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Effect of Genetic Polymorphism of SLC47A1 (MATE1) on the Therapeutic Response of Metformin in Iraqi Women with Polycystic Ovary Syndrome

A Thesis

Submitted to the Council of College of Pharmacy/ University of Kerbala as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology and Toxicology

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صدق الله العلي العظيم

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Dedication

To my source of inspiration who helped me in every stage of my life… My dear father and My kind mother…

To who supported me …My father in law and My mother in law…

To my second half who encouraged me during the period of study… My faithful husband …

To who assisted and supported me …My brothers and My sisters…

To the precious gifts and beautiful flowers … My son and My daughter …

I dedicate this thesis with love…

Maha

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List of Abbreviations

Abbreviation	Description
AE-PCOS	Androgen Excess and PCOS society
AI	Aromatase inhibitor
AMPK	Adenosine monophosphate activated protein kinase
ARMS	Amplification refractory mutation system
3-betaHSD	3-Beta hydroxyl steroid dehydrogenase
BMI	Body mass index
CLr	Renal clearance
CVD	Cardiovascular diseases
CYP 17	Cytochrome P450c 17
CYP11a1	Cytochrome P450 Family 11 Subfamily A member 1
DHT	Dihydrotestosterone
EDTA	Ethylene di amine tetra acetic acid
FAI	Free androgen index
FBS	Fasting blood sugar
FSH	Follicle stimulating hormone
GLP-1	Glucagon like peptide
GLUT-4	Glucose transporter type 4
GnRH	Gonadotrophin releasing hormone
HbA1c	Glycated hemoglobin A1c
HDL-C	High density lipoprotein-cholesterol
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IR	Insulin resistance
IVF	In vitro fertilization
LDL-C	Low density lipoprotein-cholesterol
LH	Luteinizing hormone
LKB1or STK11	Liver kinase B1 or Serine threonine kinase
MATE1	Multidrug and Toxin Extrusion Protein
MET	Metformin
Mets	Metabolic syndrome
mitGPD	Mitochondrial glycerophosphate dehydrogenase
NIH	National Institutes of Health
OCT1	Organic cation transporter

PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovary syndrome
PCO	Polycystic ovary
PCR	Polymerase chain reaction
PMAT	Plasma membrane monoamine transporter
SHBG	Sex hormone binding globulin
SLC47A1	Solute carrier Family 47 Subfamily A Member 1
SNPs	Single Nucleotide Polymorphisms
StAR	Steroidogenic acute regulatory protein
T2DM	Type 2 diabetes mellitus
TBE	Tris Borate EDTA
TG	Triglycerides
TSH	Thyroid stimulating hormone

Summary

Background: Insulin sensitizer's therapy with metformin is widely used in polycystic ovary syndrome (PCOS) , notwithstanding the treatment effectiveness that shows an individual variations in PCOS patients. Multidrug and toxin extrusion protein (MATE1) has been reported to mediate metformin excretion in kidney and bile. In the current study, the effect of the polymorphisms of MATE1 and the response to metformin has been investigated. There are at least eleven non-synonymous single nucleotide polymorphisms (SNPs) in the MATE1 (SLC47A1) gene, (rs2289669 and rs1961669) SNPs were detected in this study.

Objective: The objective of the study is to determine the clinical, hormonal and biochemical effects for three months of metformin therapy in women with polycystic ovary syndrome and study the effect of MATE1 (SLC47A1) genetic polymorphism on therapeutic response to metformin in polycystic ovary syndrome.

Patients and Methods: This study was an interventional prospective study, 231 women with polycystic ovarian syndrome were selected to participate in this study. All women enrolled in this study with the age range (18-40) years were starting metformin tablet 500 mg per oral twice daily. Blood samples were obtained from eligible patients who had given consent for genetic testing and measurement of follicle stimulating hormone , luteinizing hormone , total testosterone, sex hormone binding globulin, Estradiol, prolactin, thyroid stimulating hormone, fasting insulin, HbA1c%, fasting glucose, and lipid profiles (LDL-C, HDL-C, TG ,Total Cholesterol).

Results: The results obtained from this study demonstrated that SLC47A1 (G>A) (rs2289669) genetic polymorphism had significant effect on metformin response in patients with polycystic ovary syndrome , while SLC47A1 (A>G) (rs1961669) genetic polymorphisms had no strong effect on metformin response in hormonal and biochemical parameters in the same group of patients. The comparison in demographic and biochemical parameters between pre- and post-metformin treatment results showed improvement in clinical, hormonal and biochemical parameters.

Conclusions: This study revealed that the treatment with metformin showed improvement in menstrual regularity, hormonal and biochemical profile in Iraqi women with polycystic ovarian syndrome. In addition, there was a powerful effect of MATE1 SLC47A1(G>A) (rs2289669) genetic polymorphisms on therapeutic response to metformin in clinical, hormonal and biochemical parameters in those patients, moreover, there was an association between presence of both SNPs in SLC47A1 gene and obesity which considers as most important risk factor for PCOS.

CHAPTER ONE

Introduction

1.1. Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is an endocrine-metabolic and reproductive disorder, which is defined by a collections of signs and symptoms of androgen excess (hirsutism and/or hyperandrogenaemia) with ovarian dysfunction (oligo-ovulation and / or polycystic ovarian morphology (PCOM)), indicating that other specific diagnoses, such as non-classic congenital adrenal hyperplasia, thyroid dysfunction , have been excluded **[1]**. Polycystic ovary syndrome was first discovered by Stein and Leventhal in 1935 as a syndrome of oligo-amenorrhea and polycystic ovaries that was differently accompanied by acne, hirsutism, and obesity, affecting women of reproductive age **[2]**. PCOS is a complex disease of unknown etiology, but there is a strong indication that it can, to a large extent, be classified as a genetic disorder, characterized by multiple hormonal imbalances **[3].**

The growing attention of the scientific community in PCOS has not been paralleled by that of the international and local health authorities **[4]**. PCOS stays one of most poorly understood clinical disorders among patients, physicians and scientists **[5]**. It represents a status in which an approximately of 10 small cysts of a diameter ranging between 2 and 9 mm grow on one or both ovaries and/or the ovarian volume in at least one of the ovaries exceeds 10 ml **[6].**

1.2. Epidemiology

The prevalence of PCOS in different populations has been subsequently reported by many epidemiological studies using multiple PCOS definitions. Worldwide prevalence of PCOS ranges from 4% to 21%, depending on the criteria of diagnosis used **[5].** The prevalence of PCOS among various geographic regions ranges from 5% to 10% according to American National Institutes of Health (NIH 1990) criteria; from 10% to 15% when the Androgen Excess and PCOS society (AE-PCOS 2006) criteria were applied, and from 6% to 21% according to Rotterdam criteria **[7].**

World Health Organization (WHO) assessed that PCOS has affected 116 million women (3.4%) worldwide in 2012 **[3],** whereas, it has been estimated to affect approximately 5 million women of childbearing age in the U.S. **[8].** Subsequent studies have verified and expanded this assessment. To date, the prevalence of PCOS varies from 5% to 20%, depending on factors such as which diagnostic criterion is used, how the study population is recognized, methods that used to define each phenotypic feature, and the completeness of the phenotypic assessment and recruitment method of the populations **[9].** There is a variation in the prevalence of PCOS among adolescents based on different criteria **[10].** The prevalence under Rotterdam more than doubles that under the 1990 NIH, while the prevalence under the 2006 AES lying in-between **[11].**

In Iraq, the prevalence of syndrome is approximately (18.8%) according to the sonographic prevalence of polycystic ovary morphology among women of reproductive age group and this percentage varies according to age, BMI, and type of menstrual irregularity **[12].**

1.3. Phenotypes

The Rotterdam and AE-PCOS Society criteria recognize at least 3 unique clinical phenotypes **[13]**:

A. Frank PCOS :(oligomenorrhea, hyperandrogenism, and PCO)

B. Ovulatory PCOS :(hyperandrogenism, PCO, and regular menstrual cycles)

C. Non-PCO PCOS :(oligomenorrhea, hyperandrogenism, and normal ovaries).

The Rotterdam criteria identify a fourth phenotype, Mild or Normo androgenic PCOS, which is known by oligomenorrhea, PCO, and normal androgens **[14]**.These 4 phenotypes represent a spectrum of the same disorder, but it is currently an area of debate **[13].** Overall it seems that the presence of hyrperandrogenism, body mass index (BMI), the extent of menstrual irregularity, but not ovarian morphology, are

independent predictors of metabolic dysfunction **[15]**. However, most of studies among PCOS cohorts were determined in patients identified in the clinical setting and very little information exists regarding the characteristics or phenotype of PCOS in patients identified in the general population **[7].**

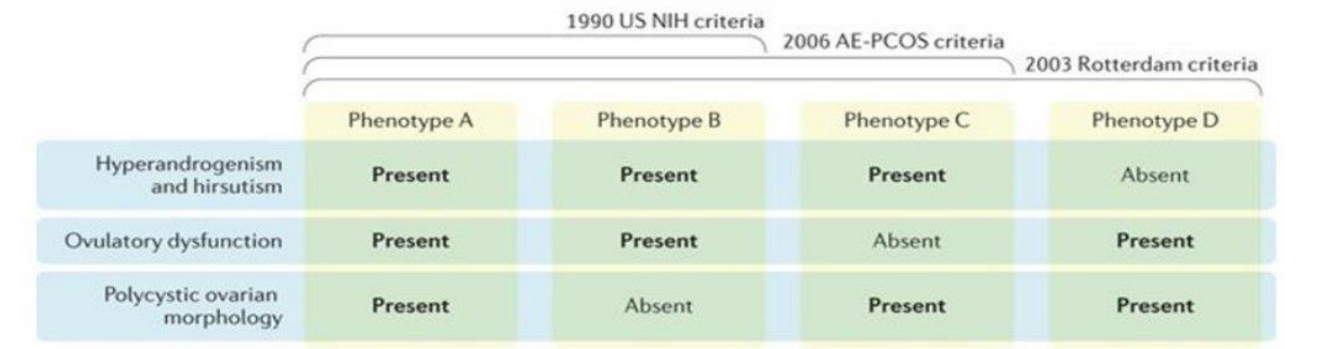


Fig. 1-1: PCOS phenotypes [16].

1.4. Predisposing Factors

PCOS as a syndrome, is reflecting multiple potential etiologies and variable clinical presentation and has a complex mode of inheritance in which genomic variants interfere with important environmental factors, including life style, for example, diet and physical inactivity leading to heterogeneous expression of the syndrome characterized by chronic anovulation or infrequent ovulation, polycystic ovaries, hyperandrogenism, obesity and insulin resistance **[17,18]**. The appearance of the disease may be affected by environmental and genetic factors that operate during earlier periods of life. Animal models suggest that fetal exposure to androgens result in PCOS-like phenotypes and associated metabolic symptoms, such that the predilection to PCOS could begin in utero through environmental or epigenetic mechanisms **[19]**. The source of intra-uterine androgens excess is unlikely to be maternal, since the fetus is protected by placental

aromatase activity and by high maternal sex hormone binding globulin (SHBG) concentrations so the expression of aromatase in the placenta of PCOS women may be diminished **[20]**. There is a strong proof for genetic predisposition to PCOS in most ethnic/racial groups studied to date, however, in spite of advances in genetic technologies, very few PCOS susceptibility genes have been established **[21]**. Positive family history of PCOS is most informative risk factor for evolution of PCOS. Based on the clustering of cases in families, PCOS is regarded to be a heritable disease. A high prevalence of PCOS or its characteristics among first-degree relatives is suggestive of genetic effects. In addition, greater conformity has been reported in monozygotic twins versus dizygotic twins. However, the form of inheritance stays elusive **[22]**.

Age at menarche in females with PCOS extends from nine years onwards, including primary amenorrhea. Earlier menarche look likes to be strongly in connection with obesity and later menarche/ amenorrhea is heavily concerning to higher androgen levels **[23]**.

PCOS is an age-related disease and the occurrence of the disease decreases with the age. The highest occurrence was seen among the age group of 18-27 years and least in the age group of 38-47 years. No patients with PCOS were above 48 years **[12]**.

There are intimate links between obesity and PCOS, obesity and weight-gain often result in clinical and biochemical manifestation of PCOS in females who are genetically predisposed to evolution of this disease so the majority of women with PCOS (38%-88%) are either overweight or obese **[24]**.

The major pathophysiological components of PCOS are gonadotropic dysfunction and insulin resistance. It has been found that both of these components are related to the BMI **[25]**.

1.5. Pathophysiology

PCOS is a result of various interactions between genetic and multiple environmental factors. Moreover, this syndrome is a multi-factorial disease, and different susceptibility of patients is probably determined by several genetic and environmental risk factors **[26]**.

1.5.1. Alterations in Gonadotrophin Releasing Hormone

Hormonal imbalances that is exemplified by elevated luteinizing hormone and normal or suppression of follicle stimulating hormone (FSH) leading to altered LH / FSH ratio **[27,28]**. Gonadotrophin releasing hormone (GnRH) induces the release of LH and FSH from the pituitary gland **[29]**. FSH controls the growth of ovarian follicles, particularly the granulosa cells. FSH acts on the granulosa cells of the ovary and transforms androgens from the theca cells of the ovary to estradiol with the support of the enzyme aromatase. Consequently, LH controls the theca cells of the ovary, which are responsible for generating androgens **[30]**. The elevation of frequency and amplitude of the production of GnRH and subsequent LH secretion is the most important pathophysiological characteristic of PCOS **[31]**.

1.5.2. Hyperandrogenemia

In vivo or functional ovarian hyperandrogenism in PCOS women is demonstrable by endocrine stimulation of ovarian theca cell LH receptors resulting in hyperandrogenic steroid hormone responses **[32]**, while in vitro maintenance of ovarian theca cells in culture reveals persisting, constitutive hyperandrogenism in theca cells obtained from women with PCOS **[33].** In addition, androgen excess within the adrenal cortex, abdominal subcutaneous adipose depots, and other extra-ovarian sources lead to supplement ovarian hyperandrogenism **[34,35]**.

Most of women with PCOS have increased androgen levels due to in large part to

the increased number of antral follicles containing thecal cells that hyper-secrete androgens **[36]**; therefore, they exhibit symptoms of excess androgen (hirsutism, acne, central adiposity) and not only experience infertility (menstrual irregularity, anovulatory infertility, miscarriage), but also have substantially increased risk of becoming obese, insulin resistant and developing type 2 diabetes, non-alcoholic fatty liver disease, dyslipidaemia and depression **[37]**.

1.5.3. Low Level of Sex Hormone Binding Globulin

Sex hormone-binding globulin (SHBG) is a 93.4-kDa glycated homo-dimeric plasma transport glycoprotein generated by hepatocytes, links and controls the levels of sex-hormones within the circulation. It organizes the bioavailability of 5-dihydrotestosterone (DHT) > testosterone > 17-estradiol in plasma via control of their respective metabolic clearance rates **[38]**. SHBG concentration is a major determinant of the metabolic clearance of these sex steroids and their access to target tissues. Serum concentrations of SHBG change substantially between individuals and are affected by different hormonal, nutritional, metabolic, growth factors, and drugs **[39]**. SHBG receptors (R_{SHBG}) are expressed in sex steroid-dependent cells and tissues such as ovaries, endometrium, colon, prostate, hypothalamus, liver, placenta, breast, epididymus, immune cells, and cardiomyoctes **[40]**. SHBG has a great importance because of its inverse correlation with polycystic ovary syndrome (PCOS), obesity, insulin resistance, metabolic syndrome, and diabetes type II **[41]**.The effects of various therapeutic agents on serum SHBG levels in PCOS patients have been studied in order to evaluate the true accuracy of SHBG in the prediction of PCOS, consequently, therapeutic interventions improved SHBG levels in PCOS women which further reduced PCOS associated complications; therefore, SHBG levels may prove to be a useful biomarker for the diagnosis and treatment of PCOS **[40]**.

1.5.4. Hyperinsulinemia and Insulin Resistance

Insulin resistance (IR) plays an important role in the pathogenesis of PCOS; ordinarily, insulin stimulates theca cells of the ovary to produce androgens both directly and indirectly as increased glucose levels inhibit the hepatic production of sex hormone-binding globulin (SHBG), leading to elevate the concentration of circulating free androgen **[42]**, Fig. (1.2)

IR is a common feature in both overweight and lean women with PCOS without dependency on their body mass index (BMI) **[43]**. Insulin resistance is popular in PCOS patients and plays an important role in the pathophysiology of metabolic and endocrine complications of PCOS patients, notwithstanding, It has been suggested that IR contributes to PCOS and increases risk of metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), and cardiovascular disease **[44]**. Insulin resistance is selective, influencing the metabolic but not mitogenic actions of insulin, serine phosphorylation of cytochrome P450c17(CYP 17), which is the rate-limiting enzyme for androgen biosynthesis that increases its activity **[45]**. It is possible that the same serine kinase phosphorylates the insulin receptor, producing insulin resistance, and P450c17 producing hyperandrogenemia. Cytokines and free fatty acids, can activate intracellular serine kinases and might play a role in the pathogenesis of PCOS **[46]**. Hyperinsulinaemia result in an increased androgen microenvironment within the ovary, which directly correlated to both ovarian androgen biosynthesis and a decrease in hepatic sex hormone-binding globulin (SHBG) synthesis **[47]**. The excess in local ovarian androgen production enhanced by hyperinsulinaemia causes premature follicular atresia and anovulation **[48]**. It is believed that IR could play a key role in the development of endothelial damage **[49]**, moreover, these risk factors are aggravated by central obesity (excess accumulation of fat in the abdominal area due to excess visceral fat) which is existing in the majority of women with PCOS which further exacerbates the

situation **[45]**, as obesity will lead to a further reduction in serum levels of SHBG, elevated levels of total testosterone, free androgen index, fasting insulin, fasting glucose and more adverse lipid profile when matched to normal weight women with PCOS **[50]**. However, it is believed that the mechanism of IR in PCOS involves many factors such as polygenic inheritance and endocrine, metabolic and immune factors that interact with environmental factors **[51]**. PCOS patients have increased magnitudes of insulin resistance when compared to ovulatory controls, when controlling age, BMI, fasting glucose and insulin levels, so quantity of excess body fat, especially patients with a BMI of at least 30 kg/m^2 is the main predictor of insulin resistance with enough magnitude to put PCOS patients at increased risk for metabolic abnormalities **[52]**.

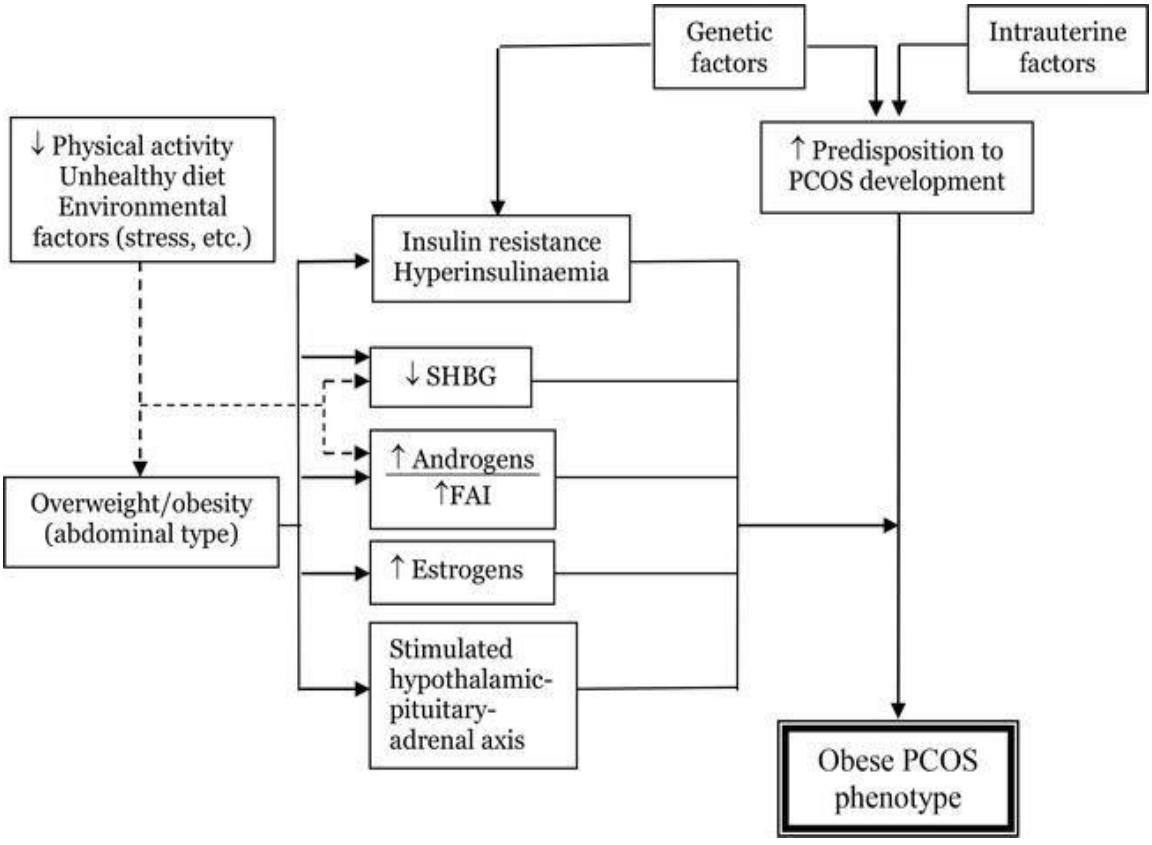


Fig. 1-2: Pathophysiology of PCOS [53].

1.6. Clinical Manifestations

PCOS is a complex condition that has great impact on quality of life. Clinical manifestations of PCO include menstrual irregularities, signs of androgen excess like (hirsutism, acne, alopecia), obesity, insulin insensitivity, and the metabolic syndrome **[54]**. Besides, oligomenorrhoea and amenorrhoea are common clinical manifestations of PCOS, oligo/amenorrhoea cycles are known as 8 or fewer cycles per year as menses occur at intervals longer than 35 days apart while amenorrhoea is the absence of period for six months or more and biochemical androgen measurements should be measured in follicular phase in patients with preserved menstrual cycles **[55]**. Excess of body weight has also been widely regarded as a key concern to women with PCOS, therefore, obesity is believed to contribute substantially to negative psychological symptoms in women with PCOS **[56]**.

Clinical manifestations of PCOS are heterogeneous and the patients may present with some of different signs and symptoms but the heterogeneity seems to be adjusted by multiple factors, such as genetic factors, nutritional condition in the uterus, prenatal androgen exposure, insulin resistance and body weight **[57]**.

1.7. Diagnosis

The diagnostic criteria for PCOS have been grouped in different classifications that have been conflicting for many years; nevertheless, the classification of Rotterdam is the most widely used, but with different frequency depending on country and medical specialties **[56]**. Rotterdam criteria specify that a woman to be PCOS must have 2 of the following 3 criteria; oligo-menorrhoea (fewer than 8 menses per 12 month period) or amenorrhoea, biochemical or clinical evidence of hyper-androgenism (specifically hirsutism and acne), and typical ultrasound characteristics of a polycystic ovary (≥12 follicles in each ovary measuring 2–9 mm in diameter and/or increased ovarian volume >10 ml) **[58]**.

Other most commonly used diagnostic classification is the American National Institutes of Health (NIH) system that defines PCOS as a combination of hyper-androgenism and ovulatory dysfunction **[59]**.

According to the Androgen Excess and PCOS Society (AE-PCOS) criteria, hyperandrogenism represents the mainstay in the diagnosis of PCOS in combination with oligo-anovulation and/or polycystic ovaries **[60].**

1.7.1. Standard Diagnostic Assessment

1.7.1.1. History-Taking

Family history should explore menstrual disorders, infertility, age of puberty and hirsutism in female relatives **[61]**. Specifically, for menstrual pattern, obesity, hirsutism and the absence of breast development, if the patient is obese, the time of onset, progression and problems should be explored, furthermore, lifestyle parameters such as diet, exercise and smoking required an evaluation, also the age of onset and progression of hirsutism and/or acne, as well as any medications used and their effects on acne and hirsutism should be taken into consideration **[62]**.

1.7.1.2. Physical Examinations

This involves general body habitus, obesity, BMI , blood pressure, existence of acne, male pattern of baldness and presence of acanthosis nigricans; moreover, the distribution and severity of hirsutism should be graded clinically **[63]**. Females with PCOS has remarkable hyperandrogenism such as clitoromegaly, deepening of the voice or a masculine body habitus should alert one to the likelihood of virilizing adrenal/ovarian tumors or congenital adrenal hyperplasia **[64]**.

1.7.1.3. Gynecologic Ultrasonography

It is used to identify and document the presence of small follicles of the ovary, so according to the Rotterdam criteria, 12 or more small follicles must be seen in an ovary on ultrasound examination as the multiple follicles contribute to the increased size of the ovaries that is 1.5 to 3 times larger than normal **[3]**.

1.7.1.4. Laboratory Investigations

Various laboratory investigations have been used for the diagnosis of PCOS, such as:

A. LH / FSH ratio: The ratio of LH (Luteinizing hormone) to FSH (Follicle stimulating hormone) is greater than 1:1 (sometimes more than 3:1), as measured on 2nd day of the menstrual cycle **[3]**.

FSH : Stimulates the growth of an egg follicle within the ovary, PCOS women show lower serum FSH levels as compared to normal cycles and which results in accumulation of antral follicles between 2 mm and 8 mm in large numbers, due to lack of adequate and timely stimulation **[65]**.

LH: Produced by pituitary gland and triggers the release of egg during ovulation, ordinarily, elevated LH concentrations are found in a large majority of PCOS patients and should be tested at 2nd day of the cycle **[66]**.

B. Prolactin: Serum prolactin should be measured in PCOS due to strong association between these two conditions, where the most common hypothesis which suggests that PCOS causes hyperprolactinemia due to its relative induction to cause hyperestrogenemia, accordingly various experimental studies have shown an increase in the level of prolactin secretion under the action of estrogen **[67]**.

C. TSH: Thyroid stimulating hormone must be measured in PCOS because it is closely related to this syndrome where multiple studies suggest that

hypothyroidism lead to reduce of sex hormone-binding globulin (SHBG) level and increment of testosterone level, consequently severe prolong hypothyroidism contributes to bigger ovarian size and/or cyst formation **[68]**.

D. **Total testosterone, SHBG and FAI :** All these parameters should be measured in PCOS where serum total testosterone is closely related to an increase level while there is a low level of serum SHBG as the majority (nearly 60%) of testosterone is bound to (SHBG), finally, free androgen index (FAI): is ratio of total testosterone to SHBG concentration multiply by 100, which often elevated in PCOS **[69]**.

E. **Fasting blood sugar (FBS), fasting insulin level and HOMA-IR**: Increased insulin levels have been helpful to predict response to drug and may signify patients who will require higher doses of metformin or the use of second medication to lower insulin, HOMA-IR is a mathematical derivation calculated from the fasting values in glucose and fasting insulin concentrations, allows a direct and moderately accurate measure of insulin sensitivity **[70]**.

F. **Lipid profile tests:** Triglyceride, low density lipoprotein–cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C) and total cholesterol should be tested at fasting state **[71]**, because multiple studies proved that women with PCOS were higher triglyceride, lower HDL-cholesterol and higher LDL-cholesterol levels than their non-PCOS counterparts **[72]**.

1.8. Comorbidities Associated with Polycystic Ovary Syndrome

There is a number of intra- and extra-ovarian factors which negatively influenced the reproductive performance of females with PCOS and interfere with oocyte maturation and ovulation **[73]**.

Diagnosis of PCOS renders the patient aware of possible fertility concerns, dysfunctional bleeding, endometrial cancer, diabetes, obesity, dyslipidemia, hypertension, and theoretical high risk of cardiovascular disease **[74]**, moreover,

women with PCOS may exist with number of metabolic, reproductive, psychological and anthropometric complications **[75]**.

1.8.1. Overweight, Obesity and Metabolic Syndrome

Obesity is a common feature of PCOS with prevalence of about 50% according to various populations and may exacerbate the reproductive and metabolic disorders associated with the syndrome, PCOS women present with increased risk for metabolic syndrome **[76,77]**.

Metabolic syndrome (in females) is defined by the presence of three out of five criteria: waist circumference > 88 cm, fasting blood sugar >100 mg/dL, systolic blood pressure >130 and/or diastolic blood pressure > 85 mmHg, HDL-C < 50 mg/dL and triglycerides >150 mg/dL **[78]**.

1.8.2. Insulin Resistance, Glucose Intolerance and T2DM

Women with PCOS at risk for increase T2DM **[77]**. Insulin resistance (IR) is a prominent feature of PCOS, progressively, IR can result in glucose intolerance, which occurs in 40% of women with PCOS after the age of 40, accordingly, within six years, half of these women may become diabetic PCOS **[79]** , therefore, even with normal weight, women with PCOS are exposed to the risk of glucose intolerance and type 2 diabetes where the risk is enlarged by obesity, furthermore, insulin resistance is mostly prevalent and severe in women with the PCOS phenotype involving hyperandrogenism and anovulation **[80]**.

