Results

and

Discussion

4 Results and Discussion

4. 1Demographic Characteristics

The demographic data (mother age, BMI, number of children, and number of abortions) for the control group, RPL without treatment group, and RPL with dydrogesterone treatment group are displayed in Table 4-1.

4. 1. 1 Age and BMI

No significant ($p \leq 0.05$) differences were found between all groups in terms of age and BMI (Table 1).

Table 4-1 Demographic characteristics of subjects

Groups / Parameters	Control (n=36)	RPL Patients Without treatment (n=35)	RPL Patients With Treatment (n=35)
Age (years)	31.92±2.95	31.60 ± 2.75	31.46 ± 2.81
Number of Abortion	NA	2.31 ± 0.47	2.49 ± 0.51
Number of Children	2.47 ± 1.08	NC	NC
BMI (kg/m^2)	25.60 ± 1.03	25.34 ± 1.35	25.13 ± 1.13

($p \leq 0.05$) is significant.
Data are represented as (Mean ± SD)
NC refers to No-Children, and NA refers to No-Abortion.

4. 2 Hormonal Analysis

The data in Table 4-2 compares the study criteria for RPL patients who received dydrogesterone treatment, those who did not receive the medication, and the control group.

Table 4- 2 Hormonal concentrations in serum of study groups

Groups / Parameters	Control (36)	RPL Patients without treatment(35)	RPL Patients with treatment(35)	p. value
Progesterone (ng/ml)	10.41 ±1.33[a]	9.31 ± 1.31 [b]	10.57 ± 1.25[c]	**0.000**
Estradiol (pg/ml)	139.55±8.34[a]	133.99± 7.45 [b]	137.62± 5.75 [c]	**0.020**
Prolactin (ng/ml)	13.13± 1.52	13.04 ± 1.32	13.05 ±1.32	0.999
FSH (mIU/ml)	6.31±1.16	6.25 ± 0.98	6.14 ± 0.67	0.875
LH (mIU/ml)	5.77 ±1.19	5.52 ± 1.01	5.53± 1.07	0.926
Testosterone (mmol/L)	0.99 ± 0.14	0.90 ± 0.58	0.91 ±0.43	0.918
($p \leq 0.05$) is significant. Data are represented as (Mean ± SD)				

4. 2. 1 Progesterone concentration

Results which were illustrated in Table 4-2 showed a significant decrease ($p \leq 0.05$) in progesterone concentration of RPL patients without treatment (9.31± 1.31 ng/ml) in a comparison with the control group (10.41± 1.33 ng/ml).

The result indicated that there was no significant ($p \leq 0.05$) difference in progesterone levels between RPL patients receiving treatment and the control group (10.57 ± 1.25, 10.41 ± 1.33 ng /ml, respectively). However, there was a statistically significant increase ($p \leq 0.05$) in progesterone concentration among RPL patients with treatment compared to those without treatment. A previous study showed that the expression levels of serum Progesterone were lower in the unexplained recurrent pregnancy

loss group than those observed in the control group but were significantly increased ($p \leq 0.05$) after dydrogesterone treatment (Di *et al*., 2023); this result was consistent with the results of our study. This study aligns with the meta-analysis, which shows that the administration of dydrogesterone to patients with recurrent pregnancy loss (RPL) resulted in significantly higher ($p \leq 0.05$) progesterone levels compared to the control group. Those findings indicate that after dydrogesterone treatment, RPL patients have the potential to achieve successful pregnancies (Guo and Lu, 2021). Experts noted that progesterone levels could potentially provide insights into prognosis and indicate the effectiveness of medication in target-oriented treatment. However, the mechanisms underlying changes in intro-placental shift and progesterone secretion capacity remain poorly understood. All experts unanimously reported that dydrogesterone is a safe treatment option. They referred to the guidelines of reputable organizations such as ESHRE, the European Progestogen Club, Russia, and China, which recommend dydrogesterone for the treatment of threatened and recurrent miscarriages (Demir *et al*., 2023).

In a previous study, there was a notable decrease in progesterone levels in the serum of women with recurrent miscarriages compared to control (Alam *et al*., 2021). During pregnancy, progesterone, a potent immunomodulator, may aid in controlling and sustaining a transition from Th1 to Th2 at the interface. Building the mother's tolerance for the growing fetus depends on this change (Szekeres-Bartho, 2018). Progesterone can be substituted in clinical practice with the oral dydrogesterone. The hormone medication duphaston is a well-recognized therapeutic option for unexplained recurrent pregnancy loss (URPL) patients since it increases luteal function and lowers the chance of miscarriage. It has a reputable safety record (Mirza *et al*., 2016).

One study has shown that dydrogesterone can induce lymphocytes to produce progesterone, which up-regulates Th2 cytokines in T helper cells (Th) and downregulates Th1 cytokines to induce blocking antibodies to protect against miscarriage (Druckmann and Druckmann, 2005). The results of other studies revealed that dydrogesterone effectively regulated and promoted the production of progesterone-induced blocking factors by lymphocytes to regulate the immune function of the maternal-fetal interface, thereby increasing the pregnancy success rate of unexplained RPL patients (Guo and Lu, 2021).

Dydrogesterone is an oral retroprogesterone that is simple, well-tolerated, and safe. It is also a selective P4 receptor agonist. Since DYD does not affect the measurement of serum P4, this medication does not require further P4level management. In fact, because of structural variations between DYD and progesterone, DYD cannot be quantified by procedures used to measure progesterone levels regularly. Dydrogesterone is used in a variety of indications worldwide such as recurrent miscarriages, and luteal insufficiency (Lecourt *et al*., 2022).

Dydrogesterone is relatively inexpensive and considered to be reassuring when used during early pregnancy; therefore, it might be appropriate to administer it in all assisted reproductive technology (ART) cases employing Frozen–thawed embryo transfer in hormone replacement therapy (HRT) cycle (Ott *et al*., 2022). Despite having normal blood progesterone levels, women with URPL may not have normal progesterone receptor levels or levels at the fetal-maternal interface. Moreover, it has been demonstrated that stress causes progesterone levels to drop dramatically (Arck *et al*., 2007).

4. 2. 2 Estradiol concentrations

A significant decrease ($p \leq 0.05$) was shown by the estradiol concentrations analysis results (Table 4-2), in RPL patients without treatment compared to the control group (133.99 ± 7.45 and 139.55 ± 8.34 pg/ml, respectively). However, the result indicated that there was no significant difference ($p \leq 0.05$) between the control group and RPL patients receiving treatment in terms of estradiol concentrations.

One of the previous studies was consistent with our study, as it showed a significant decrease ($p \leq 0.05$) in the results of estradiol levels in the serum of recurrent miscarriage women without treatment compared to control (Alam *et al*., 2021). The role of mutations in genes such as alpha and beta-estradiol receptors has been investigated in recurrent abortion (Rahman *et al*., 2018). Estradiol plays an important role in embryonic growth and development in the first trimester of pregnancy and self-estradiol secretion is unstable before the complete formation of the placenta (Yang *et al*., 2022). A drop in the level of estradiol and progesterone hormones, which ensure the course of normal biochemical and physiological processes in the muscles of the uterus, leads to an increase in the contractile activity of the myometrium, which is manifested by the phenomena of placental insufficiency in the early embryonic and early fetal periods – spotting, threat of abortion, partial separation of the chorion (Colley *et al*., 2019; Lisova *et al*., 2021).

Numerous investigations have demonstrated the role of estradiol in many facets of immunological modulation during the implantation and development of embryos (Abdulhussain *et al*., 2020; Monteiro *et al*., 2021). The placenta and fetus require an adequate supply of nutrients and oxygen for growth and development. Blood supply to the uterus and placenta must thus gradually rise. Research has indicated that estradiol

contributes significantly to the dilatation and remodeling of the uterine arteries; therefore, estradiol could contribute in some way to enhancing uterine artery blood flow and encouraging fetal development (Mandala, 2020).

The high rate of spontaneous abortions occurring before 8 weeks of gestation is consistent with low levels of progesterone and estrogen; these results imply that low levels of estrogen may be a factor in unfavorable pregnancy outcomes (Yang *et al*., 2022). Dydrogesterone does not stop ovulation at recommended doses, it does not have estrogenic or androgenic properties, and it does not cause any metabolic side effects (Stute, 2021). Estradiol has been reported to down-regulate E-cadherin in several reproductive tissues, including the uterus (Rajabi *et al*., 2014).

4. 2. 3 Prolactin, FSH, LH, and Testosterone concentrations

The concentrations of these hormones in RPL patients without treatment and RPL patients with treatment were not significantly different compared to the control group. Two studies on hormone levels in the menstrual cycle following spontaneous miscarriage have been conducted, Low levels of follicle-stimulating hormone and luteinizing hormone (LH) were associated with reduced pituitary function, according to one theory. The second suggested decreased luteal estrogen and progesterone levels and lower LH levels (Vitzthum. 2020). Reduced fertility in the post-last miscarriage cycle would result from these hormonal changes (Jukic *et al*., 2010). It's possible that the concentration of hormones does not change after treatment with dydrogesterone because dydrogesterone is a progestin that doesn't have a significant impact on hormone levels. This is because dydrogesterone specifically targets the progesterone receptors in the body, rather than directly affecting hormone production.

4. 3 Biochemical Analysis

4. 3. 1 Epithelial-cadherin concentration

The findings of this study revealed a significant decrease ($p \leq 0.05$) in the levels of E-cad between RPL patients without treatment and the control group (16.96 ± 2.11 ng /ml, 21.6 ± 2.09 ng/ml, respectively). Interestingly, there was a significant increase ($p \leq 0.05$) in the levels of E-cad among RPL patients who received treatment, as shown in Figure 4-1.

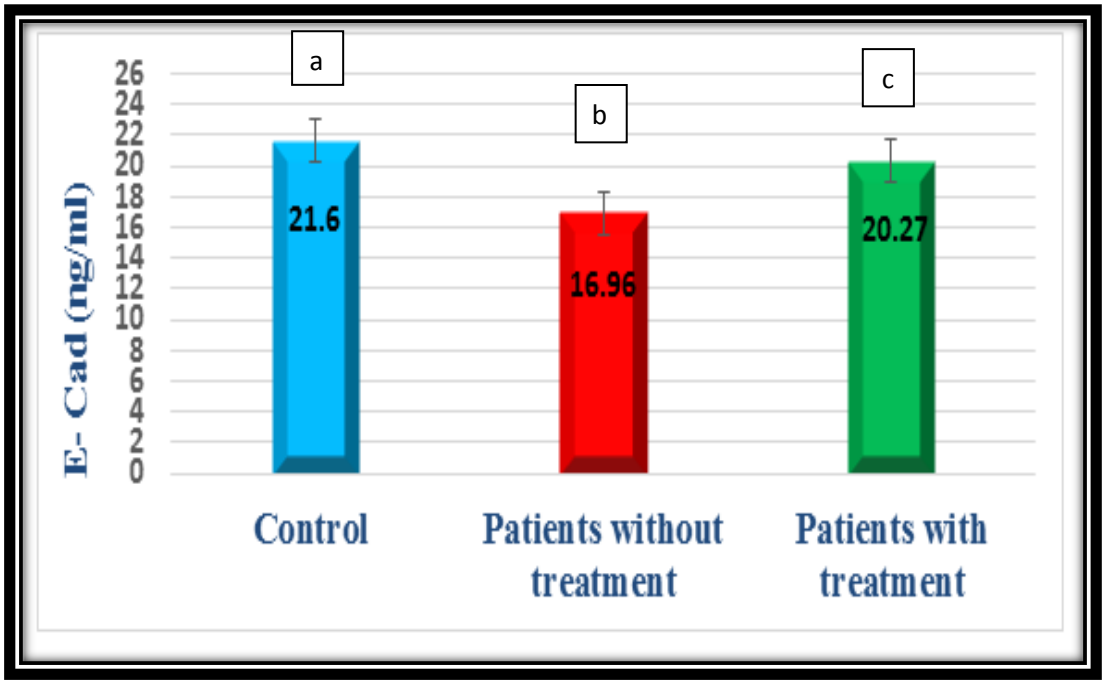


Figure 4-1 E-cadherin (Mean) (ng/ml) in control and RPL groups

This result agrees with Yang *et al*., (2017) findings which suggested a potential role of E-cadherin in the implantation processes and altered expression in women with reproductive failure and E-cad low-level association with RPL (Yang *et al*., Also the results agree with Mahdi *et al*., (2021) have showed a significant difference in E-cad concentration between RPL patients and control. Additionally, hypothesized that placental syncytiotrophoblast produces hormones for fetal development and upholds immunological tolerance in addition to being in charge of

transporting oxygen, nutrients, and wastes. When cytotrophoblasts fuse, E-cad undergoes dynamic alterations, and its downregulation occurs simultaneously with cell fusion (Wu *et al*, 2017). Likewise, Verma *et al*., (2020), have demonstrated that E-cadh expression is crucial for embryonic growth. E-cad knockout mice are unable to survive during implantation because they are unable to develop functioning trophectoderm. Also, when extravillous trophoblasts (EVTs) move or invade the cell column during epithelial-mesenchymal transition (EMT), trophoblast cells have been shown to express fewer E-cadherin levels (Deligdisch-Schor and Mareş Miceli, 2020).

A risk factor for a stable pregnancy and subsequent effects on the embryonic adhesion process may be a low serum level of E-cadherin in the RPL group (White and Plachta, 2015). The result of the present study showed a highly significant increase ($p \leq 0.05$) in E-cadherin concentration between RPL with the treatment group and RPL without the treatment group as shown in Figure 4- 1. Dydrogesterone has been widely used in the treatment of threatened miscarriage and assisted reproductive technology, showing promising outcomes (Griesinger *et al*., 2018).

