Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Kerbala
College of Pharmacy
Department of Pharmacology and Toxicology

Association of Genetic Polymorphism of STK11 Gen with Therapeutic Response of Metformin in Women with Polycystic Ovary Syndrome.

A Thesis

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

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2020 A.D. **1442 A.H.**

بسم الله الرحمن الرحيم

قَالُواْ سُبۡحَٰنَكَ

لَا عِلۡمَ لَنَآ إِلَّا مَا عَلَّمۡتَنَآۖ

إِنَّكَ أَنتَ ٱلۡعَلِيمُ ٱلۡحَكِيمُ

صدق الله العلي العظيم

سوره البقرة

الآية 32

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Dedication

TO……….....

My kind parents

My dear husband

My lovely sons

Montaha

Acknowledgements

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

I would like to thank University of Kerbala, College of Pharmacy and Pharmacology & Toxicology Department for all effort to provide the opportunity for postgraduate studies and provide laboratories and all the requirements of scientific research.

My great appreciation to the Dean of the College **Professor Dr. Ahmed Salih Sahib** for his incessant support. My deep gratitude and appreciations to my supervisors, **Assistant Professor Mazin Hamid Ouda & Consultant Gynecologist Dr. Hameedah Hadi Abdulwahid** for their continuous guidance, support and advices through the whole period of research. This work would never have been done without them.

I would like to express my deep thanks to **Assistant Professor Dr. Hassan Mahmood Abo Al-maali** for his continuous guidance regarding genetic part of the research. Thanks to all the staff members at the Kerbala Obstetrics and Gynecology Teaching Hospital also for Pharmacology and Toxicology Department at College of Pharmacy for their scientific support and help.

Finally, my gratitude to all the patients contributed in this study for their cooperation.

Montaha

Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Words
(IRS)-1 and -2	Insulin receptor substrates one and two
3β-HSD	3β-hydroxysteroid dehydrogenase
95% CI	95% Confidence interval
AE-PCOS	Androgen Excess and PCOS Society
AFC	Antral Follicle count
AGA	Androgenic alopecia
AMPK	Adenosine mono phosphate protein kinase
ANOVA	Analysis of variance
Apo A1	Apolipoprotien A-1
BMI	Body mass index
BPA	Bisphenol A
CAMP	Cyclic adenosine monophosphate
C_{max}	Peak plasma concentrations
CV	Cardiovascular
CVD	Cardiovascular disease
CYP450	Cytochrome P450
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleoside triphosphates

E2	Estradiol
ECLIA	Electrochemiluminescence immunoassay
EDTA-tube	Ethylenediaminetetra-acetic acid- tube
eGFR	Estimated glomerular filtration rate
ESHRE/ASRM	European Society of Human Reproduction and Embryology/ American Society for Reproductive Medicine
ET-1	Endothelin-1
F	Forward
F.Insulin	Fasting insulin
FAI	Free androgen index
FAS	Fatty acid synthase
FBS	Fasting blood sugar
FDA	Food and drug administration
FFA	Free fatty acids
FSH	Follicular stimulating hormone
GLP-1	Glucagon-like peptide1
GnRH	Gonadotropin-releasing hormone
HbA	Hemoglobin A
HbA1C	Glycated hemoglobin
HDL-C	High-density lipoprotein cholesterol
MATE	Multidrug and toxin extrusion

HOMA-IR	Homeostasis model assessment for IR
IGFBP-1	Insulin growth factor binding protein-1
IR	Insulin resistance
ISD	Insulin sensitizing drug
IVF	Invitro fertilization
LDL-C	Low-density lipoprotein cholesterol
LH	Luteinizing hormone
LKB1	Liver kinase B1
MiRNAs	MicroRNAs
MMTT	Mixed meal tolerance test
NAFLD	Non-alcoholic fatty liver disease
NIH	National Institutes of Health
n.	Number
NY-REN-19	Renal carcinoma antigen
SLC22A1	Solute Carrier Family 22 Member 1
OGTT	Oral glucose tolerance test
OR	Odd ratio
OCT	organic cation transporter
PAI-1	Plasminogen-activator inhibitor-1
PAP	Phosphatidate phosphatase
PCOm	Polycystic ovarian morphology

PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIk3	Phosphatidylinositol 3-kinases
PKa	Dissociation constant values
PP-14	placental protein-14
PPARα	Peroxisome proliferator-activated receptor-α
PTA	Phosphotungstic acid
Quiki	Quantitative insulin sensitivity check index
r	Ratio
R	Pearson's correlation coefficient test
R1	Reverse 1
R2	Reverser 2
RSHBG	SHBG receptors
SD	Standard deviation
SHBG	Sex hormone binding globulin
StAR, CYP11A1	The steroidogenic acute regulatory protein
STK11	Serine threonine kinase 11
T2DM	Type-II diabetes mellitus
TBE	Tries Borate EDTA

TC	Total cholesterol
TG	Triglycerides
Thr172	Threonine residue172
TNF-α	Tumor necrosis factor- α
TSH	Thyroid stimulating hormone
UV	Ultraviolet
WHO	World Health Organization

Summary

Background; Polycystic ovary syndrome (PCOS) is the most common disorder of the ovary. It is characterized by hyperandrogenism, irregular ovulatory cycle and metabolic derangement including glucose intolerance with hyperinsulinemia. Manifestations of this syndrome are heterogeneous with several consensus definitions of the disorder have been produced to describe polycystic ovary syndrome, and various emphases on clinical or biochemical hyperandrogenism, polycystic ovaries and oligoanovulation.

Diagnosis of this syndrome has at least two of the following characteristics, clinical or biochemical hyperandrogenism, oligoanovulation and polycystic ovaries on ultrasound according to Rotterdam criteria. Several genes are involved in the pathogenesis of polycystic ovary syndrome, among them a single nucleotide polymorphism of serine threonine kinase11(STK11) gene and it has been suggested to be associated with this syndrome and response to metformin therapy.

Aims of the Study; To investigate the relation of the serine threonine kinase11 (STK11) gene polymorphism (rs741765 C\T) and (rs8111699 C\G) with the response to metformin therapy for a period of three months in a polycystic ovary syndrome treated patients in Kerbala province.

Materials, Patients and Methods; This an interventional a prospective study for two hundred thirty-five Iraqi women patients, who newly diagnosed with polycystic ovary syndrome based on Roterdam criteria by physician's diagnosis during their visit to Gynecology and Obstetrics Teaching Hospital in Kerbala /Iraq. All women enrolled in this study were taken a metformin therapy (500mg) twice daily for three months. Women who were excluded from the study; those who had thyroid dysfunction, congenital adrenal hyperplasia and Cushing's syndrome, or others on hypoglycemic drugs rather than metformin and those using one or more of medication that interfere with metformin action, consequently all should be excluded before making diagnosis of PCOS.

Demographic parameters for all patients were reported, by the history-taking specifically for address, age, body mass index, blood pressure, presence of other diseases, smoking, menstrual regularity, hirsutism, martial state, number of children and times of abortion.

Blood samples were obtained from all patients. Venous blood (8ml) was withdrawn and (5ml) was placed in EDTA-tube for genetic study and for measuring fasting glucose and HbA1c; DNA extraction and polymerase chain reaction were done at the laboratory of postgraduate research in the College of Pharmacy/Kerbala University. Other (3ml) from each blood sample was placed in gel tube (EDTA-free) and serum was separated after centrifugation of blood at 3000 rpm for 10 minutes, then used for measurement of biomarkers markers.

Results; Serine-threonine kinase11(STK11) gene rs741765 and rs8111699 polymorphism were associated with metformin efficacy. Results were predicted by comparing the clinical, endocrinal and metabolic parameters among the three genotypes of each SNP; for rs741765, wild genotype (CC), heterozygous genotype(CT) and mutant genotype(TT). And for rs8111699 wild genotype (CC), heterozygous genotype (CG), mutant genotype (GG), so results revealed that the efficacy of metformin was increase with the presence of mutant alleles among the patients in both SNPs.

Present study revealed that STK11 rs8111699 SNP was associated with baseline insulin sensitivity in polycystic ovary syndrome patients and G allele carrying in the STK11 rs8111699 SNP can result in lower STK11 action and therefore lower efficiency of AMPK phosphorylation by STK11. Furthermore, the same G allele in the STK11 rs8111699 SNP like T allele in rs741765 both were associated with more metformin efficacy.

Conclusion; Polymorphism in a serine threonine kinase11 (STK11) gene, a kinase gene which expressed in liver and implicated in metformin action is associated with response to metformin treated polycystic ovary syndrome patients in Kerbala province. Present study recorded that percentage of women who responded to treatment was increased with the number of (T) allele in rs741765 and (G) allele in rs8111699, nevertheless the same mutant (G) allele in rs8111699 SNP was reported to be associated with baseline insulin sensitivity.

Chapter One

Introduction

1. Introduction

1.1 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is recognized as one of the most common endocrine disorder among infertile women, called Stein-Leventhal syndrome(1). Furthermore, PCOS affects (5–20%) of women of reproductive age worldwide within the reproductive age (15 to 44) years, but the prevalence may be twice as high under a broader Rotterdam criterion. Accordingly, it is characterized by hyperandrogenism and metabolic derangement which including; glucose intolerance and hyperinsulinemia(2).

Reproductive function impacted negatively in PCOS and predisposes to adverse obstetric outcomes, such as gestational diabetes mellitus, pre-eclampsia, fetal macrosomia and perinatal morbidity and mortality. Moreover, there is a direct link between this syndrome and endometrial carcinoma as well as type-II diabetes mellitus (T2DM)(3).

Hyperandrogenism is a cardinal clinical hallmark of the syndrome, that means hypersecretion of androgen by the stromal theca cell of the polycystic ovary. Despite of the exact cause of PCOS is not known, the syndrome can result from disturbance in the hypothalamic pituitary ovarian axis and hyperinsulinemia. Metabolic disorders and possibly cardiovascular diseases which occur in later years of hyperinsulinemia(4).

Dyslipidemia is more common in women with PCOS, with patients demonstrating higher levels of low-density lipoprotein- cholesterol (LDL-c) and triglycerides (TG) with lower levels of high-density lipoprotein cholesterol (HDL-c) compared with women without this disorder, other conditions that produce similar symptoms of PCOS include adrenal hyperplasia(5).

1.1.1 Prevalence

Depending on diagnostic criteria, PCOS prevalence is approximately (4–6.6%) in accordance with National Institutes of Health (NIH) 1990 criteria. Approximately (4–21%) when Rotterdam 2003 criteria were applied, PCOS prevalence by NIH 1990 criteria remains relatively constant(6).

Estimation the prevalence related to the population being assessed, because of ethnic differences in the clinical and biochemical features of PCOS (7). Ordinarily the reported prevalence range is between (2.2 – 26%) in various countries, depending on the recruitment method, study population, criteria used for its definition and the method used to define each criterion. The prevalence of PCOS can be as high as (30%) in women with secondary amenorrhea, (40%) in women with infertility, (75%) in women with oligomenorrhea and (90%) in women with hirsutism (8).

1.1.2 Signs and Symptoms

Common signs and symptoms of PCOS include: irregular or no menstrual cycle, oligomenorrhea (fewer than nine menstrual cycle in a year) or amenorrhea (no menstrual cycle for three or more consecutive months), or any types of menstrual disorders, so anovulation or oligovulation is a common symptom of PCOS(9).

High levels of masculinizing hormones is the most common sign of this syndrome known as hyperandrogenism, accordingly three-quarters of women with PCOS have evidence of hyperandrogenemia (10), where the visible signs of this symptom include weight gain, abdominal and subcutaneous fat, hirsutism (facial and body hair), male-pattern alopecia (hair loss), acne , thick with darker velvety skin, clitoromegaly (enlargement of the clitoris), deep voice and seborrhea (oily skin) (11). These symptoms appear because cysts produce androgens, which result in the virilization or the expression of male-like characters in the females, accordingly PCOS leads to the appearance of hyperandrogenism(12).

Skin develops light brown or black patches, a condition known as acanthosis nigricans, skin of neck, armpits, thighs, and breasts are more prone to this skin pigmentation, as well as, skin tags appear in those regions, consequently the dark pigmentation is a cutaneous marker for insulin resistance(13).

Difficulty to get pregnancy or infertility is another PCOS signs, this generally results from chronic anovulation(12). Even if implantation occurs, abortion and still birth loss risks are increased, moreover eclampsia and the small-gestational-age babies can occur (14).

Alteration in metabolic profile also occurs, where insulin resistance is a major symptom which result in hyperinsulinemia and can lead to diabetes mellitus, as high insulin level is responsible for the deposition of fat around the abdomen or central adiposity in a majority of women with PCOS resulted with body mass index (BMI) is (30 kg/m2) or higher, moreover hypertension, cardiovascular issues and dyslipidemia are co-morbidities of PCOS(15).

Patients with PCOS often display sugar cravings, frequent urination, delayed healing, fatigue, blurred vision, tingling sensation, mood swing, anxiety and depression episodes, while sleep apnea (sleep disorder in which breathing repeatedly stops and starts) is another symptom of the syndrome, due to altered sex steroid level, smooth vagina is a feature of PCOS, which can be observed during a pelvic exam due to the hormonal imbalance (16).

Poly cystic ovary syndrome can put the patient at a risk of uterine cancer, as the prevailing high estradiol level with the lack of progesterone due to ovarian malfunction increases the risk of endometrial hyperplasia(17).

1.1.3 Diagnosis

In 1935 PCOS described by Stien and Leventhal, as bilaterally enlarged ovaries with multiple cysts in many women with infertility, menstrual irregularity and hyperandrogenism**.** Where (NIH) in 1990 introduced standard criteria in PCOS for applying in researches and clinics, this definition based on clinical or biochemical hyperandrogenaemia with the exclusion of adrenal hyperplasia and hyperprolactinemia and thyroid dysfunction (18).

In 2003 Rotterdam in Netherland presented new diagnostic criteria(19) which are considered to be a compromise between those of NIH and Androgen Excess and PCOS Society (AE-PCOS), table (1-1).

Table (1-1). Diagnostic criteria for polycystic ovary syndrome (20)

NIH 1990	**1- Chronic anovulation** **2- Clinical and/or biochemical signs of hyperandrogenism** **(with exclusion of other etiologies, e.g., congenital adrenal hyperplasia)** ***(Both criteria needed)***
Rotterdam 2003	**1- Oligo- and/or anovulation** **2- Clinical and/or biochemical signs of hyperandrogenism** **3- Polycystic ovaries** ***(Two of three criteria needed)***
AE-PCOS Society 2006	**1- Oligo-anovulation and/or polycystic ovarian morphology (Ovarian dysfunction)** **2- Clinical and/or biochemical signs of hyperandrogenism** ***(Both criteria needed)***

Abbreviations: NIH, National Institutes of Health; AE-PCOS, Androgen Excess and PCOS

Polycystic ovary syndrome definition is wider, which including many more women. Currently two of the following three features; Oligoanovulation (O), Hyperandrogenemia (H) and multiple cysts on ovaries by ultrasound (P), resulted in four different phenotypes: *P* + H + O (PCOS complete) and *P* + O, H + O, *P* + H, PCOS diagnosis continues to be controversial, because of the heterogeneous nature which may change during the lifetime of the woman(21).

The Rotterdam criterion defined the syndrome as an ovarian volume of (10 ml) and/or an antral follicle reflected by cysts (2–9) mm in diameter, Antral Follicle count (AFC) of 12 follicle or more which finding in one ovary suffices, when the technology has improved, the ability to detect small follicles has increased. So it is likely that AFC of 12 follicle is too low and investigators have suggested that AFC of (18–22) follicle should be used as the lower limit for the diagnosis of PCOS(5). Nevertheless ovarian volume does not seem to change much with improved transvaginal probe frequency(13). In 2006 competing diagnostic criteria from the (AE-PCOS) was introduced (22).

Diagnosis of PCOS is important, because it is related with increased risk of insulin resistance (IR), noninsulin dependent diabetes mellitus and metabolic syndrome, all of which have long-term consequences(23).

Follicles may be oriented along the ovarian by ultrasound examination, ordinarily the periphery examination appearing like a string of pearls, figure (1-1).