1.8.3. Cardiovascular Diseases (CVD)

In post-menopausal women, a personal history of clinical hyperandrogenism or menstrual disorders expose females to an increased cardiovascular disease risk , as

classical cardiovascular risk markers are more prevalent in women with PCOS, even if they are not obese and these risks are exaggerated by obesity **[80]**. PCOS at any age is represented by greater odds for elevated CVD risk markers and these elevated markers can happen without obesity but are magnified with obesity, accordingly, PCOS women exist with higher risk of atherosclerosis **[77]**. Besides, the long-term metabolic disease of women with PCOS affects the subclinical vascular pathology markers, nevertheless, even if an elevated cardiovascular morbidity or mortality is not absolutely proved, the precautions must be taken, as a result, a life-long metabolic dysfunction can exaggerate the risk for cardiovascular disease, especially after menopause **[80]**.

1.8.4. Endometrial Cancer

Women with PCOS of all ages seem to be at higher risk of endometrial cancer **[15]**, particularly, this risk may be even elevated in premenopausal subgroup of women with PCOS as a result of hyper-estrogen production, so the increased endometrial cancer risk is due to either different metabolic risk factors or PCOS itself which is identified by many metabolic and reproductive complications that could be responsible for an increased oncology risk on endometrium **[81,82]**.

1.9. Hormonal Imbalances and Infertility in PCOS

The syndrome is a status of chronic anovulation affecting the reproductive age group of women, as well as it is always occurs as a result of an imbalance between female sex hormones **[83]**.Women with this syndrome produce abnormally elevated androgen level that leads to impairment of ovulation, skipping of menstrual cycle and difficulty in obtaining a pregnancy. Actually, the syndrome has an interesting problem for reproductive potential in young female **[84]**.

Anovulation is common among women with PCOS and represents 80-90% of WHO group II anovulatory sub-fertility **[85]**.Treatment concentrating on weight reduction, modification of life style then ovulation induction by low dose gonadotropins **[86]**, while for those women who remain refractory to previous treatments or with coexisting pathologies, assisted reproductive technologies (ARTs) can be a choice to be regarded with a closed supervision to produce required outcome of pregnancy with the sensitive polycystic ovary **[87]**. Various factors may play a role in reducing fertility in PCOS women other than anovulation, these involving the effects of increased body weight, as well as, inflammatory, endocrine and metabolic abnormalities on the quality of ova and fetal development **[88]**. Oocytes obtained from polycystic ovaries may exhibit reduced developmental competence and reduced capacity to complete meiotic division to perform successful fertilization and develop into a viable good quality embryo **[89]**.The follicular microenvironment is associated with oocyte quality, where the levels of follicular testosterone was significantly elevated in PCOS, particularly in those with incompetent meiosis **[90]**, this will badly influence the follicular microenvironment and change the interactions between granulosa cell and oocyte, lead to premature luteinization of granulosa cell, therefore, there is a decline in nuclear and/or cytoplasmic maturation of oocytes and reduce fertilization rate **[91]**. A positive correlation has been found between the number of antral follicles (2–5 mm in diameter) and testosterone concentration **[92]**. Serum androgen levels are increasing during ovarian induction especially in females with PCOS, so, this elevation is suggested to affect the outcome of pregnancy negatively **[93]**. This will contribute to reduce egg quality and increase the rate of miscarriage **[94]**.

1.10. Management of Polycystic Ovary Syndrome

Women with PCOS are at higher risk for several other health conditions such as insulin resistance, metabolic syndrome, type 2 diabetes, obesity, heart disease and high blood pressure (cardiovascular disease)**[95]**; therefore, management of PCOS include keeping a normal endometrium, antagonizing the actions of androgens on the target tissues, lowering insulin resistance (if present), and correcting anovulation **[96]**. Several interventions (pharmacological, non-pharmacological and surgical) have been presented in women with PCOS to target the reproductive, androgenic, weight-related, metabolic, and psychological outcomes related with the condition **[97]**. The interventions target the various life-stages of female from adolescence, pre-pregnancy and pregnancy to pre-menopause, accordingly, interventions of lifestyle for reducion the weight of the women which is the first choice of therapy in guidelines **[98]**.

1.10.1. Non pharmacological Treatment

Based on the relationship between obesity and PCOS, lifestyle modification especially weight loss is considered as the first-line intervention **[99]**. The first point is the treatment of obesity by dietary restriction of calories and increase physical exercise to induce weight loss can improve some aspects of health and the quality of life in patients with PCOS **[100]**. Weight loss through diet or exercise therapy and other lifestyle interventions could help restore normal ovulation, improve pregnancy rate and live birth rate of assisted pregnancy, as well as lower the risk of pregnancy complications, and improve pregnancy outcome **[101]**. Women with PCOS should follow guidelines for healthy diet and focus on low glycemic index food when selecting carbohydrate options **[102]**.

1.10.2. Pharmacological Treatment

The procedures used in PCOS treatment depend firstly on desired clinical effect: infertility treatment, menstrual disturbance regulations, reduction of the symptoms of hyper-androgenism or treatment of obesity **[103]**. Women who desired to become pregnant, clomiphene still stays first-line treatment, clomiphene citrate acts as an estrogen antagonist **[104]**. It causes an increase in the release of gonadotropins from pituitary gland by blocking negative feedback of estrogen and supports the recruitment of the follicles **[105]**.

The aromatase inhibitor (AI) Letrozole is the most effective oral medication and is considered as a drug inducer for ovulation in women suffering from anovulation with PCOS **[106].** It has been established that AIs block the transformation of androgens to estrogen, resulting in reduction of serum estrogen levels, moreover, there is a suggestion that the mechanism of AIs in ovulation induction may include increased FSH secretion in response to the reduced estrogen level **[107]**.

Gonadotropins are used as a pharmacological treatment of PCOS, they are derivatives of urinary products but recently gonadotropins have been produced by using recombinant technology **[108]**. Biological activity of gonadotropins suggested that they might be beneficial for the therapy of patients who were infertile **[109]**.

1.10.2.1. Metformin

The proof of metformin's discovery as an anti-diabetic agent spanned three centuries beginning in the herb, *Galega officinalis* in the 17^{th} century and finishing in its launch as "Glucophage" in the 20^{th} century, which is extract from the leaves of *G. officinalis* was utilized to treat many diseases such as fever, plague and symptoms of diabetes, where the herbal extract consists of guanidine and galegine as major chemical ingredient **[110]**. Despite of these compounds had an anti-diabetic effect, they were too toxic for clinical use, this development coupled with

Garcia's positive results using flumamine, a guanidine analogue, on 'flu' fever quickened the evaluation of guanidine and galegine analogues for anti-diabetic activity, these efforts culminated in the detection of metformin, presented as 'Glucophage', by Jean Sterne **[111]**.

Metformin, described chemically as 3-(diamino methylidene)-1,1-dimethyl guanidine, is an oral hypoglycemic agent belonging to the biguanides class of compounds **[112]**.

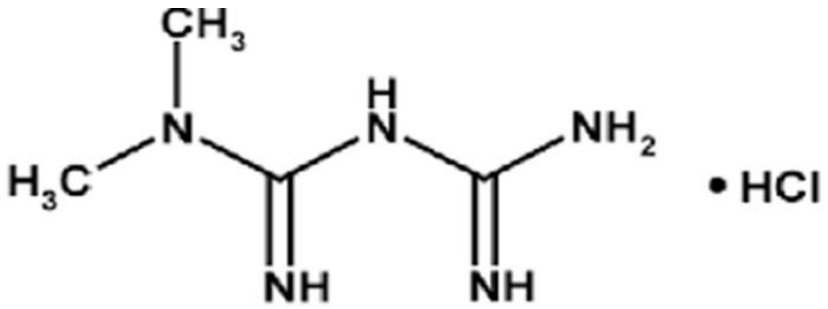


Fig. 1-3: Chemical Structure of Metformin hydrochloride [113].

1.10.2.1.A. Pharmacokinetics of Metformin

Metformin has an oral bioavailability of 40-60% and gastrointestinal absorption is approximately complete with 6 hours of ingestion. Metabolism of metformin is not occur in liver, so, it is excreted unchanged in urine , with half-life approximately 5 hr. Human mean for renal clearance (CL_r) is 510 ± 120 ml/min, where active tubular secretion in the kidney is the main route of metformin elimination **[114]**. The drug is widely distributed into body tissues including the intestine, liver, and kidney by organic cation transporters **[115]**. Intestinal absorption of metformin may be firstly mediated by plasma membrane monoamine transporter (PMAT, encoded by gene *SLC29A4*), which is expressed on the luminal side of the enterocytes **[116]**. Hepatic uptake of metformin is primarily facilitated by OCT1 (*SLC22A1*) and

possibly by OCT3 (*SLC22A3*). Both transporters are expressed on the basolateral membrane of the hepatocyte **[117]**. The uptake of metformin from the circulation into renal epithelial cells is mediated by OCT2 (gene *SLC22A2*), that is expressed predominantly at the basolateral membrane in the renal tubules. Renal excretion of metformin from the tubule cell to the lumen is mediated through MATE1 (*SLC47A1*) and MATE2-K (*SLC47A2*) **[116],** which are expressed in the apical membrane of the renal proximal tubule cells, furthermore, studies in healthy individuals proposed that they participate to the renal excretion of metformin **[118]**.

1.10.2.1.B. Pharmacodynamics of Metformin

Metformin acts mainly by inhibiting excessive hepatic glucose production, through a reduction in gluconeogenesis oxidation **[119]**; moreover, other latent effects of metformin involve an increase in glucose uptake, an excess in insulin signaling, a decrease in triglyceride and fatty acid synthesis, and an increase in fatty acid β-oxidation **[120]**. Metformin may also increase glucose utilization in peripheral tissues, and probably lower food intake and intestinal glucose absorption **[121]**. Because metformin does not stimulate endogenous insulin secretion, so it does not cause hypoglycemia or hyperinsulinemia, which are common side effects related with other antidiabetic drugs **[116]**. The molecular mechanisms of metformin action are complex, as the administration of metformin results in the phosphorylation and activation of AMP-activated protein kinase (AMPK) in the liver that may lead to different pharmacologic effects, including inhibition of glucose and lipid synthesis **[122]**. Another mechanism of metformin action has been appeared which is more recently that is independent of both AMPK activation and cellular energy charge, conversely, metformin has been found to inhibit the glycerophosphate shuttle enzyme mitochondrial glycerophosphate dehydrogenase (mitGPD) **[123]**, accordingly, this prevents glycerol from being directly utilized as a gluconeogenic substrate. It also results in an increase in the cytosolic redox state

that makes the conversion of lactate to pyruvate unfavorable and thus limits the contribution of lactate to gluconeogenic fluxes in LKB1-independent and AMPK-independent manner via decrease in hepatic energy state **[124]**.

1.10.2.1.C. Medical Uses of Metformin

1.10.2.1.C.1. Treatment of Type 2 Diabetes Mellitus

Metformin is prescribed for the management of non-insulin dependent diabetes mellitus or type 2 diabetes mellitus (T2DM); accordingly, metformin exerts hypoglycemic effects by decreasing hepatic production as well as intestinal absorption of glucose and improving insulin sensitivity **[125]**. Metformin is widely used for treating T2DM with high efficiency in lowering fasting and postprandial blood glucose and lowering glycosylated hemoglobin (HbA1c) in T2DM patients. Hypoglycemic effect of metformin is closely related to its capability in suppression of hepatic glucose production and intestinal glucose absorption, with the promotion of β-cell functions and insulin sensitivity **[126]**. Metformin is considered an "insulin-sensitizing" drug, in the liver, where metformin inhibits gluconeogenesis and glycogenolysis, and stimulates glycogenesis while in the adipose tissue and skeletal muscle metformin increases the number and affinity of the tyrosine kinase activity of the insulin receptor, and the translocation and intrinsic activity of GLUT-4 (glucose transporter type 4) **[127]**.

1.10.2.1.C.2. Weight Reduction

Many persons battle to maintain clinically applicable weight loss from way of life and bariatric surgical operation interventions; consequently, long-term follow-up from the Diabetes Prevention Program demonstrates that metformin produces long lasting weight loss, and reduced meals consumption through its fundamental weight loss mechanism **[128]**. Metformin can decrease adiposity and ameliorate obesity associated with comorbid conditions, including abnormalities in glucose

homeostasis in adolescents **[129]**, as well as decreased appetite and increase GLP-1 secretion **[130]**.

There have been a large number of studies that have examined the use of metformin as a weight-loss drug in children with obesity, inspite of each study has been unique in its design, size and the patient populations studied, the subject that emerges, as in systematic review in 2014 based on 14 randomized clinical trials, is that "metformin produces a statistically significant, but very modest reduction in BMI when combined with lifestyle interventions over the short term" **[131]**.

1.10.2.1.C.3. Treatment of Ovulatory Dysfunctions in PCOS

Insulin sensitizer such as metformin is a therapeutic choice that objectives not only insulin resistance that current in this syndrome but several other factors particularly reproductive abnormalities such as infertility, which is a predominant problem of challenge for girls within reproductive age **[132]**. Metformin has been shown to improve reproductive and metabolic defects caused by PCOS where it caused significant improvement in menstrual regularity, reduction in circulating androgen levels ,significant reduction in body weight, reducing the circulating insulin levels, inducing ovulation, reducing the risk of miscarriage as well as reducing the risk of gestational diabetes mellitus (GDM); therefore, it must be more widely used in ladies with PCOS **[133,134]**; furthermore, other studies have reported that the addition of metformin to the ovarian stimulation regime in in-vitro fertilization (IVF) improves the pregnancy outcome **[135]**.

It has been reported that Metformin has direct effects on granulosa and theca cells in human ovaries so these direct effects lead to a decrease in theca cell androstenedione production; accordingly, it decreases FSH-stimulated androgen producing enzyme activity in the granulosa cells (3betaHSD, StAR, CYP11a1, and aromatase) **[136]**, so metformin inhibits androgen and estradiol production by means of regulating steroidogenic enzymes, that regulates proliferation and is able to

phosphorylate and activate the 5′AMP-activated protein kinase , MAPK and AKT in human ovarian cells culture **[137]**. Most earlier studies indicated that metformin improved hyper-insulinemia and hyperandrogenemia with restored ovulatory function. Ovulation and pregnancy rates increase in women with PCOS due to the use of the drug alone or in combination with clomiphene citrate. In addition nonrandomized potential research counseled that metformin may reduce first-trimester spontaneous abortions in women with PCOS **[138]**. Metformin has a confirmed place in the management of PCOS being used to stimulate ovulation, limit miscarriage rates, forbidden fetal growth restriction, and improve the metabolic associations such as glucose intolerance. Since metformin can also be used as a pre-conception, it is reassuring that there is no evidence of teratogenicity **[139]**. In addition to the direct effect of metformin, it may also have indirect effects on the ovary, in the endothelium as metformin seems to enhance nitric oxide vasodilator effects. Several studies indicated that metformin is related with a statistically significant higher ovulation rate compared with placebo, metformin amplify ovulatory frequency and ameliorate hyperandrogenemia, even in non-obese girls with PCOS who show up to have regular insulin sensitivity **[140],** where the positive effect of metformin on hyperandrogenism in PCOS may be due to:

A. Reduce secretion of LH by the pituitary.

B. Reduce ovarian and / or adrenal secretion of androgens.

C. Increased hepatic levels of sex hormone-binding globulin (SHBG) **[141]**.

1.10.2.1.D. Adverse Effects of Metformin

The most common adverse effects related with metformin are mild, transient gastrointestinal symptoms, which are usually self-limiting. These gastrointestinal symptoms limit the maximum tolerated dose in over 50% of patients and about 5% of patients may be unable accepted to tolerate any dose of metformin **[115]**. Deficiency of vitamin B12 has also been associated with long term treatment of

metformin which may lead to anemia, elevated homocysteine levels and probably neurological effects **[142]**, so these side effects can be minimized by starting metformin therapy at a low dose and gradually titrating upward, and swallowing metformin with meals **[143]**. Lactic acidosis that caused by metformin is rare, and the risk of this complication may be reduced by the observance the prescribing precautions and contraindications that avoid accumulation of metformin or lactate in the body so the clinical benefits and the lack of safety risks when used with other antihyperglycemic agents have render metformin a preferred combination drug with other oral agents **[144]**.

1.11. Multidrug and Toxin Extrusion Protein 1 (MATE1) Solute Carrier Family 47 member 1 (SLC47A1) Gene

The kidney and liver play a principal role in the excretion of exogenous and endogenous compounds, both drug metabolism and transport contribute significantly to the elimination of potentially harmful compounds, subsequently, the renal drug elimination is determined not only by glomerular filtration but also by active transport processes which facilitate tubular reabsorption and secretion of drugs **[145]**.

Human multidrug and toxin extrusion protein member 1 (MATE1, SLC47A1 or also known as H^+/ organic cation antiporter member) is an electroneutral OC/H exchanger situated in the apical membrane of the renal tubule cells, encoded by (SLC47A1 gene), the class of MATE-type transporters, is the first determine to role as mediator of the cellular elimination of organic cations into renal tubular lumen **[146]**. The idea of MATE1 is essential for the renal excretion of OCs is not propped only by its special function but also by the result that much of its expression is found in the proximal tubule, which is the part of the nephron where the process of secretion of xenobiotics is supposed to happen, the pH-driven function of MATE1

suggests that the most of cationic export mediated via this transporter takes place towards the end of the proximal tubule, as micropuncture **[147]**. Experiments have shown that the luminal pH is 7.4 at the beginning of the proximal tubule, while it is thought to be much lower at distal portion **[146]**. The human MATE1 gene (SLC47A1) is situated in the tandem on chromosome 17p11.2 in which is a commonly deleted segment in patients with Smith-Magenis syndrome that is characterized by multiple congenital anomalies, mild mental retardation and behavioral issues, the relevance of MATE1 to the development of syndrome is unknown. Human MATE1encode proteins of 570, the amino acid sequence identity of MATE1 is 47.5%, the hydropathy analysis achieved suggested that MATE1 comprises of 12 transmembrane helices **[148]**. Most of current topology analysis programs predict 13 transmembrane helices with an extracellular location of the carboxyl terminus, several histidine, cysteine, and glutamate residues in various transmembrane helices of human MATE1 is involved in substrate binding and/or transport **[149]**. MATE1 is also present on the canalicular membrane of the hepatocyte and appears to act in concert with organic cation transporter 1 (OCT1) to mediate the biliary excretion of different cationic drugs and their metabolites, multiple studies have demonstrated that various drugs and environmental toxins including metformin, cimetidine, and paraquat are substrates of MATE1 **[150]**,while in the kidney, MATE1 is majorly expressed on the apical membrane of proximal tubule and may work with basolateral membrane transporter, human organic cation transporter 2 (OCT2) in the renal elimination of organic cations, in addition to the liver and kidney, other tissues that express high abundance of MATE1 are testis, adrenal gland, and skeletal muscles **[151]**. In 2005, mammalian (MATE) was identified as an orthologue of the bacterial MATE family, nevertheless, human MATE1 encoded by the SLC47A1 gene, which is primarily expressed in the kidney and liver, where it is localized to the luminal membranes of the renal tubules and bile canaliculi **[150]**. MATE1 contributes to the tissue distribution and excretion of

many drugs, so inhibition of this transporter may lead to potential drug–drug interactions (DDIs) and changes in drug exposure and accumulation in different tissues, potent in vitro inhibitors of MATE1 include: cimetidine, pyrimethamine, levofloxacin and moxifloxacin **[152]**.

Metformin is a good substrate for MATE1 (SLC47A1) which is highly expressed in the liver, kidney, and skeletal muscle that mediate the excretion of metformin from both liver and kidney **[153]**. Pharmacokinetic characterization of Mate 1(-/-) mice was performed with metformin which is a typical drug substrate of human MATE1 so after intravenous injection, renal and hepatic metformin concentrations were highly increased in the Mate1(-/-) mice as compared to wild-type mice **[149]**, also, plasma metformin levels were increased in Mate1(-/-) mice, while urinary metformin excretion was significantly decreased. These results indicate a principle role of Mate1transporter in the renal clearance of metformin and other drugs as well **[154]**.

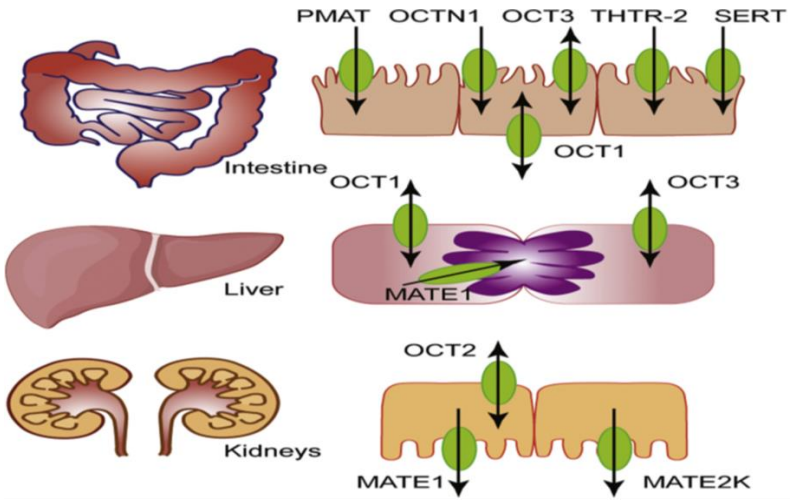


Fig. 1-4: Locations and functions of MATE1 transporter [115].

1.11.1. Single Nucleotide Polymorphism

Single nucleotide polymorphisms (SNPs) are variations in the DNA sequence that occur when a single nucleotide (A, T, C, or G) in the genome sequence is change, for example, a SNP might alter the DNA sequence AAGGCTAA to

ATGGCTAA so for a variation to be regarded a SNP, it must occur in at least 1% of the population. The SNP which make up about 90% of all human genetic variation, occur every 100 to 300 bases along the 3-billion-base human genome, where two of every three SNPs involve the replacement of cytosine (C) with thymine (T), SNPS can occur in both coding (exon) and noncoding (intron) regions of the genome and can affect proteins synthesis **[155]**.

1.11.2. Polymorphism in Multidrug and Toxin Extrusion Protein (MATE1)/(SLC47A1)

Metformin needs drug transporters for its absorption, distribution and elimination, because it poorly diffuses passively over membranes **[156]**. The drug is transported from the intestine into the bloodstream by PMAT and OCT1 **[157]**, taken up into liver by OCT1 and OCT3, into the kidneys by OCT2, and passed from proximal tubule cells into the urine via MATE1 **[116]**.

Alteration in the expression and function of these transporters may significantly contribute to drug pharmacokinetics and the inter-individual variability of drug response **[149]**. Variation in the expression of genes that involved in the transport and action of metformin may affect the response of metformin **[158]**. DNA variants in genes encoding organic ion transporters that are mediated metformin transport may provide insight into the variability of the clinical response to metformin treatment, genetic variants in SLC47A1 gene of MATE1 have been demonstrated to influence metformin response in patients with T2DM **[159]**. In MATE1 (SLC47A1) genes, there was eleven non-synonymous SNPs, where the SNPs: [SLC47A1 (G> A) (rs2289669) and SLC47A1(A> G) (rs1961669)] were detected in current study, the SNP rs2289669 is located in the 10^{th} intron of SLC47A1and this SNP does not code for amino acid change, however, a connection between the SNP rs2289669 in SLC47A1 and the glucose-lowering effect of metformin had been shown in Chinese population **[160]**. The rs1961669 was also an intronic variant of SLC47A1 gene.

1.12. Aim of Study

To investigate the molecular basis of MATE1 (SLC47A1) [(rs2289669) and (rs1961669)] genetic polymorphisms in PCOS women that taking metformin and study the effect of genetic polymorphism (rs2289669 and rs1961669) in MATE1 (SLC47A1 gene) on the therapeutic response to metformin in those patients.

CHAPTER TWO

Patients & Methods

2. Patients and Methods

2.1. Materials

The specific chemicals, kits and instruments used in this study with their suppliers, manufacture and origin are listed in tables (2.1) and (2.2).

The primers that were used for amplification analysis of MATE1 (SLC47A1) gene for SNPS identifications and alleles detection were shown in table (2.3) and (2.4).

2.1.1. The Kits and Chemicals (Table 2.1)

Table 2.1: The kits and Chemicals

Chemicals	Origin (country)
Accupower® PCR PreMix Kit	Bioneer (Korea)
Agarose powder (Pronadisa)	CONDA (Spain)
Cobas HbA1C kit	Roche (Germany)
DNA Ladder (100-1000 bp)	Intron (Korea)
Ethanol	Holland
Ethidium Bromide solution	Intron (Korea)
Elecsys FSH kit	Roche (Germany)
Elecsys LH kit	Roche (Germany)
Elecsys TSH kit	Roche (Germany)
Elecsys Estradiol III kit	Roche (Germany)
Elecsys Prolactin II kit	Roche (Germany)
Elecsys Testosterone II kit	Roche (Germany)

Genomic DNA extraction kit	Intron (Korea)
Glucose HK Gen. 3 kit	Roche (Germany)
HDL-C kit	Roche (Germany)
Isopropanol	SRL (India)
MAGLUMI Insulin (CLIA) kit	Shenzhen (China)
Nuclease free water	Promega (USA)
Primers	Bioneer (Korea)
SHBG ELISA kit	Demeditec (GmbH / Germany)
Total Cholesterol kit	Roche (Germany)
Triglyceride kit	Roche (Germany)
Tris borate EDTA (TBE) Buffer 10x	Intron (Korea)

2.1.2. Instruments (Table 2.2)

Table 2.2: The Instruments and Apparatus

Instrument	Manufacture/ origin
Autoclave	Korea
Centrifuge	Sigma 3-30k (England)
Cobas e 411 analyzer	Roche (Germany)
Cobas Integra 400 plus	Roche (Germany)
Elisys Uno	(Human) Germany
Gel Documentation System	SYNGENE (UK)
Gel Electrophoresis system	TECHNE ME (England)
Hotplate Stirrer	LabTEch (Korea)
Nanodrop	Bio Drop (England)
PCR- thermocycler	TECHNE (England)
Sensitive Balance(DENEVER)	Germany
Snibe Diagnostic Maglumi 800	(Shenzhen) China
Vortex mixer	Human Twist (Germany)
Water path	LabTEch (Korea)

2.1.3. Patients

This study is a prospective interventional study carried out at Gynecological and Obstetric Teaching Hospital / Kerbala Health Directorate / Kerbala – Iraq, out clinic patients and College of Pharmacy / University of Kerbala, during the period from July, 2019 to July, 2020 with age ranged between (18 to 40) years.

The study was conducted on three hundred and forty six women with newly diagnosed polycystic ovary syndrome. The participated women were recruited by consultation of gynecologist according to the inclusion and exclusion criteria of the study, only two hundred and thirty one women returned after three months of treatment to follow up, where the rest of females were missed because of Covid-19 pandemic (February, 2020), also thirty female became pregnant during period of treatment so they were excluded from the study.

All women enrolled in this study were starting metformin tablet 500 mg per oral twice daily for three months as standard therapy. Biochemical parameters were measured to all patients participated in this study in the follicular phase (2^{nd} day of cycle) before treatment and after the end of treatment period (3 months) for the returned patients only to demonstrate the response to metformin and this achieved according to continuous communication and follow up with patients through social media during period of treatment.

2.1.3.1. Inclusion Criteria

A. Newly diagnosed PCOS women according to Rotterdam criteria: two of three of hyperandrogenism, irregular an ovulatory periods or ultrasound polycystic ovary morphology.

B. Age of women should be within range (18-40) years.

2.1.3.2. Exclusion Criteria

A. Females less than 18 years old due to incomplete reproductive function.

B. Females more than 40 years old due to menopause and menstrual irregularities.

C. Cushing syndrome.

D. Androgen secreting adrenal tumors.

E. Thyroid disorders (hypothyroidism caused abnormal menstrual cycle)

F. Women who become pregnant during treatment course.

G. Females with impaired kidney function

H. Patients taking other hypoglycemic agents.

I. Hypertensive patients

J. Any patient with HbA1c% more than 6.5

K. Patient on any other drug that is substrate for transporter like MATE1 and OCT1.

2.1.3.3. Ethical Approval

The protocol for study was approved by the ethical research committee of College of Pharmacy, University of Kerbala. Approval was also taken from Kerbala Health Directorate and Administration of Gynecological and Obstetric Teaching Hospital. In addition, consent was taken from each patient after explaining the nature and purpose of study.

2.1.3.4. Clinical Data Collection

At the time when the blood sample was obtained, each patient was questioned whether she had used one or more of medications other than metformin, to make sure that all patients were on metformin only in order to evaluate the effects of metformin in this study. Clinical manifestations were determined by consultation of gynecologist, furthermore, demographic parameters (questionnaire) were taken from

all patients at the beginning of study before taking the treatment which were: Name, address, age, BMI, systolic blood pressure, diastolic blood pressure, job, social state (married or not), numbers of kids, numbers of abortions, hirsutism, alopecia, smoking and menstrual irregularity (Oligomenorrhoea or Amenorrhoea) . Biochemical parameters were being measured for all patients before and after three months of starting with metformin which were: FSH, LH, total testosterone, sex hormone binding globulin (SHBG), free androgen index (FAI), Estradiol, prolactin, thyroid stimulating hormone (TSH), fasting insulin, HbA1c%, fasting blood sugar, homeostatic model assessment for insulin resistance (HOMA-IR ratio) and lipid profile (LDL-C, HDL-C, TG and total cholesterol).