Estradiol and progesterone are known to have significant effects on the endometrium and regulate the expression of several genes. It is unknown if steroid hormones control the dynamic variations in E-Cad sorting in the endometrial epithelium (Canse *et al*., 2023); While in the previous study demonstrated that steroid hormones directly affect E-Cad sorting in the endometrial epithelium (Tiwari *et al*., 2021). Some studies have reported a loss of E-Cad in the endometrial epithelial cells at the site of embryo implantation; some have reported an increase in expression and/or higher apically sorted E-Cad while others have reported no change

in expression of E-Cad at the time of implantation (Tiwari *et al*., 2021; Yuan *et al*., 2021). One most normal epithelial cell E-cadherin is involved in lateral attachments between cells and regulated by intracellular calcium. The link to P4 in the endometrium is likely through the action of calcitonin, a Progesterone-induced protein in both human and rodent endometrium (Xiong *et al*., 2021). In a previous experimental study, it was found calcitonin may facilitate uterine receptivity by down-regulating the E-cad expression in rodent uterine epithelium and by inducing the tTGase expression in human endometrial epithelial cells (EECs) (Dorostghoal *et al*., 2017). This protein is one of the adhesion proteins necessary for cell adhesion in the embryo of mammal cells. After treatment, the reason for the increased cadherin concentration may be due to the effect of steroids on adhesion proteins, but the mechanism is unknown.

4. 3. 2 Progesterone Induced Blocking Factor Concentrations

The study found that RPL patients without treatment had significantly lower ($p \leq 0.05$) levels of PIBF (6.87 ± 1.45 ng/L) compared to the control (12.14 ± 1.04 ng/L). However, there was no significant difference ($p \leq 0$ .05) in PIBF between RPL patients with treatment and control (12.03 ± 1.16 ng/L, 12.14 ± 1.04 ng/L., respectively) as shown in Figure (4-2).

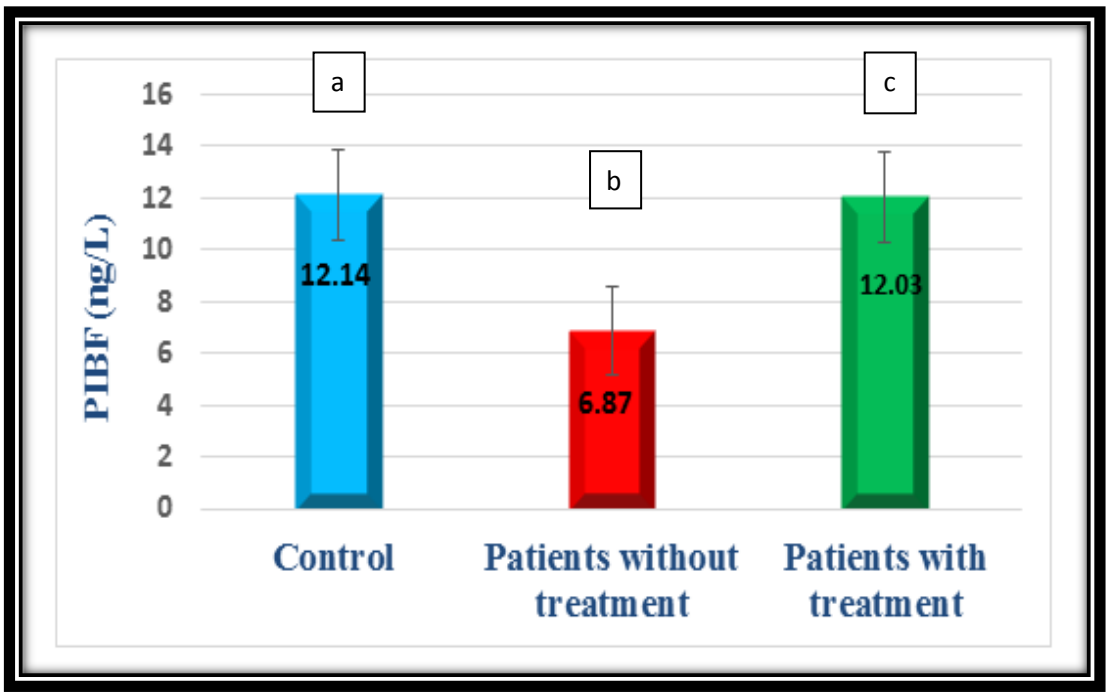


Figure 4-2 PIBF concentrations (Mean) (ng/L) in control and RPL groups

Other studies have shown that progesterone has anti-inflammatory and immunomodulatory properties, which are mediated through its nuclear and membrane progesterone receptors (PRs) (Polikarpova *et al*., 2019; Ren *et al*., 2021).

Laskarin *et al*., (2002) reported that the progesterone-induced blocking factor may participate in the immune response by regulating progesterone at the maternal-fetal interface to maintain pregnancy.

Furthermore, in response to progesterone, Gamma delta T cells produce progesterone-induced blocking factor (PIBF), which has been shown in mice to inhibit natural killer cell activity and have anti-abortive effects (Fedotcheva *et al*., 2022). According to some researchers, the fetus induces lymphocytes to make more progesterone receptors, which bind Progesterone and result in the production of PIBF (Vidal *et al*., 2023). *In vitro* experiment study, it was involved a culture of peripheral blood mononuclear cells (PBMC) mixed with testosterone, the Th1 (pro-inflammatory) cytokines IFN-γ and TNF-α are significantly reduced,

while the Th2 cytokines IL-4 and IL-6 are significantly elevated. Thus, exposure of PBMC to dydrogesterone dramatically lowers Th1/Th2 cytokine ratios, suggesting a reduction in Th1 or pro-inflammatory cytokine bias. The cytokine-modulating effects of testosterone are inhibited by the progesterone receptor antagonist RU486; this suggests that the progesterone receptor is the mediating mechanism for these effects (Raghupathy and Szekeres-Bartho, 2022).

Dydrogesterone can suppress IL-17, a powerful chemotactic and pro-inflammatory cytokine (AbdulHussain *et al*., 2020). Research conducted on animals has connected IL-17 to human miscarriage and embryo loss. It has been demonstrated that IL-17 injections during pregnancy cause embryonic loss in mice and that IL-17 levels in the decidua and peripheral blood of women who repeatedly miscarry are greater (Raghupathy and Szekeres-Bartho, 2022), and elevated serum IL-17 and Th17/Treg cell ratios in peripheral blood and at the mother-fetal interface are linked to an increased frequency of unexplained RPL (Qian *et al*., 2018).

The cellular immune effect is closely related to the development of RPL, pro-inflammatory and anti-inflammatory cytokines have a crucial influence on the success or failure of pregnancy. The DYD has been able to increase the production of (IL-10, and PIBF) and decrease the production of IFN-γ (Hudic *et al*., 2016). This study was agreed with our current study. The one of results revealed that dydrogesterone effectively regulated and promoted the production of PIBF by lymphocytes to regulate the immune function of the maternal-fetal interface, thereby increasing the pregnancy success rate of RPL patients (Guo and Lu, 2021). Dydrogesterone is not detected by anti-progesterone antibodies,

but as it binds to the progesterone receptor it can induce PIBF to the same extent as natural progesterone (Kalinka and Szekeres-Bartho, 2005).

In women who experience complications, such as recurrent spontaneous abortion, a decrease in PIBF concentrations was observed. Cohen *et al*., (2016) concluded that the presence of Progesterone without allogeneic stimulation is correlated with the increase in serum PIBF.

4. 4 Relative Gene Expression of Progesterone Receptors Genes (mPRα, mPRß and NPR)

The data presented in Table 4-3 illustrates the comparison of study parameters between patients with RPL and the control group.

Table 4-3 Relative Gene Expression of mPRα, mPRß and NPR between RPL patients and Control.

Groups / Parameters	Control (36)	RPL patients without treatments (35)	RPL patients with treatments (35)	p. value
mPRα	1.16 ± 0.60^{a}	2.49 ± 1.25^{b}	1.39 ± 0.70^{c}	**0.012**
mPRß	1.11 ± 0.54^{a}	0.67 ± 0.22^{b}	1.44 ± 0.73^{c}	**0.004**
NPR	1.06 ± 0.41	0.90 ± 0.40	1.08 ± 0.49	0.346
($p \leq 0.05$) is significant. Data are represented as (Mean $\pm$ SD).				

4. 4. 1 Relative gene expression of mPRα gene

The results of relative gene expression for progesterone membrane receptor (mPRα) (Table 4-3) (Figure 4-3) have indicated significantly higher gene expression ($p \leq 0.05$) in RPL patients without treatment and control (2.49 ± 1.25, 1.16 ± 0.60, respectively), While found none significantly increase in mPRα gene expression between RPL patients with treatment and control (2.49 ± 1.25, 1.39 ± 0.70 respectively).

4. 4. 1. 1 Relative Gene Expression of mPRα Between Control and RPL Group

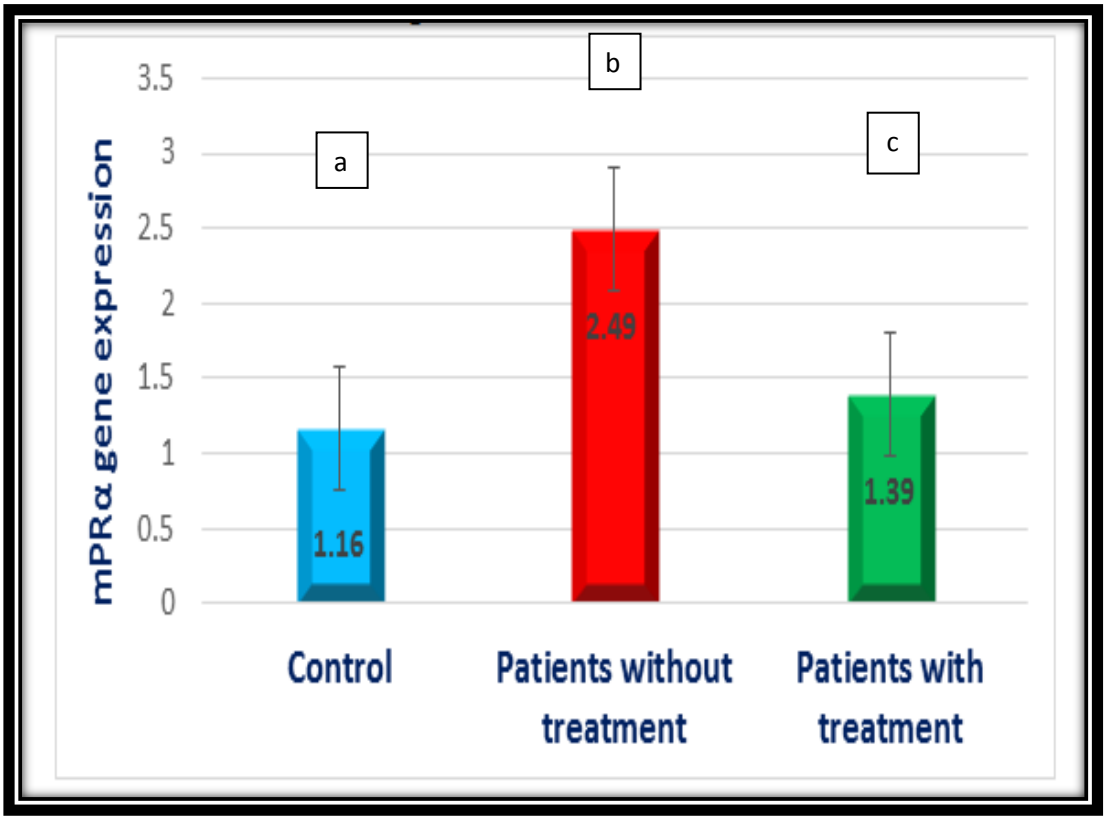


Figure 4-3 Relative gene expression of mPRα (Mean) in control and RPL groups

4. 4. 2 Relative Gene Expression of mPRß Gene

The results of (figure 4-4) patients who had repeated miscarriages without receiving dydrogesterone therapy showed a significantly lower expression of progesterone membrane receptors (mPRß) in their endometrial tissue. Additionally, there was a significant increase in gene expression of mPRß between RPL patients receiving treatment and those without treatment ($p \leq 0.05$).