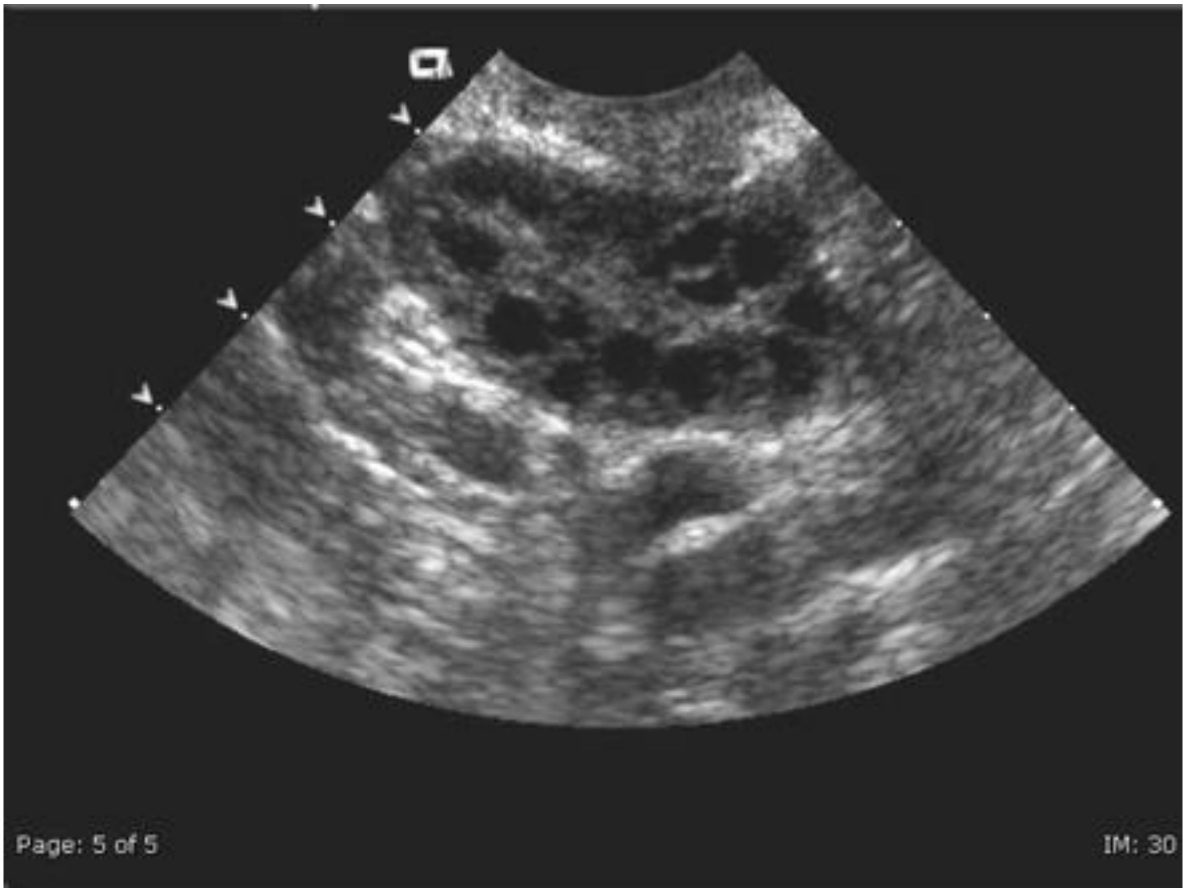


Figure (1-1). Ultrasound examination of ovary (appearing like a string of pearls(24)

20 or more follicles in at least one ovary(25), these cysts are actually immature follicles. The follicles have developed from primordial follicles and stopped (arrested) at an early antral stage(26) . A cyst is a water-filled sac containing the egg, that should have been normally discharged for possible fertilization(16). Egg conversion into a cyst termed as functional cyst and prevents an ovulation, also ovulation is blocked resulted in the disruption of the menstrual cycle causing amenorrhea, so multiple cysts are formed in the ovarian follicles due to the hormonal imbalance which is characterized as PCOS. Because of the water-retained cysts, some of which can be as big as 10 mm

wide with absence of ovulation and menstrual cycle prevents fertilization and conception, thus pregnancy will become difficult(27).

Standard assessment of PCOS done by; first, history-taking specifically for menstrual pattern, obesity, hirsutism and acne , clinical prediction rule found that these four questions can diagnose PCOS(26). Second by gynecologic ultrasonography specifically looking for small ovarian follicles, these are believed to be the result of disturbed ovarian function with failed ovulation, reflected by the infrequent or absent menstruation that is typical for the condition, while in normal menstrual cycle one egg is released from a dominant follicle, a cyst that bursts to release the egg after ovulation, so the follicle remnant is transformed into a progesterone-producing corpus luteum which shrinks and disappears after approximately (12–14) days. In PCOS there is a so-called follicular arrest; i.e., several follicles develop to a size of 5–7 mm, but not further and no single follicle reaches the preovulatory size (16 mm or more). Laparoscopic examination may reveal a thickened, smooth, pearl-white outer surface of the ovary(28).

1.1.4 Pathogenesis

The ovaries are stimulated to produce excessive amounts of androgenic hormones leading to PCOS development by either one or combination of the followings (almost combined with genetic susceptibility)(29).

First of all is the release of excessive luteinizing hormone (LH) by an anterior pituitary gland in women with ovaries are sensitive to this stimulus(30), they experience an increased frequency of hypothalamic Gonadotropin-releasing hormone (GnRH) pulses, which in turn results in an increase in the LH/FSH ratio and disturbed ovarian function(31), GnRH normally induces the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH), these two hormones are essential for the two distinct phases of menstrual cycle(32). Consequently in PCOS these hormones are scanty, so the egg is either not formed or cannot be liberated from the follicle where the cycle is disrupted and amenorrhea occurs, which can be of two types ; either primary or secondary amenorrhea(33). Primary amenorrhea is the inability to reach menarche due to chromosomal or anatomic issues, while secondary amenorrhea or hypothalamic amenorrhea is characterized by the absence of menstrual cycles for 3 or more consecutive months (34).

Second cause is the high level of insulin in the blood (hyperinsulinemia). Ordinarily obesity may play a pathogenic role in the development of the syndrome, throughout the disturbances in insulin and androgens. Adipose tissue possesses aromatase which is an enzyme that converts androstenedione to estrone and testosterone to estradiol. Accumulation of adipose tissue mass around abdomen increases the availability of metabolites that are able to affect the secretion, metabolism and peripheral action of insulin(35). Ovarian theca cells provide support to the growing follicle, assisting in mature oocyte generation, these cells are hyper responsive to the stimulatory effects of insulin in PCOS patients, figure (1-2).

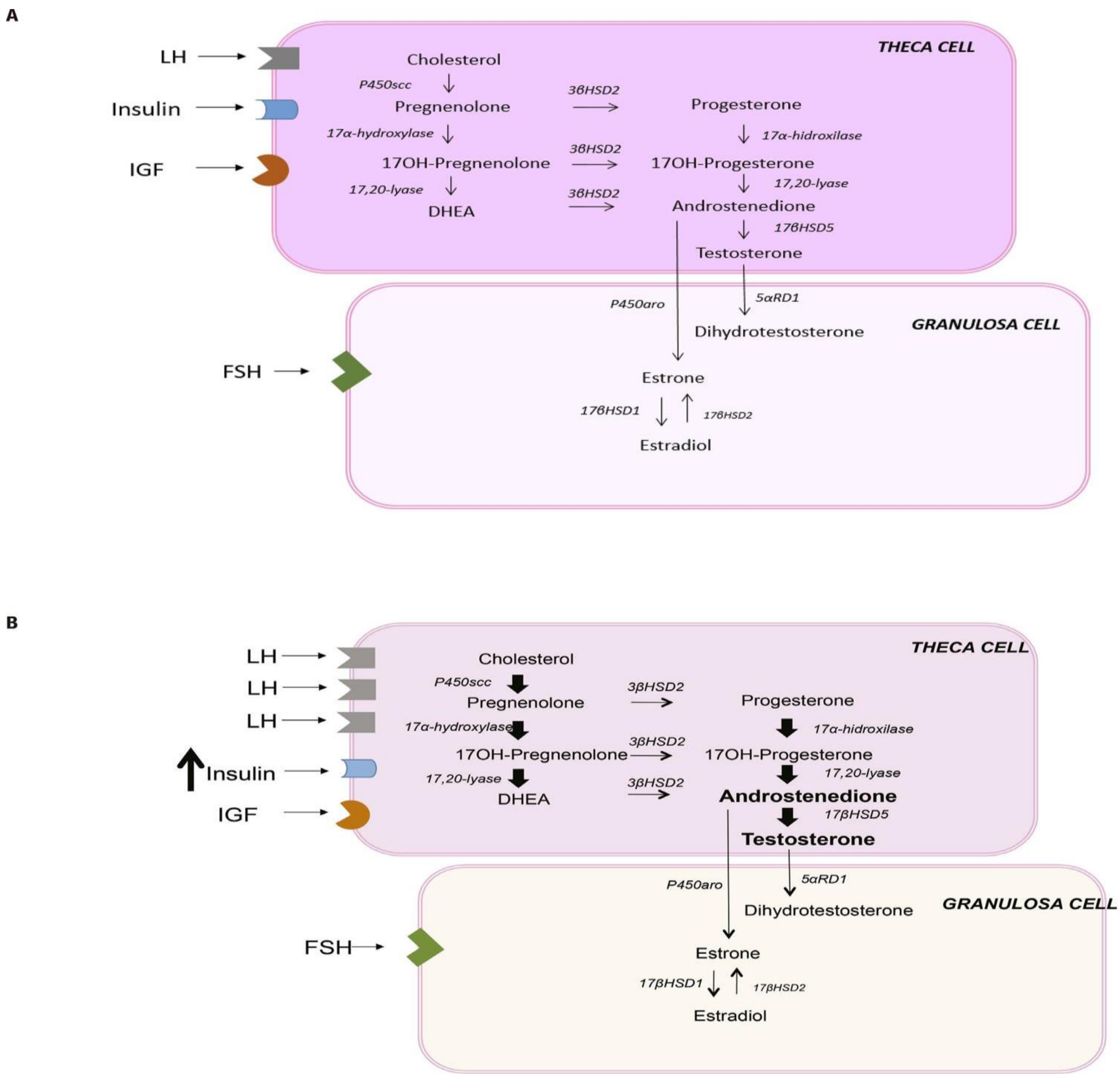


Figure (1-2). Ovarian steroidogenesis. (A) Normal ovarian steroid synthesis. (B) PCOS ovarian steroid synthesis. In comparison to normal theca cells, PCOS theca cells show increased expression of LH receptor and increased expression of CYP17A1 gene, leading to enhance of 17α-hydroxylase and 17,20-lyase activity, and amplifying androgen synthesis. Exogenous factors, such as hyperinsulinemia and IGFs are modulatory hormones that can disrupt normal intra-ovarian regulation of steroidogenesis(36)

So they proliferate causing ovarian hyperthecosis. Moreover, insulin resistance amplifies the androgenic potential in the theca cells which lead to aggravate PCOS, in addition to high sensitivity of theca cells to gonadal steroid gonadotropin stimulation aid to androgenism (16).

Sex hormone binding globulin (SHBG) is represented the third factor which is a glycoprotein regulate circulating free sexual steroid hormones and transporting to target tissues, where concentration of SHBG is regulated by: cortisol, estrogens, iodothyronine and growth factors, while decreased by androgens, insulin, prolactin and IGF-1 (21). Lower levels of SHBG in PCOS cases also aid to increased hyperandrogenism(37). SHBG decrease is affected by hyperinsulinemia and free androgens in peripheral tissues(38).

Bilateral pathologies changes represent the forth factor which include metabolic syndrome and the constant inflammations, this can lead to PCOS(39). Based on evidences, the link between non-alcoholic fatty liver disease (NAFLD) which is a chronic liver disease characterized by hepatic damage from fatty liver infiltration leading to end-stage liver disease and PCOS has been traced, that indicated a novel hepato-ovarian axis(39).

1.1.5 Etiology

Although the exact cause of PCOS is unknown, but it is understood to be a multifactorial condition with a genetic factor. Approximately (20–40%) of first-degree female relatives of women with this syndrome go on to develop PCOS (40), even if it has been never diagnosed, as with type 2 diabetes, it is likely that numerous genes each one makes a small contribution to the etiology of PCOS. Candidate genes which are complicated by epigenetic and environmental factors such as an unhealthy diet and lack of physical activity(18). Accordingly, the high prevalence of woman with this syndrome can be explained by interaction of a key genes with environmental factors(41). Thyroid dysfunction, hyperprolactinemia, androgen-secreting tumors, Cushing’s syndrome (a syndrome associated with excess cortisol levels) and congenital adrenal hyperplasia, all these diseases can drive PCOS pathogenesis(42).

Chemical exposure has been held responsible for the development of PCOS, the exposure to a number of chemicals by accidental (pesticide, vehicle exhausts, industrial pollutants etc.) or deliberate (cosmetics, household cleaning agents, chemotherapeutics etc.) (16).

Chemicals such as bisphenol A (BPA), present in packaged and canned foods, which when exposed to for a long period, can lead to reproductive issues including PCOS, as well as chemicals are included in consumer products in the form of fragrance, emulsifier, preservative, color, fixatives etc, the strong role of fragrance compounds in perturbing hormonal homeostasis and paving the path for conditions like PCOS (43).

1.1.6 Insulin Resistance as Pathological Factor in Polycystic Ovary

Majority of women with PCOS have insulin resistance and/or are obese(44). Their elevated insulin level contribute to the abnormalities seen in the hypothalamic-pituitary-ovarian axis that lead to this syndrome (45), this effect occurring by; increasing GnRH pulse frequency which result in LH over FSH dominance with increasing the sensitivity of pituitary cells to gonadotropin releasing hormone (GnRH) action by increase the number of the luteinizing hormone (LH) receptors will lead to increase the ovarian steroidogenic response to gonadotropins and reducing sex hormone binding globulin (SHBG) synthesis in the liver, so the final result is decreased follicular maturation in ovaries (41).

Insulin resistance is a common finding among women with a normal weight as well as overweight women , also adipose tissue possess aromatase, an enzyme that converts androstenedione to estrone and testosterone to estradiol (46, 47). Excess of adipose tissue in obese women creates the paradox of having both excess of androgens which are responsible for hirsutism and virilization as well as estrogens which inhibits FSH via negative feedback mechanism, accordingly hyperinsulinemia and IR are thought to be pathological key factors for PCOS and metabolic syndrome and it represent a metabolic disorder characterized by impairment of insulin function(48).

Diagnosis of IR is not standardized(49), because of many techniques are followed, for example: fasting plasma glucose, oral glucose tolerance test (OGTT), glucose/insulin ratio, homeostasis model assessment for IR (HOMA-IR), quantitative insulin sensitivity check index (Quiki) and euglycemic hyperinsulinemic clamp(50).

Acanthosis nigricans was reported in IR, it is a brown to black poorly defined velvety hyperpigmentation of the skin, present in the posterior and lateral folds of neck, axilla and groin(51), conversely the degree of hyperandrogenemia is significantly less than those observed in patients with PCOS defined by the NIH 1990 criteria. Furthermore, those patients have an increased risk for developing metabolic complications, including type 2 diabetes mellitus (T2DM) is not known, moreover there are even less data showing that women with polycystic ovaries and ovulatory dysfunction without clinical or biochemical evidence of hyperandrogenism and predicted that insulin effect on liver, adipose tissue and muscle resulted in disturbance of ovaries function (19).

1.1.7 Reproductive Consequences

1.1.7.1 Infertility

Infertility has been considered by the World Health Organization (WHO) as a public health problem and the agency defines infertility as the absence of pregnancy after two years of regular intercourse without any contraception, so a process of assessment of possible factors involved should begin (52). PCOS is characterized by anovulation due to the developmental defect of follicles beyond 10 mm in size, the clinical manifestations including infertility are related to the hypersecretion of LH in (70%) of women that suffering from hyperandrogenism which might lead to anovulatry cycle. High level of LH/FSH ratio will lead to an increase in ovarian androgen production, as consequence, there is no ovulation in most of the cycles making it essential to induce ovulation (52).

The European Society of Human Reproduction and Embryology/ American Society for Reproductive Medicine (ESHRE/ASRM) recommends that before starting any intervention a counseling before conception should emphasize the importance of lifestyle changes, especially weight loss and regular exercises in overweight women, smoking cessation and reducing alcohol consumption. Regarding ovulation inducing drugs, all are associated with the increase in multiple pregnancies, obstetric and neonatal risks (52).

About 50% of infertile women suffering from obesity and there is a clear association between obesity and menstrual irregularities, since the adipose tissue is the largest peripheral area for the aromatization of androgens to estrogens, contributing to estrogen production (52).

1.1.7.2 Ovarian Dysfunction

The assessment of ovarian follicle number has become the main item of polycystic ovarian morphology (PCOm) through the advent trans-vaginal ultrasonography. Moreover, the increase in ovarian volume is considered as accurate marker of (PCOm) provided the measurements are carried out on median sections of the ovaries. There is an almost universal consensus on the choice of follicular excess and ovarian enlargement as the main criteria to define PCOm by ultrasound (53).

1.1.7.3 Menstrual Irregularity

Polycystic ovaries patients are suffering from an occasional distressing episodes of prolonged and/or heavy vaginal bleeding. Ordinarily, adolescents frequently exhibit physiological menstrual irregularities such as oligomenorrhea, usually during the first 2 years after menarche, may due to lack of maturation of hypothalamic-hypopituitary-ovarian axis(54).