2.1.3.5. Sample Collection

Blood samples were obtained from eligible patients after taking the patient consent, (7 mL) of venous blood were withdrawn from all women that participated in this study, who are all fasting during the morning in follicular phase (cycle day 2) in patients with a cycle length shorter than 3 months (Oligomenorrhoea) while the patients with cycle length >3 months (Amenorrhoea), the blood samples had drawn on a random cycle day where (2 mL) was placed in EDTA-tube for molecular assay then were analyzed directly to obtain high purity of DNA, (2 mL) was placed in another EDTA tube for HbA1c measuring and (3mL) was placed in gel tube (EDTA-free tube) for serum analysis; serum was aspirated after centrifugation of the blood at 3000 rpm for 10 minutes; were it used for measurement.

2.1.3.6. Body Mass Index

It was calculated by measuring the patient's weight (in kilograms) divided by the square of height (in meters) **[42].**

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

2.2. Methods

2.2.1. Serum Analysis

Blood constituents determinations included serum FSH, LH, total testosterone, Estradiol, prolactin, lipid profile (LDL-C, HDL-C, TG and total cholesterol), fasting insulin, fasting glucose, thyroid stimulating hormone (TSH), sex hormone binding globulin (SHBG).The samples were put in package containing ice after collection for keeping them with suitable temperature and then transfer them to laboratory for analysis.

2.2.1.1. Determination of Follicular Stimulating Hormone

The quantitative determination of follicular stimulating hormone (FSH) in human serum is by an (Elecsys FSH) kit and Cobas e 411 analyzer and dependent upon electro chemluminescence immunoassay method **[161]**.

Principle

Sandwich principle, total duration of assay: 18 minutes.

- **1st incubation:** 40 µL of sample, a biotinylated monoclonal FSH-specific antibody, and a monoclonal FSH-specific antibody labeled with aruthenium complex form a sandwich complex.
- **2nd incubation:** After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application

of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

- Results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode**.**

Reagents

The reagent rack pack is labeled as FSH.

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL.

R1 Anti-FSH-Ab~biotin (gray cap), 1 bottle, 10 mL: Biotinylated monoclonal anti-FSH antibody 0.5 mg/L, MES buffer 50 mmol/L.

R2 Anti-FSH-Ab~Ru(bpy) (black cap), 1 bottle, 10 mL: Monoclonal anti-FSH antibody (mouse) labeled with ruthenium complex 0.8 mg/L, MES buffer 50 mmol/L.

2.2.1.2. Determination of Luteinizing Hormone

The quantitative determination of luteinizing (LH) in human serum is by an (Elecsys LH) kit and Cobas e 411 analyzer and dependent upon electro chemiluminescence immunoassay method **[162].**

Principle

Sandwich principle. Total duration of assay: 18 minutes.

- **1st incubation:** 20 μL of sample, a biotinylated monoclonal LH-specific antibody, and a monoclonal LH-specific antibody labeled with aruthenium complex form a sandwich complex.

- **2nd incubation:** After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

Reagents

The reagent rack pack is labeled as LH.

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL.

R1 Anti-LH-Ab~biotin (gray cap), 1 bottle, 10 mL: Biotinylated monoclonal anti-LH antibody (mouse) 2.0 mg/L; TRIS buffer 50 mmol/L.

R2 Anti-LH-Ab~Ru(bpy) (black cap), 1 bottle, 10 mL: Monoclonal anti-LH antibody (mouse) labeled with ruthenium complex 0.3 mg/L; TRIS buffer 50 mmol/L.

2.2.1.3. Determination of Estradiol

The quantitative determination of estradiol in human serum is by an (Elecsys Esradiol III) kit and Cobas e 411 analyzer and dependent upon electro chemilumine-scence immunoassay method **[163].**

Principle

Competition principle. Total duration of assay: 18 minutes.

- **1st incubation:** By incubating the sample (25 µL) with two estradiolspecific biotinylated antibodies, immunocomplexes are formed, the amount of which is dependent upon the analyte concentration in the sample.
- **2nd incubation:** After addition of streptavidin-coated microparticles and an estradiol derivative labeled with a ruthenium complex, the still-vacant sites of the biotinylated antibodies become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

Reagents

The reagent rack pack is labeled as E2 III.

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL.

R1 Anti-estradiol-Ab~biotin (gray cap), 1 bottle, 9 mL:

Two biotinylated monoclonal anti-estradiol antibodies (rabbit) 2.5 ng/mL and 4.5 ng/mL; mesterolone 130 ng/mL; MESb) buffer 50 mmol/L.

R2 Estradiol-peptide~Ru(bpy)²+3 (black cap) 1 bottle, 9 mL: Estradiol derivative, labeled with ruthenium complex 4.5 ng/mL; MES buffer 50 mmol/L.

2.2.1.4. Determination of Prolactin

The quantitative determination of prolactin in human serum is by an (Elecsys Prolactin II) kit and Cobas e 411 analyzer and dependent upon electro chemilumine-scence immunoassay method **[164].**

Principle

Sandwich principle. Total duration of assay: 18 minutes.

▪ **1st incubation:** 10 µL of sample and a biotinylated monoclonal prolactinspecific antibody form a first complex.

▪ **2nd incubation:** After addition of a monoclonal prolactin-specific antibody labeled with a ruthenium complexa and streptavidin-coated microparticles, a sandwich complex is formed and becomes bound to the solid phase via interaction of biotin and streptavidin.

▪ The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

▪ Results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

Reagents

The reagent rack pack is labeled as PRL II.

M. Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL.

R1. Anti-prolactin-Ab~biotin (gray cap), 1 bottle, 10 mL: Biotinylated monoclonal anti-prolactin antibody (mouse) 0.7 mg/L; phosphate buffer 50 mmol/L.

R2. Anti-prolactin-Ab~Ru(bpy) (black cap), 1 bottle, 10 mL: Monoclonal anti-prolactin antibody (mouse) labeled with ruthenium complex 0.35 mg/L; phosphate buffer 50 mmol/L.

2.2.1.5. Determination of Thyroid Stimulating Hormone

The quantitative determination of thyroid stimulating hormone (TSH) in human serum is by an (Elecsys TSH) kit and Cobas e 411 analyzer and dependent upon electro chemiluminescence immunoassay method **[165].**

Principle

Sandwich principle. Total duration of assay: 18 minutes .

- **1st incubation:** 50 µL of sample, a biotinylated monoclonal TSH-specific antibody and a monoclonal TSH-specific antibody labeled with aruthenium complex react to form a sandwich complex.
- **2nd incubation:** After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the micro particles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell
- Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrumentspecifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode**.**

Reagents

The reagent rack pack is labeled as TSH.

M. Streptavidin-coated microparticles (transparent cap), 1 bottle, 12 mL: Streptavidin-coated microparticles 0.72 mg/mL.

R1. Anti-TSH-Ab~biotin (gray cap), 1 bottle, 14 mL: Biotinylated monoclonal anti-TSH antibody (mouse) 2.0 mg/L; phosphate buffer 100 mmol/L.

R2. Anti-TSH-Ab~Ru(bpy) (black cap), 1 bottle, 12 mL: Monoclonal anti-TSH antibody (mouse/human) labeled with ruthenium complex 1.2 mg/L; phosphate buffer 100 mmol/L.

2.2.1.6. Determination of Total Testosterone

The quantitative determination of total testosterone in human serum is by an (Elecsys Testosterone II) kit and Cobas e 411 analyzer and dependent upon electro chemiluminescence immunoassay method**[166].**

Principle

Competition principle. Total duration of assay: 18 minutes.

▪ **1st incubation:** 20 µL of sample are incubated with a biotinylated monoclonal testosterone-specific antibody. The binding sites of the labeled antibody become occupied by the sample analyte (depending on its concentration).

▪ **2nd incubation:** After addition of streptavidin-coated microparticles and a testosterone derivate labeled with a ruthenium complex, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.

• The reaction mixture is aspirated into the measuring cell where the micro particles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell.

• Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

- Results are determined via a calibration curve which is an instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode .

Reagents

The reagent rack pack is labeled as TESTO II.

M. Streptavidin-coated microparticles (transparent cap), Streptavidin-coated microparticles 0.72 mg/mL.

R1. Anti-testosterone-Ab~biotin (gray cap).

Biotinylated monoclonal anti-testosterone antibody (sheep) 40 ng/mL; releasing reagent 2-bromoestradiol; MES buffer 50 mmol/L.

R2. Testosterone-peptide~Ru(bpy) (black cap), 1 bottle, 9 mL: Testosterone derivative, labeled with ruthenium complex 1.5 ng/mL; MES buffer 50 mmol/L.

2.2.1.7. Determination of Sex Hormone Binding Globulin

Serum sex hormone binding globulin (SHBG) levels was determined by (SHBG ELISA) kit and Elisys Uno**[40].**

Principle

Sandwich principle.

The micro titer wells are coated with a monoclonal mouse antibody directed towards a unique antigenic site of the SHBG molecule. An aliquot of sample containing endogenous SHBG is incubated in the coated well with enzyme conjugate, which is an anti-SHBG antibody conjugated with horse radish peroxidase. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is proportional to the concentration of SHBG in the sample. Having added the substrate solution, the intensity of color developed is proportional to the concentration of SHBG in the patient sample.

Reagents

A. Microtiter wells, 12 x 8 (break apart) srips,96 wells ; wells coated with anti-SHBG antibody (monoclonal)

B. Standard (standard 0-6), 7 vials, 0.5 ml each, ready to use; concentrations: 0-4-16-32-65-130-260 nmol/L. The standards are calibrated against the following reference material: WHO international Standard for Sex hormone Binding Globulin (08/266).

C. 0.5ml of Control low and high.

D. 125 ml of Assay buffer.

E. 14 ml of Enzyme conjugate, Anti- SHBG antibody conjugated with horseradish peroxidase.

F. 14 ml of Substrate Solution, Tetramethylbenzidine (TMB).

G. 14 ml of Stop Solution contains 0.5 M H_2SO_4

H. 30 ml of Wash Solution 40X.

2.2.1.8. Estimation of Free Androgen Index (FAI)

Free Androgen Index is a ratio used to determine abnormal androgen status in humans. The ratio is the total testosterone level divided by the sex hormone binding globulin (SHBG) level, and then multiplying by a constant, usually 100 **[167]:**

FAI = (T. testosterone/SHBG)*100

2.2.1.9. Determination of Fasting Blood Sugar (FBS)

In vitro test for quantitative determination of glucose in serum by (Glucose HK Gen. 3) kit on COBAS INTEGRA system **[168].**

Principle

Hexokinase catalyzes the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. To follow the reaction, a second enzyme, glucose-6-

phosphate dehydrogenase (G6PD) is used to catalyze oxidation of glucose-6-phosphate by $NADP^+$ to form NADPH. The concentration of the NADPH formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at 340 nm.

Reagents

COBAS Integra 400 Plus Glucose HK Liquid – 800 tests

R1 MES buffer: 5.0 mmol/L, pH 6.0 ; Mg^{2+}: 24 mmol/L; ATP: 4.5 mmol/L; $NADP^+$: 7.0 mmol/L.

R2 HEPES Buffer: 200 mmol/L, pH 8.0; Mg^{2+}: 4 mmol/L; HK (yeast): 300 ukat/L; G-6-PD (E. coli): 300 ukat/L.

2.2.1.10. Determination of Fasting Insulin

The quantitative determination of Fasting Insulin in human serum by (MAGLUMI Insulin (CLIA)) kit and Snibe Diagnostic Maglumi 800 **[169].**

Principle

The sample (or calibrator/control), buffer, magnetic microbeads coated with anti-insulin monoclonal antibody and ABEI labeled with anti-insulin monoclonal antibody are mixed thoroughly and incubated at 37℃ formed sandwich of immune-complexes. After precipitation in a magnetic field, the supernatant is decanted and then awash cycle is performed. Subsequently, the starter 1+2 are added to initiate chemiluminescence reaction. The light signal is measured by a photomultiplier within 3 seconds as relative light units (RLUs), which is proportional to the concentration of insulin present in the sample (or calibrator/control).

Reagents

Reagent Integral for 100 determinations	
Nano magnetic microbeads: Tris buffer, 1.2% (w/v), 0.2%NaN_3, coated with sheep anti-polyclonal antibody.	2.5ml
Calibrator low	2.5ml
Calibrator high	2.5ml
Anti-Insulin monoclonal antibody labeled contains BSA, 0.2% NaN_3.	10.5 ml

2.2.1.11. Estimation of Homeostasis Model of Insulin Resistance (HOMA-IR)

HOMA-IR has been widely utilized as insulin resistance index. The HOMA-IR is being used extensively for estimation of pancreas beta cell function and insulin resistance, both in clinical practice and studies **(170)** ,where is calculated by :

HOMA-IR = (FBS X fasting insulin)/405

2.2.1.12. Determination of Glycated Hemoglobin (HbA1c%)

This test determines glycated hemoglobin A1c (HbA1c%) by using COBAS HbA1c kit and COBAS INTEGRA - 400 plus, normal value of HbA1c level less than 5.7%, a level between 5.7% to 6.4% shows pre-diabetes while level above 6.5% is indicative of diabetes mellitus **[171].**

Principle:

Turbidity : Turbidmetric measurement are made with spectrophotometer to determine the concentration of particulate matter in a sample. The amount of light blocked by suspension of particles depends not on concentration but also on size, because particles tend to aggregate and settle out of suspension sample handling.

Procedure :

The HbA1c determination is based on the turbidimetrically inhibition immunoassay (TINIA for hemolyzed whole blood).

1. Sample and addition of R1 Antibody reagent**:** Glycohemoglobin (HbA1c) in the sample reacts with anti-HbA1cd antibody to form soluble antigen-antibody complexes. Since the specific HbAq2c antibody site is present only once on the HbA1c molecule, complex formation does not take place (Polyhapten reagent).
2. Addition of SR (buffer/polyhapten reagent) and start of reaction: the polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be measured turbidmetrically. Hemoglobin liberated hemoglobin in the hemolyzed sample is converted to a derivative having a characteristic absorption spectrum which is measured bichromatically during the pre-incubation phase (sample + R1) of the above immunological reaction. A separate Hb reagent is consequently not necessary. The final result is expressed as percent HbA1c and is calculated from the HbA1c/Hb ratio as follows: Standard Operating Procedure (%HbA1c acc.to DCCT/NGSP):

HbA1c (%) = (HbA1c / Hb) x 91.5 + 2.1

Reagents

Reagent cassette

R1 Antibody reagent

MES buffer: 0.025 mol/L; TRIS buffer: 0.015 mol/L, pH 6.2; HbA1c antibody (bovine serum) >0.5 mg/mL.

SR Polyhapten reagent

MES buffer: 0.025 mol/L; TRIS buffer: 0.015 mol/L, pH 6.2; HbA1c polyhapten: >8 µg/mL.

2.2.1.13. Lipid Profile Determination

2.2.1.13.1. Measurement of Total Serum Cholesterol

Determination of serum total cholesterol was by (Total Cholesterol) kit and COBAS INTEGRA - 400 plus.

Principle:

Serum total cholesterol was determined according to method that was determined by enzymatic reaction as indicated below. Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase**[172]**.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \underset{\text{Purple-blue pigment}}{\text{Quinoneimine}} + 4H_2O$$

In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to cholest-4-en-3-one a purple-blue color and hydrogen peroxide. The color intensity of the blue quinone-imine dye formed proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 583 nm **[173].** The reagents were used:

1. Buffer solution composed phosphate buffer 100 mmol/l, chloro-4-phenol 5 mmol/l, sodium chloride 2.3 mmol/l, triton X 100 1.5 mmol/l.

2. Enzymes solution composed cholesterol oxidase 100 IU/l, cholesterol esterase 170 IU/l, peroxidase IU/l, 4-amino-antipyrine 0.25 mmol/l, PEG 6000 167 μmol/l.

3. Standard solution for cholesterol 200 mg/dl (5.17 mmol/l).

The content of enzymes vial was added to buffer vial and mixed gently until complete dissolution to prepare work reagent. The procedure was carried out as in the following table:

Reagents	**Blank**	**Standard**	**Sample**
Reagent	1 ml	1 ml	1 ml
Demineralized water	10 µl	-	-
Standard	-	10 µl	-
Sample	-	-	10 µl

The tubes were mixed and then left to stand for 5minutes at 37ºC. Record absorbance at 500 nm (480-520) against blank. The colored complex is stable for 1 hour.

Sample cholesterol was obtained from the following equation:

$$\text{Total Cholesterol (mmol/l)} = \frac{A_{sample}}{A_{standard}} \times \text{Conc. of standard cholesterol}$$

2.2.1.13.2. Measurement of Serum Triglyceride

Determination of serum triglyceride was by (Triglyceride) kit and COBAS INTEGRA - 400 plus.

Principle:

Serum triglyceride concentration was determined enzymatically and the principle method associated with Trinder reaction **[174].**The absorbance of the colored complex (quinonimine) is proportional to the amount of TG in the specimen. The reagents used in this procedure are:

1. Buffer solution composed PIPES 100 mmol/l, $MgCl_2$ 9.8 mmol/l, Chloro-4-phenol 3.5 mmol/l.
2. Enzymes solution composed Lipase 1000 IU/l, peroxidase 1700 IU/l, Glycerol-3-p-oxidase 3000 IU/l, Glycerol kinase 660 IU/l, PAP 0.5 mmol/l, ATP 1.3 mm0l/l.
3. Standard solution containing glycerol equivalent to triglycerides 200 mg/dl or 2.28 mmol/l.

The content of enzymes vial was added to buffer vial and mixed gently until complete dissolution to prepare work reagent. The procedure was carried out as in the following table:

Reagents	Blank	Standard	Sample
Reagent	1 ml	1 ml	1 ml
Demineralized water	10 µl	-	-
Standard	-	10 µl	-
Sample	-	-	10 µl

The tubes were mixed and then left to stand for 5minutes at 37ºC or 10 minutes at room temerature. Record absorbance at 500 nm (480-520) against blank. The colored complex is stable for 1 hour.

$$\text{Triglyceride Concentration} = \frac{A_{Sample}}{A_{Standard}} \times 2.28$$

2.2.1.13.3. Measurement of Serum HDL-Cholesterol

Determination of serum total cholesterol was by (HDL-C) kit and COBAS INTEGRA - 400 plus.

Principle:

LDL,VLDL and chylomicron from specimen were precipitated by phosphotungstic acid and magnesium chloride. HDL-cholesterol obtained in supernatant after centrifugation is then measured with total cholesterol reagent **[175]**.The reagent used only precipitant which contained phosphotungstic acid 13.9 mmol/l and magnesium chloride 6.2 490 mmol/l. The procedure was carried out as indicated in the following table:

Reagent	Volume
Serum	0.5 ml
Precipitant	50 µl

The tubes mixed vigorously, then left to stand for 10 minutes at room temperature. Centrifuge 15 minutes at 1400-1800 x, then apply next procedure which includes measurement of cholesterol in supernatant:

Reagents	Blank	Standard	Sample
Reagent	1ml	1 ml	1 ml
Distilled water	25 µl	-	-
Standard 2.58 mmol/l	-	25 µl	-
Supernatant	-	-	25 µl

The tubes were mixed, and then left to stand for 5 minutes at 37ºC. The absorbance was measured at 500 nm (480-520) against blank. The colored complex solution is stable for 1 hour.

$$\text{HDL-C conc., mmol/L} = \frac{A_{Sample}}{A_{Standard}} \times 2.58 \text{ mmol/L}$$

2.2.1.13.4. Estimation of Serum LDL-C

Low density lipoprotein-cholesterol (LDL-C) concentration was calculated indirectly by using Friedewald and colleagues equation, which becomes the more common method in routine clinical labs **[176].**

LDL-C mmol/L = [Total-chol.] - [HDL-C] - [TG/5]

Where all concentrations are given in mg/100 ml (TG / 2.22 is used when LDL-C is expressed in mmol/L).

2.3. Molecular Analysis

Extraction of nucleic acids is the first step in most molecular analytical studies and recombinant DNA techniques. A large number of procedures, based on a variety of methods and principles, exists for the extraction and purification of nucleic acid. They all share the common requirements that the biological material needs to be lysed, cellular nucleases must be inactivated, and finally, the desired nucleic acid has to be purified from the cellular debris. Protocols differ only in the first few steps regarding tissue and cell lysis; they then share a common purification procedure as summarized below:

A. The starting material is lysed by incubation with lysis buffer and proteinase K to break open cell membranes and expose DNA and RNA.

B. Binding buffer is added to inactivate nucleases and the solution is transferred into filter tubes and briefly centrifuged.

C. After three washes with different buffers, the nucleic acid bound to the glass fiber filter is pure and can be eluted with the elution buffer that does not interfere with further reaction into a sterile 1.5 microcentrifuge tube.

D. Eluted genomic DNA can be used immediately for further molecular experiments or stored at 4 °C or frozen at -20 °C until the day of used.

2.3.1. Extraction of Genomic DNA.

The DNA extraction was conducted at College of Pharmacy / University of Kerbela / Laboratory of Molecular Biology. Genomic DNA was extracted from blood sample according to the protocol of (G-DEX™ IIb / Intron , Korea) genomic DNA extraction kit for blood.

Extraction is carried out in 4 steps that involve lyses, binding, washing and elution as the following steps:

1. 1.5 ml blood was centrifuged at 2000 rpm for 1 minute to produce buffy coat.

2. 300 µL of the formed buffy coat were added to 1.5 ml epindorff tube containing 900 µL RBC lysis solution then they were mixed toughly by vortexing and incubating for 5 minutes at room temperature.
3. The tubes were centrifuged at 10000 rpm for 1 minutes ,then the supernatant layer were removed except the white cell pellet and 50-100 µL of the remnant are remained .
4. The tube was vortexed vigorously to re-suspend the cells.
5. 300 µL of cell lysis solution were added to the re-suspend cells then pipetting up and down was done to lyse the cell.
6. Sample was chilled to room temperature then 100 µL PPT solution buffer were added to cell lysate and vortexed vigorously at high speed for 20 seconds.
7. The sample was centrifuged at 16000 rpm for 5 minutes, the precipitated proteins formed white tight pellet.
8. 300 µL of supernatant containing the DNA were transferred to 1.5 ml tube then 300 µL of 100 % isopropanol alcohol were added to the tube and the sample was mixed by inverting gently several times.
9. The sample was centrifuged at 16000 rpm for 1 minute so the DNA was visible as small white pellet.
10. The supernatant was poured off and drained on clean absorbent paper then 1ml of 70% ethanol alcohol was added to tube containing DNA and the tube was inverted several times to wash the DNA pellet.
11. The sample was centrifuged at 16000 rpm for 1 minutes, then ethanol carefully was poured off and the tube was inverted on clean absorbent paper and was allowed for air to dry about 10 -15 minutes.
12. 200 µL of DNA rehydration buffer solution were added to the tube then the DNA was rehydrated by incubating at 65c for 30 – 60 minutes or at 4 c for overnight culture. Collected DNA was stored at -20 c (deep freezing).

2.3.2. Quantitation of DNA by Spectrophotometric Method

DNA purity and concentration were measured by using spectrophotometric methods which is a Nano Drop™ system.

Principle:

The purity and concentration of extracted DNA samples were measured by absorbance method using the Nano drop instrument. The absorbance readings were done at 260 nm and at 280 nm **[177]**. At 260 nm, the DNA strongly absorbs light while at 280 nm, the protein absorbs light most strongly. DNA purity was measured by the A260/A280 ratio. The A260/A280 ratio 1.8-2.0 is commonly accepted and it signifies a high quality DNA sample **[178]**. Highly sensitive micro detector of nanodrop as blank. The micro detector was cleaned up from blank, then 1 µL of sample was applied on the micro detector of nanodrop. The concentration and A260/A280 ratio of DNA were documented from the instrument **[179]**.

2.3.3. Polymerase Chain Reactions

Polymerase chain reactions (PCR) were performed using thermocycler (Techne, England) which amplified a desired region of the genome. The concentration of desired target sequence increases from one molecule to several million copies. There are three steps to any PCR that are cycled about 25-45 times, which include:

A. **Denaturation:** This step occurs at 94-95^{o}C and entails the unwind dsDNA in to two single strands.
B. **Annealing:** This step occurs at 55-65^{o}C. A pair of short (17-26) oligonucleotide sequences (primers) anneal to the ends of the template strands of DNA and begin the reaction.
C. **Extension:** This step occurs at 72-74^{o}C and entails the extension of the primers to form a new strand that is complementary to the template strand. This occurs in the presence of the *Taq DNA polymerase*, a DNA polymerase

isolated from the organism *Thermus aquaticus* ,a bacterium that can survive at high temperatures without denaturation.

2.3.3.1. Amplification Refractory Mutation System - PCR

The allele-specific PCR also called as an amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) or PCR amplification of specific alleles (AS-PCR) used to detect the SNPs **[180]**. Tetra ARMs – PCR protocol was used for SNPs detection of MATE1 (SLC47A1) (rs2289669 **G>A**)genetic polymorphism while allele-specific PCR was used for detection of MATE1(SLC47A1) (rs1961669 **A>G**) genetic polymorphism.

Principle

The ARMS-PCR is one of the molecular techniques which was designed for creating thousands to millions copies of demanding DNA fragment**[181].** Some reagents and components are essential for ARMS-PCR, these components comprise of DNA target (DNA template) that includes the region of DNA to be amplified, primers (forward and reverse primer) which are complementary to the DNA template, *Thermus aquaticus* DNA polymerase (*Taq polymerase*) enzyme, deoxy nucleotide tri- phosphates (dNTPs) and buffer solution which make an appropriate chemical environment for maximum favorable stability and activity of the DNA polymerase.

The ARMS PCR is mostly done to identify a mutation or a polymorphism which is simply a difference in DNA sequence between two related organisms, e.g. two individual humans. Polymorphisms may be divided into those consisting of base changes and those where there is a difference in the length of the corresponding region of DNA **[182]**, it is also important that it should be able to identify whether the change in DNA is heterozygous or homozygous. A heterozygote or homozygote

is differentiated by using ARMS primers for the mutant/polymorphic and the normal (wild type) alleles. The reactions for the mutant and the normal alleles are usually carried out in separate tubes **[181].**

2.3.3.2. Primer Design

The introns of two SNPs of Mate-1 (SLC47A1 gene): [(rs2289669 G >A) and (rs1961669 A>G)] were amplified by means of specific primers to study the SNPs, the present result used primer oligonucleotide designing by using Homo sapiens (multi drugs and toxins extrusion protein1) gene, depending on https://www.ncbi.nlm.nih.gov/ websites**,** using primer blast software **[183]**.

PCR reaction was performed by using specific primers designed for SLC47A1 gene. Based on NCBI database, all genes information, sequence and SNPS details, were collected. Using specific software, primers were designed.

Primers were taken in a lyophilized state, the units of a lyophilized primer are known as a mass in picomoles. The subsequent steps were done for the reconstitution and dilution of the primers:

-The tube was centrifuged at 10000 x g for 5-10 min before decamping.

-The chosen volume from nuclease free water was added according to the manufacturer to give a primary concentration of 100 pmoles /μL so lyophilized primers were dissolved in a nuclease free water to give a (stock solution).

-For working solution: The Primers were re- mixed by suitable vortexing, then 10 Pmol of stock solution were diluted with 90 μL of nuclease free water in a 0.5 mL eppendorf tube in microcenterfuge to obtain (10 pmol) as a final concentration (working solution), the stock and working solution were kept at -20 ^{o}C, the sequence of the primers (forward and reverse) with their product size were mentioned in tables (2.3) and (2.4):

Table: 2.3. Primer sequence of SLC47A1 (G> A) (rs2289669) genetic polymorphism

Primers	Sequence (5'->3')	Tm	Ta	Product	Reference
Outer F	GAGGAACATGGTTGTGCAGC	59.76	54	410 bp	Current study
Outer_R	ACCAAGAGCCTCGGGTAAG	59.39	54		Current study
Inner_R (G allele)	CTTTGTCTAGCCGGGAACTC	57.99	54	132bp	Current study
Inner_F (A allele)	AGTTTCCACAGTAGCGTGGA	58.95	54	318 bp	Current study

Table:2.4. Primer sequence of SLC47A1 (A> G) (rs1961669) genetic polymorphism

Primers	Sequence (5'->3')	Tm	Ta	Product	Reference
Forward primer	GGTTCAGGTGAGCTTGTAGT	57.16	52	-----	Current study
R1-Allele A	AGTCTGGGTTCCTGGTTTAT	55.72	52	392	Current study
R2-Allele G	GTCTGGGTTCCTGGTTTAC	55.11	52	392	Current study

2.3.3.3. PCR Working Solution

- **Accupower® PCR PreMix –Bioneer Kit** which is ready to use PCR reagent (Add only the DNA template and primers), an individual PCR tube contains lyophilised mixture of Thermo-stable Enzyme (Top DNA polymerase) , dNTPs , Reaction buffers (Tris-HCl, KCl), Enhancer(Mgcl2), Stabilizer and Tracking Dye.

2.3.3.4. Optimization of PCR Conditions

Optimization of polymerase chain reaction was achieved after several trials.