4. 4. 2. 1 Relative Gene Expression of mPRß Between Control and RPL Group

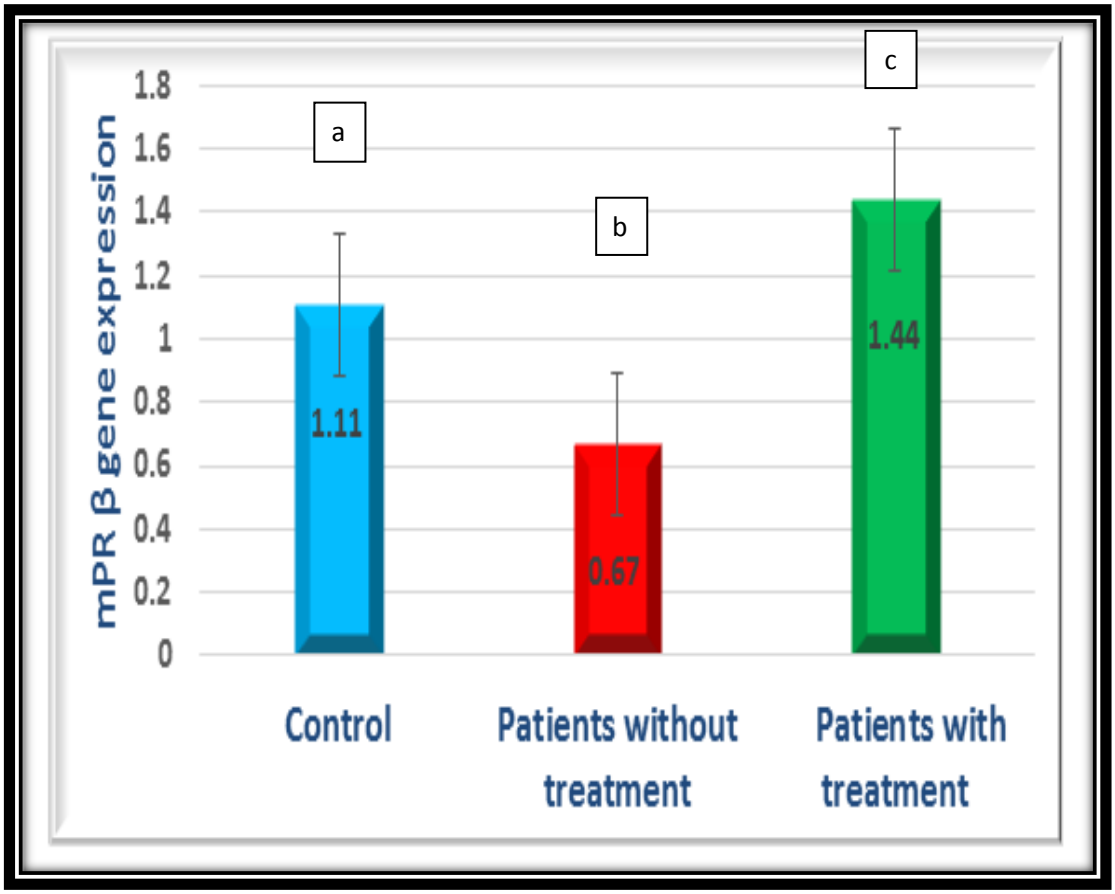


Figure 4- 4 Relative gene expression of mPRß (Mean) in control and RPL groups

4. 4. 3 Relative Gene Expression of NPR Gene

The analysis of gene expression levels of NPR showed that there was very little variation between untreated patients (0.90 ± 0.40) and the control groups (1.06 ± 0.49) (Figure 4-5). The same findings were observed when comparing the gene expression of NPR in treated and untreated patients.

4. 4. 3. 1 Relative Gene Expression of NPR between Control and RPL Group

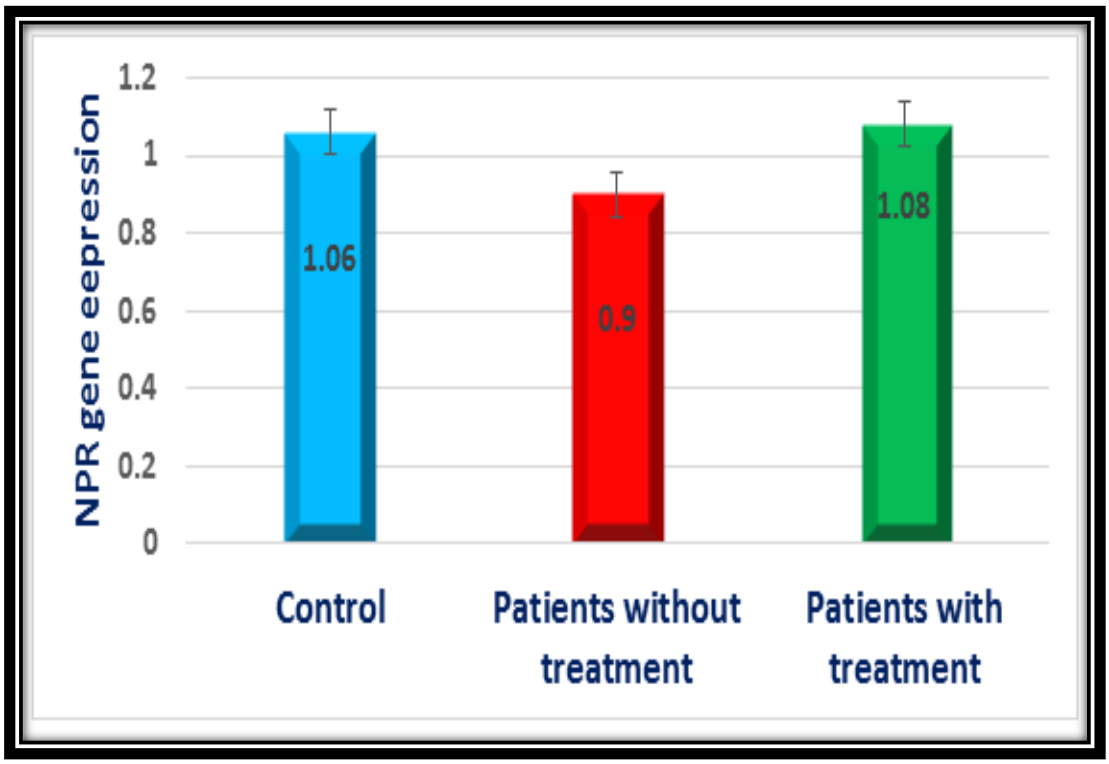


Figure 4-5 Relative gene expression of NPR (Mean) in control and RPL groups

Currently, progesterone is demonstrated to function through a genomic activity mediated via the nuclear receptors and a non-genomic activity mediated via the extranuclear receptors (Marquardt *et al*., 2019).

One supporting study proved that in the RSA patients, the expression of mPR-β in the endometrium was remarkably lower than in the normal control subject. During pregnancy, extravillous trophoblasts (EVTs) establish blood supplement for the growing fetus by invading the maternal decidua by remodelling the local vasculature (Harris, 2010; Li *et al*, 2021).

This result agrees with Rahnama *et al*., (2019) Findings that suggested a potential role of mPR-β in the implantation processes, altered expression in women with reproductive failure, and reduction in

expression of mPR-β are likely to contribute to the etiology of RPL. This result disagrees with our study that found the relative gene expression of mPRα was increased significantly ($p \leq 0.05$) in patients without treatment. In our study, we observed non-significantly ($p \leq 0.05$) lower expression of nuclear progesterone receptor in the endometrium of women with RPL as compared with the controls which agreed with a study that showed lower expression of progesterone levels and progesterone receptor in the RPL group using immunohistochemistry analysis on endometrial tissue (Tuckerman *et al*, 2004). Also in line with the present results, Salazar and Calzada, (2007) reported a significantly lower ($p \leq 0.05$) PR and ER in cases with RM compared with fertile women.

An essential steroid hormone, progesterone serves a crucial function in supporting pregnancy. Progesterone receptors (PRs), which include membrane progesterone receptors (mPRs) and nuclear progesterone receptors (NPRs), are necessary for progesterone action (Haas *et al*., 2019). One study revealed that endometrium ER and PR expression reduction might lead to a decrease in estrogen and progesterone efficiency, insufficient growth hormone secretion, or ovulation disorders, thereby affecting normal endometrium growth (Lietaer *et al*., 2021).

The reason for the decreased expression of progesterone receptors may be due to a decrease in estrogen concentration, because: Estrogen stimulates the expression of (ERa), progesterone receptor A, and progesterone receptor B, while P4 down regulates the expression of these receptors (Zhang *et al*., 2021). Disruption of these processes can result in infertility, requiring remediation by Hormonal replacement Treatment (Adda-Herzog *et al*., 2018). Nonetheless, a growing body of data from basic science and clinical trials indicates that exogenous hormone therapy

may have adverse consequences on endometrial receptivity (Fox *et al*., 2016; Salamonsen *et al*., 2016). Also, supra-physiological levels of sex steroids during Hormonal replacement Treatment may raise the risk of late obstetric complications (Mak *et al*., 2016).

As noted from previous study there is a physiological variation throughout the menstrual cycle in endometrial expression of both PR and ER, but the present study evaluated the status of the receptors in the mid luteal phase which represents the most relevant period for establishing and maintenance of pregnancy (Gibson *et al*., 2020). Aberrant PR expression and altered PRA/PRB ratio in endometrial stromal cells can disrupt endometrial progesterone signaling, resulting in increased risk of implantation failure and recurrent pregnancy loss, in addition to hormonal fluctuations (Zhou *et al*., 2016). However, most previous investigations measured hormone concentrations in serum, while hormone concentrations in local endometrial tissues could be more directly correlated with PR expression (Zhang *et al*., 2021).

The progesterone hormone is crucial to the implantation process and pregnancy maintenance. Therefore, its lack and a reduced luteal phase may cause issues with endometrial growth that are connected to RPL. In some cases, Progesterone supplementation for RPL patients does not lead to better pregnancy outcomes, as shown by other study (Coomarasamy *et al*., 2016). It is asserted that the issue extends beyond the availability of hormones and also includes the abnormalities of PRs. RPL has been associated with decreased progesterone receptor expression by the embryo and endometrium (Hickman *et al*., 2002). Additionally, a link between RPL and a variation in the PR gene's intron G is connected to implantation failure. One study found a strong correlation between the

SNPs in the PR genes and recurrent pregnancy loss (RPL) (Refeat *et al*., 2021).

Progesterone plays a vital role during pregnancy by regulating the mother's immune responses in preventing fetal miscarriage. The major role of progesterone in early pregnancy led clinicians and researchers to hypothesize those low-levels of progesterone could be the cause of miscarriages (Aruna *et al*., 2010). Progesterone is essential for the development of a receptive endometrium, it is necessary to also consider: progesterone levels (as treatment with Dydrogesterone helps in the prevention of RPL, Progesterone receptors expression levels (reduced levels have been reported in RPL women) , its transcription, its relation with estrdiol receptor (ER) and its role in immune modulation along with the gene mutations in populations of diverse ethnic and geographic backgrounds before completely ruling out the role of Progesterone receptors in the manifestation of RPL (Refeat *et al*., 2021).

The previous study did not find an increase in the concentration of serum progesterone, possibly due to negative feedback induced by dydrogesterone on progesterone secretion of the corpus luteum (Zhu *et al*., 2017). Furthermore, increasing progesterone or dydrogesterone supplementation does not proportionally increase serum levels, and uterine progesterone levels do not correlate well with serum levels (Mol *et al*., 2018). It is long known that prolonged progestin treatment leads to loss of PGR via different mechanisms including ligand-induced degradation (Yang *et al*., 2011). DYD has a favorable tolerability profile, a high selectivity for progesterone receptors, and a high oral bioavailability (Griesinger *et al*., 2020). Fechner *et al*., (2007) also showed a non-significant reduction in PGR expression by dydrogesterone. We conclude from this study the deficiency of

progesterone receptors gene expression may pose a problem in the treatment process. However, after treatment, we noticed an increase in gene expression of the receptors (NPR and mPRß), this indicates an effective effect of the treatment used on the gene expression of endometrial progesterone receptors. As for progesterone receptors (mPRα) the prolonged progestin treatment leads to loss of PGR (mPRα) via different mechanisms including ligand-induced degradation.

4. 5 The Correlation Coefficient

The correlation between studied Parameters in RPL and control was also evaluated in this study and is presented in Table 4-4.

Table 4-4 Correlations between studied variables in RPL and control cohorts

		Age	BMI	Prolactin	FSH	LH	Testosterone
Age (years)	r	1	0.169	-0.073	0.129	**-0.202***	-0.141
	p		0.084	0.456	0.187	0.038	0.148
BMI (kg/m^2)	r	0.169	1	0.074	0.021	0.001	-0.154
	p	0.084		0.448	0.832	0.989	0.114
Prolactin (ng/ml)	r	-0.073	-0.074	1	-0.155	0.122	-0.112
	p	0.456	0.448		0.112	0.211	0.255
FSH (mIU/ml)	r	0.129	0.021	-0.155	1	-0.128	-0.068
	p	0.187	0.832	0.112		0.189	0.491
LH (mIU/ml)	r	**-0.202***	0.001	0.122	-0.128	1	-0.143
	p	0.038	0.989	0.211	0.189		0.144
Testosterone	r	-0.141	-0.154	-0.112	-0.068	-0.143	1
	p	0.148	0.114	0.255	0.491	0.144	
* Correlation is significant at the 0.05 level (2-tailed)							

As shown in Table 4-4, no significant ($p \leq 0.05$) associations were found between the studied hormonal levels and age or BMI, but there was only a tendency for a significant negative correlation between LH maternal ages in women with recurrent miscarriage. Reproductive

endocrine function progressively declines with advancing age. In detail, this process seems to affect the luteinizing hormone (LH) system and androgen production (Conforti *et al*., 2021). Luteinizing hormone plays an important role in follicle growth by contributing to follicle maturation, fertilization and embryo quality. It affects the endometrium by promoting the decidualization of endometrial stromal cells and embryo implantation (Freis *et al*., 2019).

4.5.1. Correlation between Progesterone and estradiol concentration

The results of the correlation analysis in Figure 4-6 indicate a significantly positive correlation (r = 0.522) between Progesterone and estradiol concentrations in RPL patients who did not receive any treatment. Similarly, in RPL patients who received treatment, there is a significantly positive correlation (r = 0.390) between Progesterone concentration and Estradiol (pg/ml), as shown in Figure 4-7.