Menstrual irregularity can be sometimes an unreliable criterion for diagnosis of PCOS in adolescents (55), through close observation of the menstrual cycle patterns, clinicians have to differentiate physiological anovulation associated with puberty from pathological anovulation as a dysfunction identified in PCOS(56) . It has been suggested to postpone diagnosis at least 2 years after menarche to establish a persistent menstrual irregularity, accordingly the initiation of appropriate treatment will delay even in patients without menstrual symptoms, treatment may be advisable because of the long-term risk of unopposed estrogen (endometrial hyperplasia and cancer)(57).

1.1.7.4 Miscarriage

Miscarriage rates are believed to be higher in PCOS women than in normal one, a meta-analysis verified the results of several other studies and showed an increased prevalence in PCOS women of gestational diabetes, gestational hypertension, preeclampsia and premature births, consequently the infants of PCOS women were more often admitted to a neonatal intensive care unit and the perinatal mortality is higher(58).

1.1.8 Pregnancy Complications

Spontaneous abortion rate in PCOS is approximately one third of all pregnancies(59). This is at least double the rate for recognized early abortions in normal women (12–15%), the actual reasons for this complications are unclear although hypotheses include elevated LH levels, deficient progesterone secretion, abnormal embryos from atretic oocytes and an abnormal endometrium, where there are attempts to improve the live birth rate by lowering LH using GnRH agonist therapy was successful in retrospective studies(60). Morbidity will increase if the women are obese, moreover perinatal mortality is increased at least 1.5 times with the increase of pregnancy complications that include preeclampsia, diabetes, premature labor, as well as stillborn rate will increase(61).

Based on these complications and the chance of increase delivering a large infant, the C-section rate is also increased. Because most patients with PCOS have insulin resistance, so there is no surprise that the rate of gestational diabetes increases, furthermore it has recently been observed that patients with this syndrome which developed gestational diabetes, having an alterations in insulin sensitivity noted as early as the first trimester of the pregnancy (62).

1.1.9 Long-term Consequences

1.1.9.1 Obesity

Obesity is present in approximately (44%) of women with PCOS, ordinarily obesity worsens the clinical presentation of this syndrome by increasing insulin resistance and resulting in a further elevation of ovarian and adrenal androgens with free testosterone , as the treatment of obesity is one of the main goals for PCOS therapy, this may be more difficult because of insulin resistance and impaired lipolysis(63).

1.1.9.2 Type Two Diabetes Mellitus

Type two diabetes mellitus developed in (16%) of women with PCOS by the age of menopause and majority of these women have insulin resistance which is more severe in obese, therefore they are at risk to develop impaired glucose tolerance and type 2 diabetes, impaired glucose tolerance was found in (31%) of women of reproductive age with PCOS while diabetes in (7.5%) in non-obese PCOS(64).

1.1.9.3 Abnormal Lipid Profile and the Risk of Cardiovascular Diseases

Abnormal lipid and lipoprotein profile may be found in patients with PCOS, represented by patients having an elevation of; cholesterol, triglycerides and LDL-c. Conversely, there is a lowering with the levels of HDL-c and Apolipoprotien A-1(Apo A1), these variable findings depend on the obesity status, diet and ethnicity of the population(65).

Hyperandrogenism is likely to play a role in these abnormalities, as well as insulin resistance appears to be the most important contribution to these abnormalities, particularly the elevation in triglycerides, where these abnormalities are known to be highly predictive of cardiovascular disease, because hypertension which is prevalent in older women with PCOS and those who are obese(66).

1.1.10 Management

1.1.10.1 Types of Treatments

a) Non pharmacological

Healthy lifestyle is one of the most important aspects of managing PCOS successfully. Healthy diet will ensure getting an adequate intake of nutrients, vitamins and minerals, accordingly loss of excess weight may reduce the severity of some symptoms and will reduce the risk of developing type 2 diabetes and cardiovascular disease, where (5-10%) weight loss can have significant health benefits, including improved mood and fertility, with more regulation for menstrual cycles as well as a reduction in a risk of diabetes(67). The guidelines recommend that women with PCOS should be exercised sport at least 150 minutes of aerobic activity per week(68).

b) Pharmacological

Different medical therapies used to manage the symptoms of PCOS, including; infertility, excess hair, acne and weight gain, these therapies may include: clomiphene citrate which is the first choice for induction of ovulation, Clomifene is in the selective estrogen receptor modulator (SERM) family of medication and is a nonsteroidal medication. It works by causing the release of GnRH by the hypothalamus, and subsequently gonadotropin from the anterior pituitary,so making the body think that your estrogen levels are lower than they are, which causes the pituitary gland to increase secretion of follicle stimulating hormone, or FSH, and luteinizing hormone, or LH and it is associated with approximately (54–75%) ovulation rate and (22%) conception rate per cycle as well as (22.5%) of live births, but therapy with this drug may be associated with higher risk for thromboembolic events and multiple gestations(69).The other drugs which are recommended for treatment including oral contraceptive pills are commonly used to induce regular menses, protect the endometrium and ameliorate androgenic symptoms, insulin sensitizing drugs such as metformin, hormones (gonadotrophins) the use of gonadotropins [recombinant follicle-stimulating hormone (FSHr) or human menopausal gonadotropin (HMG)] , testosterone lowering drugs,the most commonly used anti-androgen for treating hirsutism is spironolactone (Aldactone), the results are modest and take at least six months to be noticeable, possible side effects include menstrual irregularity, weight loss drugs and anti-anxiety agents with antidepressants remedies(18).

1.1.10.2 Role of Insulin Sensitizer Drugs

There are several benefits of insulin sensitizer drugs like thiazolidinedione (pioglitazone) and biguanides, for the treatment of a complex disorder like PCOS: first of all is the reduction of compensatory hyperinsulinemia which in turn is expected to reduce androgens and increase sex hormone binding globulin (SHBG) synthesis, thereby reducing free androgen fraction availability in peripheral tissues, therefore it may ameliorate glucose intolerance states, and the onset of T2DM could be theoretically delayed in susceptible individuals, if not completely prevented. Improvement of insulin sensitivity and the reduction in insulin concentration can lead to increase fertility rates in otherwise infertile or hypofertile women. Other role, by decreasing insulin-mediated androgen excess and tissue availability of free androgens, through both peripheral and central mechanisms. Most of the related signs and symptoms could be at least partially improved as a result the metabolic syndrome and other associated risk factors for cardiovascular disease may be attenuated(70).

1.1.10.3 Metformin

1.1.10.3.1 General View

Metformin is a (dimethylbiguanide), figure (1-3), was discovered in 1957 and approved by Food and Drug Administration (FDA) in 1994. Guanidine derivatives, including metformin were synthesized and some (not metformin) were used to treat diabetes in the 1920 and 1930 but discontinued due to toxicity and increased availability of insulin. Metformin was rediscovered in the search for antimalarial agents in the 1940 and during clinical tests, proved useful to treat influenza when it sometimes lowered blood glucose, this property was pursued by the French physician Jean Sterne, who first reported the use of metformin to treat diabetes in 1957(71).

Metformin was marketed under the trade name Glucophage among others, it is a white, hygroscopic crystalline powder with a bitter taste. Chemically it is 1, 1 dimethyl-biguanide hydrochloride with a mode of action and uses similar to other biguanides. This small molecule is soluble in water and 95% alcohol while insoluble in ether or chloroform, its structure was generally represented in a wrong tautomeric form for several years, but it was corrected in 2005(72).

Metformin considered the first-line medication for the treatment of type 2 diabetes particularly in people who are overweight, which is also used in the treatment of PCOS. It is not associated with weight gain and it has a low risk to cause low blood sugar(73).

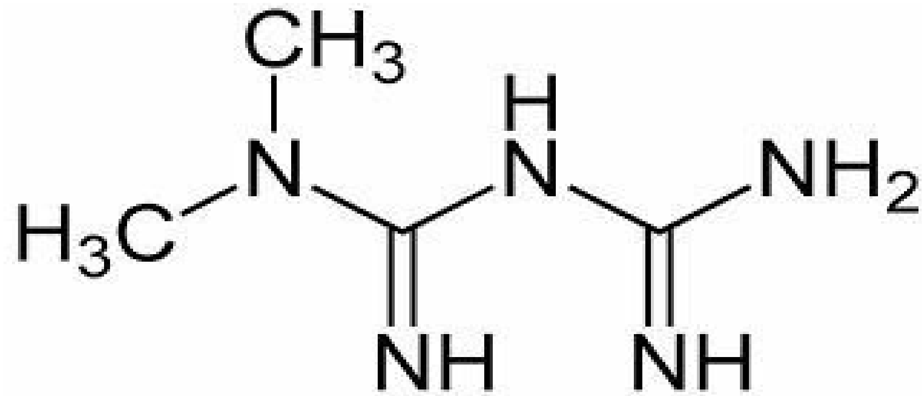


Figure (1-3). Structure of metformin (74)

1.1.10.3.2 Pharmacokinetic

Metformin has an oral bioavailability of (50–60%) under fasting conditions, which is absorbed slowly, as reflected by its very high apparent volume of distribution (650) L after a single dose , peak plasma concentration (C_{max}) is reached within one to three hours with an immediate-release formula, while extended-release formulations takes about four to eight hours. The plasma protein binding of metformin is negligible, steady state is usually reached in one or two days, metformin is not metabolized because it is cleared from the body by tubular secretion and excreted unchanged in the urine to become undetectable in blood plasma within 24 hours of a single oral dose, the average elimination half-life in plasma is 6.2 hours(75).

Metformin has acid dissociation constant values (pKa) of 12.4, so exists very largely as the hydrophilic cationic species at physiological pH value. Metformin pKa values make metformin a stronger base than most other basic medications with less than 0.01% non-ionized in blood, the lipid solubility of the non-ionized species is slight, accordingly these chemical parameters indicate low lipophilicity. Consequently, rapid passive diffusion of metformin through cell membranes is unlikely, as a result of its low lipid solubility, metformin requires the transporter SLC22A1 in order to enter cells(76).

The hepatic uptake of metformin is mediated primarily by OCT1 (*SLC22A1*) and possibly by OCT3 (*SLC22A3*). Both transporters are expressed on the basolateral membrane of hepatocytes MATE1 (*SLC47A1*) is highly expressed in the liver, kidney, and skeletal muscle , that may contribute toward the excretion of metformin from both liver and kidney(77).

1.1.10.3.3 Therapeutic Indications

Metformin, a biguanide, has been available for the treatment of type 2 diabetes mellitus, it has become the most widely prescribed antihyperglycaemic agent with a mechanism of action involves the suppression of endogenous glucose production, primarily by the liver, also the drug has an insulin sensitizing effect in peripheral tissues, such as muscle and fat. Nonetheless, because insulin levels decline with metformin use, it has been termed an insulin sensitizer(71).

Metformin has also been shown to have several beneficial effects on cardiovascular risk factors and it is the only oral antihyperglycaemic agent that associated with decreased macrovascular outcomes in patients with diabetes, cardiovascular disease, impaired glucose tolerance and PCOS are now recognized as complications of the insulin resistance syndrome. Therefore, there is growing interest in the management of this extraordinarily common metabolic disorder, while diet and exercise remain the mainstay of therapy for insulin resistance. Pharmacological intervention is becoming an increasingly viable option, according to its protective properties against diabetic complications, especially by reducing the diabetes-related death (78).

Metformin was also employed as an adjuvant therapy in cancer patients, with doses which are typical for treating diabetes. It has a strong beneficial and antiproliferative effects on breast, ovarian, prostate, lung, pancreatic, colon and breast cancers, which was confirmed in clinical trials for the latter three malignancies, in the early stage breast cancer of non-diabetic women metformin reduced fasting insulin by (22%) and improved several metabolic parameters. Anticancer metabolic effects of metformin also occurs by a direct modulation of metabolic genes and microRNAs (miRNAs)(79).

1.1.10.3.4 Mechanism of Action

1) Decrease gastrointestinal absorption with suppression of endogenous glucose production (gluconeogenesis)

Metformin works by inhibiting the production of hepatic glucose, reducing intestinal glucose absorption, and improving glucose uptake and utilization, it has a number of actions within the gut. It increases intestinal glucose uptake and lactate production, increases GLP-1 (Glucagon-like peptide-1) concentrations and the bile acid pool within the intestine, and alters the microbiome. So it has a pleiotropic action on several tissues sensitive to the primary effect of insulin or affected by insulin resistance, such as the liver, skeletal muscles, adipose tissue, endothelium and the ovaries. Primary function of metformin is to decrease hepatic glucose production mainly by inhibiting gluconeogenesis, where several mechanisms have been proposed to explain this effect; including changes in enzyme activity or a reduction in hepatic uptake of gluconeogenic substrates(80).

The mechanisms potentially involve the direct inhibition of gluconeogenic enzymes e.g. phospho-enolpyruvate carboxykinase, fructose-1,6- bisphosphatase and glucose-6-phosphatase, that reduced hepatic uptake of substrates for gluconeogenesis, and increased phosphorylation of insulin receptor substrates (IRS)-1 and -2, with the inhibition of mitochondrial respiration, moreover metformin stimulates glucose entry into the liver and glycolysis through activation of glycolytic enzymes, such as hexokinase and pyruvate kinase(81).

The activation of the AMP protein kinase (AMPK) was closely associated with the pleiotropic actions of metformin, AMPK is a phylogenetically conserved serine/threonine protein kinase which is viewed as a fuel gauge monitoring systemic and cellular energy status, which plays a crucial role in protecting cellular functions under energy-restricted conditions, nevertheless there is evidence that AMPK activation by metformin may be secondary to its effect on the mitochondria, performed through a specific inhibition of the respiratory chain complex -1(82).

2) Improve lipid metabolism.

Metformin suppresses acetyl CoA carboxylase activity, an important rate-controlling enzyme for the synthesis of malonyl-CoA, which is a critical precursor in the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation, therefore leading to decreased fatty acid synthesis and increased mitochondrial fatty acid oxidation, consequentially, metformin regulates the partitioning of fatty acids between oxidative and biosynthetic pathways(83).

The positive impact of metformin on lipid metabolism is supported by the fact that the drug has beneficial effects on fatty liver by reversing hepatic steatosis, these effects of metformin on hepatic lipid content are consistent with an increase in fatty acid oxidation and inhibition of lipogenesis, presumably mediated by AMPK activation, therefore AMPK suppresses the expression of several lipogenic genes such as fatty acid synthase (FAS), that participate in the regulation of lipogenesis by downregulating of other genes upon AMPK-mediated phosphorylation at Serine 372(84).

Metformin increases insulin-mediated glucose uptake not only in the liver, but also in the visceral fat, thereby increasing the re-esterification of free fatty acids (FFAs) will reduce lipolysis and FFA delivery to the liver, in spite of the adipose tissue is not considered a major site of metformin's action, there are several studies showing that metformin may independent of insulin action will lead to increase adipocyte glucose transport and utilization, also has appositive effect on mitochondrial and peroxisomal fatty acid β-oxidation (85).

Metformin was found to have a role in the inhibition of a tumor necrosis factor-α(TNF-α) induced lipolysis in adipocytes, which suggests that it may contribute to insulin sensitization through the decrease of systemic FFA levels, metformin may counteract adipose tissue expansion through direct inhibition of adipogenesis in the pre-adipocytes, there are data supporting the concept that metformin may selectively favor a reduction of visceral fat in the long-term, although this has been a matter of debate, in the skeletal muscles, which account for more than 80% of insulin-stimulated glucose uptake, metformin has been reported to promote a moderate increase in glucose uptake(86).

In cultures of insulin-resistant skeletal muscle cells, metformin has also been found to restore the reduction in insulin receptor substrate-1(IRS-1) phosphorylation and phosphatidyl inositol 3 kinase activity. In human chronic treatment with metformin reduces lipid accumulation in human skeletal muscle tissue, by these effects on lipid metabolism(87).

Metformin also has a direct effect at the ovarian level, its beneficial effects are partly based on the alleviation of excess insulin, as insulin was shown to directly stimulate several steroidogenic enzymes in the ovary, such as CYP (cytochrome P450)17, 3β-hydroxysteroid dehydrogenase (3β-HSD) and the steroidogenic acute regulatory protein (StAR). In this matter the gonadotropic action of insulin synergizes lutein hormone (LH). By improving insulin sensitivity, metformin reduces CYP17 activity (88).

Metformin treatment increased AMPK activity in granulosa cells, leading to subsequent reduction of steroid synthesis. It has been shown to reduce the risks of abortion in women with PCOS at high risk of pregnancy and neonatal complications by increasing some factors needed for implantation and pregnancy safekeeping, such as insulin-growth factor-binding protein-1 and glycodelin levels or uterine artery blood flow. By contrast, metformin reduces factors increasing the risk of abortion, such as endometrial androgen receptor expression, plasminogen-activator inhibitor-1(PAI-1) levels and plasma endothelin-1 (ET-1). Most of these effects are probably mediated by the metformin-induced improvement in insulin sensitivity(89).

1.1.10.3.5 Side Effects

The main side effects associated with metformin treatment are the gastrointestinal symptoms as nausea, diarrhea, flatulence, bloating, anorexia, metallic taste and abdominal pain, high blood lactic acid level is a concern if the medication is prescribed inappropriately or in large doses, these symptoms occur with variable degrees in patients and in most cases resolve spontaneously(90).