2.3.3.4.1. Optimization of SLC47A1 (G>A) (rs2289669) Genetic Polymorphism Conditions

Optimization of PCR conditions of SLC47A1 (G> A) (rs2289669) genetic polymorphism conditions was prepared by using:

- Different volumes of primer (1 µL, 2 µl)
- Different volumes of template DNA (2 µl, 3 µl, 4 µl)
- Different annealing temperatures (52°C,53°C,54°C, 55°C).

The preferred conditions which provided the best results for this SNP were addition of:

A. 1 µL outer forward primer

B. 1 µL outer reverse primer

C. 1 µL inner reverse primer

D. 1 µL inner forward primer

E. 3 µL DNA sample

F. 13 µL nuclease free water

2.3.3.4.2. Optimization of SLC47A1(A>G) (rs1961669) Genetic Polymorphism

Optimization of PCR conditions of SLC47A1(A>G) (rs1961669) genetic polymorphism were prepared by using:

- Different volumes of primer (1 µL, 2 µl)
- Different volumes of template DNA (2 µl, 3 µl ,4 µl)
- Different annealing temperatures (52°C, 57°C).

The preferred conditions which provided the best results for this SNP were addition of:

A. 1 µL forward primer

B. 1 µL reverse 1 (R1) primer

C. 1 µL reverse 2 (R2) primer in different tube

D. 3 µL DNA sample

E. 15 µL nuclease free water

A total reaction volume is 20 µL which added to the 500µl PCR tube of premix that contains 5 µL of premix which centrifuged for 10 seconds at 2000 rpm in a micro centrifuge for mixing the sample tubes and then placed in thermocycler.

2.3.3.5. PCR Program

The reaction was carried out as shown in tables (2.5) and (2.6)

Table: 2.5. Optimization conditions for SLC47A1 (G> A) (rs2289669) genetic polymorphism

Steps	Temperature	Time	Cycles
Initial Denaturation	**92**	**3 min**	**1**
Denaturation	**92**	**30 sec**	**45**
Annealing	**55**	**30 sec**	
Extension	**72**	**30 sec**	
Final extension	**72**	**5 min**	**1**
Hold	**10**	**10 min**	

Total time is 2hrs and 20 min

Table:2.6. Optimization conditions for SLC47A1 (A> G) (rs1961669) genetic polymorphism

Steps	Temperature	Time	Cycles
Initial Denaturation	**95**	**3 min**	**1**
Denaturation	**95**	**30 sec**	**45**
Annealing	**52**	**30 sec**	
Extension	**72**	**30 sec**	
Final Extension	**72**	**5 min**	**1**
Hold	**10**	**10 min**	**1**

Total time is 2 hrs and 20 min

2.3.3.5. Agarose Gel Electrophoresis

Electrophoresis is designated as a typical method to distinguish, separate and purify DNA fragments of variable sizes ranging from 100 bp to 1000 bp throughout the agarose gels. Agarose gel electrophoresis was adopted to confirm the presence of PCR amplification. PCR was completely dependable on the extracted DNA criteria **[184]**. The following factors affect the rate of DNA migration through agarose gel electrophoresis:

- The molecular size of the DNA.
- The concentration of agarose.
- The conformation of the DNA.
- The presence of ethidium bromide in the gel and electrophoresis buffer.

- The applied voltage.
- The type of agarose.

Solutions:

1x TBE buffer, DNA Ladder marker, Ethidium bromide (10 mg/ml).

2.3.3.6.1. Preparation of Solution (1x TBE solution)

1X TBE buffer (tris borate EDTA) was prepared by diluting 10X TBE buffer with deionized water (one volume of 10X TBE buffer with 9 volume of deionized water: 1:10 dilution).

2.3.3.6.2. Preparation of Agarose Gel [185,186]

- 100 ml of 1x TBE was taken in a beaker.
- 1.5 gm (for 1.5%) agarose was weighed and added to the buffer.
- The solution heated to boiling by using heater until all the gel particles were dissolved. This gel was used to identify the band of PCR product.
- The solution was stirred in order to be mixed and to avoid bubbles until the gel solution was looked clear and pure.
- The solution was cooled down at 50-60 c◦.
- 3.5 µL of stock Ethidium Bromide (10mg/ml) was added to the gel.

2.3.3.6.3. Casting of the Horizontal Agarose Gel

The gel tray was sealed with adhesive tape. A comb was pushed in the gel chamber about 1 inch from one ending of tray, then the agarose solution was poured in to the tray and the gel allowed to solidify at room temperature for approximately

30 minutes and kept at refrigerator for 15-30 minutes to harden well. The comb was carefully removed and the gel was placed in a horizontal gel electrophoresis tank. The tank was filled with 1X TBE- electrophoresis buffer (that was used for preparation of agarose gel) until the buffer reached 3-5 mm over the surface of the gel.

2.3.3.6.4. Loading of PCR Products

After the gel became clear and solid, the comb was removed and the glass plates were put in submarine horizontal gel electrophoresis chamber which was filled with 1 X TBE buffer that reached above the level of wells, 5 µL of PCR products were directly loaded to the well with great precaution to prevent damages of the wells and cross contamination of neighboring wells. The negative pole was linked to the negative side of the unit and the positive pole to another. Electrical power was turned on at 100 volt/50 Amp for 90 min or while waiting for dye indicators traveled to the appropriate distance according to the size of DNA fragment. DNA moved from cathode (negative pole) to plus Anode (positive poles). The bands that stained with Ethidium Bromide in gel were visualized by using Gel Documentation System.

2.3.3.6.5. DNA Ladder

In current study, 5 µL of DNA ladder was used (Intron, Korea) and the band size ladder was 100-1000 bp. The ladder type was used in this study can be offered directly without addition of sample loading dye.

2.3.3.6.6. Gel- Band Visualization

To visualize the DNA bands, the agarose gel was placed in the UV transilluminator device and exposed to UV light and the photos were captured by digital camera linked to PC.

2.4. Statistical Analysis

The results were expressed as mean ±SD. Statistical analysis was performed by using the PAST version 3.09, 2004. Fissure exact test and Chi- square ($\chi 2$) was used to determine any significant difference in demographic parameters between the categorical data, Paired t-test was used to examine the difference in means of biochemical parameters between pre- and post- metformin treatment results , odds ratio (OR) and confidence interval 95% (CI95%) were used to express the significance between the allele frequencies of obese and non-obese groups. In all statistical analysis that used in this study, probability value *(P value)* expressed as the significant value was (≤ 0.05) and the highly significant value was (≤ 0.01).

CHAPTER THREE

Results

3. Results

3.1. Demographic Parameters

3.1.1.Some Demographic Parameters in PCOS

Based on inclusion and exclusion criteria, 346 patients of PCOS dependent upon metformin mono therapy were enrolled in the study. For final data analysis only 231 patients were included in the study, as thirty patients became pregnant during treatment period and eighty-five patients were lost to follow up due to Covid-19 pandemic, the women which included in the study finally were with an average age ranged between (18-40) years and the mean ± SD of them were 27.2 ± 5.9 years.

Results of the present study were shown in table (3.1) and figure (3.1) using Chi-Square statistical test and Paired t-test; there was a significant difference in alopecia (*P* value ≤ 0.01) between pre- and post-metformin treatment results (χ^2 = 43.31).

The significant result (*P* value ≤ 0.01), (χ^2 = 119.6) was shown in differentiation in menstrual cycle regularity between pre- and post-metformin treatment. The result of hirsutism indicated a non-significant difference (*P* value = 0.7), (χ^2 = 0.13) between pre- and post-metformin treatment results, as well as there was no statistical difference in BMI (*P* value = 0.13) between the pre- and post -treatment period as the values were following : [(29.12 ± 5.4) and (28.35 ± 5.3)] kg/m^2 respectively as shown in table (3.1) and figure (3.2).

Table 3.1. Comparison between demographic parameters between pre- and post-metformin treatment as response to metformin

Demographic parameter		Pre-treatment n(%)	Post-treatment n(%)	Chi-Square Test (χ^2)	Probability
Hirsutism	**(+)**	209 (90.48)	120 (51.9)	χ^2= 0.13	*P* value = 0.7
	(-)	22 (9.52)	111 (48.1)		
Alopecia	**(+)**	186 (80.5)	119 (51.5)	χ^2=43.31	*P* value ≤ 0.01
	(-)	45 (19.5)	112 (48.5)		
Regularity of menstrual cycle	**(+)**	0 (0)	95 (41.1)	χ^2 =119.6	*P* value ≤ 0.01
	(-)	231 (100)	136 (58.9)		
Parameter	**Pre-metformin treatment**	**Post-metformin treatment**	**Mean difference ± S.D.**	***P* value**	
BMI kg/m^2	29.12 ± 5.4	28.35 ± 5.3	0.77 ± 1.5	0.13	

Chi-square test, Paired t-test, *P* value ≤ 0.01: highly significant change, (+) : present, (-) : not present, BMI: body mass index

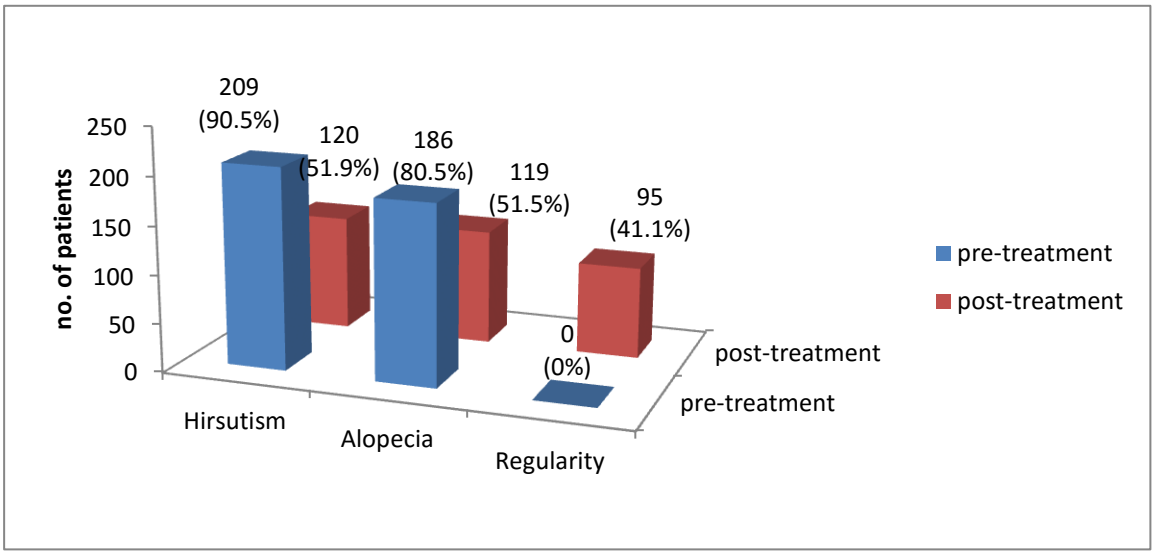


Fig. 3.1.: Response in demographic parameters in PCOS patients pre- and post-metformin treatment

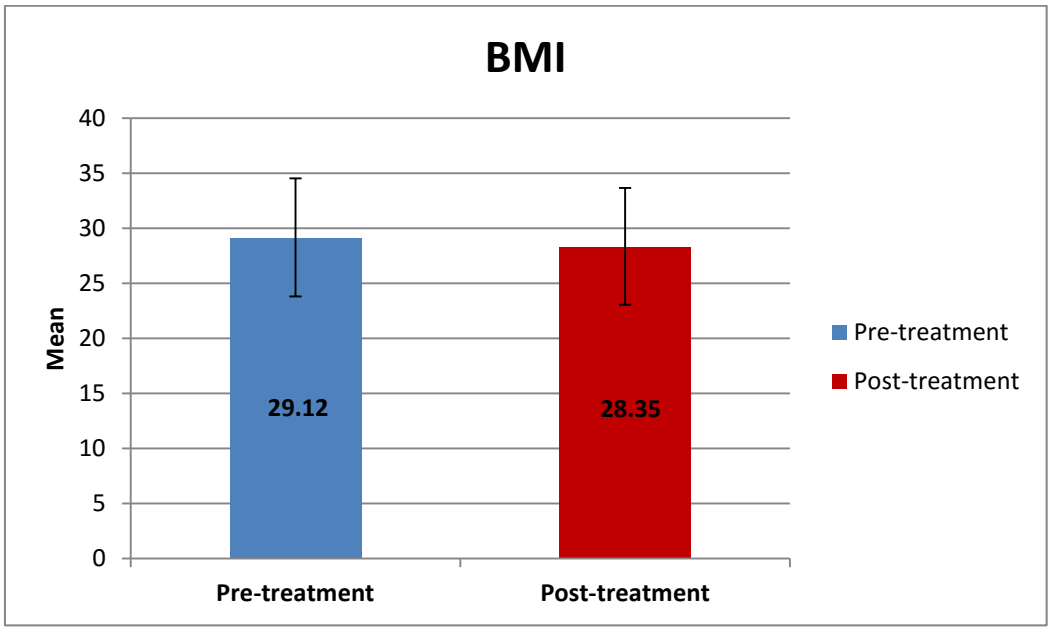


Fig. 3.2.: Change in BMI (kg/m^2) as a response to metformin between pre- and post-metformin treatment

3.2. Studies of Biochemical Markers

3.2.1. Some Biochemical Parameters in PCOS

The results of present study were shown in table (3.2) using statistical paired t-test; there was a highly significant increase in the mean of FSH level (*P* value ≤ 0.01) between pre- and post- metformin treatment [(5.7 ± 1.99),(6.5 ± 2.4)] mIU/mL respectively while a highly significant reduction in the mean of LH level (*P* value ≤ 0.01) has been recorded as the value before treatment was (10.13 ± 6.19) mIU/mL and the value after treatment was (8.5 ± 4.8) mIU/mL. as a result, LH/FSH ratio indicated highly significant decrease (*P* value ≤ 0.01) in values between pre- and post- treatment where the results as follows :(1.85 ± 1.08) and (1.4 ± 0.8) , as shown in Figure (3.3).

In current study, there was a non-significant change in TSH level (*P* value = 0.99) where the value before treatment was (2.27 ± 0.97) ulU/mL and after treatment was (2.3 ± 1.9) ulU/mL while prolactin recorded highly significant decrease (*P* value ≤ 0.01) and its value was (23.9 ± 13.6) ng/mL before treatment while its value after treatment was (20.13 ± 9.9) ng/mL.

The results of total testosterone level before and after treatment were [(0.6 ± 0.35),(0.45 ± 0.3)] ng/mL respectively as there was highly significant decrease between them (*P* value ≤ 0.01) , while (SHBG) level showed highly significant elevation (*P* value ≤ 0.01) where the value before treatment was (41.84 ± 22.6) nmol/L and after treatment was (51.3 ± 21.8) nmol/L. FAI recorded highly significant reduction (*P* value ≤ 0.01) between pre- and post- metformin treatment period where the results were as follows: (9.6 ± 17.7) and (4.5 ± 6.7) , as shown in Figure (3.5).

Estradiol showed no significant reduction (*P* value= 0.3) with values of pre-treatment in comparison with post-treatment [(64.4 ± 29.3),(61.5 ± 31.6)] pg/mL respectively as demonstrated in Figure (3.4).

The results of the current study of FBS level were highly significant reduction (*P* value ≤ 0.01) when comparing the outcomes values for the pre- and post-treatment period values (96.03 ± 12.5) and (92.45 ± 12.97) mg/dL. Non-significant reduction in fasting insulin level (*P* value = 0.23) between pre- and post-metformin treatment has been observed [(20.02 ± 14.5),(18.6 ± 11.7)] µUl/mL respectively, furthermore, HOMA-IR ratio indicated a non-significant decrease (*P* value = 0.13) as the values before treatment was (4.8 ± 3.7) while the value after treatment was (4.3 ± 2.96). In contrast, the results of HbA1c% before and after treatment were [(4.95 ± 0.69) , (4.6 ± 0.65)] respectively as there was an important highly significant decrease between them (*P* value ≤ 0.01) as shown in Figure (3.6).

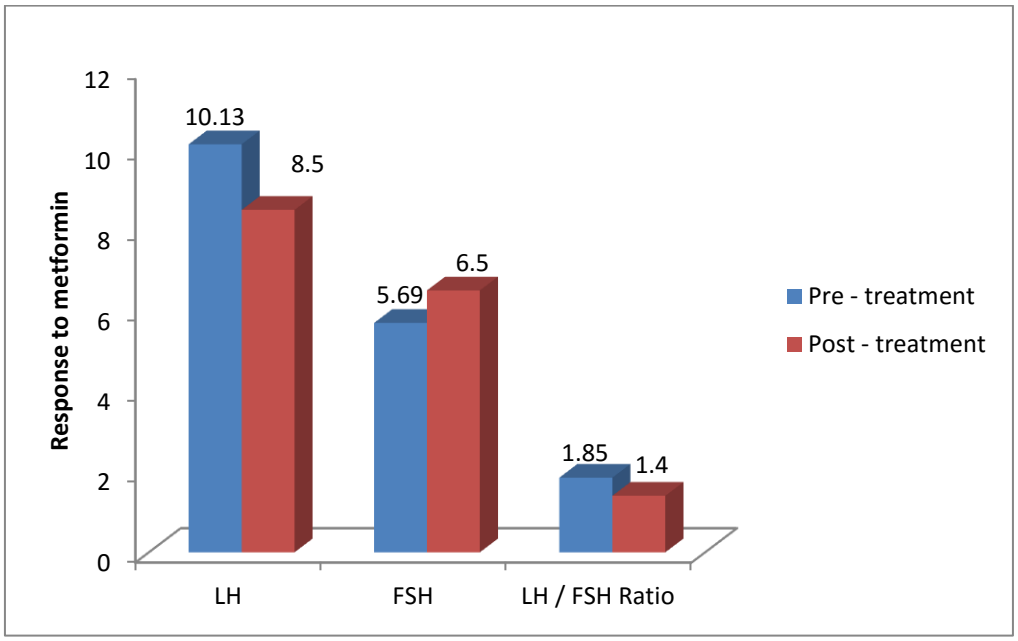
Regarding lipid profile , the results of the current study of TG level were highly significant decrease (*P* value ≤ 0.01) when comparing the outcome values for the pre- and post- treatment period [(127.6 ± 49.4) and (113.2 ± 39)] mg/dL. LDL-C level recorded highly significant reduction (*P* value ≤ 0.01) and its value was (98.06 ± 66.4) mg/dL before treatment while its value was (85.1 ± 25.5) mg/dL after treatment while there was no statistical increase for HDL-C (*P* value = 0.1) between pre- and post- treatment period as the values were as follows: [(44.8 ± 9.96) and (46.28 ± 9.46)] mg/dL. Total cholesterol showed a highly significant reduction (*P* value ≤ 0.01) between pre- and post- metformin treatment [(160.7 ± 43.8),(146.6 ± 44.03)] mg/dL respectively as shown in Figure (3.7).

Table 3.2. Comparison between biochemical parameters in PCOS patients pre- and post-metformin treatment as response to metformin

Parameters	Pre–treatment (Mean ± SD)	Post–treatment (Mean ± SD)	Mean difference ± SD	*P* value
FSH (mIU/mL)	5.7 ± 1.99	6.5 ± 2.4	-0.8 ± 2.2	≤ 0.01
LH (mIU/mL)	10.13 ± 6.19	8.5 ± 4.8	1.6 ± 4.5	≤ 0.01
LH / FSH	1.85 ± 1.08	1.4 ± 0.8	0.1 ± 0.55	≤ 0.01
TSH (ulU/mL)	2.27 ± 0.97	2.3 ± 1.9	0.00087±0.55	0.99
Prolactin (ng/mL)	23.9 ± 13.6	20.13 ± 9.9	3.8 ± 9.7	≤ 0.01
T.testosterone (ng/mL)	0.6 ± 0.35	0.45 ± 0.3	0.14 ± 0.25	≤ 0.01
SHBG (nmol/L)	41.84 ± 22.6	51.3 ± 21.8	-9.4 ± 15.1	≤ 0.01
FAI	9.6 ± 17.7	4.5 ± 6.7	5.06 ± 15.6	≤ 0.01
Estradiol (pg/mL)	64.4 ± 29.3	61.5 ± 31.6	2.97 ± 37.3	0.3
FBS(mg/dL)	96.03 ± 12.5	92.45 ± 12.97	3.6 ± 11.3	≤ 0.01
F. Insulin level (µUl/mL)	20.02 ± 14.5	18.6 ± 11.7	1.46 ± 9.09	0.23
HOMA-IR	4.8 ± 3.7	4.3 ± 2.96	0.48 ± 2.48	0.13
HbA1c%	4.95 ± 0.69	4.6 ± 0.65	0.36 ± 0.56	≤ 0.01
TG (mg/dL)	127.6 ± 49.4	113.2 ± 39	14.4 ± 25.99	≤ 0.01
LDL-C (mg/dL)	98.06 ± 66.4	85.1 ± 25.5	12.96 ± 59.5	≤ 0.01
HDL-C (mg/dL)	44.8 ± 9.96	46.28 ± 9.46	-1.48 ± 6.25	0.1
T. Cholesterol (mg/dL)	160.7 ± 43.8	146.6 ± 44.03	14.08 ± 28.4	≤ 0.01

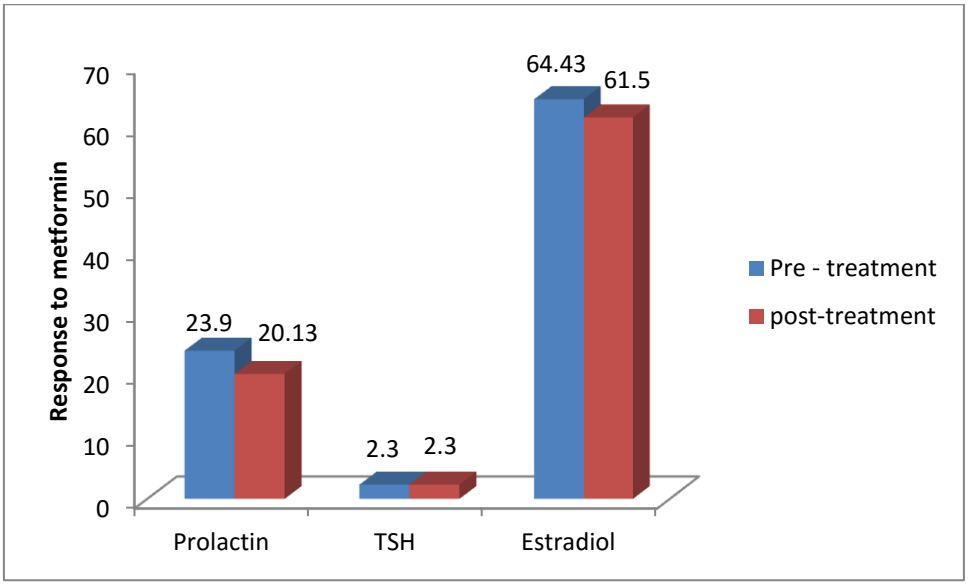
Paired t-test, *P value* ≤ 0.01: highly significant change

FSH: follicular stimulating hormone, LH: luteinizing hormone, TSH: thyroid stimulating hormone, SHBG: sex hormone binding globulin, FAI: free androgen index, FBS: fasting blood sugar, HOMA-IR: homeostatic model assessment for insulin resistance, HbA1c%: glycated hemoglobin, TG: triglycerides, LDL-C: low density lipoprotein, HDL-C: high density lipoprotein.



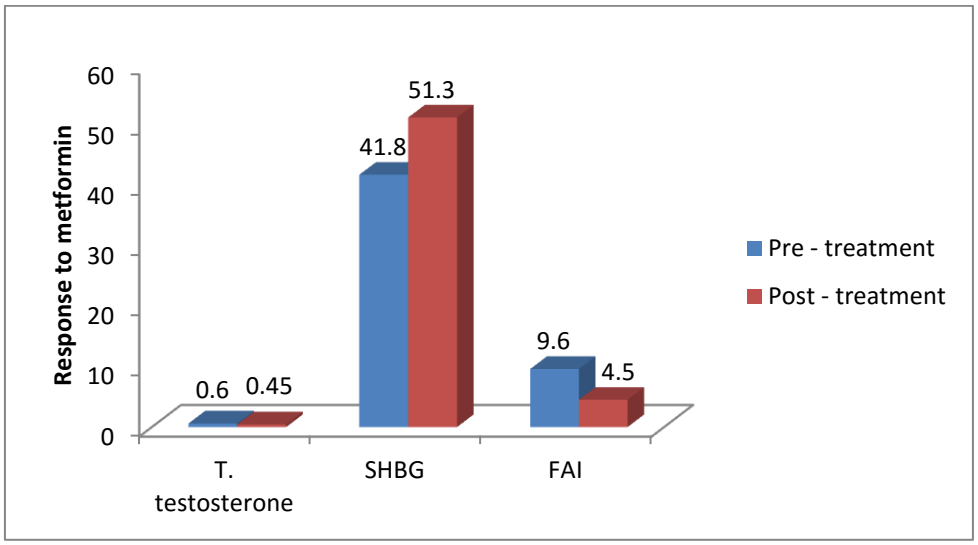
FSH: follicular stimulating hormone, LH: luteinizing hormone

Fig. 3.3. Change in mean of LH (mIU/mL), FSH (mIU/mL) and LH/FSH ratio as a result to metformin treatment between pre- and post-treatment.



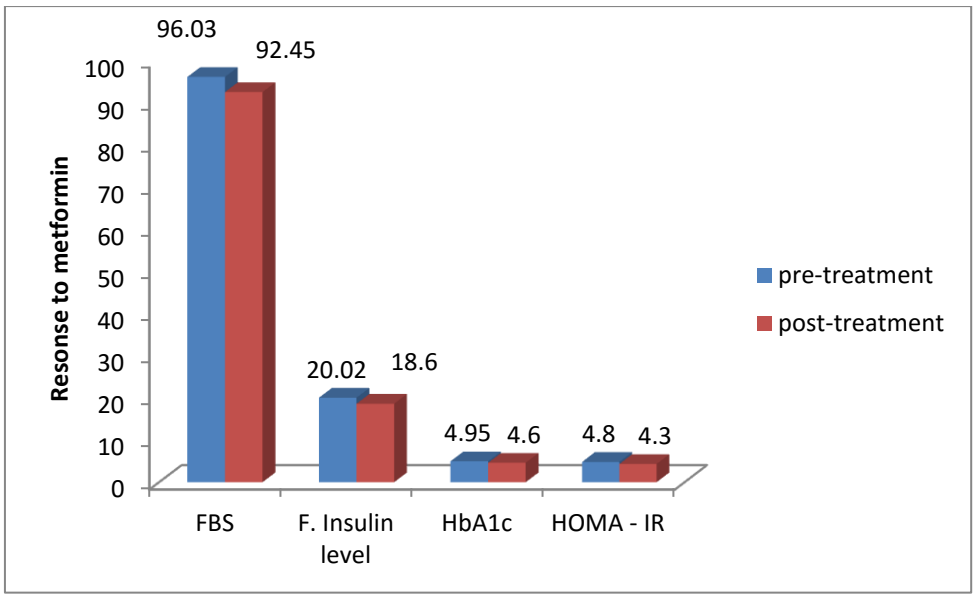
TSH: thyroid stimulating hormone

Fig. 3.4. Change in mean of prolactin (ng/mL), TSH (ulU/mL) and Estradiol (pg/mL) as a result to metformin treatment between pre- and post-treatment.



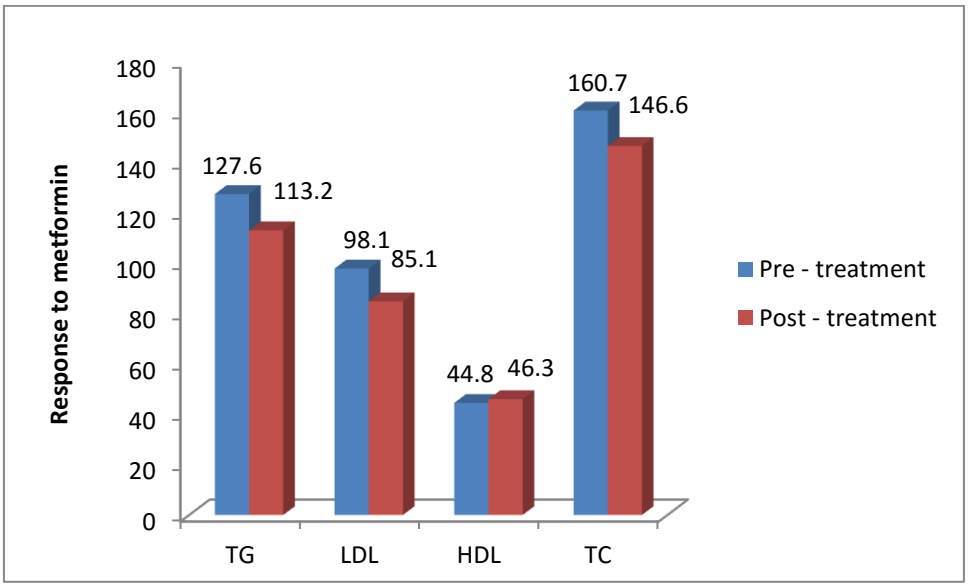
SHBG: sex hormone binding globulin, FAI: free androgen index

Fig. 3.5. Change in T. testosterone (ng/mL), SHBG (nmol/L) and FAI as a result to metformin between pre- and post- treatment



FBS: fasting blood sugar, HOMA-IR: homeostatic model assessment for insulin resistance, HbA1c%: glycated hemoglobin

Fig. 3.6. Change in mean of FBS (mg/dL), fasting insulin (µUl/mL), HOMA-IR and HbA1c% as a response to metformin between pre- and post-treatment



TG: triglycerides, LDL-C: low density lipoprotein, HDL-C: high density lipoprotein

Fig. 3.7. Change in mean of lipid profile (mg/dL) as a result to metformin treatment between pre- and post-treatment.