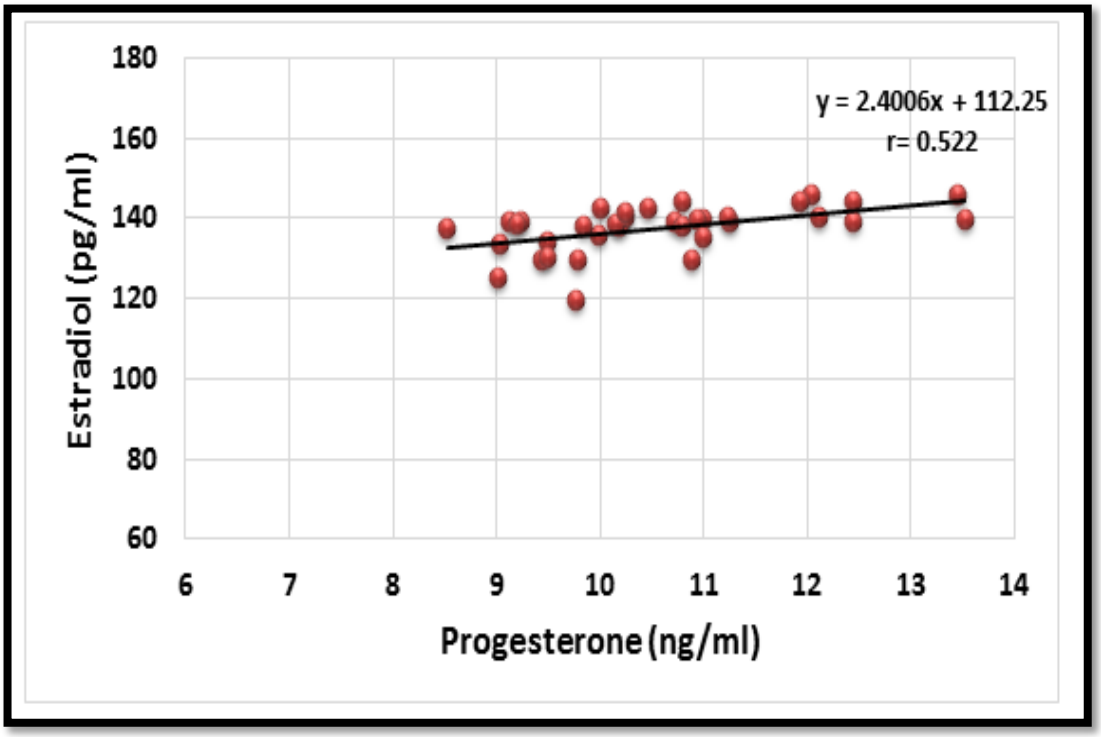


Fig (4-6): Correlation between Progesterone concentration (ng/ml) and Estradiol (pg/mL) in RPL patients without treatment.

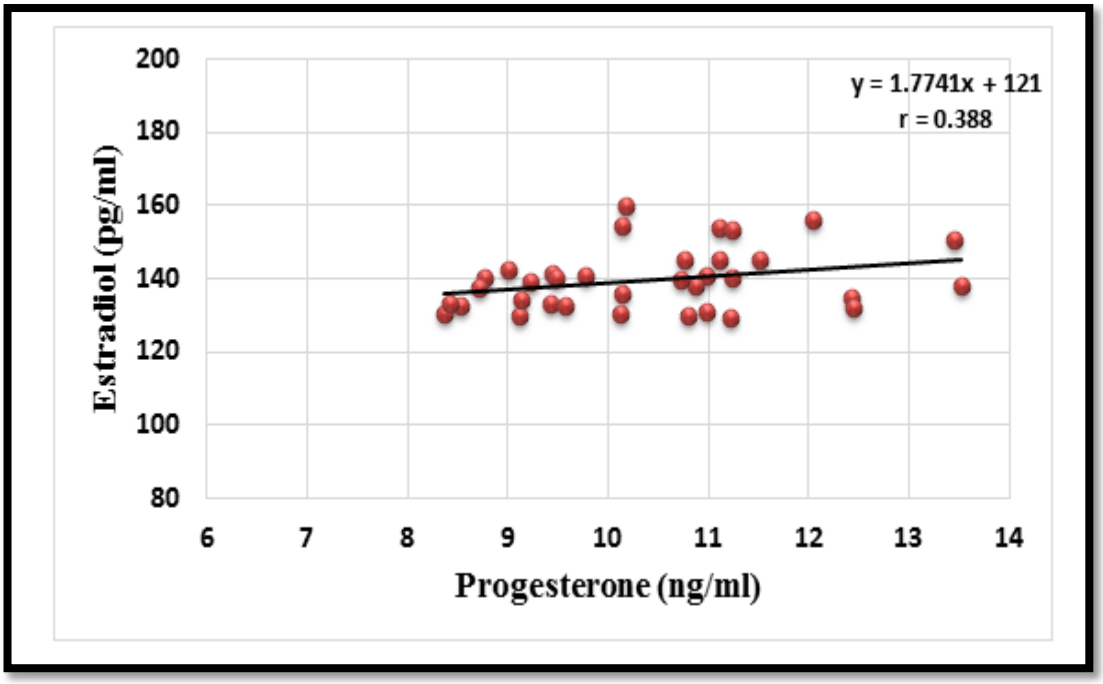


Fig (4-7): Correlation between Progesterone concentration (ng/ml) and Estradiol (pg/ml) in RPL patients with treatment.

During the luteal phase, hormonal changes are mainly regulated by progesterone and estrogen. These hormones collaborate to support the growth and maintenance of the endometrial lining, preparing it for potential egg implantation. Additionally, elevated progesterone levels inhibit further ovulation as the body readies itself for a possible pregnancy (Muneeba *et al*., 2023).

Furthermore, High Estrogen levels have also been associated with miscarriage. It binds to uterine receptors, stimulating PR expression in uterine cells and enhancing the endometrial response to progesterone. (Gomaa *et al*., 2023).

4. 5. 2 Correlation between Progesterone and FSH

The data presented in Figures 4-8 demonstrate a noteworthy significantly negative correlation (r = - 0.46) between Progesterone and FSH levels in RPL patients without treatment. In addition, Figure 4-9 indicates a comparable significantly negative correlation (r = - 0.390)

between Progesterone concentrations and FSH (mIU/ml) in RPL patients who experienced treatment.

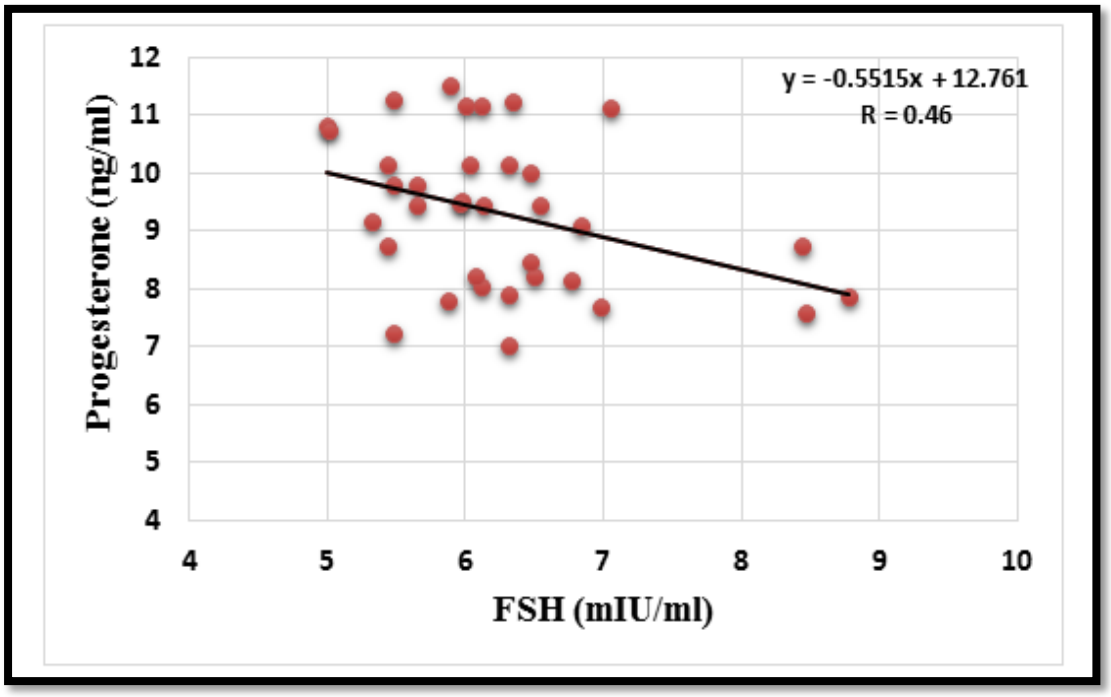


Fig (4-8): Correlation between Progesterone (ng/ml) and FSH (mIU/ml) in RPL Patients without Treatment.

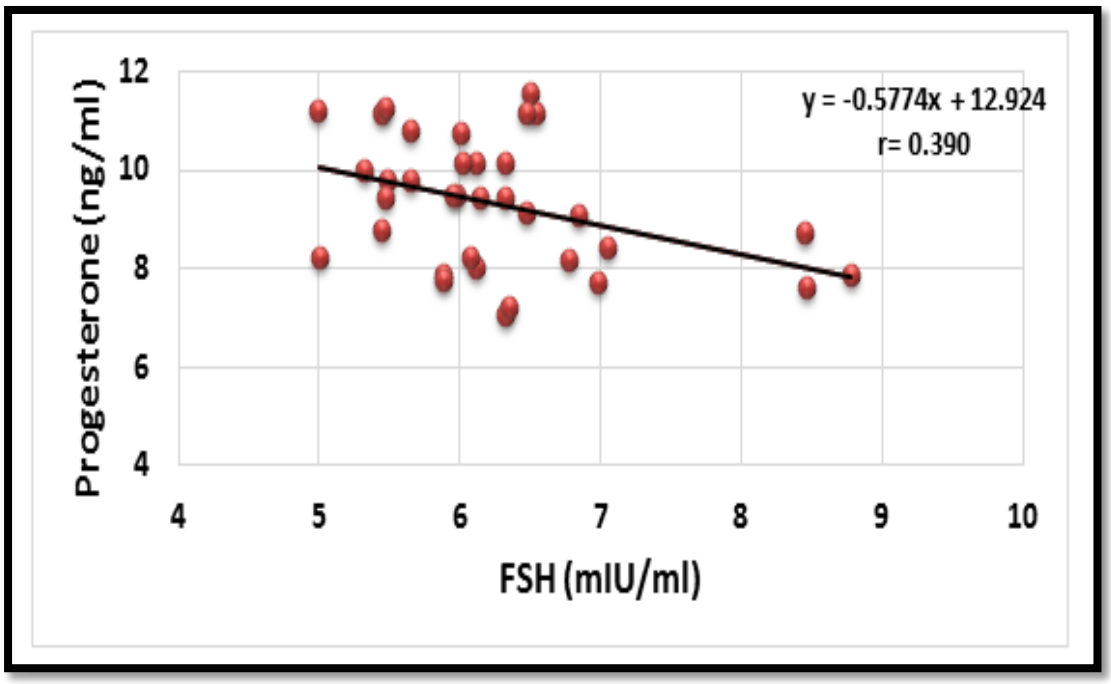


Fig (4-9): Correlation between Progesterone concentration (ng/ml) and FSH (mIU/ml) in RPL patients with treatment.

The progesterone is synthesized and released largely by the luteal phase (Allaway *et al*., 2017). In this stage, the secretion of progesterone and estradiol rises and falls significantly. Regression of the luteal phase is linked to a decrease in hormone output. At the so-called luteal-follicular transition, the concentration of FSH begins to grow in the late luteal phase (Broer *et al*., 2011).

The presence of estradiol and progesterone in the luteal phase results in negative feedback on both FSH and LH secretion. Because of this negative feedback, the levels of FSH and LH are relatively low in the luteal phase. The progesterone: estrogen ratio slows down the GnRH pulse generator to about one pulse every 4 h. This inhibits FSH release and thus restricts follicular development during the luteal phase (Shaw *et al*., 2010).

4. 5. 3 Correlation between Progesterone with PIBF and E-cad

According to the current study, there is a significantly positive correlation (r = 0.382) between the concentration of progesterone and PIBF in RPL patients who did not receive any treatment (as shown in Fig 4-10). Moreover, the study also demonstrated a significantly positive correlation (r = 0.660) between the concentration of PIBF (ng/ml) and progesterone (ng/ml) in RPL patients who received treatment (as depicted in Fig 4-11).

There is no significantly positive correlation (r = 0.237) between Progesterone and E-cad concentration in RPL patients without treatment (Fig 4- 12).

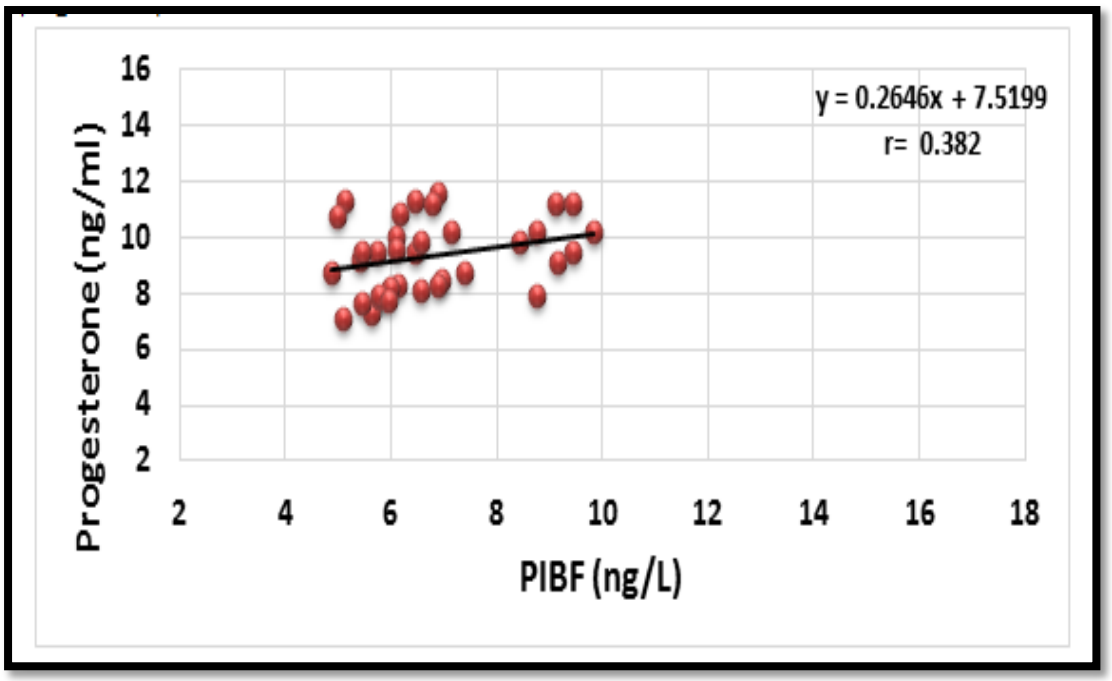


Fig (4-10): Correlation between Progesterone concentration (ng/ml) and PIBF (ng/L) in RPL patients without treatment.