The severity of side effects can be reduced by gradual administration of metformin and titrating the dose increase guided by the severity of symptoms, starting dose of 500 mg daily during the main meal of the day for (1–2) weeks can lessen the side effects and allow tolerance to develop, weekly or biweekly increase by 500 mg a day can then be pursued as required until a maximum dose of (2500–2550) mg/day is reached depending on the clinical benefit and side effects, if the dose increase results in worsening of the side effects, the current dose can be maintained for (2–4) weeks until tolerance is developed (91).

1.1.10.3.6 Contraindication

Metformin is contraindicated in people with severe renal impairment with estimated glomerular filtration rate (eGFR) below (30ml/min/1.73m2), also those with known hypersensitivity to metformin and acute or chronic metabolic acidosis, including diabetic ketoacidosis with or without coma(71).

Metformin has a very low risk of birth defects and complications for the baby, making this drug safe to take before and during pregnancy. Metformin is also safe to take during breastfeeding(92). While with COVID-19, the observational evidence grows that prior metformin use may reduce mortality in hospitalized patients with COVID-19, researchers have continued to stress that randomized trials are needed(93).

1.1.10.3.7 Role of Metformin in Polycystic Ovary

Metformin cause a reduction of serum androgens in women with PCOS and inhibits hepatic gluconeogenesis will lead to increase the glucose uptake by peripheral tissues and reduces fatty acid oxidation. Furthermore, it was the first insulin sensitizing drug (ISD) to be used in this syndrome to investigate the role of insulin resistance in the pathogenesis of PCOS, metformin has a positive effect on the endothelium and adipose tissue independent of its action on insulin and glucose level (94).

High triglycerides and low level of (HDL-C) are the most prominent abnormalities that represent strong predictors of cardiovascular disease (CVD) and myocardial infarction, metformin can theoretically influence dyslipidemia, either directly through its action on fatty acid metabolism in the liver or indirectly by improving hyperinsulinemia(95).

Metformin does not replace the need for lifestyle modification among obese and overweight PCOS women, as the evidence categorically does not encourage its use to help weight loss, although it may be useful in redistributing adiposity according to some evidence, it takes a longer time to help with ovulation induction hence it fared worse than clomiphene citrate, metformin supplemented with lifestyle changes may prove superior, its benefit in invitro fertilization (IVF) patients is only confirmed with regard to reduction of the incidence of ovary hyper-stimulation syndrome(OHSS) which is important given its high risk among PCOS patients, it has a role in reducing the risk of miscarriage and the available evidence regarding gestational diabetes mellitus (GDM) prevention is encouraging(96).

1.2 Serine Threonine Kinase 11

1.2.1 Function

Serine/threonine kinase 11 (STK11) also known as liver kinase B1 (LKB1) or renal carcinoma antigen NY-REN-19, which is a protein kinase that in humans is encoded by the *STK11* gene, the *LKB1/ STK11*gene which encodes a member of the serine/threonine kinase family, regulates cell polarity and functions as a tumor suppressor(97).

LKB1 is a primary upstream kinase of adenosine monophosphate-activated protein kinase (AMPK) in the liver, glucose uptake and fatty acid oxidation in muscle, that regulate energy balance in the hypothalamus. (STK11)– (AMPK) signaling pathway is a master regulator of glucose and lipid metabolism with pleiotropic actions in liver, skeletal muscle, pancreas and brain. These functions of STK11 are thought to be achieved by direct phosphorylation of the AMPK family of proteins(98).

The STK11-AMPK signaling pathway is one of the key regulators of insulin sensitivity and glucose homeostasis, playing critical roles in metabolic processes such as fatty acid synthesis and gluconeogenesis not only via an allosteric mechanism by cyclic AMP, but also through phosphorylation of a key threonine residue (Thr172) on the a-catalytic subunit, the latter is catalyzed by STK11 and metformin is frequently used in research as an AMPK agonist then activate a necessary element in cell metabolism that is required for maintaining energy homeostasis(99).

LKB1 exerts its growth suppressing effects by activating a group of 14 other kinases comprising AMPK and AMPK-related kinases, consequently the activation of AMPK by LKB1 suppresses growth and proliferation when energy and nutrient levels are scarce. Activation of AMPK-related kinases by LKB1 plays vital roles maintaining cell polarity thereby inhibiting inappropriate expansion of tumor cells, new researches are emerging that loss of LKB1 leads to disorganization of cell polarity and facilitates tumor growth under energetically unfavorable condition, accordingly mutations in this gene have been associated with Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by the growth of polyps in the gastrointestinal tract, pigmented macules on the skin and mouth and other neoplasms(100).

The LKB1 gene was also found to be mutated in lung cancer of sporadic origin, predominantly adenocarcinomas. Further, new researches have uncovered a large number of somatic mutations of the LKB1 gene that are present in cervical, breast, intestinal, testicular, pancreatic and skin cancer(101). LKB1 is activated allosterically by binding to the pseudokinase STRAD and the adaptor protein MO25. The LKB1-STRAD-MO25 heterotrimeric complex represents the biologically active unit that is capable of phosphorylating and activating AMPK and at least 12 other kinases that belong to the AMPK-related kinase family(102), figure (1-4) showed the location of STK11 gene on chromosome 19.

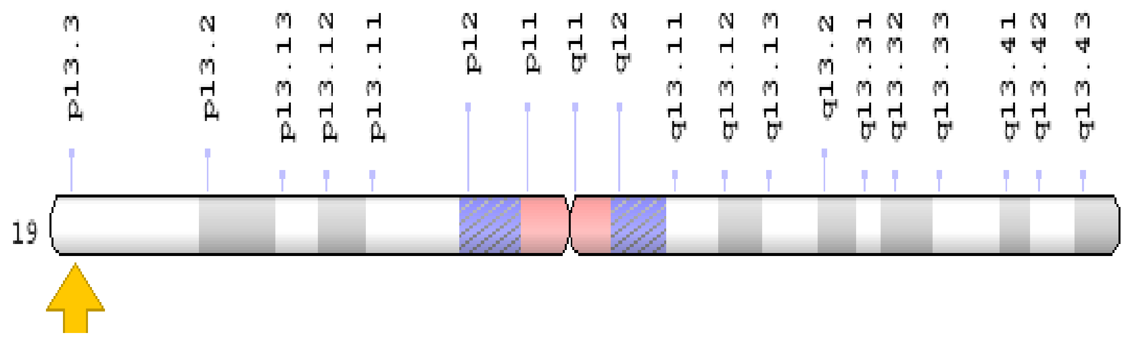


Figure (1-4). Location of STK11 gene on chromosome 19(103)

1.2.2 Role of Serine Threonine Kinase11 in Insulin Sensitization

STK11-AMPK signaling pathway is one of the key regulators of insulin sensitivity and glucose homeostasis, it was playing critical roles in metabolic processes such as fatty acid synthesis, gluconeogenesis in liver, glucose uptake, fatty acid oxidation in muscle and regulation of energy balance in the hypothalamus (104).

AMPK is known to be activated not only via an allosteric mechanism by cyclic AMP, but also through phosphorylation of a key threonine residue (Thr172) on the catalytic subunit. STK11 knockout in mice liver was result in increased gluconeogenesis (105), while it deletion in pancreatic Beta cells (β cells) caused an increase in β cells mass and higher insulin secretion in response to glucose, also STK11 deletion in white adipose tissue reduced the levels of insulin receptor 1 (106).

STK11 has been implicated in the control of cell polarity and energy metabolism in adult pancreatic β cells (107). Different gene variants in STK11 being associated with insulin resistance like (C/G) RS8111699, (C/T) rs741765 and (T/C) rs1172545 were reported in study of single nucleotide polymorphisms (SNPs) and associated with response to drugs used in insulin sensitization (108).

1.2.3 Molecular Action of Metformin

Potential molecular mechanisms of metformin action. Metformin activates AMP-activated protein kinase (AMPK) by increasing its phosphorylation state and serine-threonine kinase 11(LKB1/STK11) seems to be implicated in this process. Increased AMPK activity is associated with the translocation of glucose transporter (GLUT4) to the membrane and with the stimulation of glucose uptake. Further, AMPK inactivates Acetyl-CoA carboxylase (ACC), decreasing fatty acid synthesis.

Activation of AMPK by metformin also reduces the expression of sterol response element binding protein-1 (SREBP-1), a transcription factor that induces the expression of lipogenic genes, favoring the inhibition of fatty acid synthesis. Additionally, the activation of AMPK also promotes the inhibition of target of rapamycin (TOR) pathway, and, consequently, suppresses gluconeogenesis and protein synthesis(109),figure(1-5).

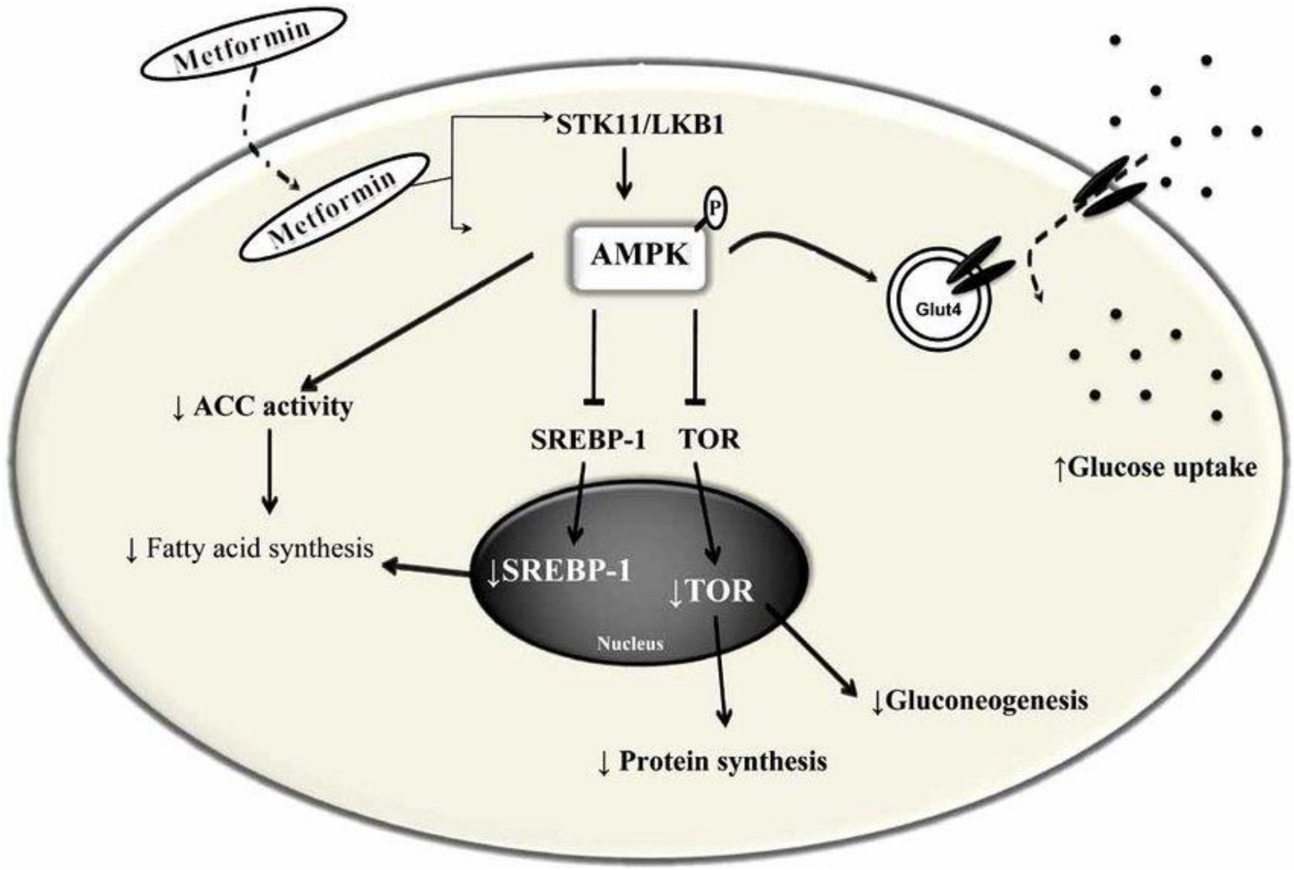


Figure (1-5) Molecular mechanism of metformin

1.2.4 Stk11 Gene Polymorphism

A gene is said to be polymorphic if more than one allele occupies that gene's locus within a population. In addition to having more than one allele at a specific locus, each allele must also occur in the population at a rate of at least 1% to generally be considered polymorphic. Gene polymorphisms can occur in any region of the genome. The majority of polymorphisms are silent, meaning they do not alter the function or expression of a gene.A polymorphic variant of a gene can lead to the abnormal expression or to the production of an abnormal form of the protein; this abnormality may cause or be associated with disease (110).

Polycystic ovary syndrome, a common, complex, genetic disorder, is the most common endocrinopathy affecting women. Ovulatory response to treatment of polycystic ovary syndrome is associated with a polymorphism in the STK11 gene and STK11 genotyping appears to be able to identify subgroups of women with PCOS with low, intermediate and high likelihood of response to metformin(111). The C allele of a single nucleotide polymorphism in the STK11 rs8111699 gene is associated with a significantly decreased chance of ovulation in PCOS women treated with metformin(112)

1.3 Aim of the Study

To investigate the relation of a single nucleotide polymorphisms (C/T) rs741765 and (C/G) rs8111699 of STK11 gene with response to metformin treatment in polycystic ovary patients in the population of Kerbala province.

Chapter TWO

Materiales, Patients & Method

2.1 Materials

The specific chemicals, kits and instruments used in this study with their suppliers and manufacture origin are listed in tables (2-1) and (2-3). The primers which used in the amplification analysis of STK11 gene for both SNP were; Forward (F) and Reveres (R1, R2). The position of rs741765 (C\T) SNP on STK11 gene was illustrated in figure (2-1) and for rs8111699 (C\G) in figure (2-2).

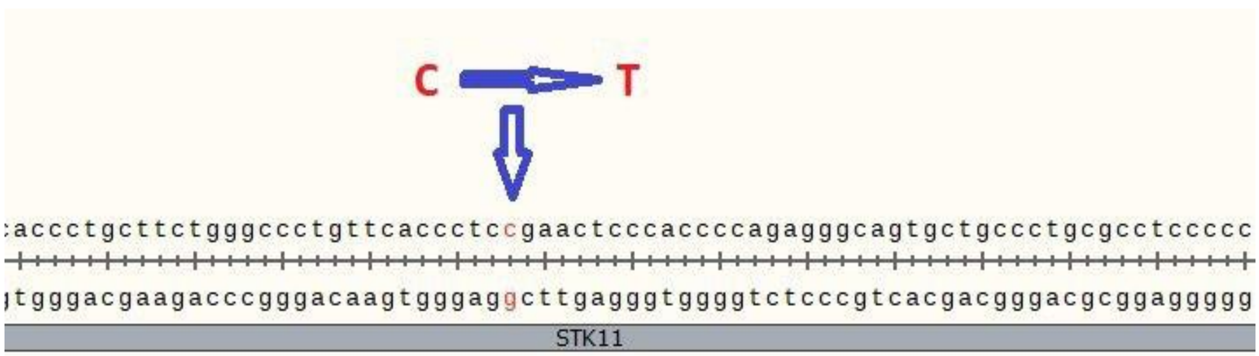


Figure (2-1). **Position of SNP rs741765 (C\T) on gene sequence of STK11(113)**

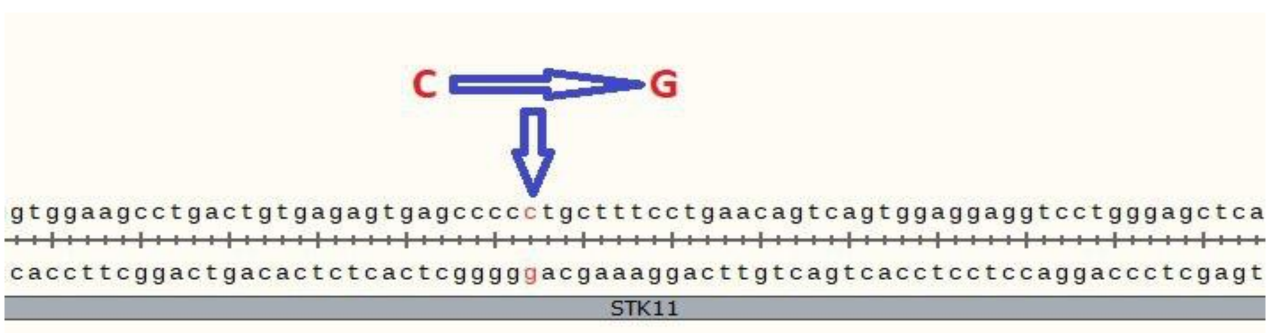


Figure (2-2). **Position of SNP rs8111699 (C\G) on gene sequence of STK11** (114)

2.1.1 Kits and Chemicals

Kits and chemicals used in study were shown in table (2-1).