3.3. Genotyping of SLC47A1 (G>A) (rs2289669) and (A> G) (rs1961669) Genetic Polymorphism

3.3.1. Results of Amplification Reaction

The amplification of SNPs of MATE1: SLC47A1 (G>A) (rs2289669) was shown in 318 bp, 132 bp as in Figure (3.8) and SLC47A1 (A> G) (rs1961669) genetic polymorphism was shown in 392 bp as in Figure (3.9).

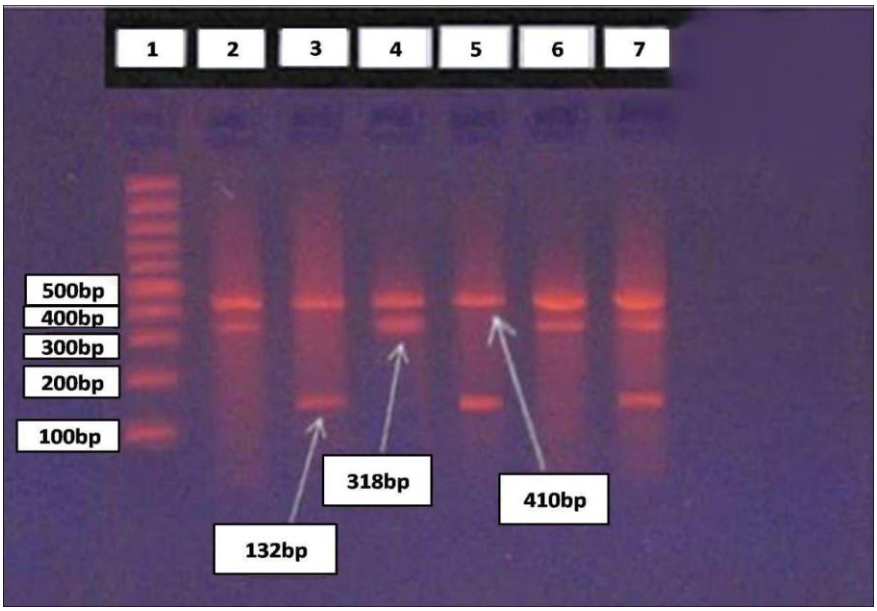


Fig. 3.8.: The Amplification Refractory Mutation System (ARMS) of (MATE1) SLC47A1 (G> A) (rs2289669) genetic polymorphism showed: Lane 1: Represented DNA ladder 100 - 1000 bp, Lane 2, 4 and 6 : Represented AA genotype (mutant) were showed in (318 bp), Lane 3 and 5: Represented GG genotype (wild) were showed in (132 bp) and Lane 7: Represented heterozygous GA genotype.

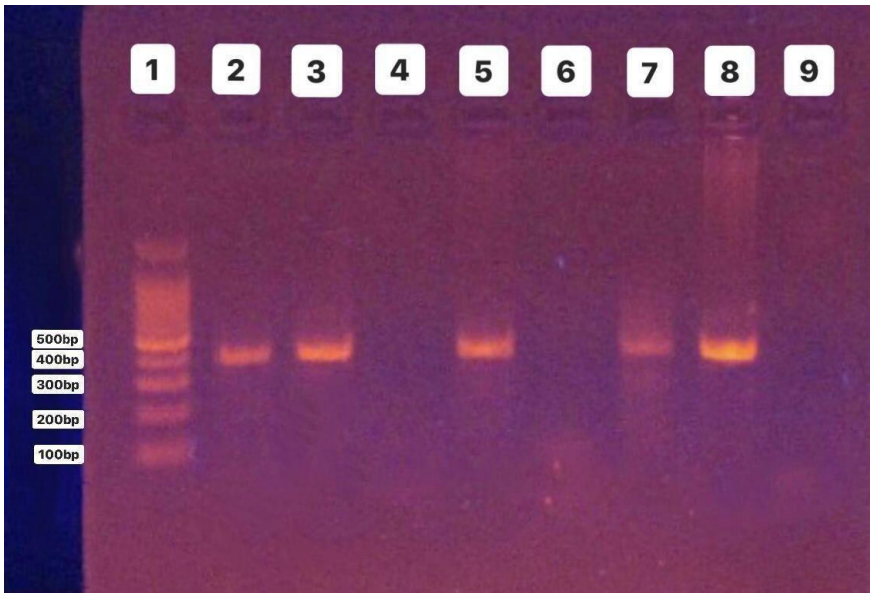


Fig. 3.9.: Allele Specific PCR of MATE1(SLC47A1) (A> G) (rs1961669) genetic polymorphism showed: Lane 1: Represented DNA ladder 100 - 1000 bp, Lane 2, 3: Represented (one sample) heterozygous GA genotype were shown in (392 bp), Lane (4 , 5) and Lane (6 , 7): Each two lanes Represented (one sample) which was GG genotype (mutant) were shown in (392 bp) and Lane 8, 9: Represented (one sample) AA genotype (wild) were shown in (392 bp).

3.3.1.1. Distribution of Allele Frequencies of SLC47A1 Gene Polymorphism (G>A) (rs2289669) in Iraqi PCOS Females :

The patients that enrolled in present study were classified into three genotypes, for SLC47A1 (G> A) (rs2289669) genetic polymorphism, first: homozygous for the G allele (GG) wild type, second: heterozygous (AG) and the third was homozygous for the A allele (AA) mutant type.

Among 231 patients, there were 119 heterozygous (AG) genotypes (51.52%),73 (GG) genotypes (31.6%) and 39 (AA) genotypes (16.88%) for the SNP rs2289669 in the SLC47A1gene, so minor allele frequency was 42.64% for allele A.

Table (3.3) and Figure (3.10) summarize genotyping of the patients of study according to genetic polymorphism of (G> A) (rs2289669) of the SLC47A1 gene.

Table 3.3: Allele frequencies of SLC47A1 gene polymorphism of (G>A) (rs2289669) in PCOS patients

Genotype		n. (%)
Wild	**GG**	73(31.6)
Heterozygous	**AG**	119(51.52)
Mutant	**AA**	39(16.88)
Allele		
G		133(57.4%)
A		98(42.6%)
Total		231(100)

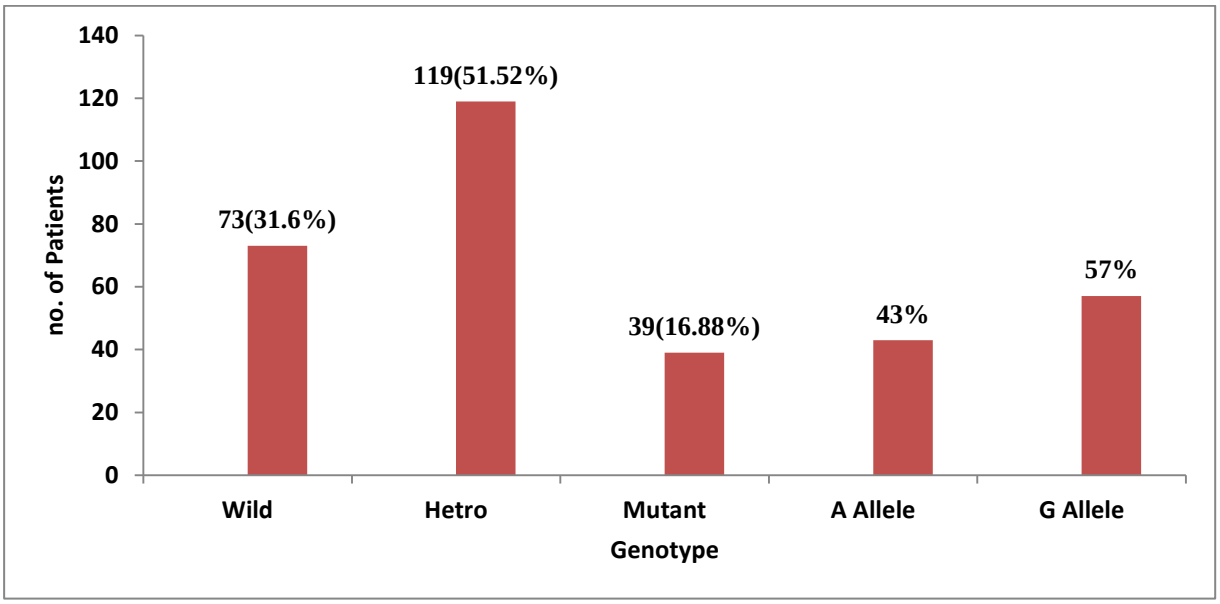


Fig. 3.10: Genotype and Allele frequencies of SLC47A1 (G> A) (rs2289669) genetic polymorphism

3.3.1.2. Distribution of Allele Frequencies of SLC47A1 Gene Polymorphism (A>G) (rs1961669) in Iraqi PCOS Females MATE1(SLC47A1)

The patients that enrolled in present study were classified into three genotypes, for SLC47A1(A> G) (rs1961669), first: homozygous for the A allele (AA) wild type, second: heterozygous (GA) and the third was homozygous for the allele G (GG) mutant type.

Among 231 patients, there were 152 (AA) genotypes (65.8%), 67 (GA) genotypes (29%) and 12 (GG) genotypes (5.2%) of SNP rs1961669 in the SLC47A1gene, so minor allele frequency for G allele was 19.7%.

Table (3.4) and Figure (3.11) summarizes genotyping of the patients of study according to genetic polymorphism of (A> G) (rs1961669) in the SLC47A1 gene.

Table 3.4.: Allele frequencies of SLC47A1 gene polymorphism of (A> G) (rs1961669) in PCOS patients

Genotype		n. (%)
Wild	**AA**	152(65.8)
Heterozygous	**GA**	67(29)
Mutant	**GG**	12(5.2)
Allele		
A		177(80.3)
G		54(19.7)
Total		231(100)

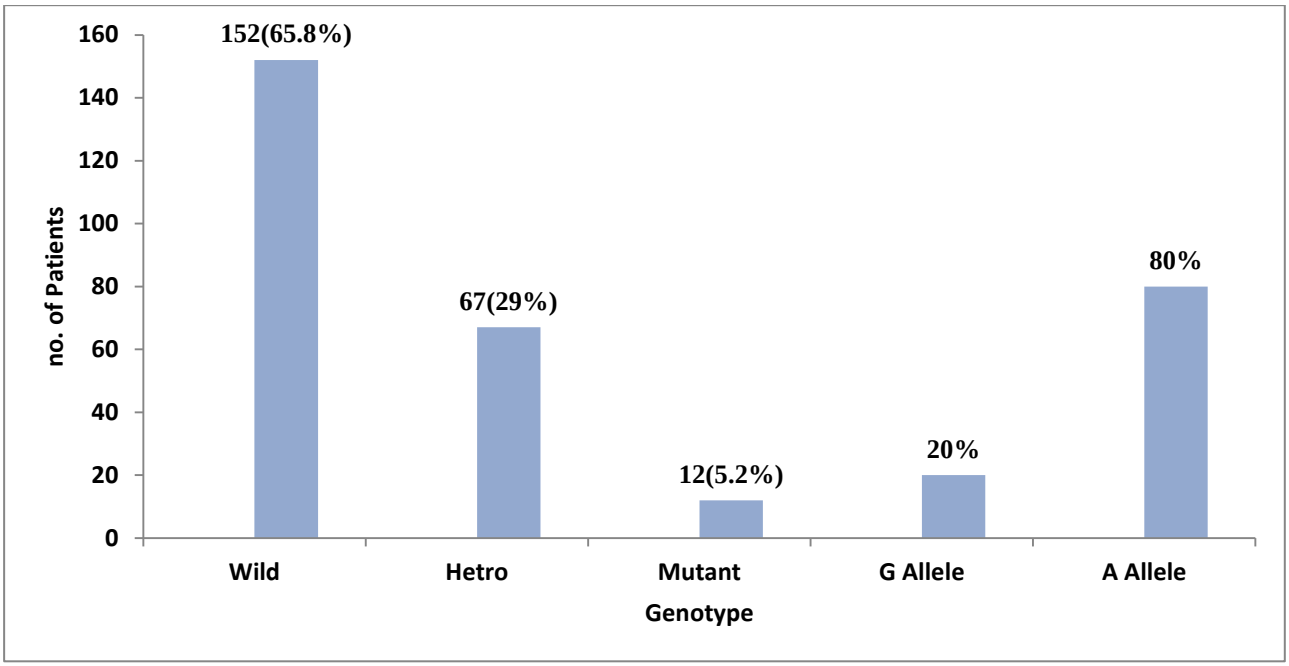


Fig. 3.11: Genotype and Allele frequencies of SLC47A1 (A>G) (rs1961669) genetic polymorphism

3.3.2. Effect of MATE1(SLC47A1)(G>A)(rs2289669) Genetic Polymorphism on Metformin Response in Polycystic Ovary Syndrome

3.3.2.1. Comparison of the Clinical Response to Metformin in Demographic Parameters across SLC47A1 (G>A) (rs2289669) Genotypes

The results of the present study were shown in table (3.5) using Fissure exact test, Chi-Square and Paired t-test, where the table showed demographic parameters before and after initiation of metformin therapy in each genotype of SLC47A1 (G> A) (rs2289669), there was a highly significant response to metformin in hirsutism for (GG) genotype (*P* value ≤ 0.01) , (AG) genotype was also recorded a highly significant response for hirsutism (*P* value ≤ 0.01), χ^2 = 20.4 while the study indicate no statistically significance in (AA) genotype with (*P* value = 0.5), χ^2 = 0.75.

The current study demonstrated that there was a highly significant response to metformin for alopecia in (GG) genotype (*P* value ≤ 0.01), χ^2 = 65.8, moreover, highly significant response was shown in (AG) genotypes (*P* value ≤ 0.01), χ^2 = 6.56 while there was no statistical difference for alopecia in (AA) genotype (*P* value = 0.4), χ^2 = 0.68.

Menstrual regularity in the current study indicated highly significant response to metformin treatment in all of three genotypes of SLC47A1 (G> A) (rs2289669) as (GG) genotype (*P* value ≤ 0.01), (GA) genotype (*P* value ≤ 0.01) and (AA) genotype (*P* value ≤ 0.01).

Regarding BMI, the present study showed significant response to metformin (*P* value ≤ 0.01) in BMI in (AG) genotype between pre- and post- metformin treatment (30.9 ± 5.5, 27.9 ± 5.8) kg/m^2 respectively while a non-significant response has shown in both (AA) genotype (*P* value = 0.3) and (GG) genotype (*P* value= 0.57).

Table 3.5. Effect of genetic polymorphism of SLC47A1 of (G>A) (rs2289669) on response to metformin in demographic parameters in Iraqi PCOS women (n=231)

Parameter	Genotype	Number	Response	Pre-metformin treatment n (%)	Post - metformin treatment n (%)	Statistical Analysis	Probability
Hirsutism	GG	73	(+)	68 (93.15)	10 (13.7)	Fissure exact test	P value ≤ 0.01
			(-)	5 (6.85)	63 (86.3)		
	AG	119	(+)	107 (90)	78 (65.5)	$\chi^2 = 20.4$	P value ≤ 0.01
			(-)	12 (10)	41 (34.5)		
	AA	39	(+)	34 (87.2)	32 (82)	$\chi^2 = 0.75$	P value = 0.5
			(-)	5 (12.8)	7 (18)		
Alopecia	GG	73	(+)	62 (85)	13 (17.8)	$\chi^2 = 65.8$	P value ≤ 0.01
			(-)	11 (15)	60 (82.2)		
	AG	119	(+)	93 (78.2)	75 (63)	$\chi^2 = 6.56$	P value ≤ 0.01
			(-)	26 (21.8)	44 (37)		
	AA	39	(+)	32 (82)	29 (74.4)	$\chi^2 = 0.68$	P value = 0.4
			(-)	7 (18)	10 (25.6)		
Regularity of menstrual cycle	GG	73	(+)	0 (0)	58 (79.5)	Fissure exact test	P value ≤ 0.01
			(-)	73 (100)	15 (20.5)		
	AG	119	(+)	0 (0)	30 (25.2)	Fissure exact test	P value ≤ 0.01
			(-)	119 (100)	89 (74.8)		
	AA	39	(+)	0 (0)	7 (18)	Fissure exact test	P value ≤ 0.01
			(-)	39 (100)	32 (82)		

Parameter	Genotype	Pre-metformin treatment	Post - metformin treatment	P Value
BMI (kg/m^2)	GG	27.15 ± 5.53	26.43 ± 5.49	0.57
	AG	30.9 ± 5.5	27.9 ± 5.8	≤ 0.01
	AA	28.6 ± 5.1	27.96 ± 5.02	0.3

Fissure exact test, Chi-squre test, paired t-test, *P value* ≤ 0.01: highly significant change, (+) : present, (-) : not present, BMI: body mass index

3.3.2.1.1. Association Between Allele Frequencies in Obese and Non-obese Women with SLC47A1(rs2289669)(G > A) Genetic Polymorphism

In the present study, the women that enrolled in final data analysis were subdivided into obese (BMI ≥ 30 kg) and (non-obese BMI< 30) kg according to their BMI, then comparison between these two subgroups related to their genotyping were made, the results is best presented in table (3.6) which showed that the odd ratio (OR) of AG genotype is 1.9 , CI 95% is 1.04 – 3.4 and ($P \leq 0.05$) as compared to wild (reference) which represented by GG genotype, while the AA genotype of this SNP has the odd ratio of 3.05, CI 95% is 1.34 – 6.9 and ($P \leq 0.01$) as compared to wild genotype(reference).

Table 3.6. Comparison between allele frequencies in obese and non-obese patients with SLC47A1(rs2289669)(G > A) genetic polymorphism

Genotype	Obese BMI ≥ 30 kg n. (%)	Non-obese BMI< 30 kg n.(%)	Odds ratio	CI 95%	P value
GG (Reference)	42 (18.2)	31 (13.4)	-	-	-
AG	50 (21.7)	69 (29.9)	1.9	1.04 – 3.4	≤ 0.05
AA	12 (5.2)	27 (11.7)	3.05	1.34 – 6.9	≤ 0.01

Odds ratio, CI 95% , *P value*≤ 0.05: significant change, *P value*≤ 0.01: highly significant change, BMI: body mass index

3.3.2.2. Comparison of the Clinical Response to Metformin in Biochemical Parameters Across SLC47A1 (G>A) (rs2289669) Genotypes

The results of the present study were shown in table (3.7) using Paired t-test , the table showed biochemical parameters before and after initiation of metformin therapy in each genotype of SLC47A1 (G> A) (rs2289669). To examine the individual differences in patient response, the effect of MATE1 polymorphisms on metformin treated PCOS patients should be investigated and the association between genetic polymorphism and metformin treatment response in PCOS should be analyzed, accordingly, the association between genotypes and the levels of hormones including (FSH, LH, TSH, prolactin, T. testosterone, Estrogen) and biochemical markers including (FBS, Insulin, HbA1c% and lipid profiles) should be detected.

The results of current study of LH level were highly significant reduction (*P* value≤ 0.01) when comparing the outcome values for the pre- and post-treatment period (11.02 ± 6.5 and 7.7 ± 3.9) mIU/mL in patients of heterozygous carriers of SLC47A1 Of (G> A) (rs2289669). In contrast, AA genotype indicated non-significant reduction in LH level between pre- and post-metformin treatment (*P* value=0.24) while GG genotype showed non-significant increase in LH (*P* value =0.26).

FSH recorded highly significant increase (*P* value≤ 0.01) in AG genotype as the value before treatment was (5.6 ± 2.07) mIU/mL while the value after treatment was (7.13 ± 2.4) mIU/mL, while AA genotype showed non-significant increase (*P* value= 0.5), a non-significant decrease in FSH (*P* value=0.7) has been revealed in GG genotype between pre- and post- treatment with metformin, as a result LH/FSH ratio also indicated highly significant reduction (*P* value ≤ 0.01) in AG genotype where the value of ratio was 2.03 ± 1.15 before treatment and 1.15 ± 0.6

after treatment while there was a non-significant decrease (*P* value=0.15) in the same ratio in AA genotype and non-significant increase in GG genotype (*P* value = 0.18).

Regarding TSH hormone level, there was a non-significant change in AG, AA and GG genotypes [*P* value = 0.6, *P* value = 0.8 and *P* value=0.24] respectively.

The results of prolactin before and after treatment in (AG) genotype were (23.3 ± 12.8 and 18.8 ± 9.2) ng/mL respectively, as there was a highly significant reduction (*P* value≤ 0.01) between them, while (AA) and (GG) genotypes showed a non-significant reduction in prolactin level [*P* value = 0.08, *P* value=0.26] respectively.

There was significant reduction in Estradiol level (*P* value ≤ 0.05) in (GG) genotype as the value before treatment was (62.5 ± 27.5) pg/mL compared with the value after treatment which was (51.7 ± 26.98) pg/mL, but there was non-significant decrease in (AA) genotype (*P* value=0.84) and (AG) genotype recorded non-significant increase for Estradiol level between per- and post-metformin therapy (*P* value = 0.8).

Total testosterone level recorded highly significant reduction (*P* value ≤ 0.01) in (AG) genotype as its value before treatment was (0.5 ± 0.36) ng/mL while its value after treatment was (0.37 ± 0.27) ng/mL, in (AA) group, total testosterone was also showed significant reduction *(P* value ≤ 0.05) and its value before treatment was (0.6 ± 0.3) ng/mL while it's value after treatment was (0.4 ± 0.25) ng/mL. In contrast to this, there was non-significant decrease in (GG) genotype (*P* value = 0.6).

SHBG level indicated highly significant increment (*P* value≤ 0.01) in (AG) genotype between pre- and post-metformin treatment (39.1 ± 23.03,51.4 ± 21.97) nmol/L respectively while non-significant increase in both (AA) and (GG) genotypes has been recorded [*P* value = 0.086 , *P* value = 0.16] respectively.

FAI showed significant reduction in AG genotype (*P* value≤ 0.01) as its value

before treatment was (9.88 ± 17.4) while its value after treatment was (3.3 ± 3.5), on the other hand, there was non-significant reduction in FAI in both (AA) and (GG) genotypes [P value= 0.09 , *P* value = 0.33] respectively.

Regarding FBS, the study revealed a significant decrease (*P* value≤ 0.05) in AG genotype between pre- and post-metformin therapy (96.2 ± 11.4,92.1 ± 10.7) mg/dL respectively while there was non- significant decrease in FBS in both groups of (AA) and (GG) genotypes [*P* value = 0.12 , *P* value=0.4] respectively.

Fasting insulin level indicated a significant decrease (*P* value ≤ 0.05) between pre- and post- metformin therapy in (AG) genotype only (19.45 ± 15.4 ,15.98 ± 9.9) µUl/mL respectively while non-significant decrease in Fasting insulin in (AA) genotype has been shown (*P* value = 0.88) and there was non-significant increase in F. insulin in GG genotype (*P* value = 0.6) between pre- and post- treatment period, as a result HOMA-IR indicated a significant reduction (*P* value≤ 0.05) as the its value before treatment was (4.7 ± 3.9) while its value after treatment was (3.7 ± 2.5), however, there was non-significant reduction in HOMA-IR in (AA) genotype *(P* value = 0.7) and non-significant increase in GG group (*P* value = 0.7) between pre- and post- treatment period.

The results of current study for HbA1c% were significant decrease (*P* value ≤ 0.05) in (AG) and (AA) genotypes when comparing the outcome values for the pre- and post- treatment period, as for (AG) group, its value was (5.05 ± 0.65) before treatment while its value was (4.6 ± 0.6) after treatment, for, as well as, for (AA) group, HbA1c% , recorded 4.9 ± 0.7 before treatment and 4.4 ± 0.66 after treatment but (GG) group indicated non-significant reduction in HbA1c% between pre- and post-treatment with metformin (*P* value = 0.4).

Regarding lipid profile , the results of TG level in (AG) genotype before and after treatment were as follows: (130.96 ± 57.5 and110.12 ± 42.4) mg/dL respectively as there was a highly significant reduction (*P* value ≤ 0.01) between

them while both (AA) and (GG) group demonstrated non-significant decrease for TG [*P* value = 0.18 and *P* value=0.4] respectively.

LDL-C also recorded highly significant reduction (*P* value≤ 0.01) in AG genotype, as its value was (106.5 ± 88.9) mg/dL before treatment while its value after treatment was (85.01 ± 27.9) mg/dL, nevertheless, (AA) and (GG) genotypes showed non-significant reduction in LDL-C [*P* value=0.2 and *P* value=0.5] respectively.

There was a significant increase in HDL-C level (*P* value ≤ 0.05) only in AG group between pre- and post-treatment period as the values were as follows: (44.2 ± 10.9 and 46.96 ± 9.7) mg/dL while (AA) and (GG) genotypes results were statistically non- significant for HDL-C (*P* value = 0.5 and *P* value=0.8) respectively.

The results of present study of total cholesterol level were highly significant reduction (*P* value ≤ 0.01) in (AG) genotype when comparing the outcome value of the pre- and post- treatment period (164.9 ± 48.99 and 141.8 ± 47.7) mg/dL while (AA) and (GG) genotypes indicated non-significant decline in TC [*P* value=0.49 and *P* value=0.55] respectively.

Table 3.7. Effect of Genetic Polymorphism of SLC47A1 of (G>A) (rs2289669) on Response to Metformin in Biochemical Parameters in Iraqi PCOS Women (n.=231)

Genotypes	GG n.=(73)			AG n.=(119)			AA n.=(39)		
Parameters	**Pre metformin treatment**	**Post metformin treatment**	***P* value**	**Pre metformin treatment**	**Post metformin treatment**	***P* value**	**Pre metformin treatment**	**Post metformin treatment**	***P* value**
LH(mIU/mL)	8.8 ± 5.45	9.9 ± 5.7	0.26	11.02 ± 6.5	7.7 ± 3.9	≤ 0.01	9.8 ± 6.12	8.35 ± 4.9	0.24
FSH(mIU/mL)	5.9 ± 1.9	5.8 ± 2.1	0.7	5.6 ± 2.07	7.13 ± 2.4	≤ 0.01	5.6 ± 1.8	5.9 ± 2.2	0.5
LH/FSH Ratio	1.55 ± 0.9	1.8 ± 0.99	0.18	2.03 ± 1.15	1.15 ± 0.6	≤ 0.01	1.8 ± 1.09	1.5 ± 0.9	0.15
TSH(ulU/mL)	2.33 ± 0.99	2.15 ± 0.8	0.24	2.26 ± 0.98	2.4 ± 2.45	0.6	2.18 ± 0.9	2.14 ± 0.9	0.8
Prolactin(ng/mL)	25.07 ± 15.4	22.6 ± 10.9	0.26	23.3 ± 12.8	18.8 ± 9.2	≤ 0.01	23.9 ± 12.3	19.5 ± 9.5	0.08
Estradiol(pg/mL)	62.5 ± 27.5	51.7 ± 26.98	≤ 0.05	66.8 ± 31.24	68 ± 33.5	0.8	60.9 ± 26.6	59.7 ± 29.4	0.84
T.testosterone(ng/mL)	0.6 ± 0.35	0.6 ± 0.37	0.6	0.5 ± 0.36	0.37 ± 0.27	≤ 0.01	0.6 ± 0.3	0.4 ± 0.25	≤ 0.05
SHBG(nmol/L)	46.4 ± 21.3	51.5 ± 22.2	0.16	39.1 ± 23.03	51.4 ± 21.97	≤ 0.01	41.65 ± 22.8	50.2 ± 20.8	0.086
FAI	8.96 ± 17.5	6.6 ± 9.8	0.33	9.88 ± 17.4	3.3 ± 3.5	≤ 0.01	9.7 ± 19.2	4.2 ± 5.9	0.09
FBS(mg/dL)	97.6 ± 13.15	95.9 ± 12.7	0.4	96.2 ± 11.4	92.1 ± 10.7	≤ 0.05	92.7 ± 13.9	87.14 ± 17.4	0.12
F. Insulin(µUl/mL)	22.6 ± 14.7	23.9 ± 13.1	0.6	19.45 ± 15.4	15.98 ± 9.9	≤ 0.05	16.9 ± 10.3	16.5 ± 10.8	0.88
HOMA-IR	5.5 ± 3.9	5.7 ± 3.3	0.7	4.7 ± 3.9	3.7 ± 2.5	≤ 0.05	3.9 ± 2.5	3.6 ± 2.7	0.7
HbA1c%	4.8 ± 0.7	4.7 ± 0.7	0.4	5.05 ± 0.65	4.6 ± 0.6	≤ 0.05	4.9 ± 0.7	4.4 ± 0.66	≤ 0.05
TG(mg/dL)	122.5 ± 39.08	116.9 ± 35.2	0.4	130.96 ± 57.5	110.12 ± 42.4	≤ 0.01	126.8 ± 38.5	115.5 ± 34.6	0.18
LDL-C(mg/dL)	88.1 ± 26.5	85.2 ± 24.4	0.5	106.5 ± 88.9	85.01 ± 27.9	≤ 0.01	90.98 ± 18.3	85.2 ± 19.6	0.2
HDL-C(mg/dL)	45.15 ± 9.6	44.7 ± 10.1	0.8	44.2 ± 10.9	46.96 ± 9.7	≤ 0.05	45.95 ± 7.4	47.15 ± 7.2	0.5
Total cholesterol(mg/dL)	157.3 ± 37.3	153.5 ± 39.4	0.55	164.9 ± 48.99	141.8 ± 47.7	≤ 0.01	154.12 ± 37.3	148.2 ± 39.5	0.49

Paired t-test, *P value* ≤ 0.05 : significant change, *P value* ≤ 0.01 :highly significant change

3.3.3. Effect of MATE1 (SLC47A1) (A> G) (rs1961669) Genetic Polymorphism on Response to Metformin in Polycystic Ovary Syndrome

3.3.3.1. Comparison of the Clinical Response to Metformin in Demographic Parameters Across SLC47A1 (A> G) (rs1961669) Genotypes

The results of the present study were displayed in table (3.8) using Fissure exact test, Chi-Square and Paired t-test. The table showed demographic parameters before and after initiation of metformin therapy in each genotype of SLC47A1(A> G) (rs1961669).