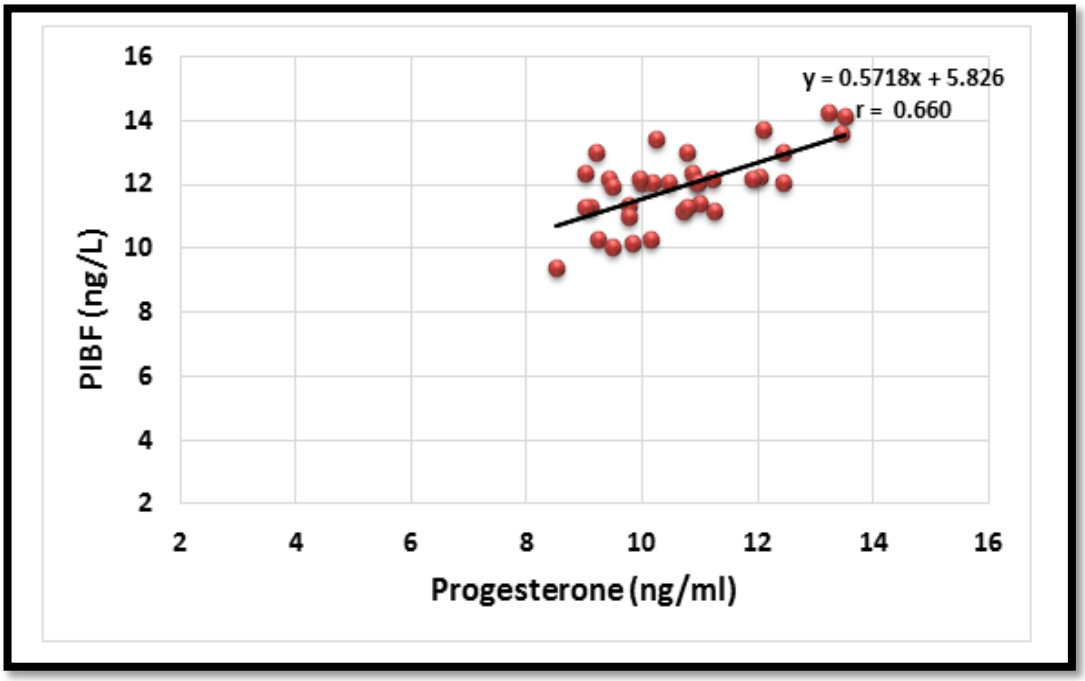


Fig (4-11): Correlation between Progesterone concentration (ng/ml) and PIBF concentration (ng/ml) in RPL patients with treatment.

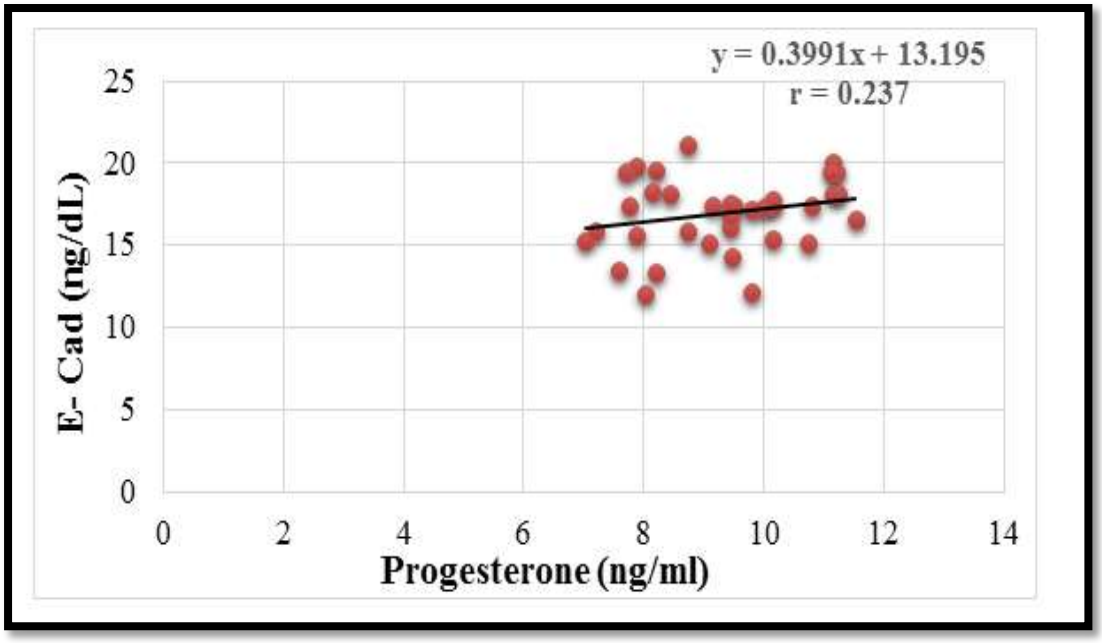


Fig (4-12): Correlation between Progesterone (ng/ml) and E-Cad concentration (ng/dl) in RPL patients without treatment.

Progesterone can influence T-cell activation through PIBF and LIF secretion, regulating it via membrane PR (mPR). It is known that T-cell activation is associated with a rapid increase in the intracellular free calcium concentration. This process is thought to be associated with mPR, and progesterone is capable of regulating the intracellular free calcium concentration (Cantonero *et al*., 2020). The activation of PGRMC1 (progesterone receptor membrane component 1) by progesterone attenuates this Ca2+ signaling pathway, which leads to a decrease in the Ca2+-dependent NFAT1 nuclear accumulation. This progesterone and PRGMC1-related pathway can probably suppress T-cell activation (Fedotcheva *et al*., 2022).

Other previous study showed that dydrogesterone effectively regulated and promoted the production of progesterone-induced blocking factors by lymphocytes to regulate the immune function of the maternal-fetal interface, thereby increasing the pregnancy success rate of RPL patients (Guo and Lu, 2021).

Both progesterone and PIBF alter the cytokine balance in favour of a Th2 response. In the uterus progesterone induces the differentiation of naïve T cells upon antigen recognition into Th2 memory cells. Lymphocytes from pregnant women respond to progesterone treatment with decreased production of Th1 cytokines and an increased production of Th2 cytokines. Lymphocytes from women with recurrent miscarriage or pre-term delivery tend to produce elevated levels of Th2 cytokines in the presence of PIBF. These data indicate that progesterone and PIBF alter the cytokine balance and contribute to decreased cell-mediated responses during pregnancy (Szekeres-Bartho and Schindler, 2019).

Progesterone, probably via endometrial calcitonin induction leading to increased intracellular calcium, could regulate E-cadherin expression. Calcitonin is known to be a potential regulator of implantation. Thus, E-cadherin may possess a dual function. In the preliminary phases, its expression at the cell surface is required to ensure adhesiveness. In contrast, E-cadherin may be subsequently down-regulated to enable epithelial cell dissociation and blastocyst invasion (Al-Jubouri *et al*., 2021).

Estradiol and progesterone are known to have profound effects on the endometrium and modulate the expression of many genes, whether the dynamic changes in E-Cad sorting in the endometrial epithelium are regulated by steroid hormones is unknown (Deligdisch-Schor and Mareş Miceli, 2020)

4.5.4 Correlation between Progesterone and progesterone receptors gene expression

An inverse relationship (r = - 0.132) was found between mPRα gene expression and Progesterone in untreated RPL patients (Fig 4-13). There is a negative correlation (r = - 0.014) between the mPRα gene expression

and Progesterone in RPL patients who did receive treatment (as shown in Fig 4-14).

The Current study found a significantly positive correlation between mPRß gene expression and progesterone in both untreated (r = 0.380, Fig 4-15) and treated RPL patients (r = 0.156, Fig 4-16). Additionally, there was a significantly positive correlation between NPR gene expression and progesterone concentration in untreated (r = 0.319, Fig 4-17) as well as treated RPL patients (r = 0.346, Fig 4-18).

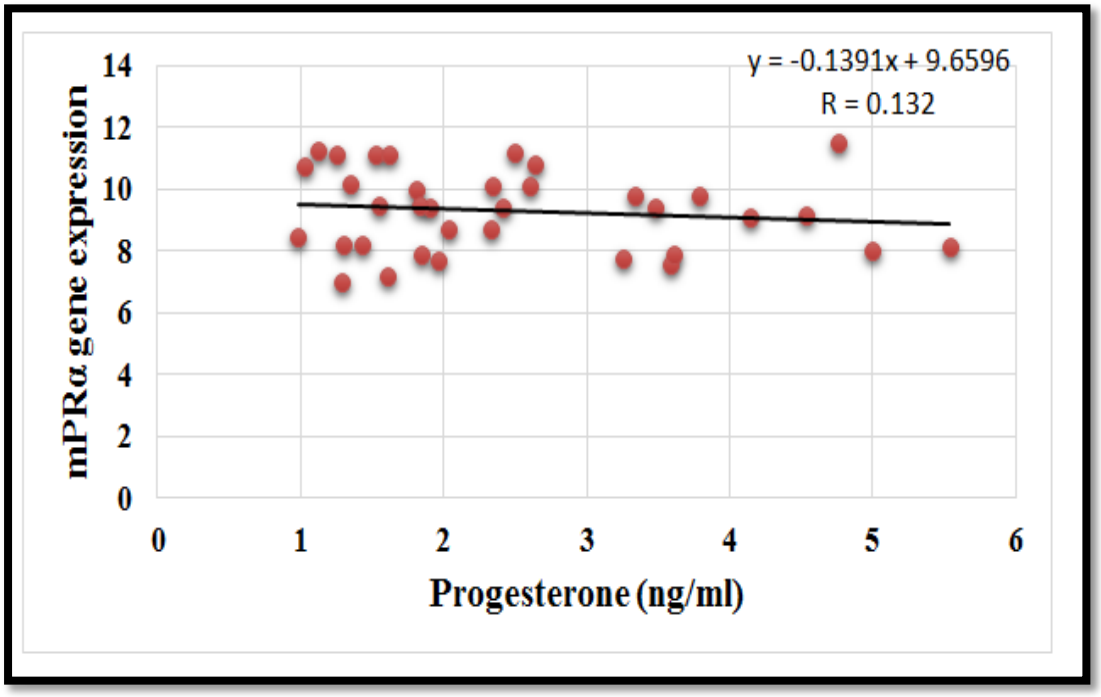


Fig (4-13): Correlation between mPRα gene expression and Progesterone (ng/mL) in RPL patients without treatment.

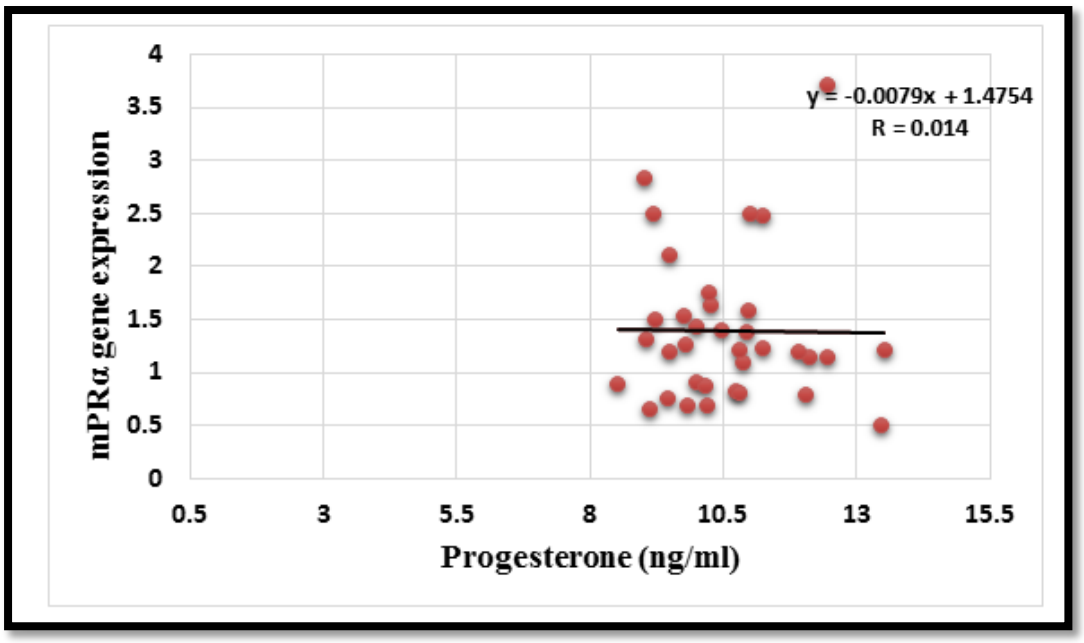


Fig (4-14): Correlation between mPRα gene expression and progesterone concentration (ng/mL) in RPL Patients with Treatment.