Table (2-1). Kits and chemicals

n.	Chemicals	Company/Country
1	Agarose	BDH/ England
2	DNA ladder (100bp)	Bioneer/ KOREA
3	Elecsys Estradiol III Kit	ROCHE/Germany
4	Elecsys insulin Kit	ROCHE/Germany
5	Elecsys Progesterone III Kit	ROCHE/Germany
6	Elecsys Prolactin II Kit	ROCHE/Germany
7	Elecsys reagent kit FSH Kit	ROCHE/Germany
8	Elecsys reagent kit LH Kit	ROCHE/Germany
9	Elecsys reagent kit TSH Kit	ROCHE/Germany
10	Elecsys Testosterone II Kit	ROCHE/Germany
11	Ethanol 95%	Systerm/ Malaysia
12	Ethidium Bromide	Sigma / USA
13	G-DEX tmIIb Genomic DNA Extraction Kit	Bioneer/ KOREA
14	Glucose kit	BAIOLABO / France
15	Hemoglobin(HbA1c)Kit	Stan bio lab. / USA
16	Isopropanol	BDH / England
17	Kit of HDL-Cholestrol	Randox (US)
18	Metformin tablet (500mg)	Merck
19	Nuclease Free Water	Promega/USA
20	PCR Premix	Bonier/ KOREA
21	Primers	Bonier/ KOREA
22	SHBG ELISA Assay Kit	DiaMetra/Italy
23	TBE (Tries Borate EDTA) buffer solution	Bio basic/ Korea
24	Total cholesterol MR kit	Linear / Spain
24	Triglyceride kit	BAIOLABO / France

2.1.2 Equipments

Equipments and apparatus used in this study are shown in table (2-2).

Table (2-2). Equipments and Apparatus

n.	Apparatus & Equipments	Company/Country
1	Autoclave	LabTek/ Korea
2	Balance	Precis / Switzerland
3	Clean View – UV Cabinet	Cleaver / USA
4	Cobas e 411 analyzer	ROCHE/Germany
5	Digital camera	Canon/ England
6	Electrophoresis apparatus	Consort / Belgium
7	Electrophoresis power supply	Consort / Belgium
8	Electrophoresis tank	Consort / Belgium
9	High speed Centrifuge	Mikro 200R Hettich / Germany
10	Hot Plate Magnetic Stirrer	LabTech DAIHAN / Korea
11	Micro-Centrifuge	BIONEER / Korea
12	Micropipettes	Eppendorf / Germany
13	Nano drop UV- Spectrophotometer	Quawell Q5000 / USA
14	Sensitive balance	AND/ Taiwan
15	Shaking Water bath	Grant / England
16	Thermo cycler PCR instrument	Cleaver / USA
17	UV-VIS Spectrophotometer	UV-VIS /JAPAN
18	Vortex – mixture	(Karlkole) / Germany

2.2 Study Design

This is an interventional prospective study carried out at Gynecology and Obstetrics Teaching Hospital in Kerbala province, during the period from July, 2019 till the end of April, 2020. The protocol for the study was approved by the Ethical Committee of College of Pharmacy in Kerbala and verbal consent was given by each patient after explaining the nature and purpose of study. The study was conducted on two hundred thirty- five Iraqi women with newly diagnosed polycystic ovary syndrome. All women agreed to have 500 mg of metformin twice daily in the open-label follow period of the study. Patients were relied on self-report for medication compliance and the patients reporting consistent use at the regular study visits.

The participated women were recruited by consultation of an infertility according to the inclusion and exclusion criteria of the study.

2.2.1 Patients selection

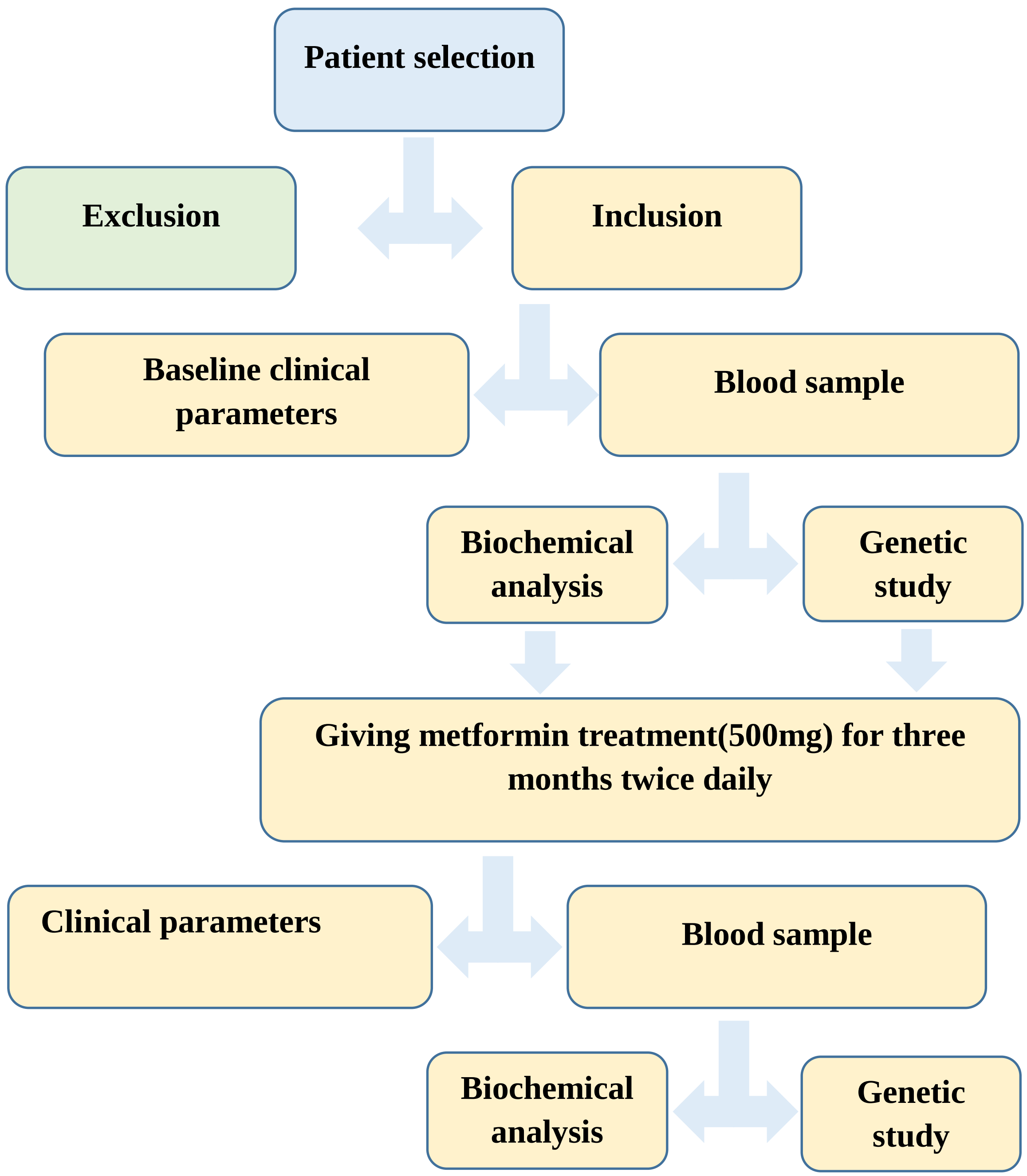
Inclusion criteria included all women enrolled in this study should be newly diagnosed with PCOS. After a selection of patients, metformin tablets (500mg) was given twice daily for three months, this criterion was carried out according to the Rotterdam criteria when two of three of the following symptoms: hyperandrogenism, irregular an ovulatory periods or ultrasound polycystic ovary (PCOS) morphology. The age of the women should be within (18-40) years old.

Exclusion criteria included contraindications to metformin treatment, also those using hypoglycemic drugs, oral contraceptive within the previous 2 months and women taking medication known to affect weight loss.

Patients with functional defect of gonadal and adrenal glands with metabolic disturbance of carbohydrate and lipid were also excluded. Furthermore, women had thyroid dysfunction, diabetes mellitus, Cushing's syndrome and congenital adrenal hyperplasia, all those should be excluded in current study before making the diagnosis of PCOS.

2.2.2 Study Protocol

Protocol of the study was done as the following scheme;



2.3 Method

2.3.1 Clinical Data Collection

At the time when the blood samples were obtained, each patient was questioned about demographic parameters by history-taking, specifically for; address, age, body mass index, blood pressure, presence of other diseases, smoking, menstrual regularity, hirsutism, alopecia, marital status, number of children and times of abortion.

Clinical manifestations were determined by consultation of an infertility. Furthermore, other data were extracted from the medical records of consenting patients are data of diagnosis or by gynecologic ultrasonography , specifically looking for small ovarian follicles. These are believed to be the result of disturbed ovarian function with failed ovulation, reflected by the infrequent or absent menstruation that is typical of the condition.

Patients were divided according to regularity as being: oligomenorrhoeic about (a cycle interval of longer than 35 days but less than six months), amenorrhoeic (less than two menstrual cycles in the year) or hypomenorrhoeic (bleeding lasting less than 1 day).

Hirsutism and alopecia are major clinical feature of hyperandrogenism in PCOS patients, where hirsutism (thicker hair on face and body in face, chest and back) based on the amount and location of hair growth on a woman. While alopecia (hair thinning with loss), the examination of hirsutism and alopecia was done by the physical examination.

Body mass index (BMI) is a metric currently using for defining anthropometric height/weight characteristics in adults and for classifying (categorizing) them into groups. Body mass index (BMI) was calculated by dividing the weight measured in kg by height per square meter(115).

BMI (kg/m2) = Body weight (kg) / Height (m^2)

For comparison between the patients, BMI was also categorized into four groups according to the conventional WHO classification: underweight (<18.5 kg/m2), normal weight (18.5–24.9 kg/m2), overweight (25–29.9 kg/m2), and obese (≥30 kg/m2).

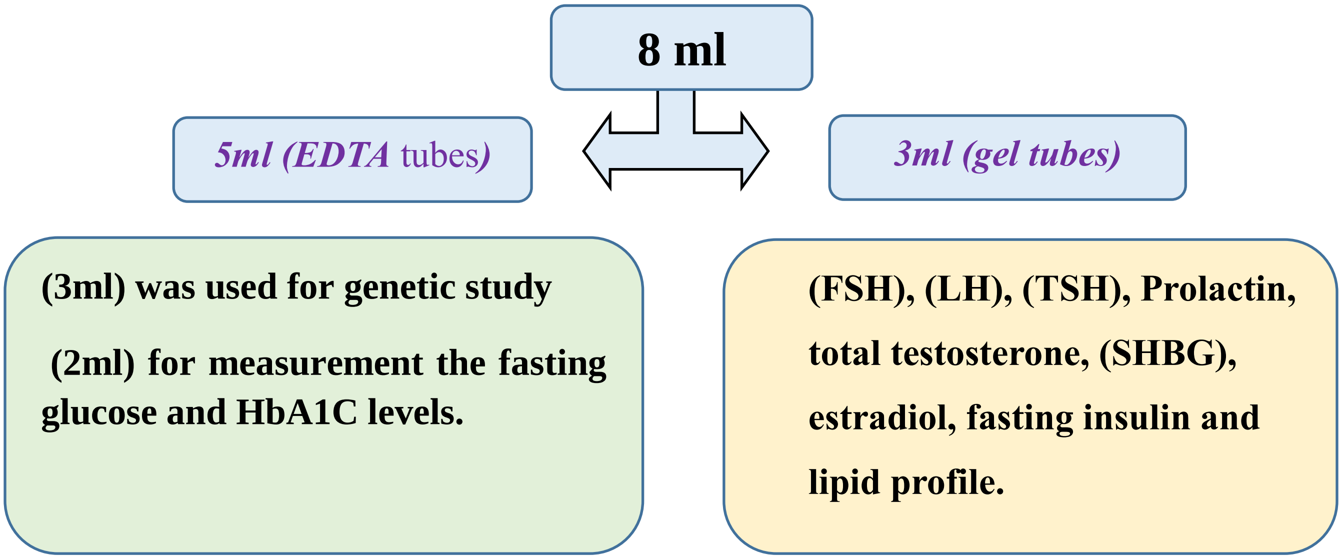
Patients were divided according to BMI into two groups overweight\obese patients with BMI ≥ 25 kg/m2 and non-overweight \ obese patients BMI < 25 kg/m2.

2.3.2 Collection and Preparation of the Blood Samples

After approval by the Ethical Committee of College of Pharmacy in Kerbala province, blood samples were obtained from all patients. Venous blood about (8ml) was withdrawn from each patient who participated in this study.

From withdrawn blood sample of each patient when the medical checkup was done for them in early follicular phase of the menstrual cycle, (5ml) was placed in EDTA-tube from which (3ml) was used for genetic study and (2ml) for the measurement of fasting glucose and HbA1C levels, remaining (3ml) from withdrawn blood sample was collected in gel tubes (EDTA-free) and used for serum separation after centrifugation of blood at 3000 rpm for 10 minutes to measure the following markers: follicular stimulating hormone (FSH), (LH), (TSH), Prolactin, total testosterone, (SHBG), estradiol, fasting insulin, and lipid profile including; triglycerides (TG), (LDL-C), (HDL-C) and total cholesterol (TC), as shown in figure(2-3). Medical checkup was done for the patients after the end of therapy period

(3-months), this checkup was identical to that before metformin administration,as shown in the following scheme.



2.3.3 Biochemical Analysis

2.3.3.1 Serum Lipid Profile Assessment

2.3.3.1.1 Measurement of Serum Total Cholesterol Concentration

Serum total cholesterol (TC) is determined by utilizing a readymade laboratory kit for this purpose. Principle of determination is based on the enzymatic hydrolysis according to the following reaction(116):

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

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Choles-2-ene-3-one} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-amino-antipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinonimine} + 2H_2O$$

The quantity of the former red dye quinonimine is proportional to the cholesterol concentration. The absorbance of quinonimine was read at 500 nm spectrophotometrically.

Reference range: (150-220mg/dl)

Reagents

Table (2-3). Reagents used for total cholesterol assay

Reagents	Composition	Concentration
Reagents 1 (Buffer)	**Phosphate buffer**	**100 mmol/L**
	Chloro-4-phenol	**5.0 mmol/L**
	Sodium chloride	**2.3 mmol/L**
	Triton x100	**1.5 mmol/L**
Reagents 1 (Enzymes)	**Cholesterol oxidase**	**100 IU/L**
	Cholesterol esterase	**170 IU/L**
	Peroxidase	**1200 IU/L**
	PAP	**0.25 mmol/L**
	PEG 6000	**167 µmol/L**
Reagents 1 (Standard)	**Cholesterol 200mg/Dl**	**5.17 mmol/L**

Procedure

The content of vial reagent 2 (enzymes) was added to vial reagent 1 (buffer), mix gently until complete dissolution (approximately 2 minutes) to prepare work reagent. The procedure was carries out as in the following:

Table (2-4). Procedure of total cholesterol assessment

Reagents	Blank	Standard	Sample
Reagent	1.0 Ml	1.0 Ml	1.0 Ml
Demineralized Water	10 µL	-	-
Standard	-	10 µL	-
Sample	-	-	10 µL

The tubes were mixed, and then let stands for 5 minutes at 37 ˚C. Record absorbance at 500 nm (480-520) against blank. The color was stable for 1 hour.

Calculation

$$cholesterol\left({}^{mg}/_{dl}\right) = sample\,\frac{absorbance(at500nm)}{standard\ absorbance} \times 200$$

2. 3.3.1.2 Measurement of Serum Triglyceride Concentration

Triglycerides are enzymatically hydrolyzed to glycerol and fatty acids according to following equations(117)

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol + free fatty acids}$$

$$\text{Glycerol + ATP} \xrightleftharpoons{\text{Glycerol Kinase}} \text{Glycerol-3-phosphate + ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightleftharpoons{\text{Glycerol-3- phosphate Oxidase}} \text{Dihydroxyaceto-phosphate} + H_2O_2$$

$$H_2O_2 + \text{ParachloroPhenol} + \text{4-aminoantipyrine} \rightarrow \text{Quinonimine} + 2H_2O + HCL$$

Absorbance was read at 500 nm spectrophotometric ally.

Reference range is: (60-160 mg/dl).

Reagents

Table (2-5): Reagents used for triglycerides assay

Reagents	Composition	Concentration
Reagents 1 (Buffer)	PIPES magnesium	100 mmol/L
	Chloride	9.8 mmol/L
	Chloro-4-phenol Preservative	3.5 mmol/L
(Enzymes)	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 mmol/L
Reagents 1 (Standard)	Glycerol equivalent to triglycerides	2.28 mmol/L

Reagents preparation

The content of reagent 2 (enzymes) was added promptly into reagent 1 (buffer) and mixed gently until complete dissolution (approximately 2 minutes).