There was highly significant response to metformin in hirsutism in (AA) genotype (*P* value ≤ 0.01), χ^2 = 95.79 while there was non- significant response in (GA) and (GG) groups (*p* value=0.15), χ^2= 2.1 and (*P* value=0.5) respectively.

The current study also demonstrated that there is highly significant response to metformin for alopecia in (AA) and (GA) genotypes (*P* value ≤ 0.01) between pre- and post- treatment period where the results were as follows: for (AA), χ^2 = 74.97 and for (GA), χ^2= 6.56. In contrast, there was non-significant response in (GG) genotype with (*P* value = 0.4), χ^2 = 0.68.

Menstrual regularity in the current study recorded statistically highly significant response (*P* value ≤ 0.01) between pre- and post- treatment period in both (AA) and (GA) groups while (GG) genotype showed non-significant response in regularity of menstrual cycle (*P* value= 0.06).

The results of current study showed highly significant reduction in BMI (*P* value ≤0.01) in (AA) group between pre- and post- therapy (29.8 ± 5.6 vs 27.5 ± 5.4) kg/m^2 respectively, while both (GA) and (GG) indicated non-significant reduction between pre- and post- treatment [*P* value=0.6 and *P* value=0.2]respectively.

Table 3.8. Effect of genetic polymorphism of SLC47A1(A>G) (rs1961669) on response to metformin in demographic parameters in Iraqi PCOS women n.=(231)

Parameter	Genotype	Number	Response	Pre-metformin treatment n(%)	Post - metformin treatment n(%)	Statistical Analysis	Probability
Hirsutism	AA	152	(+)	138 (90.8)	56 (36.8)	χ^2=95.79	P value ≤0.01
			(-)	14 (9.2)	96 (63.2)		
	GA	67	(+)	60 (89.5)	54 (80.6)	χ^2 = 2.1	P value =0.15
			(-)	7 (10.5)	13 (19.4)		
	GG	12	(+)	11 (91.7)	10 (83.3)	Fissure exact test	P value = 0.5
			(-)	1 (8.3)	2 (16.7)		
Alopecia	AA	152	(+)	128 (84.2)	54 (35.6)	χ^2=74.97	P value ≤0.01
			(-)	24 (15.8)	98 (64.4)		
	GA	67	(+)	48 (71.7)	56 (83.6)	χ^2 = 2.75	P value = 0.097
			(-)	19 (28.3)	11 (16.4)		
	GG	12	(+)	10 (83.3)	9 (75)	Fissure exact test	P value = 0.6
			(-)	2 (16.7)	3 (25)		
Regularity of menstrual cycle	AA	152	(+)	0 (0)	79 (52)	Fissure exact test	P value ≤0.01
			(-)	152 (100)	73 (48)		
	GA	67	(+)	0 (0)	13 (19.4)	Fissure exact test	P value ≤0.01
			(-)	67 (100)	54 (80.6)		
	GG	12	(+)	0 (0)	3 (25)	Fissure exact test	P value =0.06
			(-)	12 (100)	9 (75)		
Parameter	**Genotype**			**Pre-metformin treatment**	**Post - metformin treatment**	**P value**	
BMI(kg/m$^{2)}$	AA			29.8 ± 5.6	27.5 ± 5.4	≤ 0.01	
	GA			28.2 ± 4.8	27.1 ± 4.6	0.2	
	GG			26.1 ± 5.6	24.99 ± 5.2	0.6	

Fissure exact test, Chi-square test, paired t-test, *P value* ≤ 0.01: highly significant change, (+) : present, (-) : not present, BMI: body mass index

3.3.3.1.1. Association Between Allele Frequencies in Obese and Non-obese Women with SLC47A1(rs1961669)(A>G) Genetic Polymorphism

Regarding SLC47A1(rs1961669)(A> G), comparison between obese and non-obese women in the present study revealed that the odd ratio (OR) of GA genotype of SLC47A1(rs1961669) (A> G) is 1.44 , CI 95% was 0.8 – 2.6 and ($P \leq 0.05$) as compared to wild (reference) which is represented by AA genotype, while the GG genotype of this SNP has the odd ratio of 2, CI 95% is 1.03 – 22.97 and ($P \leq 0.05$) as compared to wild genotype as shown in table (3.9).

Table 3.9. Comparison between allele frequencies in obese and non-obese patients with SLC47A1(rs1961669)(A > G) genetic polymorphism

Genotype	Non-obese BMI<30kg n. (%)	Obese BMI ≥30kg n. (%)	Odds ratio	CI 95%	*P* value
AA (Reference)	75 (32.5)	77 (33.3)	-	-	-
GA	27 (11.7)	40 (17.3)	1.44	0.8 – 2.6	≤ 0.05
GG	2 (0.9)	10 (4.3)	2	1.03 – 22.97	≤ 0.05

Odds ratio, CI 95% , *P value*≤ 0.05: significant change, BMI: body mass index

3.3.3.2. Comparison of the Clinical Response to Metformin in Biochemical Parameters Across SLC47A1 (A> G) (rs1961669) Genotypes

The results of the present study were shown in table (3.10) using paired t-test , the table showed biochemical parameters before and after initiation of metformin therapy in each genotype of SLC47A1 (A> G) (rs1961669). The association between genetic polymorphism and metformin treatment response in PCOS should

be analyzed so the relation between genotypes and response in mean levels of hormones including (FSH, LH, TSH, estrogen, prolactin, T. testosterone and SHBG) and biochemical markers including (FBS, Insulin, HbA1c% and lipid profiles) should be detected.

The results of current study demonstrated that the reduction in LH level between pre- and post- metformin treatment are highly significant in patients of heterozygous and homozygous carriers of SLC47A1 Of (A> G) (rs1961669) [(10.4 ± 5.3 vs 7.6 ± 3.7) mIU/mL(*P* value ≤ 0.01) and (17.9 ± 9.3 vs 10.3 ± 5.1) mIU/mL(*P* value≤ 0.05)] respectively. In contrast, AA genotype showed non-significant reduction (*P* value = 0.33) between pre- and post- treatment period.

A highly significant increase in FSH in both GA (*P* value≤ 0.01) and GG (*P* value≤ 0.05) genotypes has been revealed as the result in GA group for FSH before therapy was (5.7 ± 2) mIU/Ml while after therapy the result was (7.1 ± 2.2) mIU/Ml, furthermore, in GG group, the result before treatment was (5.6 ± 2.1) mIU/Ml while after treatment, the result for FSH was (7.6 ± 2.5)mIU/mL. However, AA genotype showed non-significant increase (*P* value = 0.08) in mean of FSH level.

LH/FSH ratio also indicated significant reduction in GA and GG genotypes (*P* value≤ 0.05) between pre- and post- metformin treatment[(1.9 ± 0.8 vs 1.1± 0.6) and (3.3 ± 1.2 vs 1.4 ± 0.6)] respectively while there was non-significant decrease in the same ratio in AA genotype between pre- and post- treatment period (*P* value = 0.087).

There was no statistical change for TSH level between pre- and post- treatment period in all genotypes groups (AA, GA and GG genotypes) as [*P* value = 0.4, *P* value = 0.6 and *P* value=0.9] respectively.

The results of the current study of prolactin were significant in all three groups (AA, GA and GG) as (*P* value ≤ 0.05) between pre- and post- treatment period,

the results in AA group was (24.9 ± 14.1) ng/mL before treatment while the results after treatment was (21.4 ± 10.6) ng/mL, in GA group the result for prolactin was (21.4 ± 12.4) ng/mL while the results after treatment was (17.7 ± 8.3) ng/mL, in GG group, the result before metformin therapy was (26.1 ± 12.15) ng/mL while after therapy, the result was (18.2 ± 5.2) ng/mL.

There is non-significant change in Estradiol level in all groups AA, GA and GG (*P* value = 0.5, *P* value=0.15 and *P* value = 0.5) respectively between pre- and post-treatment period.

The data of the current study revealed a significant reduction in total testosterone level (*P* value ≤ 0.05) between pre- and post- metformin therapy in GA and AA genotype [(0.6 ± 0.35 vs 0.5 ± 0.3) and (0.5 ± 0.3 vs 0.4 ± 0.2) ng/mL] respectively while there is non-significant decrease in GG genotype (*P* value=0.09).

SHBG level recorded highly significant increase (P value ≤ 0.01) in both GA and AA genotypes so in GA group, its value before treatment was (38.4 ± 20.6) nmol/L while its value after treatment was (52.2 ± 22.1)nmol/L, and in AA group, the result was(43.2 ± 22.97) nmol/L before treatment while the result was (49.95 ± 21.3) nmol/L after treatment. In contrast, a non-significant increase for SHBG in GG genotype (*P* value = 0.1) has been shown.

The results of FAI in this study before and after treatment were (7.95 ± 9.8 and 3.4 ± 5.1) for GA group respectively and the results for AA group were (10.2 ± 20.4 and 5.1 ± 7.4) as there was highly significant reduction between them (*P* value ≤ 0.01) while there is non-significant reduction in FAI in GG genotype (*P* value= 0.066).

In the present study, FBS recorded significant decrease (P value≤ 0.05) in both GA and AA genotypes between pre- and post- metformin therapy [(94.96 ± 12.5 , 90 ± 10.95) and (96.5 ± 12.6, 93.4 ± 14)] mg/dL respectively, while a non-significant decrease in FBS has been shown in GG genotype (*P* value = 0.7).

There was no statistical difference for fasting insulin level between pre- and post- treatment period in all genotypes AA, GA and GG as the values were: [*P* value = 0.6 , *P* value=0.15 and *P* value=0.6]respectively, accordingly, HOMA-IR indicated non-significant reduction the same as fasting insulin in all genotypes : AA, GA and GG between pre- and post- treatment period [*P* value = 0.48, *P* value = 0.076 and *P* value= 0.6] respectively.

Regarding HbA1c, it recorded highly significant reduction (*P* value ≤ 0.01) in AA genotype where the value before treatment was (4.9 ± 0.7) while the value after treatment was (4.6 ± 0.7) , GA group showed significant decrease in HbA1c as the value in GA before treatment was (5.01 ± 0.6) while the value after treatment was (4.5 ± 0.6) , however, GG group displayed non-significant reduction in HbA1c% (*P* value=0.1) between pre- and post- treatment period with metformin.

The results of current study showed that TG recorded highly significant decrease (*P* value ≤ 0.01) in TG level in GA and AA genotypes when comparing the outcome values between pre- and post- treatment period as for GA group (131.9 ± 63.7and 108.6 ± 43.1) mg/dL and for AA group (126.6 ± 40.4 and 116.4 ± 36.2)] mg/dL respectively while GG group revealed non-significant change in TG (*P* value= 0.4.)

LDL-C also showed a significant decrease (*P* value ≤ 0.05) in both GA and AA genotypes between pre- and post- metformin treatment [(96.7 ± 37.8 vs 83.6± 30.7) and (98.4 ± 77.3 vs 85.4 ± 22.5)] mg/dL respectively, while GG genotypes indicated non-significant reduction in LDL (*P* value=0.4).

HDL-C level recorded highly significant increase (*P* value ≤ 0.05) only in (GA) group between pre- and post- metformin treatment (42.2 ± 10.8 vs 45.9 ± 9.98) mg/dL respectively while there was no statistical change in both AA and GG groups [(*P* value = 0.5) and (*P* value = 0.9)] respectively.

In current study, a significant reduction in total cholesterol in both GA (*P* value ≤ 0.01) and AA (P value ≤ 0.05) genotypes has been obtained as the value in GA group was (154.5 ± 46.9) mg/dL before treatment while the value after treatment was(131.9 ± 43.6) mg/dL, and in AA group, the results before treatment was (162.5 ± 41.8) mg/dL while the result after treatment was(152.2 ± 42.4) mg/dL but GG genotype showed non-significant change in TC (*P* value=0.47) .

Table 3.10. Effect of genetic polymorphism of SLC47A1(A>G) (rs1961669) on response to metformin in biochemical parameters in Iraqi PCOS women (n.=231)

Genotypes	AA n.=(152)			GA n.=(67)			GG n.=(12)		
Parameters	**Pre metformin treatment**	**Post metformin treatment**	***P* value**	**Pre metformin treatment**	**Post metformin treatment**	***P* value**	**Pre metformin treatment**	**Post metformin treatment**	***P* value**
LH(mIU/mL)	9.4 ± 5.9	8.8 ± 5.1	0.33	10.4 ± 5.3	7.6 ± 3.7	≤ 0.01	17.9 ± 9.3	10.3 ± 5.1	≤ 0.05
FSH(mIU/mL)	5.7 ± 1.98	6.2 ± 2.4	0.08	5.7 ± 2	7.1 ± 2.2	≤ 0.01	5.6 ± 2.1	7.6 ± 2.5	≤ 0.05
LH/FSH Ratio	1.7 ± 1.1	1.5 ± 0.9	0.087	1.9 ± 0.8	1.1 ± 0.6	≤ 0.05	3.3 ± 1.2	1.4 ± 0.6	≤ 0.01
TSH(ulU/mL)	2.2 ± 0.95	2.1 ± 0.9	0.4	2.3 ± 1	2.5 ± 3.2	0.6	2.7 ± 0.8	2.6 ± 0.8	0.9
Prolactin (ng/mL)	24.9 ± 14.1	21.4 ± 10.6	≤ 0.05	21.4 ± 12.4	17.7 ± 8.3	≤ 0.05	26.1 ± 12.15	18.2 ± 5.2	≤ 0.05
Estradiol (pg/mL)	63.3 ± 29.96	60.8 ± 33.3	0.5	66.3 ± 26.7	59.8 ± 25.1	0.15	68.5 ± 36.4	79.1 ± 38.9	0.5
T.testosterone (ng/mL)	0.6 ± 0.35	0.5 ± 0.3	≤ 0.05	0.5 ± 0.3	0.4 ± 0.2	≤ 0.05	0.6 ± 0.4	0.4 ± 0.2	0.09
SHBG (nmol/L)	43.2 ± 22.97	49.95 ± 21.3	≤ 0.01	38.4 ± 20.6	52.2 ± 22.1	≤ 0.01	44.1 ± 27.9	62.4 ± 24.4	0.1
FAI	10.2 ± 20.4	5.1 ± 7.4	≤ 0.01	7.95 ± 9.8	3.4 ± 5.1	≤ 0.01	10.6 ± 14.6	2.4 ± 2.01	0.066
FBS(mg/dL)	96.5 ± 12.6	93.4 ± 14	≤ 0.05	94.96 ± 12.5	90 ± 10.95	≤ 0.05	95.6 ± 10.3	94.3 ± 7.4	0.7
F. Insulin (µUl/mL)	21.1 ± 15.4	20.3 ± 12.3	0.6	18.1 ± 12.4	15.2 ± 9.5	0.15	19.7 ± 13.5	15.3 ± 9.95	0.6
HOMA-IR	5.1 ± 3.9	4.8 ± 3.1	0.48	4.4 ± 3.3	3.5 ± 2.4	0.076	4.3 ± 3.5	3.6 ± 2.5	0.6
HbA1c%	4.9 ± 0.7	4.6 ± 0.7	≤ 0.05	5.01 ± 0.6	4.5 ± 0.6	≤ 0.01	5.1 ± 0.5	4.7 ± 0.7	0.1
TG(mg/dL)	126.6 ± 40.4	116.4 ± 36.2	≤ 0.05	131.9 ± 63.7	108.6 ± 43.1	≤ 0.01	116.3 ± 62.9	97.8 ± 46.5	0.4
LDL-C (mg/dL)	98.4 ± 77.3	85.4 ± 22.5	≤ 0.05	96.7 ± 37.8	83.6 ± 30.7	≤ 0.05	101.9 ± 38.3	89.7 ± 30.9	0.4
HDL-C (mg/dL)	45.6 ± 9.3	46.3 ± 9.5	0.5	42.2 ± 10.8	45.9 ± 9.98	≤ 0.05	49.2 ± 11.1	48.9 ± 5.5	0.9
Total cholesterol (mg/dL)	162.5 ± 41.8	152.2 ± 42.4	≤ 0.05	154.5 ± 46.9	131.9 ± 43.6	≤ 0.01	172.7 ± 49.5	157.8 ± 51.02	0.47

Paired t-test, *P value* ≤ 0.05 : significant change, *P value* ≤ 0.01 :highly significant change

CHAPTER
Four

Discussion

4. Discussion

Metformin, an insulin sensitizing agent, is commonly used for the treatment and improvement of symptoms associated with polycystic ovarian syndrome **[141]**. Metformin clinical response is highly variable among patients, and the identification of contributing factors of this variability is important, particularly in the field of personalized medicine. Metformin needs drug transporters for its absorption, distribution and elimination, because it poorly diffuses passively across membranes **[156]**. Transporter that involved in the excretion of metformin is multi drug and toxin extrusion protein (MATE1) that encoded by SLC47A1 gene where is located on the luminal side of the renal proximal tubules of kidney and bile canaliculi of liver as involved in transportation of this drug out of the cell to the bile and urine **[187]**. Genetic variants in SLC47A1 gene of MATE1 have been demonstrated to influence metformin response in patients with PCOS **[188]**.

This is the first study which has been done to demonstrate and assess the effect of MATE1 (SLC47A1) genetic polymorphism on metformin response in Iraqi population with PCOS.

4.1. Demographic studies

In this prospective study, the most common clinical manifestations of PCOS are hirsutism, alopecia and menstrual irregularity which occur as a result of abnormal steroidgenesis that included excessive LH secretion and hyperandrogenism **[47]**.

Regarding alopecia and menstruation, the study showed a significant improvement following three months of metformin treatment and the women exhibited more regular cycle and less hair loss where this improvement was due to the fact that metformin improves insulin resistance which lead to decrease theca cells hypertrophy, consequently decrease LH production from these cells and in

turn decrease ovarian androgen production and ovarian (cytochrome P450c17α) enzyme activity with a consequent decline in the serum testosterone which is the main responsible factor of hair loss **[189,190].**

It has been suggested that metformin can improve the cycle frequency of ovulation and menstruation which tend to be disturbed in PCOS women **[191].** Similar results were obtained from some studies which showed that metformin reduce hair loss in women with PCOS **[190,192].** However, worsening of alopecia was shown by **[193],** and this may because metformin has the ability to cause significant decrease in mean of vitamin B12 level by inhibition of calcium-dependent ileac absorption of B12-intrinsic factor complex that reverse the calcium supplementation as well as metformin decreases folate concentration in patients with PCOS after 6 months of treatment and this side effect of metformin is duration and dose dependent **[142,194].**

The effect of metformin on menstruation is best represented in table (3.1), which showed that following metformin treatment for three months, the cycle tended to be more regular than before treatment so these results agreed with these studies**[191,195],** that indicated improvement in cyclicity after metformin treatment.

Hirsutism response to metformin in this study was non-significant due to the fact that the hair growth cycle needs a period of three months, accordingly to achieve the highest effect of metformin on hirsutism, treatment is required for more than six months **[196]**.

4.2. Biochemical Studies

Regarding the effect of metformin on the hormonal profile in PCOS women, it is best represented in table (3.2), the study demonstrated that metformin treatment resulted in a significant reduction in serum level of LH, total testosterone,

prolactin along with significant decrease in LH/FSH ratio as well as this study clarified a significant increase in FSH, SHBG level.

As shown in table (3.2) and figure (3.3), the observed data indicate a significant increase in FSH level after 3 months of treatment. In PCOS, an increase in GnRH pulse frequency results in decrease of FSH production and reduction in stimulation of granulosa cell, this contributes to poor egg development and inability or difficulty of ovulation that improved by metformin treatment which reduce exaggerated GnRH pulse frequency, rebalanced the hormones level, consequently, induces the ovulation **[197]**, so these results are in agreement with studies**[196,198],** that showed a significant increase in FSH level while the current study disagreed with the study **[199],** which demonstrated that FSH levels remain unaffected after treatment with metformin.

Women with PCOS exhibit increased expression of cytochrome P450c17α (CYP 17), which results in increase the activity of 17α-hydroxylase and 17,20-lyase in ovarian theca cells where this dysregulation of CYP 17 enhances the production of 17α-hydroxyprogesterone, androstenedione and testosterone which previously increased by insulin so metformin decreases ovarian cytochrome P450c17α activity secondary to a reduction in insulin **[200]**, these findings were compatible with the study **[200],** that showed a similar result, while the study **[201]** showed that the reduction in LH was more limited or non-significant.

The observed data also showed that the reduction in values of LH/FSH ratio after metformin treatment period was highly significant as LH has decreased significantly and there is significant change in FSH, so LH / FSH ratio is also decreased significantly.

Prolactin was recorded a significant reduction in its value after 3 month of treatment. High levels of estrogen in PCOS which are produced by conversion of androgen by the enzyme aromatase would stimulate prolactin production so

metformin reduces elevated prolactin levels as a result of reducing the level of estrogen **[196]**. In addition, metformin can cross the blood brain barrier and stored locally in the pituitary gland resulting in the reduction of prolactin synthesis and/or release by affecting dopaminergic regulation of lactotroph function as well as metformin is improving local dopamine action, partially restoring normal function of these cells, consequently, decreasing circulating prolactin levels **[202,203]**, so these results are in line with the study **[195]** and not compatible with other study **[196],** which showed that metformin has no effect on prolactin level after 6 months of treatment.

Data observed in Figure (3.5), described a significant reduction in total testosterone levels and significant increase in SHBG levels due to that metformin induces insulin level reduction which is associated with an increase in SHBG production, IGFBP-I availability to ovaries and a reduced IGF-I/IGFBP-I ratio, which may be partly responsible for the reduction of plasma androgen levels in PCOS patients**[201,204]**, similar results were obtained from other studies **[205,206],** that showed a significant reduction in total testosterone and significant increment in SHBG after metformin therapy while other studies interfered with these results demonstrated a non-significant decrease and increase in total testosterone and SHBG respectively after metformin therapy **[207,208]**. Free androgen index (FAI) in current study showed a significant reduction after three months of metformin treatment as there was a significant decrease in total testosterone and significant increase in SHBG, accordingly FAI decreased significantly , these findings are in agreement with these studies **[205,209]**.

Results of current study indicated that metformin treatment also improved biochemical profile (i.e.) there was a significant decrease in fasting blood sugar level (FBS), HbA1c% and lipid profile.

The effect of metformin on FBS is well represented in figure (3.6) which indicated that fasting blood sugar (FBS) in current study demonstrated a significant reduction after metformin therapy as the drug suppresses liver production of glucose, increases the sensitivity of liver, muscle, fat and cells to insulin and decrease the absorption of carbohydrates **[196]**, therefore, current results agreed with the study **[196]**, and disagreed with other **[210]**, which showed non-significant reduction of FBS.

The Present study showed a significant reduction in HbA1c% level after metformin treatment where HbA1c% level reflects the average blood glucose levels from the last 3 months because once glucose has attached to hemoglobin in a red blood cell, it stays there for the life of the red blood cell, which is around 3 months which were the same period of treatment of present study so metformin decrease glucose production from liver and absorption of glucose from intestine and result in decrease HbA1c% after three months treatment **[210]**, these outcomes are compatible with the study **[210]** and interfere with other **[211],** that indicted a non-significant reduction in HbA1c% after metformin treatment.

Regarding lipid profiles data that are observed in Figure (3.7) which showed a significant reduction of TG, LDL-C and total cholesterol after three months of metformin treatment as the treatment led to a reduction in the activity and expression of several products or enzymes that involved in the lipid synthesis, such as acetyl-CoA carboxylase, Sterol regulatory element-binding protein-1 (SREBP-1), fatty acid synthase, and HMG-CoA reductase so metformin improved lipid profiles by its glucose lowering effects, these outcomes are in agreement with studies which showed similar results **[207,212]**.

4.3. Molecular Studies

Various studies regarding the effects of variants in MATE1 (SLC47A1 gene) on metformin response in T2DM patients **[213,214].** This study was the first research project performed which assessed MATE1(SLC47A1gene) polymorphisms effects follow –up on metformin response in Iraqi women with PCOS.

4.3.1. MATE1 (SLC47A1) (G>A) (rs2289669) Genetic Polymorphism

In current study as shown in table (3.3), the genotypic distribution of MATE1 and the allele frequency of SLC47A1 gene rs2289669 in Iraqi populations was approximately the same as those found in Iranian populations **[215]**. There was a significant response in hirsutism and alopecia after three months of treatment with metformin in AG and GG genotypes as compared to AA genotype i.e. AG and GG genotypes had better response to metformin than homozygous carriers of SLC47A1 rs2289669 A- allele, since the clinical response to metformin required duration of treatment for at least six months to be optimal or maximum as hair cycle growth lasts for three months **[196]**.

Regularity of menstrual cycle in current study indicated a significant response to metformin after three months treatment in the three groups of genotypes. The presented work demonstrated a significant reduction in BMI after three months of metformin therapy in AG genotypes in comparison with GG and AA genotypes of PCOS women.

Regarding association between allele frequencies in obese and non-obese PCOS women , it is well represented in table (3.6) which revealed that odds ratio value that results from comparing AG genotypes of SLC47A1 (G>A) (rs2289669) of both obese and non-obese with the wild genotype GG (reference) was more than

one, it means that there was an association between these two genotypes in both groups as well as the odds ratio which observed from comparing AA genotypes of both obese and non-obese women with GG indicated that there was an association between these genotypes as odds ratio value was more than one which leads to a fact that the presence of SLC47A1 (G>A) (rs2289669) genetic polymorphism had an association with the obesity which considers as important risk factor for PCOS.

In the current study , as shown in table (3.7), there was highly significant reduction in LH level among heterozygous carriers of SLC47A1 (G>A) (rs2289669) genetic polymorphism after metformin therapy, FSH level showed significant increment in AG genotype only as a response to three months of metformin therapy , as a result LH/FSH ratio indicated significant reduction in AG genotype in comparison to other genotypes.

In present study, patients with AG genotype had the highest significant reduction in prolactin level, FAI, fasting insulin, FBS and HOMA-IR in response to metformin. AG genotype indicated significant increase in SHBG level in this study. Lipid profile (TG, LDL-C and TC) demonstrated significant reduction in AG genotype while HDL-C also showed significant increase in AG genotype in response to metformin treatment. Total testosterone level and HbA1c% indicated significant reduction in both homozygous and heterozygous carriers of SLC47A1 (G>A) (rs2289669) genetic polymorphism (i.e.) A-allele carriers in comparison with the wild GG genotype.

In the current prospective study, the MATE1 SLC47A1 (G>A) (rs2289669) genetic polymorphism was found to influence the response to metformin in patients with PCOS . There were great individual variations among PCOS women which carrying different MATE1 SLC47A1 (G>A) (rs2289669) genotypes. After three months follow-up, the conclusion was that SLC47A1 gene rs2289669 (G>A) variants had a powerful influence on PCOS patients. There were various

explanations: first, the A-allele frequency was approximately 43% in all population. Second, patients carrying rs2289669 A/A homozygous and A/G heterozygous had remarkably better glucose-lowering effect after three-months of treatment with oral metformin, that agreed with the study **[216].**

Several studies indicated a link between SLC47A1 (rs2289669) genetic polymorphism and HbA1c% reduction by metformin in T2DM patients **[160,215-217]**. The present study was in line with the study **[215],** that showed highest HbA1c% reduction in AG genotype after six-months on metformin treatment in Iranian population**.** In addition, the study **[217],** reported that homozygous carriers of SLC47A1 (G> A) (rs2289669) A- allele have two folds reduction in HbA1c% during six months of metformin treatment in Caucasian population in comparison with the rest of diabetic patients, the present study was also in agreement with the results obtained by the study **[216]**, which demonstrated 116 metformin users in the prospective study and found an increase of metformin effect by 0.3% in A-allele carriers of this variant. The study **[160],** showed that the AA homozygous carriers of SLC47A1 rs2289669 (G>A) polymorphism had the lower clearance of metformin than carriers of wild type in Chinese patients.