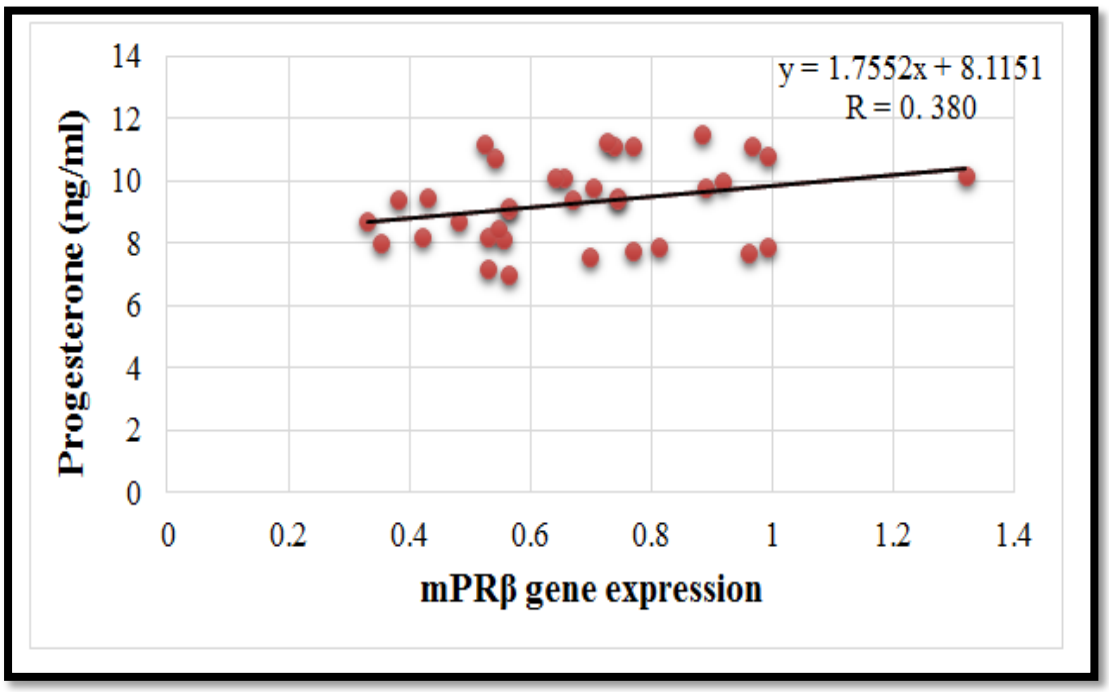


Fig (4-15): Correlation between mPR β gene expression and Progesterone concentration (ng/mL) in RPL patients without treatment.

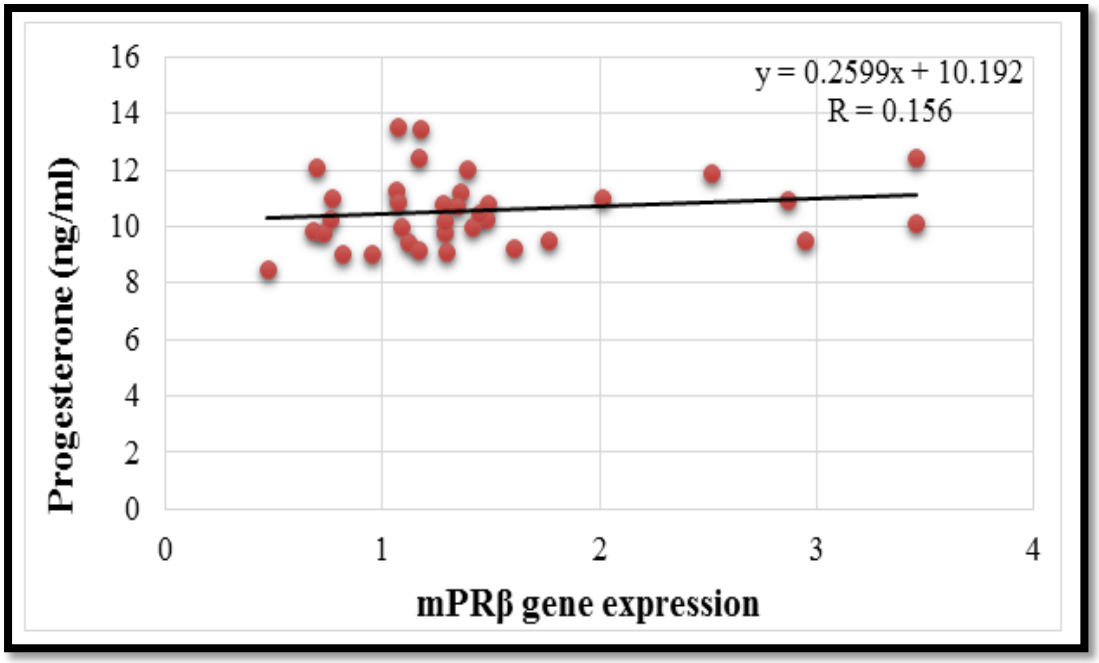


Fig (4-16): Correlation between mPRß gene expression and Progesterone concentration (ng/ml) in RPL patients with treatment.

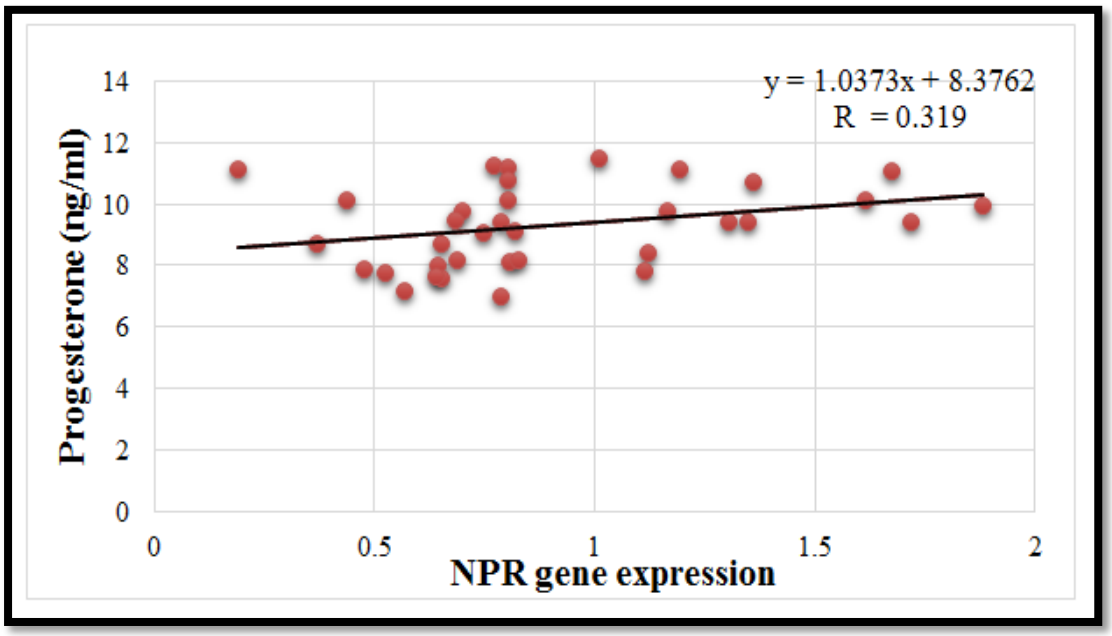


Fig (4-17): Correlation between NPR gene expression and progesterone concentration (ng/ml) in RPL patients without treatment.

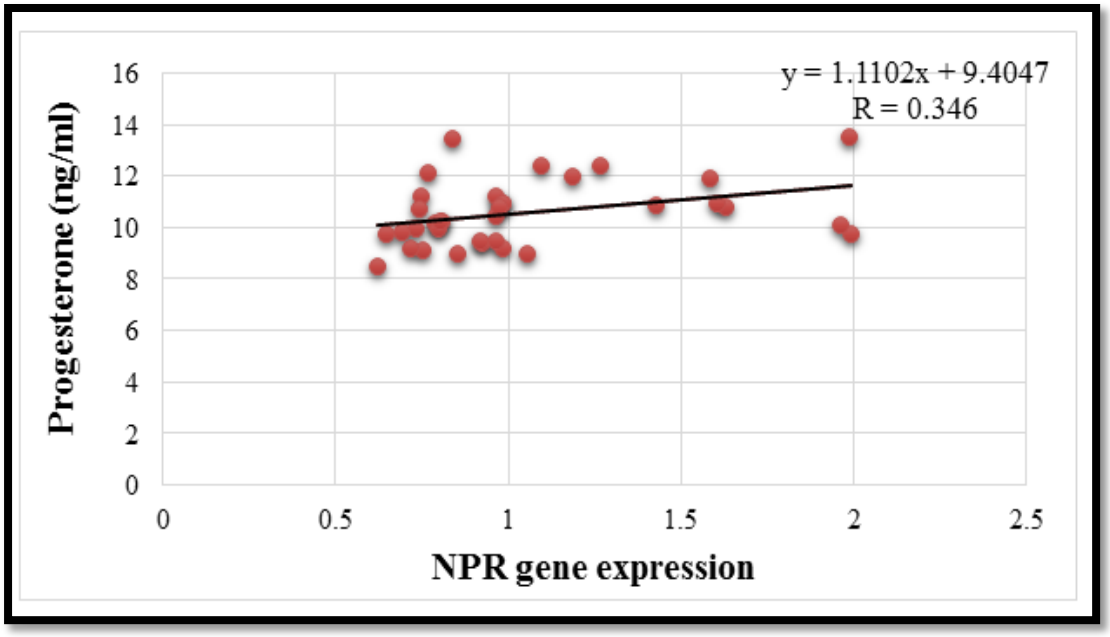


Fig (4-18): Correlation between NPR gene expression and progesterone concentration (ng/mL) in RPL patients with treatment.

The progesterone physiologic effects are mediated by an interaction with its receptors called progesterone receptors (PRs). The therapeutic application of progesterone is targeted to prevent pregnancy complications such as recurrent miscarriage. There is yet controversy in the usage of this clinical method. Some studies have indicated the benefit of progesterone in the treatment of RPL; whereas other studies revealed negative results. The latter asserts that the inefficiency of progesterone is due to its responsiveness rather than its availability. In fact, in these cases, the expression or function of progesterone receptors is involved (Rahnama *et al*., 2019).

Progesterone is known to contribute significantly to the crosstalk between different cells in the uterus and placenta to affect different processes. Progesterone influences decidualization by controlling the differentiation of endometrial stromal cells, and disruption of this signaling can lead to pregnancy complications such as recurrent miscarriage and pre-eclampsia, emphasizing the importance of

progesterone in this cellular crosstalk (Raghupathy and Szekeres-Bartho, 2022).

A defect in progesterone function has been attributed to dysregulation of PR rather than the availability of the hormone and disturbed receptor expression has been linked to pregnancy loss, although the exact mechanism is poorly understood. Dysregulation of PR may be associated with reduced receptor expression or linked to genetic polymorphisms of PR affecting its response to progesterone; Furthermore, RM may be linked to progesterone resistance as a result of epigenetic modification (Gomaa *et al*., 2023).

Recurrent pregnancy loss might be linked to reduced endometrial progesterone receptors and appears to be more related to the non-genomic activity of progesterone. Endometrial receptor expression correlates to tissue hormonal level rather than to serum hormonal level (Salazar and Calzada, 2007).

4. 5. 5 Correlation between progesterone receptors gene expressions with PIBF

The study found an inverse correlation between mPRα gene expression and PIBF in both untreated (r = - 0.147, Fig 4-19) and treated RPL patients (r = - 0.152, Fig 4-20). Additionally, there was a positive correlation between mPRβ gene expression and PIBF in untreated RPL patients (r = 0.075), also, a similar positive correlation was observed for mPRß gene expression and PIBF in treated RPL patients (r = 0.0297, Fig 4-22). Furthermore, the study revealed a positive Correlation with NPR gene Expression and PIBF in both untreated (r = 0.250) and treated RPL patients (r = 0.184).

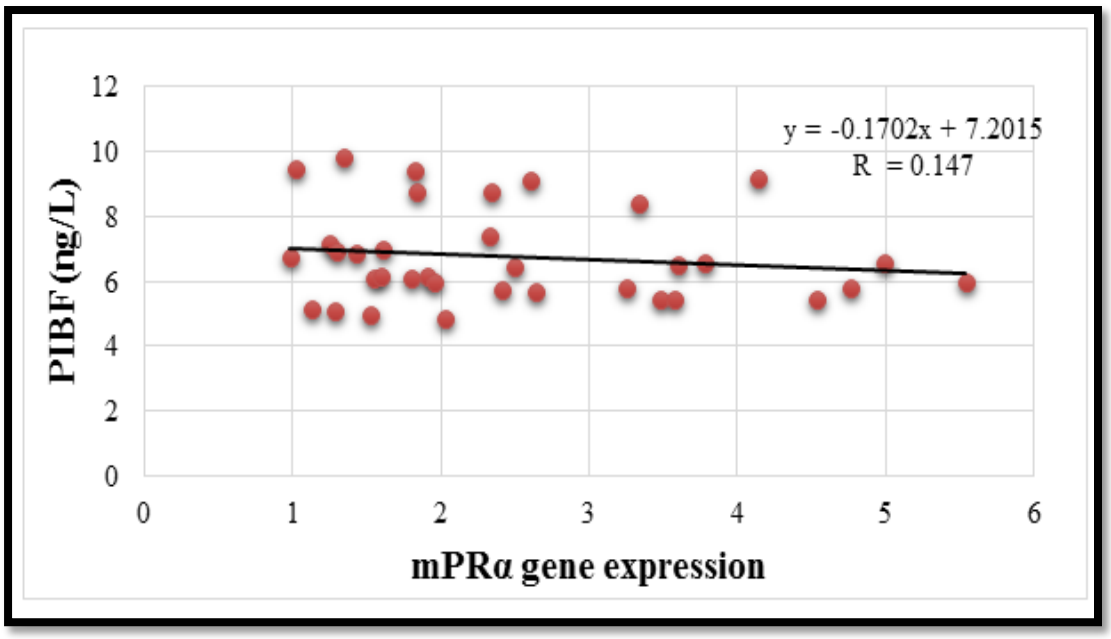


Fig (4-19): Correlation between mPRα gene expression and PIBF concentration (ng/L) in RPL patients without treatment.