Procedure

Three sets of tubes used include blank, standard and sample. The volumes added to each tube are as follows:

Table (2-6). Procedure of triglycerides assessment

Reagents	Blank	Standard	Sample
Reagent	1.0 Ml	1.0 Ml	1.0 Ml
Demineralized Water	10 µL	-	-
Standard	-	10 µL	-
Sample	-	-	10 µL

The tubes were mixed, and then let stands for 5 minutes at 37 ˚C. or 10 minutes at room temperature. record absorbance at 500 nm (480-520) against blank. The color was stable for 1 hour.

Calculation

$$TG\ concentration\left({}^{mg}/_{dl}\right) = sample\,\frac{absorbance(at500nm)}{standard\ absorbance} \times 200$$

2. 3.3.1.3 Measurement of High Density Lipoprotein Cholesterol Concentration

Low density lipoprotein- cholesterol (LDL-C), very low density lipoprotein (VLDL-C), and chylomicrons fraction are precipitated quantitatively by the addition of phosphotungstic acid which contains magnesium chloride at pH 6.2. After centrifugation, the supernatant contains cholesterol concentration in the HDL fraction which is determined by using cholesterol kit(118).

Reagents

Table (2-7). Reagents used for high density lipoprotein- cholesterol assay

Reagents	Composition	Concentration
Reagents 1	**Phosphotungstic acid (PTA)**	**13.9 mmol/L**
	Magnesium chloride	**570 mmol/L**
Reagents 1	**HDL-cholesterol**	**2.58 mmol/L**

Reference range is: (more than 40 mg/dl) for male & female.

Procedure

Three sets of tubes used include blank, standard and sample. The volumes added to each tube are as follows:

Table (2-8). Procedure of high density lipoprotein- cholesterol assessment

Reagents	Blank	Standard	Sample
Reagent	**1 Ml**	**1 Ml**	**1 Ml**
Demineralized Water	**25 µL**	**-**	**-**
Standard	**-**	**25 µL**	**-**
Sample	**-**	**-**	**25 µL**

The tubes were mixed, and then let stands for 5 minutes at 37 Cº record absorbance at 500 nm against blank.

Calculation

$$HDL - C\ \left({}^{mg}/_{dl}\right) = sample\,\frac{absorbance(at500nm)}{standard\ absorbance} \times St.conc \times 1.$$

2.3.3.1.4 Determination of Low Density Lipoprotein Cholesterol Concentration

Low density lipoprotein -cholesterol (LDL–C) can be measured mathematically from the total cholesterol, triglycerides and the HDL-C concentration using Friedwald's formula(119).

This formula is applicable to TG conc. <5mmole/l (400 mg/dl).

LDL-C, mmol / l = Total cholesterol – (TG / 2.2) – HDL-C

LDL-C, mg / dl = Total cholesterol – (TG / 5) – HDL-C

When TG less than 5 mmol/l (400mg/dl)

Reference range is: (less than 180 mg/dl) or (3.37- 4.12 mmol/l) for male & female

2. 3.3.2 Determination of Glycosylated Hemoglobin Concentration

Glycosylated hemoglobin(HbA1c) concentration was calculated in the method presented, a preparation of hemolysis whole blood is mixed with a weakly binding cation-exchange resin. The non-glycosylated hemoglobin (HbA) binds to the resin, leaving free to be removed by means of the resin separator in the supernate. The present of HbA1 is determined by measuring the absorbance values at 415 nm of the HbA1fraction and the total Hb fraction, calculation the ratio of absorbance (R) and comparing this ratio to that of a glycohemoglobin standard carried through the same procedure (118).

Reagents

- Glycohemoglobin ion – exchange resin.

- Glycohemoglobin lysing reagent.

- Glycohem oglobin standard (lyophilized)

Procedure

- Hemolysate preparation

1- A volume of 0.5 ml of lysing reagent was added into appropriately labeled tube.
2- A volume of 0.1 ml of blood sample was added into above mentioned tube and mixed well then left for 5 minutes.

- Glycohemoglobin separation

1- A volume 0.1ml was pipetted on the prepare hemolysate into appropriately labeled resin tube.
2- The resin position was separated in the tubes approx. 1cm above ion exchange resin.
3- The tubes were mixed for 5 minutes on a hematology mixer.
4- After the incubation, the resin separation has to be into the tube until the ion exchange resin is being firmly packed in bottom of 13 mm tube.
5- The supernatant was added to be poured into a cuvette. The absorbance was read at 415 nm against water (=△A HbA1)

- Total Hemoglobin

1- A volume 5ml distilled water was dispensed to each tube.
2- A volume 0.02ml was pipetted of hemolysated to each tube, and mixed well.
3- The absorbance was read at 415 nm against distilled water.

Calculation

For each standard and unknown, the ratio (R) of the Glycohemoglobin absorbance to the hemoglobin absorbance calculated as follows:

$$R = \frac{A\text{- Glycosylated}}{A\text{-Total}}$$

$$\text{Glycohemoglobin} = \frac{R(Test)}{R(standard)} x7.6$$

2.3.3.3 Determination of Fasting Serum Glucose Concentration

Glucose is oxidized by glucose oxidase to gluconate and hydrogen peroxide according to the following equation(120).

Reagents

Table (2-9). Reagents used for fasting serum glucose assay

Reagents	Composition	Concentration
Reagent 1 Buffer solution	Tris buffer PH 7	100 mmol/L
	Phenol	0.3 mmol/L
Reagent 2 Enzymes	Glucose oxidase	10000 U/L
	Peroxidase	1000 U/L
	4-Amino-antipyrine	6.2 mmol/L
Reagent 3Standard	Standard glucose	100 mg/Dl 5.56 mmol/L

Procedure

Table (2-10). procedure of fasting serum glucose assessment

Reagent	Blank	Standard	Sample
Working Solution	1.0 Ml	1.0 Ml	1.0 Ml
Standard	-	10 μL	-
Sample or Unknown	-	-	10 μL

Tubes were mixed and incubated for 10 minutes at 37 ˚C. Or 30 minutes at 25 ˚C. Then they were measured at wavelength of (505 nm) at room temperature.

Calculation

$$\text{Glucose concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{Conc. of standard glucose}$$

Concentration of standard glucose solution = 100mg/dl

2.3.3.4 Determination of Follicular Stimulating Hormone Concentration

The electrochemiluminescence immunoassay “ECLIA” is intended for use on Elecsys and cobas e immunoassay analyzers(121).

Test 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 a ruthenium complexa 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 2point calibration and a master curve provided via the reagent barcode or e-barcode(122).

Reagents - Working Solutions

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

R1 Anti-FSH-Ab~biotin (gray cap), 1 bottle, 10 mL: Biotinylated monoclonal anti FSH antibody (mouse) 0.5 mg/L, MES buffer 50 mmol/L, pH 6.0; preservative.

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, pH 6.0; preservative.

Reagent handling

The reagents in the kit have been assembled into a ready for use unit that cannot be separated. All information required for correct operation is read in from the respective reagent barcodes.

Storage and stability

Store at 2-8 °C. Do not freeze.

Store the Elecsys reagent kit upright in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in mIU/mL or in IU/L)

Measuring range

The range of (0.100-200) mIU/mL (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 0.100 mIU/mL. Values above the measuring range are reported as > 200 mIU/mL.

2.3.3.5 Determination of Luteinizing Hormone Concentration

The electrochemiluminescence immunoassay "ECLIA" is intended for use on Elecsys and cobas e immunoassay analyzers(123).

Test 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 a ruthenium 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 2point calibration and a master curve provided via the reagent barcode or e-barcode.

Reagents - Working Solutions

The reagent rackpack is labeled as LH. M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL; preservative. 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, pH 8.0; preservative. 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, pH 8.0; preservative.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in mIU/mL or IU/L).

Measuring range

0.100-200 mIU/mL (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 0.100 mIU/mL. Values above the measuring range are reported as > 200 mIU/mL.

2.3.3.6 Determination of Thyroid Stimulating Hormone Concentration

Intended use Immunoassay for the in vitro quantitative determination of thyrotropin in human serum and plasma. The electrochemiluminescence immunoassay "ECLIA" is intended for use on Elecsys and cobas e immunoassay analyzers(124).

Test 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 a ruthenium 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 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 instrumentspecifically generated by 2point calibration and a master curve provided via the reagent barcode or e-barcode(122).

Reagents - Working Solutions

The reagent rackpack is labeled as TSH.

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

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, pH 7.2; preservative.

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, pH 7.2; preservative.

Expected values

Expected value about (0.270-4.20) µIU/Ml.

2 .3.3.7 Determination of Prolactin Concentration

Intended use Immunoassay for the in vitro quantitative determination of prolactin in human serum and plasma. The electrochemiluminescence immunoassay “ECLIA” is intended for use on Elecsys and cobas immunoassay analyzers(125). When determining prolactin, it should be remembered that the measured concentration is dependent upon when the blood sample was taken, since the secretion of prolactin occurs in episodes and is also subject to a 24 hour.

The release of prolactin is inhibited by dopamine, L-dopa and ergotamine derivatives. A number of publications report the presence of macroprolactin in the serum of female patients with various endocrinological diseases or during pregnancy. Differing degrees of detection of the serum macroprolactins relative to monomeric prolactin (22-23 kDa) by various immunoassays have also been described. This could lead to a false diagnosis of hyperprolactinemia depending on the immunoassay used. In case of implausible high prolactin values a precipitation by polyethylene glycol (PEG) is recommended in order to estimate the amount of the biological active monomeric prolactin. For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings(126).

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: 10 µL of sample and a biotinylated monoclonal prolactin specific 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 2point calibration and a master curve provided via the reagent barcode or e-barcode(127).

Reagents - Working Solutions

The reagent rackpack is labeled as PRL II.

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

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, pH 7.0; preservative

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, pH 7.0; preservative.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in µIU/mL, ng/mL or in mIU/L). Conversion factors: µIU/mL (mIU/L) x 0.047 = ng/mL ng/mL x 21.2 = µIU/mL (mIU/L).

Measuring range

The range of (1.00-10000) µIU/mL or (0.0470-470) ng/mL (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 1 µIU/mL or < 0.0470 ng/mL. Values above the measuring range are reported as > 10000 µIU/mL or > 470 ng/mL (or up to 100000 µIU/mL or 4700 ng/mL for 10-fold diluted samples) (128).

2 .3.3.8 Determination of Testosterone Concentration

Intended use Immunoassay for the in vitro quantitative determination of testosterone in human serum and plasma. The electrochemiluminescence immunoassay “ECLIA” is intended for use on Elecsys and cobas e immunoassay analyzers(129).

The Elecsys Testosterone II assay is based on a competitive test principle using a high affinity monoclonal antibody (sheep) specifically directed against testosterone. Endogenous testosterone released from the sample by 2-bromoestradiol competes with the added testosterone derivative labeled with a ruthenium complexa for the binding sites on the biotinylated antibody.

Test principle

Competition principle and 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 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(122).

Reagents - working solutions

The reagent rackpack is labeled as TESTO II.

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

R1 Anti-testosterone-Ab~biotin (gray cap), 1 bottle, 10 mL: Biotinylated monoclonal anti-testosterone antibody (sheep) 40 ng/mL; releasing reagent 2-bromoestradiol; MES buffer 50 mmol/L, pH 6.0; preservative.

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, pH 6.0; preservative.

Measuring range

The ranges of (0.025-15.0) ng/mL or (0.087-52.0) nmol/L (defined by the Limit of Detection and the maximum of the master curve). Values below the Limit of Detection are reported as < 0.025 ng/mL or < 0.087 nmol/L. Values above the measuring range are reported as > 15.0 ng/mL or > 52.0 nmol/L.

2. 3.3.9 Determination of Estradiol Concentration

Intended use Immunoassay for the in vitro quantitative determination of estradiol in human serum and plasma. The electrochemiluminescence immunoassay “ECLIA” is intended for use on Elecsys and cobas e immunoassay analyzers

The Elecsys Estradiol III assay employs a competitive test principle using two monoclonal antibodies specifically directed against 17β-estradiol. Endogenous estradiol released from the sample by mesterolone competes with the added estradiol derivative labeled with a ruthenium complexa for the binding sites on the biotinylated antibody(130).

Test principle

Competition principle with total duration of assay: 18 minutes.

- 1st incubation: By incubating the sample (25 μL) with two estradiol-specific 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 instrumentspecifically generated by 2point calibration and a master curve provided via the reagent barcode or e-barcode.

Reagents - Working Solutions

The reagent rackpack is labeled as E2 III.

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

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, pH 6.0; preservative.

R2 Estradiol-peptide~Ru(bpy) (black cap), 1 bottle, 9 mL: Estradiol derivative, labeled with ruthenium complex 4.5 ng/mL; MES buffer 50 mmol/L, pH 6.0; preservative

Limits and ranges

Measuring range 18.4-11010 pmol/L

(5-3000 pg/mL) (defined by the Limit of Detection and the maximum of the master curve). Values below the Limit of Detection are reported as < 18.4 pmol/L or < 5 pg/mL. Values above the measuring range are reported as > 11010 pmol/L or > 3000 pg/mL (or up to 110100 pmol/L or 30000 pg/mL for 10-fold diluted samples)(122).

2. 3.3.10 Determination of Fasting Insulin Concentration

Intended use Immunoassay for the in vitro quantitative determination of human insulin in human serum and plasma. The determination of insulin is utilized in the diagnosis and therapy of various disorders of carbohydrate metabolism, including diabetes mellitus and hypoglycemia. The electrochemiluminescence immunoassay “ECLIA” is intended for use on Elecsys and cobas e immunoassay analyzers(131).

Test principle

Sandwich principle.

Total duration of assay: 18 minutes.

- 1st incubation: Insulin from 20 µL sample, a biotinylated monoclonal insulin specific antibody, and a monoclonal insulin specific antibody labeled with a ruthenium complexa) 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 instrumentspecifically generated by 2 point calibration and a master curve provided via the reagent barcode or e-barcode

Reagents - Working Solutions

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

R1 Anti-insulin-Ab~biotin (gray cap), 1 bottle, 10 mL: Biotinylated monoclonal anti-insulin antibody (mouse) 1 mg/L; MESb) buffer 50 mmol/L, pH 6.0; preservative.

R2 Anti-insulin-Ab~Ru(bpy) (black cap), 1 bottle, 10 mL: Monoclonal anti-insulin antibody (mouse) labeled with ruthenium complex 1.75 mg/L; MES buffer

50 mmol/L, pH 6.0; preservative Not necessary due to the broad measuring range(132).

Table (2-11). Expected values of insulin (122).

Insulin level (pmol/L)	Insulin level(mIU/L)	
<174	<25	Fasting
208-1597	30-230	30 minute after glucose administration
125-1917	18-276	1hourse after glucose administration
111-1153	16-166	2 hourse after glucose administration

2. 3.3.11 Determination of Sex – Hormone- Binding Globulin Concentration

Sex – hormone- binding globulin ELISA Assay Kit is a direct immunoenzymatic colorimetric method for quantitative determination of SHBG concentration in human serum or plasma. SHBG kit is intended for research use only and is not intended for diagnostic procedures(133, 134).

Principle

The test of this Kit is based on the simultaneous binding of human SHBG to two monoclonal antibodies, one immobilized on microwell plates and the other conjugated with horseradish peroxidase (HRP). After incubation, the bound/free separation is performed by a simple solid-phase washing. Then the enzyme HRP in the bound-fraction reacts with the Substrate (H_2O_2) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H_2SO_4) is added. The color intensity is proportional to the SHBG concentration in the sample. The SHBG concentration in the sample is calculated through a calibration curve(135).

Reagents and materials supplied in the kit.

1. SHBG Calibrators (5 vials, 1 mL each) CAL0 REF DCE002/8706-0 CAL1 REF DCE002/8707-0 CAL2 REF DCE002/8708-0 CAL3 REF DCE002/8709-0 CAL4 REF DCE002/8710-0

2. SHBG Control (1 vial, 1 mL, ready to use) Concentration of Control is Lot specific and is indicated on Quality Control report REF DCE045/8703-0

3. 5X Assay buffer (1 vial, 50 mL) Hepes buffer; BSA 10 g/L, stabilisers REF DCE049-0

4. Conjugate (1 vial, 12 mL) Monoclonal antibody anti SHBG conjugated with horseradish peroxidase (HRP) REF DCE002/8702-0

5. Coated Microplate (1 breakable microplate) Monoclonal antibody anti SHBG adsorbed on microplate REF DCE002/8703-0

6. TMB Substrate (1 vial, 15 mL) H_2O_2-TMB 0.26g/L (avoid any skin contact) REF DCE004-0

7. Stop Solution (1 vial, 15 mL) Sulphuric acid 0.15 mol/L (avoid any skin contact) REF DCE005-0

8. 50X Conc. Wash Solution (1 vial, 20 mL) NaCl 45g/L; Tween-20 55g/L REF DCE006-0

Procedure

1. Preparation of the Calibrators (C0…C4).

The Calibrators have the following concentrations:

Table (2-12). Concentrations of calibrators in SHBG assessment

	C0	C1	C2	C3	C4
nmol/L	**0**	**2.78**	**27.8**	**111.1**	**277.8**

The Calibrators and Control have already been prediluted and are ready to use. Once opened, the Calibrators are stable 3 months at 2-8°C.