The data of the current study was disagree with the study **[218],** which showed that the SNP rs2289669 in SLC47A1 did not influence the glycemic response to metformin in patients of South India.

Estradiol level showed significant decrease in GG genotype in response to metformin in comparison with other genotypes which result in conclusion that there was no influence of SLC47A1 (G>A) (rs2289669) A- allele variant on Estradiol level in response to three months of metformin in women with PCOS.

The mechanism by which this polymorphism affects actions of metformin is not clear, because, this SNP is located in an intron, and the SNP does not code for an amino acid change, most likely , the A-variant of the SNP rs2289669 is in linkage

disequilibrium with a SNP that causes reduction in MATE1 functioning, although we cannot exclude that it has a direct effect, e.g., by affecting gene expression **[216]**.

A reduced efflux of metformin in the renal brush border due to an impaired MATE1 transporter will lead to an increase in metformin plasma levels and possibly to a larger decrease in glucose levels. Similarly, a reduced efflux from the hepatocyte will lead to higher metformin levels in the hepatocyte and a stronger inhibition of the gluconeogenesis, resulting in lower glucose levels.

The rs2289669 G>A polymorphism was associated with an increased glucose-lowering effect, implying that the gene with the A allele encodes a MATE1 efflux transporter less effective in transporting metformin (i.e.) the SNP causes loss of function of MATE1 transporter resulting in increment in metformin concentration and less metformin excreted in urine and bile as a result more metformin action in patients ,accordingly ,more glucose lowering effect, reduction in FBG and HbA1c ,better enhancement in insulin sensitivity and reduction of insulin resistance which leads to decrease in LH/FSH ratio, total testosterone, consequently, increase of SHBG resulting in improvement in PCOS symptoms especially menstrual irregularity, therefore; there was an effect of the SNP rs2289669 in the SLC47A1, encoding the MATE1 transporter on the glucose-lowering effect of metformin.

4.3.2. MATE1 (SLC47A1) (A>G) (rs1961669) Genetic Polymorphism

Table (3.8) showed a significant response in both hirsutism and alopecia in homozygous wild A-allele carriers after three months treatment with metformin in comparison with homozygous and heterozygous carriers of SLC47A1 (A>G) (rs1961669), it means that AA genotype had better response to metformin in hirsutism and alopecia than GG and AG genotypes. Regularity of menstrual cycle

in current study indicated significant response to metformin after three months of treatment in AA and GA genotypes in comparison with GG genotype. Present study demonstrated significant reduction in BMI after three months of metformin therapy in AA genotypes in comparison with GA and GG genotypes of PCOS women, so homozygous carriers of common A- allele had best response to metformin as related to BMI so the G-allele variant of SLC47A1 (A>G) (rs1961669) had no association with demographic characterizations in Iraqi patients with PCOS.

In the current study, the association between allele frequencies in obese and non-obese PCOS women was shown in table (3.9) which demonstrated that odds ratio value resulting from comparison of (GA) genotypes of both obese and non-obese with the wild genotype (AA) (reference) was more than one lead to a fact that there was an association between these two genotypes in both groups. Besides, the odds ratio which concluded from comparing GG genotypes (mutant) in both obese and non-obese women with the same reference also indicated that there was an association between these genotypes because odds ratio value was more than one, these results demonstrated that the obesity in patients with PCOS was associated with presence of SLC47A1 (A> G) (rs1961669) genetic polymorphism in those patients.

Regarding the effects of the study genotypes of SLC47A1 (A> G) (rs1961669) on the response to metformin in biochemical parameters which was best represented in table (3.10), there was a significant reduction in LH level among GG and GA genotypes after metformin therapy, FSH showed significant increase in GG and AG genotype in response to metformin compared with wild A-allele, as a result LH/FSH ratio indicated significant reduction in GG and AG genotype compared to other genotypes.

In the present study, prolactin level showed significant reduction in all

genotypes in response to metformin , while total testosterone level indicated significant reduction in GA and AA genotypes as compared with GG genotype. The current study demonstrated that SHBG had significant increase in GA and AA genotypes as a result FAI showed significant decrease in GA and AA genotypes after metformin treatment. Lipid profile included TG, LDL-C and TC showed significant reduction in GA and AA genotypes in comparison with GG genotypes.

Fasting blood glucose and HbA1c% demonstrated significant reduction in GA and AA genotypes so GA and AA groups had better response to metformin than GG genotype, while GA genotype only had highest increase in HDL-C level in response to metformin. Most of these results indicated that there was no association of G-allele variant of SLC47A1 (A>G) (rs1961669) with demographic and biochemical parameters in Iraqi PCOS populations, these results were in line with the findings of the study **[216],** which involve study the effect of SLC47A1 (A> G) (rs1961669) gene polymorphism and other SNPs of MATE1 (SLC47A1 gene) on HbA1c% change in diabetic Caucasian populations.

The current study is the first study performed to assess the effect of SLC47A1 (A>G) (rs1961669) genetic polymorphism on metformin response in Iraqi women with PCOS. The SNP rs1961669 is an intronic SNP which occurs in non-coding region of DNA, accordingly; it doesn't cause amino acid changes. Minor or G-allele frequency was 19.7% (i.e.) approximately (0.2) which approximately similar to minor allele frequency or G-allele variant in Caucasian populations in the study **[216],** which showed that the frequency of minor allele was 17% and the SNP was in linkage disequilibrium. Because of the low frequency of minor allele for this SNP and being an intronic one, the SNP rs1961669 had no strong effect on metformin response in demographic and biochemical markers in Iraqi females with PCOS who were taking the mentioned drug.

Conclusions
&
Recommendations

Conclusions

1. The current study indicated that the treatment with metformin showed improvement in menstrual irregularity, hormonal and biochemical profile in Iraqi women with polycystic ovarian syndrome.
2. The application of molecular biology to study the transporters of metformin has made a significant progress in determining the factors that lead to the development in understanding of metformin efficacy in PCOS.
3. The MATE1 transporter of metformin in women with PCOS is relatively polymorphic, so the response to metformin was different in PCOS patients as MATE1 transporter could be important for metformin therapeutic action; therefore , genetic polymorphisms of MATE1 might contribute to differences in the effectiveness of metformin treatment in patients with polycystic ovarian syndrome.
4. The AG genotype of SLC47A1 (G> A) (rs2289669) genetic polymorphism is more predominant than other genotypes which were AA and GG.
5. The AG genotype of SLC47A1 (G> A) (rs2289669) had the highest response to metformin treatment in hormonal and biochemical parameters as observed by significant reduction in mean of LH/FSH ratio, Free androgen index, fasting insulin, FBG, insulin resistance represented by HOMA-IR and lipid profile (TG, LDL-C and TC) with significant increase in SHBG and HDL-C levels.
6. The AA/AG genotypes of SLC47A1 (G> A) (rs2289669) had the highest response to metformin that represented by significant reduction in total testosterone and HbA1c% levels so A-allele variant of SLC47A1 (G> A) (rs2289669) was associated with biochemical parameters in Iraqi women with Polycystic ovarian syndrome.

7. The SLC47A1 (A> G) (rs1961669) genetic polymorphism had no strong effect on metformin response in demographic and most biochemical parameters in Iraqi women with Polycystic ovarian syndrome.
8. The odd ratio indicated an association between presence of both SNPs in SLC47A1 gene and obesity which considers as most important risk factor for PCOS.

Recommendations

1. Classify Patients to groups with different doses of metformin according to genetic bases.
2. Study another SNPs of SLC47A1 gene and their effects on MATE1 transporter and the response to metformin.
3. Using more advanced molecular techniques for detection of other MATE1 (SLC47A1) genetic polymorphisms like DNA sequencing and Real time PCR.
4. Increase the period of treatment with metformin to at least (6 months) in order to obtain better results or response in clinical parameters and BMI.
5. Adjustment in dose of metformin must be done in populations which had A-allele variant of SLC47A1 (G> A) (rs2289669) and who have abnormal kidney functions.

Future Work

The response to metformin has long been variable so continuous examination of genetic variants influencing metformin transport and action and unbiased genome wide studies of genetic polymorphisms associated with metformin response will identify genetic markers that predict treatment response in PCOS women as these studies will be important for personalized treatment.

References

References

1. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. Nature Reviews Endocrinology. 2018 ; 14(5) : 270-284.

2. Azziz R, Adashi EYJAjoo, gynecology. Stein and Leventhal: 80 years on. 2016 ; 214(2) : 247. e1-. e11.

3. Kabel AMJPB. Polycystic ovarian syndrome: insights into pathogenesis, diagnosis, prognosis, pharmacological and non-pharmacological treatment. 2016 ; 4(1) : 7-12.

4. Brakta S, Lizneva D, Mykhalchenko K, Imam A, Walker W, Diamond MP, et al. Perspectives on polycystic ovary syndrome: is polycystic ovary syndrome research underfunded? 2017 ; 102(12) : 4421-7.

5. Dokras A, Saini S, Gibson-Helm M, Schulkin J, Cooney L, Teede HJF, et al. Gaps in knowledge among physicians regarding diagnostic criteria and management of polycystic ovary syndrome. 2017 ; 107(6) : 1380-6. e1.

6. El-Hayek S, Bitar L, Hamdar LH, Mirza FG, Daoud GJFip. Poly cystic ovarian syndrome: an updated overview. 2016 ; (124)7 : 1-15.

7. Lizneva D, Suturina L, Walker W, Brakta S, Gavrilova-Jordan L, Azziz RJF, et al. Criteria, prevalence, and phenotypes of polycystic ovary syndrome. 2016 ; 106(1) : 6-15.

8. Ndefo UA, Eaton A, Green MRJP, Therapeutics. Polycystic ovary syndrome: A review of treatment options with a focus on pharmacological approaches. 2013 ; 38(6) : 336-355.

9. Bozdag G, Mumusoglu S, Zengin D, Karabulut E, Yildiz BOJHR. The prevalence and phenotypic features of polycystic ovary syndrome: a systematic review and meta-analysis. 2016 ; 31(12) : 2841-55.

10. Naz MSG, Tehrani FR, Majd HA, Ahmadi F, Ozgoli G, Fakari FR, et al. The prevalence of polycystic ovary syndrome in adolescents: A systematic review and meta-analysis. 2019 ; 17(8) : 533-542.

11. Ding T, Hardiman PJ, Petersen I, Wang F-F, Qu F, Baio GJO. The prevalence of polycystic ovary syndrome in reproductive-aged women of different ethnicity: a systematic review and meta-analysis. 2017 ; 8(56) : 96351-96358.

12. Jamal AF, Ismael RAJZJoMS. Ultrasonographic prevalence of polycystic ovarian morphology among women of reproductive age group. 2019 ; 23(1) : 57-65.

13. Clark NM, Podolski AJ, Brooks ED, Chizen DR, Pierson RA, Lehotay DC, et al. Prevalence of polycystic ovary syndrome phenotypes using updated criteria for polycystic ovarian morphology: an assessment of over 100 consecutive women self-reporting features of polycystic ovary syndrome. Reproductive Sciences. 2014 ; 21(8) : 1034-43.

14. Moghetti P, Tosi F, Bonin C, Di Sarra D, Fiers T, Kaufman J-M, et al. Divergences in insulin resistance between the different phenotypes of the polycystic ovary syndrome. 2013 ; 98(4) : E628-E37.

15. Brower M, Brennan K, Pall M, Azziz RJTJoCE, Metabolism. The severity of menstrual dysfunction as a predictor of insulin resistance in PCOS. 2013 ; 98(12) : E1967-E71.

16. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JS, Legro RS, et al. Polycystic ovary syndrome. Nature reviews Disease primers. 2016 ; 2(1) : 1-18.

17. Li S, Zhu D, Duan H, Tan Q. The epigenomics of polycystic ovarian syndrome: from pathogenesis to clinical manifestations. Gynecological Endocrinology. 2016 ; 32(12) : 942-6.

18. Dumesic DA, Oberfield SE, Stener-Victorin E, Marshall JC, Laven JS, Legro RS. Scientific statement on the diagnostic criteria, epidemiology, pathophysiology, and molecular genetics of polycystic ovary syndrome. Endocrine reviews. 2015;36(5):487-525.

19. Abbott DH, Nicol LE, Levine JE, Xu N, Goodarzi MO, Dumesic DAJM, et al. Nonhuman primate models of polycystic ovary syndrome. 2013 ; 373(1-2) : 21-8.

20. Maliqueo M, Lara HE, Sánchez F, Echiburú B, Crisosto N, Sir-Petermann TJEJoO, et al. Placental steroidogenesis in pregnant women with polycystic ovary syndrome. 2013 ; 166(2) : 151-5.

21. Strauss JF, Modi BP, McAllister JM. The Genetics of Polycystic Ovary Syndrome: From Genome-Wide Association to Molecular Mechanisms. Reproductive Medicine for Clinical Practice: Springer; 2018. p. 25-33.

22. Sirmans SM, Pate KAJCe. Epidemiology, diagnosis, and management of polycystic ovary syndrome. 2014 ; 6 :1-13.

23. Morin-Papunen LJDG. Risk factors in adolescence for the development of polycystic ovary syndrome. 2016 ; 49(11) : 838-46.

24. Barber T, McCarthy M, Wass J, Franks SJCe. Obesity and polycystic ovary syndrome. 2006 ; 65(2) : 137-45.

25. Moran C, Arriaga M, Rodriguez G, Moran SJIjoe. Obesity differentially affects phenotypes of polycystic ovary syndrome. 2012 ; 2012(317241) : 1-8.

26. Mohammad MB, Seghinsara AMJAPjocpA. Polycystic ovary syndrome (PCOS), diagnostic criteria, and AMH. 2017 ; 18(1) : 17-21.

27. Le MT, Le VNS, Le DD, Nguyen VQH, Chen C, Cao NT. Exploration of the role of anti-Mullerian hormone and LH/FSH ratio in diagnosis of polycystic ovary syndrome. Clinical endocrinology. 2019 ; 90(4) : 579-85.

28. Begum GS, Shariff A, Ayman G, Mohammad B, Housam R, Khaled NJd. Assessment of risk factors for development of polycystic ovarian syndrome. 2017 ; 4(1) : 164-167.

29. Kumar P, Sharma A. Gonadotropin-releasing hormone analogs: Understanding advantages and limitations. Journal of human reproductive sciences. 2014 ; 7(3) : 170-174.

30. Baldauff NH, Witchel SFJCOiE, Diabetes, Obesity. Polycystic ovary syndrome in adolescent girls. 2017 ; 24(1) : 56-66.

31. Shaaban Z, Khoradmehr A, Shirazi MRJ, Tamadon AJIjobms. Patho-physiological mechanisms of gonadotropins–and steroid hormones–related genes in etiology of polycystic ovary syndrome. 2019 ; 22(1) : 3-16.

32. Rosenfield RL, Ehrmann DAJEr. The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. 2016 ; 37(5) : 467-520.

33. Abbott DH, Dumesic DA, Levine JE. Hyperandrogenic origins of polycystic ovary syndrome–implications for pathophysiology and therapy. Expert review of endocrinology & metabolism. 2019 ; 14(2) : 131-43.

34. O'Reilly MW, Kempegowda P, Walsh M, Taylor AE, Manolopoulos KN, Allwood JW, et al. AKR1C3-mediated adipose androgen generation drives lipotoxicity in women with polycystic ovary syndrome. 2017 ; 102(9) : 3327-39.

35. Kraynak M, Flowers MT, Shapiro RA, Kapoor A, Levine JE, Abbott DHJAJoP-E, et al. Extraovarian gonadotropin negative feedback revealed by aromatase inhibition in female marmoset monkeys. 2017 ; 313(5) : E507-E14.

36. Puurunen J. ANDROGEN SECRETION AND CARDIOVASCULAR RISK FACTORS IN WOMEN WITH AND WITHOUT PCOS 2015 ;1299 :1-114.

37. Rodgers RJ, Suturina L, Lizneva D, Davies MJ, Hummitzsch K, Irving-Rodgers HF, et al. Is polycystic ovary syndrome a 20th Century phenomenon? 2019 ; 124 : 31-4.

38. Wallace IR, McKinley MC, Bell PM, Hunter SJ. Sex hormone binding globulin and insulin resistance. Clinical endocrinology. 2013 ; 78(3) : 321-9.

39. Kashif Zaidi S, Shen W-J, Azhar S. Impact of aging on steroid hormone biosynthesis and secretion. Open Longevity Science. 2012 ; 6(1) :1-30.

40. Deswal R, Yadav A, Dang AS. Sex hormone binding globulin-an important biomarker for predicting PCOS risk: A systematic review and meta-analysis. Systems biology in reproductive medicine. 2018 ; 64(1) : 12-24.

41. Polak K, Czyzyk A, Simoncini T, Meczekalski B. New markers of insulin resistance in polycystic ovary syndrome. Journal of endocrinological investigation. 2017 ; 40(1) : 1-8.

42. Maqbool M, Dar MA, Gani I, Geer MI. Insulin Resistance and Polycystic ovary Syndrome: A Review. Journal of Drug Delivery and Therapeutics. 2019 ; 9(1-s) : 433-6.

43. Oliva MM, Zuev V, Lippa A, Carra M, Lisi FJErfm, sciences p. Efficacy of the synergic action of myoinositol, tyrosine, selenium and chromium in women with PCOS. 2019 ; 23(19) : 8687-94.

44. Mayer SB, Evans WS, Nestler JEJWsH. Polycystic ovary syndrome and insulin: our understanding in the past, present and future. 2015 ; 11(2) : 137-49.

45. Battaglia C, Battaglia B, Mancini F, Paradisi R, Fabbri R, Venturoli SJAoegS. Ultrasonographic extended-view technique for evaluation of abdominal fat distribution in lean women with polycystic ovary syndrome. 2011 ; 90(6) : 600-8.

46. Moghetti P. Insulin resistance and polycystic ovary syndrome. Current pharmaceutical design. 2016 ; 22(36) : 5526-34.

47. Rosenfield RL, Ehrmann DA. The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocrine reviews. 2016 ; 37(5) : 467-520.

48. Catteau-Jonard S, Dewailly D. Pathophysiology of polycystic ovary syndrome: the role of hyperandrogenism. Polycystic Ovary Syndrome. 40: Karger Publishers ; 2013. p. 22-7.

49. van den Oever IA, Raterman HG, Nurmohamed MT, Simsek SJMoi. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. 2010 ; 2010(792393) :1-15.

50. Coghlan E, Hart RJ. Integrated Strategies for Enhancement of Fertility in PCOS. Infertility in Women with Polycystic Ovary Syndrome: Springer; 2018. pp. 289-304.

51. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz RJNre. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. 2011 ; 7(4) : 219-231.

52. Dahan MH, Reaven GJE. Relationship among obesity, insulin resistance, and hyperinsulinemia in the polycystic ovary syndrome. 2019 ; 64(3) : 685-9

53. Orbetzova MM. Clinical Impact of Insulin Resistance in Women with Polycystic Ovary Syndrome. InPolycystic Ovarian Syndrome 2020 Jan 10. IntechOpen.

54. Kim J, Mersereau JE, Khankari N, Bradshaw PT, McCullough LE, Cleveland R, et al. Polycystic ovarian syndrome (PCOS), related symptoms/sequelae, and breast cancer risk in a population-based case–control study. 2016 ; 27(3) : 403-14.

55. McCook JG, Bailey BA, Williams SL, Anand S, Reame NEJTjobhs, research. Differential contributions of polycystic ovary syndrome (PCOS) manifestations to psychological symptoms. 2015 ; 42(3) : 383-94.

56. Dewailly DJBP, Obstetrics RC, Gynaecology. Diagnostic criteria for PCOS: is there a need for a rethink? 2016 ; 37 : 5-11.

57. Allahbadia GN, Merchant R. Polycystic ovary syndrome and impact on health. Middle East Fertility Society Journal. 2011;16(1):19-37.

58. Keen MA, Shah IH, Sheikh G. Cutaneous manifestations of polycystic ovary syndrome: A cross-sectional clinical study. Indian dermatology online journal. 2017 ; 8(2) : 104-110.

59. Raperport C, Homburg RJCMIRH. The Source of Polycystic Ovarian Syndrome. 2019 ; 13(10) : 1-6.

60. Lauritsen MP, Bentzen J, Pinborg A, Loft A, Forman JL, Thuesen L, et al. The prevalence of polycystic ovary syndrome in a normal population according to the Rotterdam criteria versus revised criteria including anti-Müllerian hormone. 2014 ; 29(4) : 791-801.

61. Diamanti-Kandarakis EJBp, obstetrics rC, gynaecology. PCOS in adolescents. 2010 ; 24(2) : 173-83.

62. Kyrou I, Weickert MO, Randeva HS. Diagnosis and management of polycystic ovary syndrome (PCOS). Endocrinology and diabetes: Springer; 2015. p. 99-113.

63. Chang RJ, Dumesic DA. Polycystic ovary syndrome and hyperandrogenic states. Yen and Jaffe's reproductive endocrinology: Elsevier; 2019. p. 520-55. e13.

64. Tharian K, Warner JJP, health c. Polycystic ovarian syndrome in adolescent girls. 2011 ; 21(7) : 309-14.

65. Arora B, Arora V, Patel S. Polycystic Ovarian Syndrome: An awareness guide for women. Journal of Pharmacy Research. 2014 ; 8(7) : 884-92.

66. Guastella E, Longo RA, Carmina E. Clinical and endocrine characteristics of the main polycystic ovary syndrome phenotypes. Fertility and sterility. 2010 ; 94(6) : 2197-201.

67. Delcour C, Robin G, Young J, Dewailly D. PCOS and Hyperprolactinemia: what do we know in 2019? Clinical Medicine Insights: Reproductive Health. 2019 ; 13(10) :1-7.

68. Abdelsalam KEA, Ibrahim W. Relationship between TSH, T4, T3 and Prolactin in overweight and lean Sudanese PCOS Patients. International Journal of Biomedical Research. 2015 ; 6(2) : 108-12.

69. Al Kindi MK, Al Essry FS, Al Essry FS, Mula-Abed W-AS. Validity of serum testosterone, free androgen index, and calculated free testosterone in women with suspected hyperandrogenism. Oman medical journal. 2012 ; 27(6) : 471.

70. Lunger F, Wildt L, Seeber BJGE. Accurate screening for insulin resistance in PCOS women using fasting insulin concentrations. 2013 ; 29(6) : 541-4.

71. Domke I, Grawitz AB, Cole T, Fresi P. Multicenter Evaluation of a new HDL-Cholesterol Reagent on Roche/Hitachi and COBAS INTEGRA® Systems. Clinical Chemistry. 2006 ; 52(S6) : A113.

72. Wild RA. Dyslipidemia in PCOS. Steroids. 2012 ; 77(4) : 295-9.

73. Wolf WM, Wattick RA, Kinkade ON, Olfert MDJIjoer, health p. Geographical prevalence of polycystic ovary syndrome as determined by region and race/ethnicity. 2018 ; 15(11) : 2589-2602.

74. Glintborg D, Andersen M. MANAGEMENT OF ENDOCRINE DISEASE: Morbidity in polycystic ovary syndrome. European journal of endocrinology. 2017 ; 176(2) : R53-R65.

75. Gilbert EW, Tay CT, Hiam DS, Teede HJ, Moran LJ. Comorbidities and complications of polycystic ovary syndrome: an overview of systematic reviews. Clinical endocrinology. 2018 ; 89(6) : 683-99.

76. Diamanti-Kandarakis E, Mara Spritzer P, Petermann T, Beatriz Motta A. Insulin resistance and polycystic ovary syndrome through life. Current pharmaceutical design. 2012 ; 18(34) : 5569-76.

77. Fauser B, Tarlatzis B, Rebar R, Legro R, Balen A, Lobo R, et al. Consensus on womens health aspects of polycystic ovary syndrome. 2012 ; 27(1) : 14-24.

78. Spritzer PMJRBdGeO. Primary and secondary prevention of metabolic and cardiovascular comorbidities in women with polycystic ovary syndrome. 2015 ; 37(1) : 1-4.

79. Moran LJ, Misso ML, Wild RA, Norman RJJHru. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. 2010 ; 16(4) : 347-63.

80. Peigne M, Dewailly D, editors. Long term complications of polycystic ovary syndrome (PCOS). Annales d'endocrinologie; 2014 ; 75(4) : 194-9.

81. Barry JA, Azizia MM, Hardiman PJJHru. Risk of endometrial, ovarian and breast cancer in women with polycystic ovary syndrome: a systematic review and meta-analysis. 2014 ; 20(5) : 748-58.

82. Palomba S, Santagni S, Falbo A, La Sala GBJIjowsh. Complications and challenges associated with polycystic ovary syndrome: current perspectives. 2015 ; 7 : 745.

83. Group REASPCW. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Human reproduction. 2004 ; 19(1) : 41-7.

84. Hassan M.F., Abdul-Kadim H. Oocyte dysmorphism in women with PCOS and its' effects on empryo development following ICSI.Wjpmr. 2018 ; 4(12) : 78-84.

85. Sunanda B, Nayak S. A study to assess the knowledge regarding PCOS (polycystic ovarian syndrome) among nursing students at NUINS. Nitte University Journal of Health Science. 2016 ; 6(3) : 24-26.

86. Neven ACH, Laven J, Teede HJ, Boyle JA, editors. A summary on polycystic ovary syndrome: diagnostic criteria, prevalence, clinical manifestations, and management according to the latest international guidelines. Seminars in reproductive medicine. 2018 ; 36: 5-12 .

87. Shah D. Challenges during Assisted Reproductive Technology in Polycystic Ovary Syndrome. Practical Guide in Assisted Reproductive Technology. 2018 ; 35 :1805-1815.

88. Balen AH, Morley LC, Misso M, Franks S, Legro RS, Wijeyaratne CN, et al. The management of anovulatory infertility in women with polycystic ovary syndrome: an analysis of the evidence to support the development of global WHO guidance. 2016 ; 22(6) : 687-708.

89. O'Shea LC, Mehta J, Lonergan P, Hensey C, Fair T. Developmental competence in oocytes and cumulus cells: candidate genes and networks. Systems biology in reproductive medicine. 2012 ; 58(2) : 88-101.

90. Palomba S, Daolio J, La Sala GBJTiE, Metabolism. Oocyte competence in women with polycystic ovary syndrome. 2017 ; 28(3) : 186-98.

91. Lebbe M, Taylor AE, Visser JA, Kirkman-Brown JC, Woodruff TK, Arlt W. The steroid metabolome in the isolated ovarian follicle and its response to androgen exposure and antagonism. Endocrinology. 2017 ; 158(5) : 1474-85.

92. Namik JJ, Alalaf SK, Al Tawil NG. Anti-mullerian hormone and antral follicle count in polycystic ovary syndrome and non-polycystic ovary syndrome women. Zanco Journal of Medical Sciences. 2018 ; 22(3) : 292-9.

93. Bekhatroh RA, Adel IN. How Polycystic Ovary Syndrome (PCOS) Affects the Fertility Potential of Reproductive Age Women? International Journal of Caring Sciences. 2019 ; 12(3) : 1906-1912.

94. Poshdar M, Imani M, Ahmadi AJTJoUUoMS. Investigation of the effect of experimental polycystic ovarian syndrome induced by stradiolvalerate on oocyte quality and in vitro fertilization potential and evaluation of vitamin E supplementation to emryo culture in mouse model. 2018 ; 29(7) : 539-48.

95. Soni A, Singla S, Goyal S. polycystic ovary syndrome: pathogenesis, treatment and secondary associated diseases. Journal of Drug Delivery and Therapeutics. 2018 ; 8(5) : 107-12.

96. Bates GW, Legro RS. Longterm management of polycystic ovarian syndrome (PCOS). Molecular and cellular endocrinology. 2013 ; 373(1-2) : 91-7.

97. Teede HJ, Misso ML, Deeks AA, Moran LJ, Stuckey BG, Wong JL, et al. Assessment and management of polycystic ovary syndrome: summary of an evidence-based guideline. Medical Journal of Australia. 2011 ; 195 : S65-S112.

98. Pundir J, Charles D, Sabatini L, Hiam D, Jitpiriyaroj S, Teede H, et al. Overview of systematic reviews of non-pharmacological interventions in women with polycystic ovary syndrome. 2019 ; 25(2) : 243-56.

99. Panidis D, Tziomalos K, Papadakis E, Vosnakis C, Chatzis P, Katsikis I. Lifestyle intervention and anti-obesity therapies in the polycystic ovary syndrome: impact on metabolism and fertility. Endocrine. 2013 ; 44(3) : 583-90.

100. Speelman DL. Nonpharmacologic Management of Symptoms in Females With Polycystic Ovary Syndrome: A Narrative Review. The Journal of the American Osteopathic Association. 2019 ; 119(1) : 25-39.

101. Melo AS, Ferriani RA, Navarro PA. Treatment of infertility in women with polycystic ovary syndrome: approach to clinical practice. Clinics. 2015;70(11):765-9.

102. Boyd M, Ziegler J. Polycystic Ovary Syndrome, Fertility, Diet, and Lifestyle Modifications: A Review of the Current Evidence. Topics in Clinical Nutrition. 2019 ; 34(1) : 14-30.

103. Bednarska S, Siejka A. The pathogenesis and treatment of polycystic ovary syndrome: What's new. Adv Clin Exp Med. 2017 ; 26(2) : 359-67.