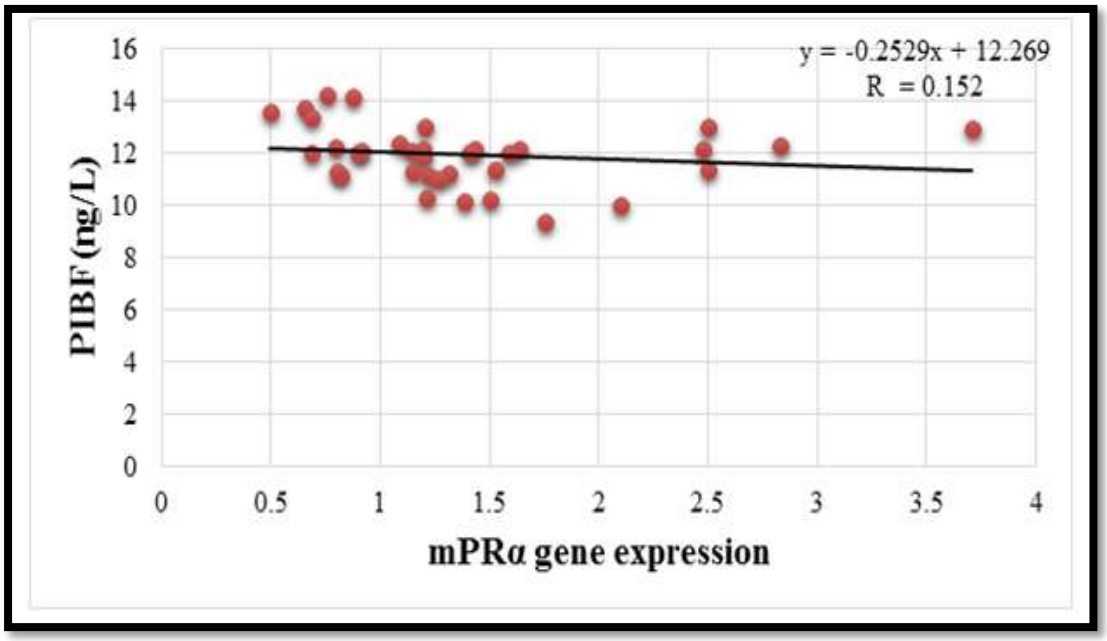


Fig (4-20): Correlation between mPRα gene Expression and PIBF Concentration (ng/L) in RPL patients with treatment.

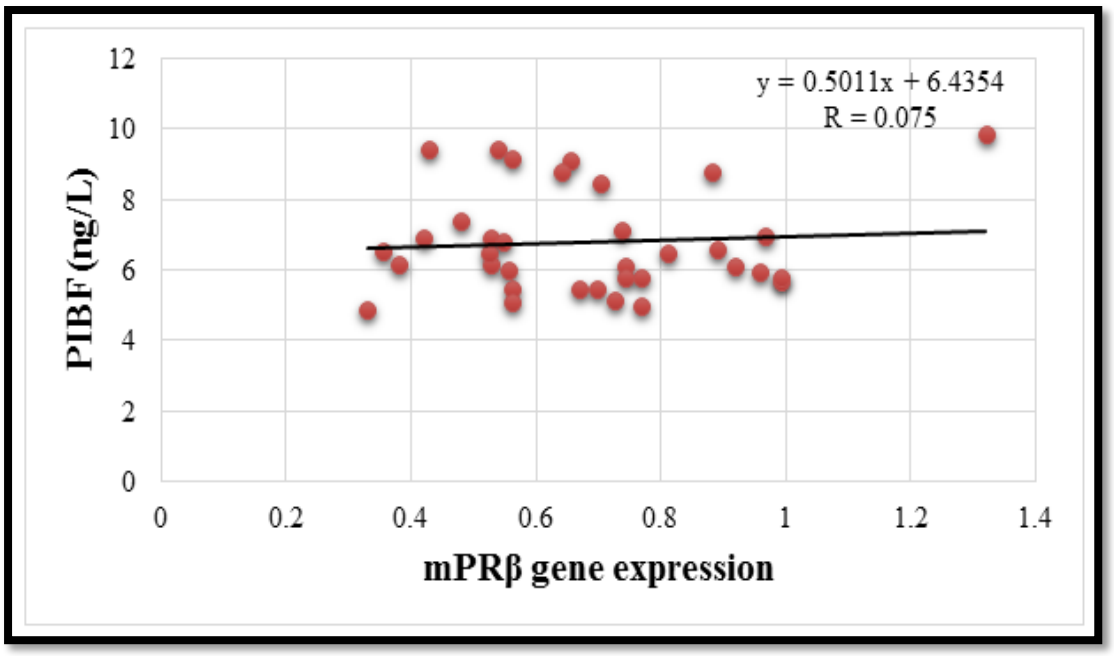


Fig (4-21): Correlation between mPRβ gene expression and PIBF concentration (ng/L) in RPL patients without treatment.

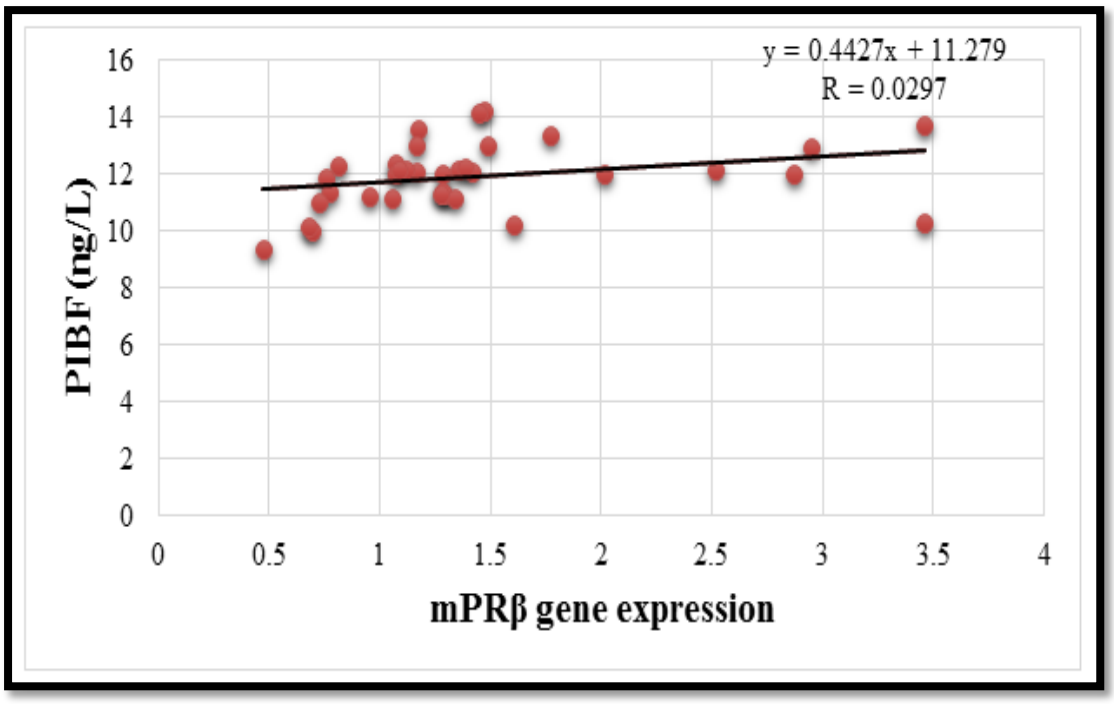


Fig (4-22): Correlation between mPRß gene expression and PIBF concentration (ng/L) in RPL patients with treatment.

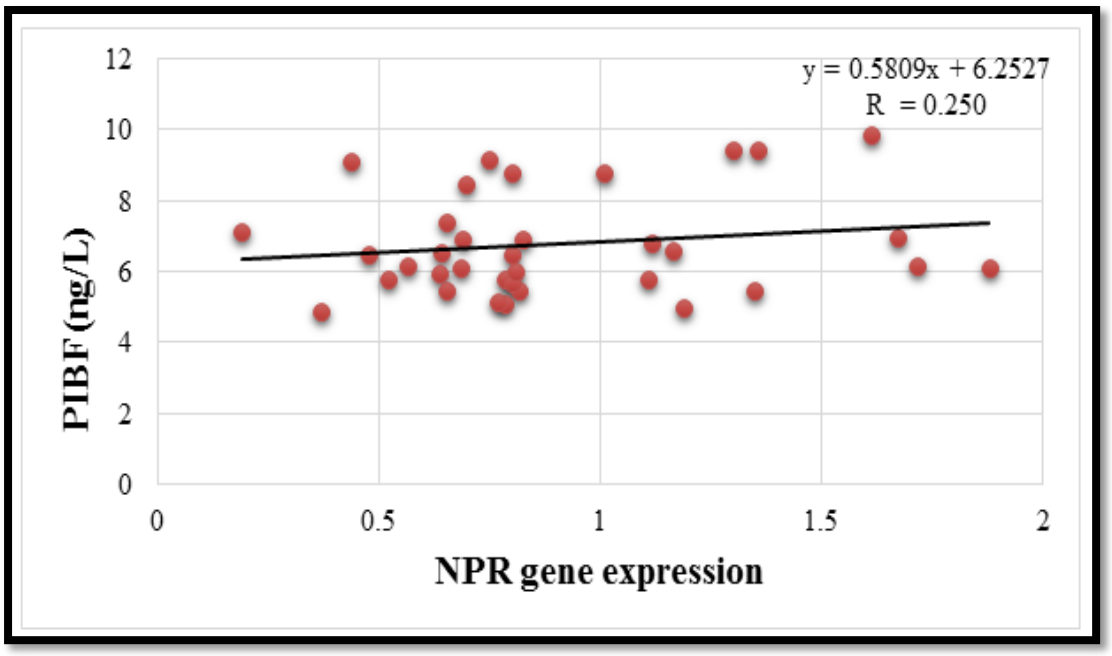


Fig (4-23): Correlation between NPR gene expression and PIBF concentration (ng/L) in RPL patients without treatment.

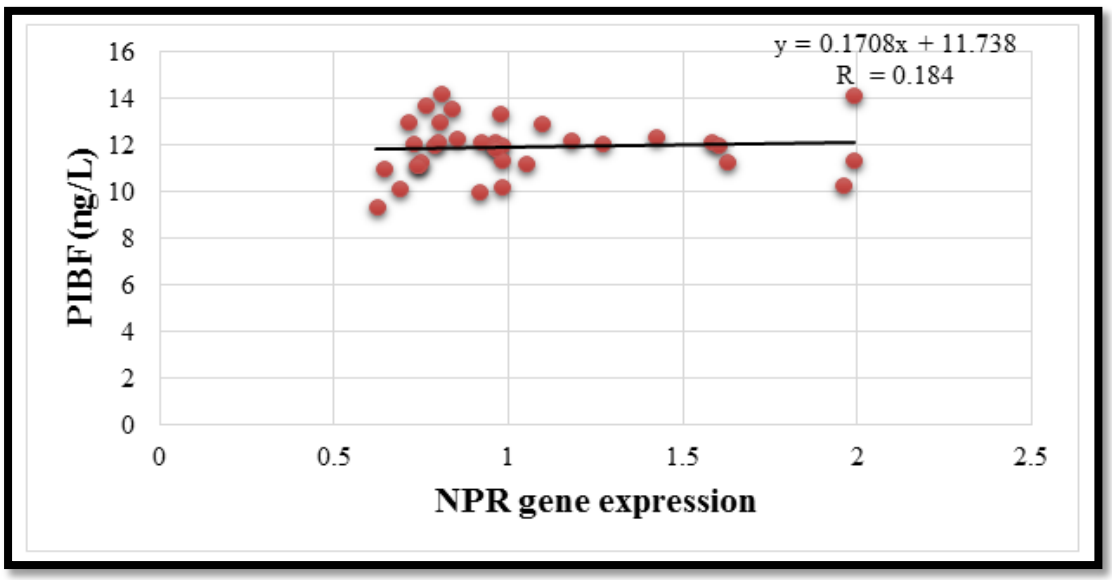


Fig (4-24): Correlation between NPR gene expression and PIBF concentration (ng/L) in RPL patients with treatment.

The fetal antigens are recognized by the maternal immune system, thereby resulting in lymphocyte activation and the induction of progesterone receptors (PRs) in immune cells. Upon binding of progesterone to PRs on lymphocytes, a downstream mediator called progesterone-induced blocking factor is produced. PIBF induces an

increased production of Th2 cytokines and inhibits the degranulation of NK cells, and by regulating the maternal immune response, it contributes to successful implantation and maintenance of pregnancy (Szekeres-Bartho and Schindler, 2019).

Membrane progesterone receptors (mPRs) may correlate with the immunomodulatory properties of Progesterone on T cells. Variation in the expression of mPRs may influence Progesterone regulatory performance during pregnancy. On the other hand, PIBF increases in pregnant normal women compared to women who have experienced abortion (Rafiee *et al*., 2021). Human and animal studies suggest that inducing PIBF production could be the indirect mechanism by which dydrogesterone improves pregnancy outcomes (Motomura *et al*., 2023).

Conclusions

And

Recommendations

Conclusions

Based on the results of the study, the following conclusions can be drawn:

1. Evaluating the endocrine profile before conceiving is crucial for preventing unwanted pregnancy loss and promoting women's overall health.
2. Women who experience recurrent spontaneous abortion of the first type exhibit reduced levels of progesterone and estrogen. This decrease may be a contributing factor to the occurrence of miscarriage. However, after undergoing treatment with dydrogesterone, there is a noticeable increase in serum levels of estradiol and progesterone. This is due to the drug's direct impact on raising progesterone levels.
3. The decrease in serum levels of E-Cadherin and PIBF strongly indicates a positive correlation with progesterone levels, providing compelling evidence that these proteins significantly contribute to the pathogenesis of this medical condition.
4. It is plausible that the decline in mPRß gene expression and the rise in mPRα could be linked to the recurrence of pregnancy loss, implying that these receptors may have a role to play in this condition. Moreover, the alterations in the gene expression of progesterone receptors suggest that dydrogesterone impacts the expression of these receptors.