2. Preparation of Wash Solution

Dilute the contents of each vial of the buffered Wash Solution Concentrate (50X) with distilled water to a final volume of 1000 mL prior to use. For smaller volumes respect the 1:50 dilution ratio. The diluted wash solution is stable for 30 days at 2÷8°C.

3. Preparation of the Sample

Serum or plasma can be used. Specimen can be stored at 2-8°C for at short time (max two days). For longer storage the specimen should be frozen at -20°C. Avoid repeated freezing and thawing cycles. Proceed as follows:

1. Dilute the 5X Assay Buffer (1:5):

Dilute 50 mL of assay buffer (reagent 3) with 200 mL of distilled H2O; for smaller volumes respect the dilution ratio 1:5. Mix gently leaving in a rotating shaker at least 5 minutes.

2. Dilute the samples (1:100): Dilute 10 μL of sample with 990 □L of diluted assay buffer; for different volumes respect the dilution ratio 1:100. Mix gently avoiding the use of vortex. x solution.

Procedure

- Allow all reagents to reach room temperature (22-28°C).

- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.

- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.

- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C0-C4), two for each Control, two for each sample, one for Blank

Table (2-13). Procedure of SHBG assessment

Reagent	Calibrator	Sample/ Control	Blank
Calibrator C0-C5	**25 µL**	**-**	**-**
Diluted Samples/ Control	**-**	**25 µL**	**-**
Conjugate	**100 µL**	**100 µL**	**-**
Incubate at room temperature (22-28°C) for 1 hours. Remove the content from each well. Wash the wells 3 times with 300 µL of diluted Wash Solution.			
TMB Substrate	**100 µL**	**100 µL**	**100 µL**
Incubate at room temperature (22-28°C) for 15 minutes in the dark.			
Stop Solution	**100 µL**	**100 µL**	**100 µL**
Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank within 5 minutes			

Results

1. Mean Absorbance

Calculate the mean of the absorbancies (Em) corresponding to the single points of the calibration curve (C0-C4) and of each sample.

2. Calibration curve

Plot the values of absorbance (Em) of the Calibrators (C0-C4) against concentration. Draw the best-fit curve through the plotted points. (es: Cubic Spline or Four Parameter Logistic).

3. Calculation of Results

Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in nmol/L.

Table (2-14). Reference values SHBG (136).

nmol/L	Men	Women
Mean	**43**	**63**
Absolute range	**15-100**	**15-120**

2.3.4 Determination of Free Androgen Index

Free Androgen Index (FAI) is a ratio used to determine abnormal androgen status in humans, and it is calculated by:

FAI = T (total testosterone) (nmol/L)/SHBG (nmol/L) × 100. To apply the equation, T was converted to SI units using the following conversion factor: T (nmol/L) = T (ng/mL) × 3.467(137).

2.3.5 Determination of Homeostasis Model Assessment for IR

Homeostatic model assessment index (HOMA-IR) is a good indication for insulin resistance in human body and can be calculated by:

HOMA-IR = (fasting insulin in μU/mL × fasting glucose in mmol/L)/22.5

glucose (mmol/L) = glucose (mg/dL) × 0.0555(137).

2.3.6 Genotyping

2.3.6.1 DNA Extraction

The DNA extraction was carried out at College of Pharmacy in Kerbala province. DNA Genome was extracted from blood sample according to protocol G-DEX tmIIb Genomic DNA Extraction Kit.

Principle

DEX tmIIb Genomic DNA Extraction Kit provides a quick, simple technique for preparation of purified and intact DNA from mammalian blood. Samples are processed using a binding column in a micro-centrifuge tube. Up to 200 µl of blood can be processed per purification. The DNA of the isolated genome is of high quality and can be used in common applications such as agarose gel analysis, restriction enzyme process and PCR analysis.

DNA Extraction is carried out in 5 steps that involve RBC & Cell Lysis Step, RNase Treatment Step (Optional), Protein Precipitation (PPT) Step, DNA Precipitation Step, DNA Rehydration Step:

1st RBC & Cell Lysis Step

1. 1 ml whole blood (or bone marrow) to a 15 ml tube containing 3 ml RBC Lysis Solution was added. Invert to mix and incubate 5 minutes at room temperature. Invert again at least once during the incubation.

2. Centrifuge at 2,000 x g for 5 minutes. supernatant leaving behind the white cell pellet was removed and remain about 100 µl of the residual liquid.

3. The tube was vortex vigorously to resuspend the cells. (note3) In order to dissolve the pellet completely, vortex carefully

4. 1 ml Cell Lysis Solution was added to the resuspended cells and pipet up and down to lyse the cells.

3rd Protein Precipitation (PPT) Step

1. Sample was cooled to room temperature. Add 333 µl PPT buffer to the cell lysate. And Vortex vigorously at high speed for 20 seconds. (note7) In order to remove the protein contamination, in some case, put the sample to the ice during 5min.

2. Centrifuge at 2,000 x g for 5 minutes. The precipitated proteins will form a tight dark brown pellet. (note8) If the protein pellet is not tight, repeat Step 1 followed by incubation on ice for 5 minutes and then repeat Step.

4th DNA Precipitation Step

1. The supernatant containing the DNA was poured (leaving behind the precipitated protein pellet) into a 15 ml tube containing 1 ml 100% Isopropanol (2-propanol). Mix the sample by inverting gently several times. (note9) When the low DNA yield, add the glycogen(200mg/ml) which is 1/100$\mu\ell$ of Cell Lysis Buffer before adding the isopropanol .2-Propanol volume is 90~95% of supernatant when pipetting. It is good to add 2propanol about the same volume of supernatant.

2.Centrifuge at 2,000 x g for 3 minutes; the DNA will be visible as a small white pellet.

3.The supernatant was poured out and drain tube briefly on clean absorbent paper. Add 1 ml 70%Ethanol and invert the tube several times to wash the DNA pellet. And centrifuge at2,000 x g for 3 minute. Carefully pour off the ethanol. Pellet may be loose so pour slowly and watch pellet.

4.The tube was inverted and drained on clean absorbent paper and allow to air dry 10-15minutes. (note10) When discard the supernatant, be careful not to discard the

DNA pellet. Avoid the over dry because too much dried DNA is not dissolved very well in DNA Hydration buffer.

5th DNA Rehydration Step

1. 100 µl DNA Rehydration Buffer was added (100 µl will give a concentration of 300 µg/ml if the total yield is 30 µg DNA). (note11) When the high DNA yield, it is very difficult to dissolve the DNA. In this case, add the proper volume of the solution.

2. Rehydrate DNA by incubating at 65°C for 1 hour or 2 hours. If possible, tap tube periodically to aid in dispersing the DNA.

3. For storage, sample may be centrifuged briefly and then transferred to a 1.5 ml micro tube. For long-term storage, store at -20°C or -80°C.

4. Measure the DNA purity O.D 260:280 ratios.

2. 3.6.2 Determination the Concentration and Purity of the Extracted DNA

Spectrophotometric methods were used to estimate the concentration and purity of extracted DNA. The purity and concentration of DNA were measured by absorbance method using the Nano drop instrument. The absorbance readings were done at 260 nm and at 280 nm. At 260 nm the DNA strongly absorbs light while at 280 nm the protein absorbs light most strongly. DNA purity was measured by theA260/A280 ratio. The A260/A280 ratio 1.8-2.0 is commonly accepted. Initially 1 µL of nuclease free water was smeared on the highly sensitive micro detector of nano-drop as blank. The micro detector was cleaned up from blank. Then 1 µL of DNA sample was applied on the micro detector of nano-drop. The concentration and A260/A280 ratio of DNA were documented from the instrument.

2. 3.6.3 Amplification of DNA

In order to amplify the target gene (STK11), ALLEL SPECIFIC -PCR was used with a specific primer. The ALLEL SPECIFIC -PCR reactions were performed in 25 µl volumes in PCR tubes under sterile conditions, all the volume of the reaction mixture was completed to 25 µl with using DDH2O and the premix which contained optimum concentrations of reaction requirements (MgCl2 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM) has been used, table (2-15).

Table (2-15). Components of master mix for detection of STK11 gene

No.	Material	Volume(µl)
1	Premix	12.5
2	Forward	1.5
3	Reverse	1.5
4	Template DNA	5
5	DDH2O	4.5
	Total	25 µl

2.3.6.4 Primers

A primer is a short single strand of DNA fragments consisting from (18-22) bases known as oligonucleotides that are a complementary sequence to the target DNA region. It used to start the process of amplification, without such substance the reaction is not initiated on a single DNA molecule. Thus it should be first annealed to the single strands obtained from denaturation of the double stranded DNA(138).

Polymerase chain reaction was performed using a specific primer pairs designed for STK11 gene. Based on NCBI database, all gene information and SNPs detail.

Preparation of the primers in the following steps: -

Materials: Lypholized primers, Sterile dH20

1. The tube was spin down before opening the cap.
2. Prepare Master Stock, pmoles/µl, the desired amount of sterile dH20 was added according to the manufacturer to obtain a 100 pmoles/µl (Master Stock).
3. The tube was mixed properly to re-suspended the primers equally.
4. Preparing Working Stock, 10 pmoles/µl, ten microliters of the master stock were transferred to a 0.5 ml eppendorf tube that contains 90µl of sterile dH20 to obtain a 10 pmoles/µl (Working Stock).
5. The master stock was stored at -20 C°.

The sequence of primers used for PCR amplification of STK11 gene rs741765 and rs8111699 was illustrated in Tables (2-16) and (2-17) respectively. The primers were designed by Assistant Professor Dr. Hassan Mahmood Abo Al-maali, College of Pharmacy/ University of Kerbala.

Table (2-16). Specific primers of STK11 gene rs741765

Primer	Sequence (5´ –3´)	Allele	Size(bp)	Company Country
Forward	**TGTGCAGAAATGTAGGGTT**		177	BIONEER/ Korea
Reverse 1	**CCTCTGGGGTGGGAGTTCG**	C	177	
Reverse2	**CCTCTGGGGTGGGAGTTCA**	T	177	

Table (2-17). Specific primers of STK11 gene rs8111699

Primer	Sequence (5´ –3´)	Allele	Size(bp)	Company Country
Forward	**CCACGAGGCCTTTCTGATGT**		693	BIONEER/ Korea
Reverse 1	**CACTGACTGTTCAGGAAAGCAG**	C	693	
Reverse 2	**CACTGACTGTTCAGGAAAGCAC**	G	693	

2. 3.6.5 Polymerase Chain Reaction

Polymerase chain reaction (PCR) has become one of the most important techniques in modern biological and medical science (139). It amplifies a specific region of a DNA strand to generate thousands to millions of copies of a particular DNA sequence (140).

A basic PCR usually requires the following:

1) DNA template containing the target DNA region.

2) Two primers to initiate DNA synthesis.

3) A thermostable DNA polymerase to catalyze DNA synthesis.

4) Deoxynucleoside triphosphates (dNTPs, the building blocks of new DNA strand).

5) Buffer including bivalent cations, usually Mg^{2+}.

There are three steps of a PCR that are cycled about 25-35 times(141), this steps including the following:

- Denaturation: This step includes separation of the double DNA strands into two single strands are accomplished by heating for about 94-95°C.

- Annealing: At lower temperature (55-65°C), DNA primers (which are short single strand DNA fragments) attach to the ends of each strands of the DNA and initiates the reaction.

- Extension: This step occurs at 72-74°C, where each primer binding to the DNA template will be extended complementary to the template DNA. This process is carried out in the presence of the Taq DNA polymerase, because of its ability to operate efficiently at high temperatures.

2. 3.6.6 Optimization of PCR Conditions

Different volumes of primer (0.5 µl,1 µl, 1.5 µl,) with different volumes of template DNA (1 µl,2 µl,3 µl,4 µl,5 µl, 6 µl) and different experiments of the reaction conditions were trailed in order to optimize the conditions of the reaction. PCR tube centrifuged for 30 seconds at 2000 xg in a micro-centrifuge in order to mix solutions well at room temperature then tubes are placed in the thermocycler to start the reaction. Programs of the PCR protocol reaction for STK11 gene polymorphism for each rs741765 and rs8111699 were illustrated in tables (2-18) and (2-19).

Table (2-18). Allele specific –PCR program for detection of STK11 gene rs741765 SNP

No.	Stage	Cycle	Step	Temp.	Time
1	Initial Denaturation	1	1	92 °C	2min.
2	Denaturation	45	1	92 °C	30 sec.
3	Annealing	45	2	45 °C	30 sec.
4	Extension	45	3	72 °C	20 sec.
5	Final Extension	1	1	72 °C	5 min.
6	Hold Phase	1	1	10 °C	Open

Table (2-19). Allele specific –PCR program for detection of STK11 gene rs8111699 SNP

No.	Stage	Cycle	Step	Temp.	Time
1	Initial Denaturation	1	1	92 °C	2min.
2	Denaturation	45	1	92 °C	30 sec.
3	Annealing	45	2	40 °C	30 sec.
4	Extension	45	3	72 °C	45sec.
5	Final Extension	1	1	72 °C	5 min.
6	Hold Phase	1	1	10 °C	Open

Two PCR reaction were performed, one with primer rs741765 C(R1) and rs741765(F) producing (PCR C) and second with primer rs741765 T(R2) and rs741765 (F) to produce (PCR T) for STK11 rs741765 SNP. The same thing with rs8111699 SNP one with primer rs8111699 C (R1) and rs8111699(F) resulted in (PCR C), and second with primer rs8111699 G (R2) and rs8111699 (F) to result (PCR G), and run side by side on an agarose gel. In rs741765 (177) bp band indicated the presence of the allele; if amplification failure, this mean the absence of an allele in the gel and the same for SNP rs8111699 in (693) bp, table (2-20).

Table (2-20). Bands obtained from the amplification product of STK11, rs741765 and rs 8111699 SNPs

Genotype			No. of bands	Size of bands (bp)	
Wild type	rs741765	rs8111699		rs741765	rs8111699
	CC	CC	1	177 C allele	693 C allele
Heterozygous	CT	CG	2	177 C allele 177 T allele	693 C allele 693 G allele
Homozygous	TT	GG	1	177 T allele	693 G allele

2. 3.6.7 DNA electrophoresis

Electrophoresis through agarose is a standard method used to separate, identify and purify DNA fragments. The technique is simple and rapid to perform and capable of resolving fragments of DNA that cannot be separated adequately by other procedures(142).

The agarose gel electrophoresis was done according to Harisha method(143) and ethidium bromide staining was added (2-3 μl) , which can bind with DNA by forming close van der Walls contacts with the base pairs and that's why it binds to the hydrophobic interior of the DNA molecule. Molecules that bind in this manner are called intercalating agents because they intercalate into the compact array of stacked bases, 2% agarose was prepared using this same protocol.

2.3.6.7.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis is the standard method for separating and identifying nucleic acid fragments (144). It is an effective way of separating DNA fragments of varying sizes ranging from 100 bp to 25 kb(145) . The separation in this way uses an electric current to migrate the biomolecules through a porous gel matrix at a rate that is proportional to the particle charge, size and shape(146).

Procedure

One hundred milliliters of a 2% agarose solution were prepared by the following steps(147).

A. Preparation of solution

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

B. Preparation of the agarose gel

1. Two grams of agarose were weighted and placed inside a conical flask and 100 mL of 1x buffer (TBE) was added with gentle mixing.
2. The solution was placed in the microwave for one minute until the agarose was completely dissolved and the solution became clear and then allowed to cool to about 55 °C before pouring.
3. Ethidium Bromide dye solution (2µl) was added to the solution.
4. The gel tray ends were closed with tape.
5. The comb was placed in the gel tray about 1 inch from one end of the tray.

6. The gel solution was poured into the chamber and allowed to solidify for 30 minutes at room temperature.

7. The comb was removed from the gel and the chamber was placed in a horizontal electrical system and was covered (only until the wells were flooded) with the same buffer TBE used to prepare the gel.

8. About (6 µl) from the samples were loaded on each well with extreme care to avoid damage to wells and contamination of adjacent wells.

9. Cathode pole was connected to the well side of the unit and the anode to the other side.

10. Electrophoresis was performed at 75 V, for 60-100 min or until the dye markers were migrated at an appropriate distance, depending on the size of the DNA to be visualized.

2. 3.6.8 Photo Documentation

The agarose gel was placed over an ultraviolet transilluminator device and subjected to ultraviolet light and the images were captured using a digital camera and conceived by a computer connected to the transilluminator.

2.4 Limitation of Study

1- Time limit.

2- Cost of kits.

3-freguent electric current cuts during the work.

4-Pandemic of covid 19.

2.5 Statistical Analysis

Microsoft Excel program (Version 2016) with Statistical package for the social sciences (SPSS) (Version 25) were used in current study. Chi-square test was used for comparison of demographic parameters. All values were expressed as mean± standard deviation (M±SD). Student-t-test was used to collectively indicate the presence of any significant difference between the two groups for a normally distributed quantitative variable. And paired t- test was used in comparison the levels of biochemical markers at baseline and after treatment with p value less than 0.05 was considered to be significant. Anova test was used for comparison among three group, Pearson's correlation analysis was used to show the strength and direction of association between two quantitative variables(148).