104. Diamond MP, Legro RS, Coutifaris C, Alvero R, Robinson RD, Casson P, et al. Letrozole, gonadotropin, or clomiphene for unexplained infertility. New England Journal of Medicine. 2015 ; 373(13) : 1230-40.

105. Sachdeva G, Gainder S, Suri V, Sachdeva N, Chopra SJJor, infertility. Prediction of Responsiveness to Clomiphene Citrate in Infertile Women with PCOS. 2019 ; 20(3) : 143-150.

106. Kar S. Clomiphene citrate or letrozole as first-line ovulation induction drug in infertile PCOS women: A prospective randomized trial. Journal of human reproductive sciences. 2012 ; 5(3) : 262-265.

107. Hadnott T, Chang RJJotES. SAT-222 Hormone Responses Associated with Letrozole Ovulation Induction in Anovulatory Women with PCOS. 2019 ; 3(Supplement-1) : SAT-222.

108. Thomas S, Sudharshini S. Polycystic ovarian syndrome: Treatment options for infertility. Current Medical Issues. 2016 ; 14(4) : 87-93.

109. Lunenfeld B, Bilger W, Longobardi S, Alam V, D'Hooghe TM, Sunkara SJFie. The development of gonadotropins for clinical use in the treatment of infertility. 2019 ; 10 : 429.

110. Patade GR, Marita ARJJoO, Research M. Metformin: A Journey from countryside to the bedside. 2014 ; 1(2) : 127-130.

111. Bailey CJ. Metformin: historical overview. Diabetologia. 2017 ; 60(9) : 1566-76.

112. Yunoos M, Sankar DG. Stability indicating quantitative RP-HPLC method development and validation for simultaneous determination of metformin hydrochloride and saxagliptin in bulk and combined tablet dosage form. J Chem Pharm Res. 2015 ; 7(4) : 346-55.

113. Havele S, Dhaneshwar S. Development and validation of a HPLC method for the determination of metformin hydrochloride, gliclazide and piogliglitazone hydrochloride in multicomponent formulation. 2010 ; 1(10) : 1-16.

114. Graham G, Punt J, Arora M, Day R, Doogue M. Duong 176. JK, et al. Clinical pharmacokinetics of metformin Clin Pharmacokinet. 2011 ; 50 : 81-98.

115. Liang X, Giacomini KM. Transporters involved in metformin pharmaco-kinetics and treatment response. Journal of pharmaceutical sciences. 2017 ; 106(9) : 2245-50.

116. Gong L, Goswami S, Giacomini KM, Altman RB, Klein TE. Metformin pathways: pharmacokinetics and pharmacodynamics. Pharmacogenetics and genomics. 2012 ; 22(11) : 820-827.

117. Hagenbuch BJCP, Therapeutics. Drug uptake systems in liver and kidney: a historic perspective. 2010 ; 87(1) : 39-47.

118. Kusuhara H, Ito S, Kumagai Y, Jiang M, Shiroshita T, Moriyama Y, et al. Effects of a MATE protein inhibitor, pyrimethamine, on the renal elimination of metformin at oral microdose and at therapeutic dose in healthy subjects. 2011 ; 89(6) : 837-44.

119. Madiraju AK, Qiu Y, Perry RJ, Rahimi Y, Zhang X-M, Zhang D, et al. Metformin inhibits gluconeogenesis via a redox-dependent mechanism in vivo. Nature medicine. 2018 ; 24(9) : 1384-94.

120. Dumitrescu R, Mehedintu C, Briceag I, Purcărea V, Hudita D. Metformin-clinical pharmacology in PCOs. Journal of medicine and life. 2015; 8(2) : 187-92.

121. Rhee S-j, Choi Y, Lee S, Oh J, Kim S-J, Yoon SH, et al. Pharmacokinetic and pharmacodynamic interactions between metformin and a novel dipeptidyl peptidase-4 inhibitor, evogliptin, in healthy subjects. Drug design, development and therapy. 2016 ; 10 : 2525-3534.

122. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia. 2017 ; 60(9) : 1577-85.

123. Baur JA, Birnbaum MJ. Control of gluconeogenesis by metformin: does redox trump energy charge? Cell metabolism. 2014 ; 20(2) : 197-9.

124. Pryor R, Cabreiro FJBJ. Repurposing metformin: an old drug with new tricks in its binding pockets. 2015 ; 471(3) : 307-22.

125. Prameela KL, Veni P, Krishna R, Satyanarayana P, Babu BH. RP-HPLC method for the simultanous assay of metformin and benfotiamine : Development and validation: International Journal of Pharmaceutical Sciences and Research.. 2018 ; 9(1) : 264-70.

126. Zhou T, Xu X, Du M, Zhao T, Wang JJB, Pharmacotherapy. A preclinical overview of metformin for the treatment of type 2 diabetes. 2018 ; 106 : 1227-35.

127. Miceli DD, Vidal PN, Batter MFC, Pignataro O, Castillo VAJOvj. Metformin reduces insulin resistance and the tendency toward hyperglycaemia and dyslipidaemia in dogs with hyperadrenocorticism. 2018 ; 8(2) : 193-9.

128. Malin SK, Kashyap SR. Effects of metformin on weight loss: potential mechanisms. Current Opinion in Endocrinology, Diabetes and Obesity. 2014 ; 21(5) : 323-9.

129. Yanovski JA, Krakoff J, Salaita CG, McDuffie JR, Kozlosky M, Sebring NG, et al. Effects of metformin on body weight and body composition in obese insulin-resistant children: a randomized clinical trial. Diabetes. 2011 ; 60(2) : 477-85.

130. Cicero AF, Tartagni E, Ertek S. Metformin and its clinical use: new insights for an old drug in clinical practice. Archives of medical science: AMS. 2012 ; 8(5) : 907-917.

131. Kaplowitz PJP. Is there a role for metformin in the treatment of childhood obesity? 2017 ; 140(1) : e20171205.

132. Khan A, Karim N, Ainuddin JA. The Triad Of PCOS, Infertility And Metformin. JBUMDC. 2019 ; 9(1) : 67-71.

133. Liu W-W, Li D-H, Luo X-Z, Tang L-L, Shi Y-L. Therapeutic effect of metformin on patients with polycystic ovary syndrome with normal insulin sensitivity: A retrospective study. Reproductive and Developmental Medicine. 2019 ; 3(3) : 153-158.

134. Lashen H. Role of metformin in the management of polycystic ovary syndrome. Therapeutic advances in endocrinology and metabolism. 2010 ; 1(3) : 117-28.

135. Tso LO, Costello MF, Albuquerque LET, Andriolo RB, Macedo CR. Metformin treatment before and during IVF or ICSI in women with polycystic ovary syndrome. Cochrane Database of Systematic Reviews. 2014; (11) : 1-41.

136. Thomas I, Gregg BJPd. Metformin; a review of its history and future: from lilac to longevity. 2017 ; 18(1) : 10-6.

137. Di Pietro M, Velazquez C, Matzkin ME, Frungieri MB, Peña MG, de Zúñiga I, et al. Metformin has a direct effect on ovarian cells that is dependent on organic cation transporters. Molecular and cellular endocrinology. 2019 ; 499 : 1-10.

138. Fraga F, Romeiro GACS, de Sá LBPC, Arbex AK. Polycystic Ovary Syndrome and the Role of Metformin in Ovulation Induction. Health. 2018 ; 10(05) : 565-576.

139. Hyer S, Balani J, Shehata H. Metformin in pregnancy: Mechanisms and clinical applications. International journal of molecular sciences. 2018 ; 19(7) : 1954-1967.

140. Artini PG, Obino MER, Sergiampietri C, Pinelli S, Papini F, Casarosa E, et al. PCOS and pregnancy: a review of available therapies to improve the outcome of pregnancy in women with polycystic ovary syndrome. Expert review of endocrinology & metabolism. 2018 ; 13(2) : 87-98.

141. Genazzani AD, Ricchieri F, Lanzoni C. Use of metformin in the treatment of polycystic ovary syndrome. Women's Health. 2010 ; 6(4) : 577-93.

142. Mazokopakis EE, Starakis IK. Recommendations for diagnosis and management of metformin-induced vitamin B12 (Cbl) deficiency. Diabetes research and clinical practice. 2012 ; 97(3) : 359-67.

143. Panahi Y, Mojtahedzadeh M, Zekeri N, Beiraghdar F, Khajavi M-R, Ahmadi A. Metformin treatment in hyperglycemic critically ill patients: another challenge on the control of adverse outcomes. Iranian journal of pharmaceutical research: IJPR. 2011 ; 10(4) : 913-919.

144. Lalau J-D. Lactic acidosis induced by metformin. Drug safety. 2010 ; 33(9) : 727-40.

145. Corsini A, Bortolini M. Drug-induced liver injury: The role of drug metabolism and transport. The Journal of Clinical Pharmacology. 2013 ; 53(5) : 463-74.

146. Meyer zu Schwabedissen HE, Verstuyft C, Kroemer HK, Becquemont L, Kim RB. Human multidrug and toxin extrusion 1 (MATE1/SLC47A1) transporter: functional characterization, interaction with OCT2 (SLC22A2), and single nucleotide polymorphisms. American Journal of Physiology-Renal Physiology. 2010 ; 298(4) : F997-F1005.

147. Tsuda M, Terada T, Asaka J-i, Ueba M, Katsura T, Inui K-i. Oppositely directed H+ gradient functions as a driving force of rat H+/organic cation antiporter MATE1. American Journal of Physiology-Renal Physiology. 2007 ; 292(2) : F593-F8.

148. Otsuka M, Matsumoto T, Morimoto R, Arioka S, Omote H and Moriyama Y. A human transporter protein that mediates the final excretion step for toxic organic cations. Proceedings of the National Academy of Sciences. 2005 ; 102(50) : 17923-8.

149. Nies AT, Koepsell H, Damme K, Schwab M. Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy. Drug Transporters: Springer; 2011. p. 105-67.

150. Motohashi H, Inui K-i. Multidrug and toxin extrusion family SLC47: physiological, pharmacokinetic and toxicokinetic importance of MATE1 and MATE2-K. Molecular aspects of medicine. 2013 ; 34(2-3) : 661-8.

151. Nies AT, Damme K, Schaeffeler E, Schwab M. Multidrug and toxin extrusion proteins as transporters of antimicrobial drugs. Expert opinion on drug metabolism & toxicology. 2012 ; 8(12) : 1565-77.

152. Wittwer MB, Zur AA, Khuri N, Kido Y, Kosaka A, Zhang X, et al. Discovery of potent, selective multidrug and toxin extrusion transporter 1 (MATE1, SLC47A1) inhibitors through prescription drug profiling and computational modeling. Journal of medicinal chemistry. 2013 ; 56(3) : 781-95.

153. Ito S, Kusuhara H, Kuroiwa Y, Wu C, Moriyama Y, Inoue K, et al. Potent and specific inhibition of mMate1-mediated efflux of type I organic cations in the liver and kidney by pyrimethamine. Journal of Pharmacology and Experimental Therapeutics. 2010 ; 333(1) : 341-50.

154. Toyama K, Yonezawa A, Masuda S, Osawa R, Hosokawa M, Fujimoto S, et al. Loss of multidrug and toxin extrusion 1 (MATE1) is associated with metformin-induced lactic acidosis. British journal of pharmacology. 2012 ; 166(3) : 1183-91.

155. Dotsev A, Sermyagin A, Gladyr E, Deniskova T, Wimmers K, Reyer H, et al. Population structure and genetic diversity of Russian native cattle breeds. 2017 ; 95 : 80.

156. Proctor WR, Ming X, Bourdet D, Han TK, Everett RS, Thakker DR. Why does the intestine lack basolateral efflux transporters for cationic compounds? A provocative hypothesis. Journal of pharmaceutical sciences. 2016 ; 105(2) : 484-96.

157. Han TK, Proctor WR, Costales CL, Cai H, Everett RS, Thakker DR. Four cation-selective transporters contribute to apical uptake and accumulation of metformin in Caco-2 cell monolayers. Journal of Pharmacology and Experimental Therapeutics. 2015 ; 352(3) : 519-28.

158. Zhou K, Donnelly L, Yang J, Li M, Deshmukh H, Van Zuydam N, et al. Heritability of variation in glycaemic response to metformin: a genome-wide complex trait analysis. The Lancet Diabetes & Endocrinology. 2014 ; 2(6) :481-7.

159. Pau CT, Cheang KI, Modi BP, Kasippillai T, Keefe CC, Shulleeta M, et al. The role of variants regulating metformin transport and action in women with polycystic ovary syndrome. Pharmacogenomics. 2016 ; 17(16) : 1765-73.

160. He R, Zhang D, Lu W, Zheng T, Wan L, Liu F, et al. SLC47A1 gene rs2289669 G> A variants enhance the glucose-lowering effect of metformin via delaying its excretion in Chinese type 2 diabetes patients. Diabetes research and clinical practice. 2015 ; 109(1) : 57-63.

161. Gudeloglu A, Parekattil SJ. Update in the evaluation of the azoospermic male. Clinics. 2013 ; 68 :27-34.

162. ESHRE TR, Group A-SPCW. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertility and sterility. 2004 ; 81(1) : 19-25.

163. Melmed S, Polonsky KS, Larsen PR, Kronenberg HM. Williams Textbook of Endocrinology E-Book. Elsevier Health Sciences; 2015 Nov 11.

164. Whyte M, Pramodh S, Srikugan L, Gilbert J, Miell J, Sherwood R, et al. Importance of cannulated prolactin test in the definition of hyperprolactinaemia. Pituitary. 2015 ; 18(3) : 319-25.

165. Kronenberg H. Williams textbook of endocrinology. Elsevier Health Sciences; 2007 Nov 30.

166. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. The Journal of Clinical Endocrinology & Metabolism. 2007 ; 92(2) : 405-13.

167. Cocksedge K, Saravelos S, Wang Q, Tuckerman E, Laird S, Li T. Does free androgen index predict subsequent pregnancy outcome in women with recurrent miscarriage? Human reproduction. 2008;23(4):797-802.

168. Finley PR, Tietz NW, editors. Clinical guide to laboratory tests. WB Saunders company; 1996.

169. Joslin EP, Kahn CR, editors. Joslin's Diabetes Mellitus: Edited by C. Ronald Kahn...[et Al.]. Lippincott Williams & Wilkins; 2005.

170. Biswas S, Dey SK. A Comparative study for Diagnostic Evaluation of Polycystic Ovarian Syndrome (PCOS) by Estimation of High Sensitivity C-reactive protein (hsCRP) and Insulin Resistance. JMSCR. 2018 ; 6(5) : 69-74.

171. Farrukh A, Shafique S, Sajjad A, Naeem S. HbA1c levels among Tertiary Health Care patients with type 2 Diabetes. Pakistan Journal of Medical and Health Sciences. 2018 ; 12(3) : 1151-3.

172. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clinical chemistry. 1974 ; 20(4) : 470-5.

173. Mantovani A, Garlanda C, Doni A, Bottazzi B. Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. Journal of clinical immunology. 2008 ; 28(1) : 1-13.

174. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clinical chemistry. 1982 ; 28(10) : 2077-80.

175. Carl A, Edward R. Tietz text book of clinical Biochemistry.1999.

176. Carl A, Edward R. Tietz text book of clinical Biochemistery and Molecular Diagnostics; 4th. 2006.

177. Park J. Lab Math: A Handbook of Measurements, Calculations, and Other Quantitative Skills for Use at the Bench. The Yale Journal of Biology and Medicine. 2003;76(4-6):197.

178. Tataurov AV, You Y, Owczarzy R. Predicting ultraviolet spectrum of single stranded and double stranded deoxyribonucleic acids. Biophysical chemistry. 2008 ; 133(1-3) : 66-70.

179. Sharma A, Tapadia K, Sahin R, Shrivas K. Surfactant-Assisted Nanodrop Spectrophotometer Determination of Iron (III) in a Single Drop of Food, Biological, and Environmental Samples. Journal of Applied Spectroscopy. 2016 ; 82(6) : 1064-71.

180. Darawi MN, Ai-Vyrn C, Ramasamy K, Hua PPJ, Pin TM, Kamaruzzaman SB, et al. Allele-specific polymerase chain reaction for the detection of Alzheimer's disease-related single nucleotide polymorphisms. BMC medical genetics. 2013 ; 14(1) : 1-8.

181. McWilliams D. Genetic Amplification: Quantitative Polymerase Chain Reaction and Its Problems and Uses. 2016.

182. Hedrick P. Genetics of populations. Jones & Bartlett Learning; 2011 Aug 24.

183. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC bioinformatics. 2012 Dec 1;13(1):134-144.

184. Yılmaz M, Ozic C, Gok İ. Principles of nucleic acid separation by agarose gel electrophoresis. Gel Electrophoresis–Principles and Basics. 2012 Apr 4;33.

185. Lee PY, Costumbrado J, Hsu C-Y, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. JoVE (Journal of Visualized Experiments). 2012 ; (62) : e3923.

186. Russell DW, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 2001.

187. Becker ML, Visser LE, van Schaik RH, Hofman A, et al. Interaction between polymorphisms in the OCT1 and MATE1 transporter and metformin response. Pharmacogenetics and genomics. 2010;20(1):38-44.

188. Chang HH, Hsueh Y-S, Cheng YW, Ou H-T, Wu M-H. Association between polymorphisms of OCT1 and metabolic response to metformin in women with polycystic ovary syndrome. International journal of molecular sciences. 2019;20(7):1720-1730.

189. McKellar L. Stories of the cystorhood: Exploring women's experiences with polycystic ovarian syndrome: Implications for education, self-perception, and medicalization (Doctoral dissertation) 2016.

190. Palomba S, Falbo A, Zullo F, Orio Jr F. Evidence-based and potential benefits of metformin in the polycystic ovary syndrome: a comprehensive review. Endocrine Reviews. 2009;30(1):1-50.

191. Hwang KR, Choi YM, Kim JJ, Chae SJ, Park KE, Jeon HW, et al. Effects of insulin-sensitizing agents and insulin resistance in women with polycystic ovary syndrome. Clinical and experimental reproductive medicine. 2013 ; 40(2) : 100-105.

192. Ou H-T, Chen P-C, Wu M-H, Lin C-Y. Metformin improved health-related quality of life in ethnic Chinese women with polycystic ovary syndrome. Health and quality of life outcomes. 2016;14(1):119-129.

193. Franks S. When should an insulin sensitizing agent be used in the treatment of polycystic ovary syndrome? Clinical endocrinology. 2011 ; 74(2) : 148-51.

194. Esmaeilzadeh S, Gholinezhad-Chari M, Ghadimi R. The effect of metformin treatment on the serum levels of homocysteine, folic acid, and vitamin B12 in patients with polycystic ovary syndrome. Journal of human reproductive sciences. 2017 ; 10(2) : 95-101.

195. Velija-Ašimi Z. Evaluation of endocrine changes in women with the polycystic ovary syndrome during metformin treatment. Bosnian Journal of basic medical sciences. 2013 ; 13(3) : 180-185.

196. Kazerooni T, Dehghan-Kooshkghazi M. Effects of metformin therapy on hyperandrogenism in women with polycystic ovarian syndrome. Gynecological endocrinology. 2003 ; 17(1) : 51-6.

197. Omran MYS. Metformin and polycystic ovary syndrome. International journal of health sciences. 2007;1(1):75-80.

198. Aruna J, Mittal S, Kumar S, Misra R, Dadhwal V, Vimala N. Metformin therapy in women with polycystic ovary syndrome. International Journal of Gynecology & Obstetrics. 2004 ; 87(3) : 237-41.

199. Jindal P, Mehandiratta P, Sharma A, Takkar V, Kapila PT. Effect of metformin on FSH, LH and prolactin levels in patients with polycystic ovarian syndrome. Int J Med Res Prof. 2016 ; 2(5) : 176-79.

200. Kurzthaler D, Hadziomerovic-Pekic D, Wildt L, Seeber BE. Metformin induces a prompt decrease in LH-stimulated testosterone response in women with PCOS independent of its insulin-sensitizing effects. Reproductive Biology and Endocrinology. 2014 ; 12(1) : 98-103.

201. De Leo V, La Marca A, Orvieto R, Morgante G. Effect of metformin on insulin-like growth factor (IGF) I and IGF-binding protein I in polycystic ovary syndrome. The Journal of Clinical Endocrinology & Metabolism. 2000 ; 85(4) : 1598-600.

202. Ghaneei A, Jowkar A, Ghavam MRH, Ghaneei ME. Cabergoline plus metformin therapy effects on menstrual irregularity and androgen system in polycystic ovary syndrome women with hyperprolactinemia. Iranian journal of reproductive medicine. 2015;13(2):93-100.

203. Krysiak R, Kowalcze K, Szkrobka W, Okopien B. The effect of metformin on prolactin levels in patients with drug-induced hyperprolactinemia. European Journal of Internal Medicine. 2016;30:94-8.

204. Li R, Yu G, Yang D, Li S, Lu S, Wu X, et al. Prevalence and predictors of metabolic abnormalities in Chinese women with PCOS: a cross-sectional study. BMC endocrine disorders. 2014 ; 14(1) : 76-83.

205. Mrozinska S, Wajda A, Czekanska P, Zdzierak B, Kialka M, Milewicz T. Metformin treatment decreased free androgen index among PCOS women. In19th European Congress of Endocrinology 2017 May 3 (Vol. 49). BioScientifica.

206. Nawrocka J, Starczewski A. Effects of metformin treatment in women with polycystic ovary syndrome depends on insulin resistance. Gynecological Endocrinology. 2007 ; 23(4) : 231-7.

207. Singh B, Panda S, Nanda R, Pati S, et al. Effect of metformin on hormonal and biochemical profile in PCOS before and after therapy. Indian Journal of Clinical Biochemistry. 2010 ; 25(4) : 367-70.

208. Diamanti-Kandarakis E, Alexandraki K, Protogerou A, Piperi C, Papamichael C, Aessopos A, et al. Metformin administration improves endothelial function in women with polycystic ovary syndrome. European Journal of Endocrinology. 2005 ; 152(5) : 749-56.

209. Hahn S, Quadbeck B, Elsenbruch S, Gärtner R, Finke R, Mann K, et al. Metformin, an efficacious drug in the treatment of polycystic ovary syndrome. Deutsche medizinische Wochenschrift (1946). 2004 ; 129(19) : 1059-64.

210. Jakubowska J, Bohdanowicz-Pawlak A, Milewicz A, Szymczak J, et al. Plasma cytokines in obese women with polycystic ovary syndrome, before and after metformin treatment. Gynecological Endocrinology. 2008 ; 24(7) : 378-84.

211. Bredella MA, McManus S, Misra M. Impact of metformin monotherapy versus metformin with oestrogen-progesterone on lipids in adolescent girls with polycystic ovarian syndrome. Clinical endocrinology. 2013 ; 79(2) : 199-203.

212. Kocer D, Bayram F, Diri H. The effects of metformin on endothelial dysfunction, lipid metabolism and oxidative stress in women with polycystic ovary syndrome. Gynecological Endocrinology. 2014 ; 30(5) : 367-71.

213. Stocker SL, Morrissey KM, Yee SW, Castro RA, et al. The effect of novel promoter variants in MATE1 and MATE2 on the pharmacokinetics and pharmacodynamics of metformin. Clinical Pharmacology & Therapeutics. 2013 ; 93(2) : 186-94.

214. Christensen MM, Brasch-Andersen C, Green H, Nielsen F, et al. The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c. Pharmacogenetics and genomics. 2011 ; 21(12) : 837-50.

215. Mousavi S, Kohan L, Yavarian M, Habib A. Pharmacogenetic variation of SLC47A1 gene and metformin response in type2 diabetes patients. Molecular Biology Research Communications. 2017 ; 6(2) : 91-94.

216. Becker ML, Visser LE, Van Schaik RH, Hofman A, et al. Genetic variation in the multidrug and toxin extrusion 1 transporter protein influences the glucose-lowering effect of metformin in patients with diabetes: a preliminary study. Diabetes. 2009 ; 58(3) : 745-9.

217. Tkáč I, Klimčáková L, Javorský M, Fabianová M, et al. Pharmacogenomic association between a variant in SLC47A1 gene and therapeutic response to metformin in type 2 diabetes. Diabetes, obesity and metabolism. 2013 ; 15(2) : 189-91.

218. Raj GM, Mathaiyan J, Wyawahare M, Priyadarshini R. Lack of effect of the SLC47A1 and SLC47A2 gene polymorphisms on the glycemic response to metformin in type 2 diabetes mellitus patients. Drug metabolism and personalized therapy. 2018 ; 33(4) : 175-85.

Appendices

Questionnaire of PCOS Patients
Demographic Characterizations

General Information: **No. :** ☐

Name: **Address:**

- Age : ( ) years ( ) months
- Residence: ( ) urban ( ) rural
- Age of menarche: ( ) years
- BMI : ( ) Kg/m²
- Blood pressure: systolic: ( ) Diastolic: ( )

- Hirsutism Yes ☐ No ☐

- Alopecia Yes ☐ No ☐

- Marital status:
  - ❖ Married ☐ No. of kids ( ) times of abortion ( )
  - ❖ Non married ☐

- Jop ( ) Education ( )

- Smoking
  - ❖ Never ☐ daily ☐ occasionally ☐

- Presence of other diseases yes ☐ No ☐
- 1- 2- 3- 4-

- Menstrual cycle
  - ❖ Regular ☐ Irregular ☐ frequency ( )

الخلاصة

خلفية الدراسة : يستخدم العلاج بمحفزات الانسولين (المتفورمين) على نطاق واسع في متلازمة تكيس المبايض المتعدد (PCOS)، وبالرغم من ذلك فان فعالية العلاج تظهر تباينا فرديا في مرضى متلازمة تكيس المبايض وقد اشارت الدراسات ان البروتين الناقل متعدد الادوية والسموم (MATE1) هو المسؤول عن افراز المتفورمين في الكلى والصفراء، اما بالنسبة للدراسة الحالية فقد فحص الارتباط بين تعدد النمط الجيني ل MATE1 وفعالية علاج المتفورمين في مرضى متلازمة تكيس المبايض.
هنالك ما لا يقل عن أحد عشر من الطفرات المترادفة (SNPs) في جين (SLC47A1) وقد شخصت طفرتين منها في هذه الدراسة وهما : (rs1961669 , rs2289669).

الهدف من الدراسة : صممت هذه الدراسة لتحديد التأثيرات السريرية والهرمونية والكيميائية الحيوية لثلاثة أشهر من العلاج بالميتفورمين في النساء المصابات بمتلازمة تكيس المبايض المتعدد PCOS ودراسة ارتباط تعدد النمط الجيني MATE1(SLC47A1) مع استجابة المتفورمين في متلازمة تكيس المبايض.

المرضى والوسائل : اعدت هذه الدراسة كدراسة استطلاعية تداخلية قائمة على الملاحظة ، و اختير مئتان وواحد وثلاثون امرأة مصابة بمتلازمة تكيس المبايض المتعدد للمشاركة في هذه الدراسة، كانت جميع النساء المشمولات في هذه الدراسة من الفئة العمرية (18-40) سنة وتم تناول أقراص الميتفورمين 500 ملغ فمويا مرتان يوميًا وقد تم سحب عينات دم من المرضى المؤهلين الذين أعطوا موافقة لإجراء الاختبارات الجينية ، وقياس الهرمون المنبه للجريب ، والهرمون اللوتيني ، التستوستيرون الكلي ، الكلوبيولين المرتبط بالهرمونات الجنسية ، الإستراديول ، البرولاكتين ، هرمون الغدة الدرقية ، الأنسولين الصائم ، السكر التراكمي ، الكلوكوز الصائم والدهون بانواعها الثلاثية والمفيدة والضارة و الكوليستيرول الكلي.

النتائج : أظهرت نتائج الدراسة أن وجود الطفرة (rs2289669) (G> A) له ارتباط كبير باستجابة الميتفورمين في المرضى المصابين بمتلازمة تكيس المبايض ، بينما وجود الطفرة الاخرى (A> G) (rs1961669)لم يكن له ارتباط كبير باستجابة الميتفورمين في نفس الشريحة من المرضى ، كما أظهرت المقارنة في العلامات الديموغرافية والكيميائية الحيوية بين نتائج العلاج قبل استخدام الميتفورمين وبعده تحسنًا في المعايير المذكورة أعلاه.

الاستنتاجات : بينت هذه الدراسة ان دواء المتفورمين أظهر تحسنا ملحوظا في انتظام الدورة الشهرية وكذلك الجوانب الهرمونية والحيوية في النساء العراقيات المصابات بمتلازمة تكيس المبايض بالإضافة الى ذلك هنالك أرتباط قوي بين وجود الطفرة (rs2289669) (G> A) (SLC47A1) في البروتين الناقل (MATE1) والاستجابة السريرية والهرمونية والحيوية لعلاج المتفورمين في هذه الشريحة من المرضى.

جمهورية العراق
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جامعة كربلاء
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تأثير تعدد الأشكال لجين MATE1 (SLC47A1) على الأستجابة لعقار المتفورمين في متلازمة تكيس المبايض المتعدد للمرأة العراقية.

رسالة

مقدمة الى كلية الصيدلة بجامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الادوية والسموم

من قبل

مها محمد كاظم مجيد آل طعمة

بكالوريوس صيدلة (جامعة الكوفة 2007)

بإشراف

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