Recommendations

1. Progestogens, such as dydrogesterone, are effective in treating threatened and recurrent pregnancy loss. However, further studies are required to gain a better understanding of patient-specific factors, such as treatment approaches for intrauterine abnormalities, immune system status, and thyroid function, which could potentially improve pregnancy outcomes.

2. Studying the expression of progesterone receptors in the peripheral blood of individuals with recurrent abortions and comparing them to those of healthy individuals can also yield valuable insights.

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Appendices

Appendix 1
Ethical roles

وزارة الصحة
دائرة صحة كربلاء
مركز التدريب والتنمية البشرية
لجنة البحوث

استمارة رقم ٢٠٢١/٠٣

رقم القرار: ١١٥

تاريخ القرار: ٢٠٢٢/٥/١٥

قرار لجنة البحوث

درست لجنة البحوث في دائرة صحة كربلاء مشروع البحث ذي الرقم (٢٠٢٢٠١١٥/كربلاء) المعنون:

تقييم بعض العوامل الهرمونية والجزيئية عند النساء اللواتي يعانين من الاجهاض المتكرر ويتلقين عقار الديدروجستيرون في محافظة كربلاء

والمقدم من الباحثة **زهراء جاسب حميد** الى وحدة ادارة البحوث والمعرفة في مركز التدريب والتنمية البشرية في دائرة صحة كربلاء بتاريخ ٢٠٢٢/٤/٢٤ وقررت:

قبول مشروع البحث اعلاه كونه مستوفيا للمعايير المعتمدة في وزارة الصحة والخاصة بتنفيذ البحوث ولا مانع من تنفيذه في مؤسسات الدائرة.

الدكتور
نعيم عبيد المشهداني
مقرر لجنة البحوث
15/05/2022

وزارة الصحة
دائرة صحة كربلاء المقدسة
لجنة البحوث

المرفقات:

ملاحظات:

- تم تخويل عضو لجنة البحوث (د.تقوى خضير عبد الكريم) او مقرر اللجنة (د.نعيم عبيد هلال) للتوقيع على هذا القرار استنادا الى النظام الداخلي للجنة البحوث.
- الموافقة تعني ان مشروع البحث قد استوفى المعايير الاخلاقية والعلمية لإجراء البحث والمعتمدة في وزارة الصحة. اما التنفيذ فيعتمد على التزام الباحث بتعليمات المؤسسة الصحية التي سينفذ فيها البحث.

Appendix 2
Comprehensive examination committee

Republic of Iraq
Ministry Of Higher Education and Scientific Research
College of Science – University of Kerbala
Division of Postgraduate studies

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء - كلية العلوم
شعبة الدراسات العليا

العدد/ع/16/ 3734
التاريخ/ 28/ 10/ 2021

أمـــر إداري

استناداً إلى مصادقة السيد رئيس الجامعـــة المحترم على أصـــل محضـــر الجلسة الثالثة المفتوحة لمجلس كليتنا للعـــام الدراسي 2021-2022 والتي عقدت للفترة من 14- 2021/10/20 والمرسل إلينا بكتاب امانة مجلس الجامعة ذي العدد /ج/ 1408 بتاريخ 2021/10/26 والمبلغ الينا بكتاب امانة مجلس الكلية ذي العدد م.ك/247 في 2021/10/27 واستنادا للصلاحيات المخولة لنا تقرر:

تشكيل لجنة من التدريسيين المدرجة أسماؤهم في الجدول أدناه لاجراء الامتحان الشامل لطالبة الدراسات العليا/الدكتوراه/ قسم علوم الحياة (زهراء جاسب حمود).

على ان يكون موعد الامتحان التحريري في يوم الثلاثاء الموافق 2021/12/7 والامتحان الشفهي في يوم الثلاثاء الموافق 2021/12/21.

ت	اسم التدريسي	اللقب العلمي	المادة الامتحانية المقترحة	المنصب	مكان العمل
1	د. نصير جواد حمد المختار	استاذ	Fertility and sterility	رئيسا	كلية الطب / جامعة بابل
2	د.داخل غاني عمران	استاذ	Blood Physiology	عضوا	كلية علوم بنات /جامعة بابل
3	د. نرجس هادي منصور	أستاذ	Metabolic pathways	عضوا	كلية العلوم /جامعة كربلاء
4	د. جاسم حنون هاشم	أستاذ مساعد	Hormones	عضوا	كلية العلوم /جامعة كربلاء
5	د.كوكب عبد الله حسين	أستاذ مساعد	Molecular techniques	عضوا	كلية العلوم / جامعة كربلاء

أ. م.د. جاسم حنون هاشم العوادي
العميد وكالة
2021 / /

نسخة منه إلى
- مكتب السيد العميد / للتفضل بالعلم مع التقدير .
- جامعة بابل/كلية الطب / للتفضل بالموافقة على اشراف الأستاذ الدكتور نصير جواد حمد ... مع التقدير ..
- جامعة بابل /كلية العلوم للبنات/للتفضل بالموافقة على اشراف الاستاذ الدكتور داخل غاني عمران ... مع التقدير ..
- جامعة كربلاء /كلية العلوم / للتفضل بالموافقة على اشراف الاستاذ الدكتور نرجس هادي منصور .. مع التقدير ..
- جامعة كربلاء/كلية العلوم/للتفضل بالموافقة على اشراف الأستاذ مساعد الدكتور جاسم حنون هاشم ..مع التقدير ..
- جامعة كربلاء/كلية العلوم / للتفضل بالموافقة على إشراف الأستاذ مساعد الدكتور كوكب عبد الله حسين ...مع التقدير ..
- وزارة التعليم العالي والبحث العلمي / قسم شؤون المنتسبات للعلم.
- السيد رئيس قسم علوم الحياة. مع التقدير.
- الدراسات العليا مع الأوليات .
- الصفرة

طباعة: 2021/10/31A.Khalaf

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العنوان: العراق-محافظة كربلاء المقدسة – المدينة الجامعية – كلية العلوم ص.ب 1125

Appendix 3
Questionnaire

استمارة جمع العينة والاستبانة

رقم العينة : العمر :

الاجهاض المتكرر الاولي: تاريخ اخذ العينة :

- عدد الولادات :
- عدد الاسقاطات :
- مؤشر كتلة الجسم :
- انتظام الدورة الشهرية :
- هل تعاني المريضة من نقص في هرمون البروجسترون :
- هل تم اجراء عملية جراحية للمريضة برفع احد المبيضين :
- هل تعاني من امراض وراثية :
- هل توجد حالة مماثلة اخرى في العائلة تعاني من نفس المرض :
- هل تعاني المريضة من تشوهات خلقية في الرحم :
- نوع العلاج المستخدم :
- هل المريضة منتظمة في اخذ العلاج :
- مدة العلاج :
- الية اخذ العلاج :

موافقة المريضة :

رقم الهاتف:

الخلاصة

فقدان الحمل المتكرر Recurrent Pregnancy Loss (RPL) وهي حالة يتم تعريفها على أنها فقدان الحمل ثلاث مرات متتالية قبل 20 أسبوعًا من آخر دورة شهرية؛ قد يكون سبب فقدان الحمل المتكرر، جزئيًا، هو انخفاض مستويات هرمون البروجسترون خلال المرحلة الأصفرية. يهدف الديدروجستيرون (Dydrogesterone)، وهو بروجستيرون اصطناعي، إلى تعزيز تقبل بطانة الرحم ومنع فقدان الحمل.

شملت الدراسة (106) امرأة من مستشفى النسائية والتوليد التعليمي، مستشفى خديجة الكبرى، وعيادات العقم الخارجية في محافظة كربلاء، تم تقسيم المشاركات إلى ثلاث مجموعات: المجموعة الأولى ضمت (35) مريضة تعانين من فقدان الحمل المتكرر وتلقت الديدروجستيرون لمدة ستة أشهر، المجموعة الثانية (35) مريضة تعانين من فقدان الحمل المتكرر دون علاج، والمجموعة الثالثة ضمت 36 امرأة سليمة كمجموعة سيطرة. تمت مطابقة جميع المشاركات في الدراسة حسب العمرالذي تراوح من (25 إلى 38) سنة ومؤشر كتلة الجسم Body Mass Index (BMI) . تم تطبيق الدراسة الحالية لتقييم تراكيز بعض الهرمونات (البروجستيرون، الاستراديول، البرولاكتين، الهرمون المنبه للجريبات، الهرمون الملوتن والتستوستيرون، والعوامل البيوكيميائية (عامل الحصر المستحث بالبروجسترون والكادهارين الظهاري) والتعبير الجيني النسبي لمستقبلات البروجسترون (النووية والغشائية) لجميع عينات الدراسة.

أنخفضت مستويات هرمون البروجسترون والاستراديول بشكل ملحوظ ($p \geq 0.05$) في مريضات فقدان الحمل المتكرر بدون علاج مقارنة مع السيطرة. الى جانب ذلك، لايوجد فرق كبير في مستويات البروجسترون بين مريضات RPL اللواتي يتلقين العلاج ومجموعة السيطرة. ولكن لوحظت زيادة ذات دلالة إحصائية ($P \geq 0.05$) في تركيز البروجسترون بين المريضات اللواتي تعانين من فقدان الحمل المتكرر مع العلاج مقارنة مع اللواتي لا يتناولن العلاج. ايضا،هنالك علاقة إيجابية معنوية ($r = 0.522$) بين البروجسترون والاستراديول في مريضات فقدان الحمل المتكرر بدون علاج. قدرت الدراسة تراكيز البرولاكتين، الهرمون المنبه للجريب، الهرمون الملوتن والتستوستيرون لدى النساء، ولم تختلف هذه المعايير معنويا ($P \geq 0.05$) بين جميع مجموعات هذه الدراسة. أظهرالارتباط وجود علاقة عكسية بين تركيز البروجسترون وهرمون FSH في مرضى RPL بدون علاج ومع العلاج ($r = - 0.46$، $r = - 0.390$، على التوالي).

أشارت البيانات المتعلقة بتركيزات عامل منع البروجسترون progesterone induced blocking factor (PIBF) و الكادهارين الظهاري (E-Cad) إلى انخفاض كبير ($p \geq 0.05$) في مريضات فقدان الحمل المتكرر RPL دون علاج مقارنة بمجموعة السيطرة. ومع ذلك، كان هناك ارتفاع ملحوظ في مستويات PIBF والكادهارين الظهاري بين مريضات RPL دون علاج و اللاتي يتلقين العلاج. علاوة على ذلك، لوحظ وجود علاقة ايجابية معنوية بين البروجسترون وPIBF في كل من مريضات RPL غير المعالجات واللاتي يتلقين العلاج.

أشارت نتائج التعبير الجيني النسبي لمستقبلات البروجسترون الغشائية (mPRα) إلى زيادة كبيرة في التعبير الجيني ($p \geq 0.05$) في مريضات RPL بدون علاج ومجموعة السيطرة. كما وجدت نفس النتيجة بين مريضات RPL مع العلاج و مجموعة بدون علاج. تم العثور على علاقة عكسية بين التعبير الجيني mPRα وتركيز البروجسترون في مريضات RPL غير المعالجات ومريضات RPL الخاضعات للعلاج ($r = - 0.132$، $r = - 0.014$ على التوالي). أظهرت نتائج المريضات اللاتي تعرضن للإجهاض المتكرر دون تلقي علاج الديدروجستيرون انخفاضًا ملحوظًا في مستقبلات البروجسترون الغشائية (mPRß) في أنسجة بطانة الرحم. بالإضافة إلى ذلك، كانت هناك زيادة كبيرة في التعبير الجيني لـ mPRß بين مريضات RPL المتلقيات للعلاج وغير المتلقيات ($P \geq 0.05$). أظهرت نتائج الارتباط وجود علاقة إيجابية معنوية ($r = 0.380$) بين التعبير الجيني mPRß والبروجستيرون في مريضات RPL بدون علاج وفي مريضات RPL اللاتي يخضعن للعلاج ($r = 0.156$). أظهرت تحليل مستويات التعبير الجيني لـ NPR أن هناك اختلافًا طفيفًا جدًا بين المريضات غير المعالجات ومجموعة السيطرة. وقد لوحظت نفس النتائج عند مقارنة التعبير الجيني لـ NPR في المريضات المعالجات وغير المعالجات، كان هناك ارتباط إيجابي ($r = 0.319$) بين التعبير الجيني NPR وتركيز البروجسترون في مريضات RPL غير المعالجات وفي مريضات RPL المعالجات ($r = 0.346$).

تشير النتائج إلى أن انخفاض التعبير الجيني لـمستقبلات البروجسترون الغشائية (mPRβ) التعبير الجيني المرتفع لـمستقبلات البروجسترون الغشائية (mPRα) لدى مريضات RPL قد يلعب دورًا مهمًا في التسبب في فقدان الحمل المتكرر RPL. وانخفاض التعبير الجيني mPRα وزيادة التعبير الجيني mPRβ بعد العلاج بالديدروجستيرون، مما يشير إلى أن الدايدروجسترون يؤثر بشكل مباشر على التعبير الجيني لمستقبلات البروجسترون في بطانة الرحم.

جامعة كربلاء

كلية العلوم

قسم علوم الحياة

تقييم بعض العوامل الهرمونية والجزيئية عند النساء اللواتي يعانين من الإجهاض المتكرر ويتلقين عقار الديدروجستيرون في محافظة كربلاء

اطروحة مقدمة الى

مجلس كلية العلوم- جامعة كربلاء

وهي جزء من متطلبات نيل درجة الدكتوراه في علوم الحياة

من قبل

زهراء جاسب حميد كاظم

بكالوريوس علوم حياة / جامعة بابل , 2011

ماجستير علوم حياة /علم الحيوان / جامعة بابل , 2015

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