Chapter Three

Results

3. Results

3.1 Patients Data

The present study included 350 patients diagnosed with PCOS, but only 235 of them participated in the study. From the total number of participants about 115 patients were excluded from the study because 28 (8%) of them became pregnant in the second month of the follow-up period, others unable to contact, science the pandemic of Covid-19 virus, so all of them were dropped out from the study, and thus were excluded from further analyses.

3.2 Demographic Features of the Study Patients

The mean age of patients was (27.1±5.21) years with a range of (18 – 40) years old. In current study PCOS reported to be more prevalent among younger ages (<30) years old with percentage (78.3%) compare to (21.7%) in patients with (>30) years old. In Iraq, diagnosis of PCOS often associated with infertility and mostly start in the first year after the marriage and majority of the patients in the present study were married (88.5%), even though some symptoms may start at menarche age, table (3-1).

Table (3-1). Distribution the age groups among the patients.

Age	Frequency (%)
(15-20)years	80 (34.1%)
(21-25) years	67 (28.5%)
(26-30) years	37 (15.7%)
(31-35) years	30 (12.7%)
(36-40) years	21 (8.9%)
Total	235

Higher proportion was reported from urban population 202 (85.9%) in comparison to rural 33(14.04%) counterparts in current study. Present study revealed that high percentage of patients have school education 127 patients (54.1%) and less with university education 108 patients (45.9%).

Majority of PCOS patients in present study had their age at menarche in the 12 years old, table (3-2).

Table (3-2). Distribution of patients according to age at menarche.

Age of M	**Frequency** %
10 Year	6 (2.5%)
11 Year	27 (11.4%)
12 Year	93 (39.5%)
13 Year	72 (30.6%)
14 Year	30 (12.7%)
15 Year	7 (2.9%)
Total	235

Infertile patients or those with no children were high (48.5%), with exclusion of unmarried patients from this percentage**,** the number of patients or their percentage in presented study was decreased by increasing a number of children, table (3-3).

Infertility in present study was divided into two types, either primary or secondary type. Higher percentage of the patients were recorded with secondary infertility compare to primary one.

Table (3-3). Distribution the number of children among married patients.

n. of children	Patients frequency (%)
0	101 (48.5 %)
1	51 (24.51 %)
2	36 (17.3 %)
3	16 (7.69 %)
4	3 (1.44 %)
7	1 (0.48 %)
Total	208

One of the PCOS complications is abortion(149), (28.36%) in present study were suffering from repeated miscarriages, table (3-4).

Table (3-4). Distribution the number of abortions among married patients.

Abortion	Patients frequency (%)
0	149 (71.63%)
1	31 (14.9%)
2	16 (7.6%)
3	11 (5.28%)
7	1 (0.48%)
Total	208

3.3 Clinical Features of the Studied Patients

3.3.1 Menses Regularity

Irregular menstruation is clear phenomena of all patients in the current study, where none of the PCOS women had normal menstrual cycles. Patients were divided according to regularity as being: oligomenorrhoeic 66% (a cycle interval of longer than 35 days but less than six months), amenorrhoeic 25% (less than two menstrual cycles in the year) or hypomenorrhoeic 9% (bleeding lasting less than 1 day). Restoration of regular menstrual cycle with metformin was reported in 96 (41%)patients.

3.3.2 Hirsutism and Alopecia

Hirsutism and alopecia are major clinical feature of hyperandrogenism in PCOS patients, so in current study revealed a high percentage of patients with hirsutism (thicker hair on face and body) and alopecia (hair thinning with loss). The percentages which reported among the patients were (91.96%, 80.86 %) of hirsutism and alopecia respectively, also we found increased frequency of this features among obese patients in comparison with non-obese one. Upon a period of metformin therapy, hirsutism and alopecia were recorded only among (48.6%, 42.8 %) from the patients, figure (3-1).

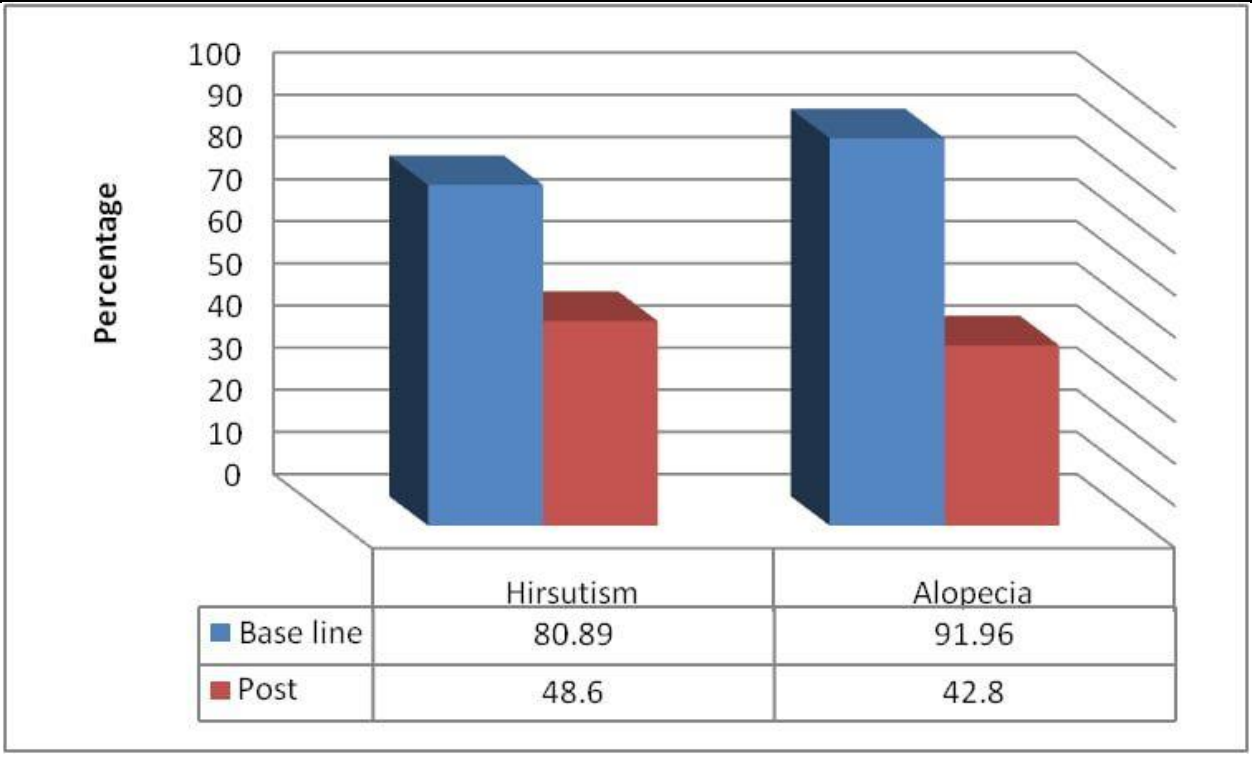


Figure (3-1). Percentages of hirsutism and alopecia among the patients.

Hirsutism and alopecia were found to be associated with levels of androgen to peripheral tissues, table (3-5).

Table (3-5). Comparison the baseline levels of FAI and HOMA-IR between the patients with and without alopecia and hirsutism

Biomarke	Hirsutism			Alopecia		
	Present	Not present	P value	Present	Not present	P value
FAI mean ± SD	8.84±3.5	7.88±2.23	0.03	9.85±5.67	6.2±2.11	0.01
HOMA-IR mean ±SD	5.02±1.73	4.01±1.46	0.007	6.1±1.13	3.2±1.02	0.003

Student t-test, p<0.05 statistically significant, SD=standard deviation, (FAI) free androgen index, (HOMA-IR) Homeostasis model assessment for IR.

The level of FAI in the patients with hirsutism was (8.84±3.5) compare to (7.88±2.32) in patients without hirsutism, p value 0.03, while in patients with alopecia, the level was (9.85± 5.67) in comparison to (6.62±2.11) in patients without alopecia, p value 0.01, nevertheless they were also associated with insulin resistance, so HOMA –IR level in patients with hirsutism was (5.02±1.73) in comparison to (4.01± 1.64) in patients without hirsutism, p value 0.007 and (6.1±1.13) in patients with alopecia compare with (3.2±1.02) in those without alopecia p value 0.003.

3.3.3 Effect of Metformin on Body Mass Index

The percentage of overweight and obese patients in present study were high (31.4%, 44.6%) respectively, table (3-6).

Table (3-6). Distribution of patients according to BMI phenotypes.

Classification	BMI	Patients number (%)
Underweight	<18.5	5 (2.12 %)
Normal weight	18.5—24.9	51 (21.7%)
Overweight	25 ---29.9	74 (31.4%)
Obese	>30	105 (44.6%)
Total		235

Mean BMI level before treatment was (28.8±5.62) kg/m2 and significantly decreased after treatment to (27.2± 5.66) kg/m2, p value 0.03, accordingly the percentage of patients presented with obesity having BMI ≥ 25 kg/m2 was reduced from 76.1% to 42.5%. Nevertheless, obesity was worsened and BMI was increased in 10 patients (4.3%). Body mass index was associated with dyslipidemia in women with

PCOS, table (3-7), were the Pearson's correlation coefficient test (r) value between BMI and lipid profile was measured as following, TG (0.27), LDL-C (0.15), TC (0.43), HDL-C (-0.01).

Table (3-7). Correlation of body mass index with the lipid profile.

DATA	r (Pearson's correlation coefficient test)				
	BMI	**TG**	**LDL**	**TC**	**HDL**
BMI(kg/m2)	1	-	-	-	-
TG(mg/dl)	0.27	1	-	-	
LDL(mg/dl)	0.15	0.2556	1	-	-
T.cholestrole(mg/dl)	0.43	0.3667	0.3055	1	-
HDL(mg/dl)	-0.01	-0.1033	-0.3044	-0.1113	1

BMI = body mass index, TG = triglycerides, TC = total cholesterol, HDL-C = high density lipoprotein-cholesterol, LDL-C = low density lipoprotein-cholesterol.

3.4 Effect of Metformin on Biochemical Characteristics

Biochemical characteristics of all enrolled patients were represented in tables (3-8) and (3-9). By comparison of the baseline and post- data, the differences of all tested biochemical markers (endocrinal and metabolic) were significant at p value ≤ 0.05.

3.4.1. Effect of Metformin on Endocrinal Parameters

The comparison of endocrinal parameters among the patients in current study was illustrated in table (3-8).

Table (3-8). Comparison of endocrinal parameters (baseline and post treatment)

Clinical and endocrinal parameters	baseline. (n. =235)	POST. (n. =235)	P Value
FSH (mIu/ml)	5.73±1.996	6.49±2.51	0.004
LH(mIu/ml)	10.01±5.92	8.55±4.86	0.006
LH/FSH ratio	1.807±0.96	1.4±0.86	0.008
TSH(µlU/Ml)	2.33±0.97	2.159±0.87	0.004
PROLACTIN(ng/ml)	23.74±9.68	20.48±10.87	0.005
TESTESTERONE(ng/ml)	0.576±0.35	0.456±0.326	0.006
SHBG(nmol/L)	41.85±22.63	51.91±21.62	0.001
FAI	8.053±10.39	3.6±7.823	0.002
ESTRADIOL(pg/ml)	57.88±49.2	68.1±47.45	0.004

Clinical and endocrinal characteristics of patients before and after treatment. Paired t-test, $p<0.05$ statistically significant, SD=standard deviation, LH luteinizing hormone, FSH follicular stimulating hormone, TSH thyroid stimulating hormone, SHBG sex hormone binding globulin, FAI free androgen index.

Luteinizing hormone (LH) value above (8) mIU/ml was diagnosed in (81.8%) of patients and mostly among an overweight or obese in comparison with non-obese patients at a base line level measurement. Mean LH level before treatment was (10.01±5.92) mIU/ml and after treatment was (8.55±4.86) mIU/ml. This improvement recorded in (65.95%) of patients. Statistical analysis was showed the difference in mean value of LH was significant at p value 0.006.

Follicular stimulating hormone (FSH) may be the more immediate cause of anovulation in PCOS(150), mean FSH level before and after treatment was (5.73±1.99 , 6.49± 2.51) mIU/ml. Increase in mean value of FSH was statically significant p value 0.004, accordingly the level was increased in (61.27%) of the patients.

The ratio of LH/FSH in present study was elevated in 83 patients (35.4%), those with a ratio of more than 2 in a blood sample taken at follicular phase (ie, day 4 to 6) of the menstrual cycle. The mean LH/FSH ratio level before and after treatment was (1.807±0.96 ,1.4± 0.86) respectively. Statistical analysis showed that decrease in the mean value was significant p value 0.008, after a period of metformin treatment, the dropping in a ratio was recorded in 131patients (55.74%).

Thyroid stimulating hormone (TSH) level of all enrolled patients in the study was within normal value, science patients suffering from thyroid dysfunction were previously excluded from the study. Mean TSH level before treatment was (2.33±0.97) µlU/Ml and after treatment (2.4±0.159) µlU/Ml p value 0.004, and it was statistically significant, serum decrement in hormone (but remain within normal range) occurred in about 153 patients (65.1%), metformin suppressive effect for TSH more reported in overweight patients more than in non-obese one.

Prolactin level greater than (25)ng/mL was found in about 31 patients(13%) in present study, after exclusion of a prolactin-secreting pituitary adenoma that occurs in premenopausal women and is associated with menstrual irregularities (151). Mean prolactin level before and after treatment was (23.74±9.68 ,20.48± 10.87) ng/mL respectively, statistical analysis was showed that decrease in the mean value of prolactin was significant p value 0.005, the dropping in the hormone was occurred in 161 patients (68.51%). Metformin action on prolactin correlated with the degree of a reduction in HOMA-IR, their presence suggests that some signal pathways regulating prolactin production and release with signal pathways affected by insulin level may overlap to some extent, so, in present study the dropping in prolactin level revealed in patients with BMI >25 more than non-obese due to more improvement in insulin sensitivity.

Total testosterone level was elevated in present study in 139 patients or (59.14%) and there was a clear association existing between serum insulin and total testosterone demonstrated the increased level of insulin will lead to increase the endocrinological and morphological response of the ovaries, figure (3-2).

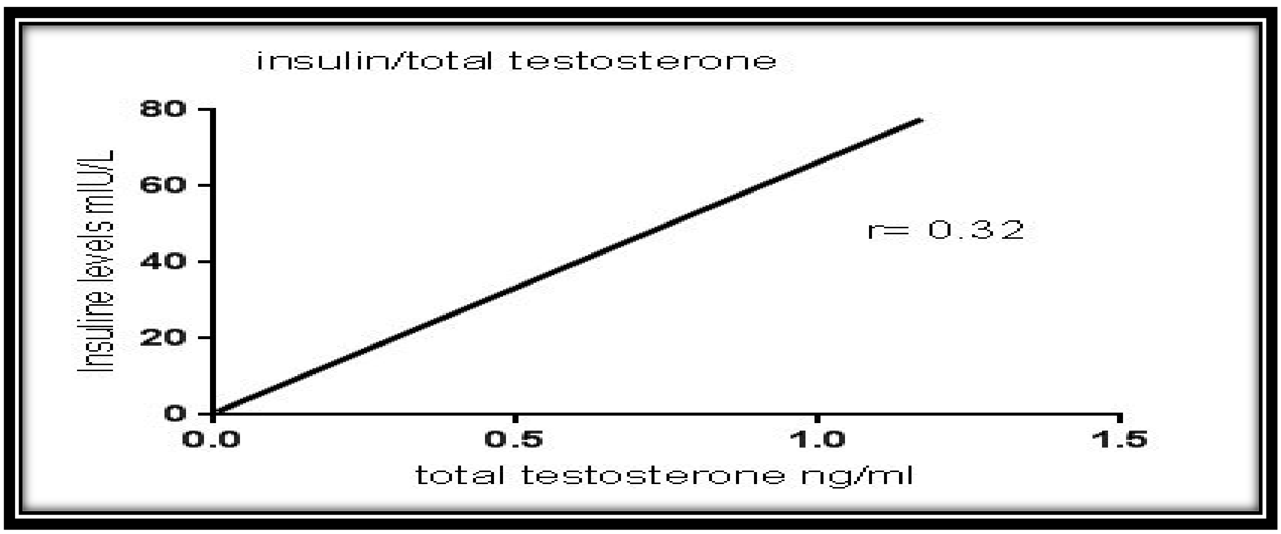


Figure (3-2). Correlation of total testosterone with a fasting insulin
r = 0.32; (Pearson's correlation coefficient test)

The mean testosterone level before treatment was (0.5765± 0.3504) ng/mL and after treatment was (0.4569± 0.3261) ng/mL, significant at p value 0.006 with a dropping in hormone was reported in 146 patients (62.12%).

Reduction of sex hormone binding globulin (SHBG) level is commonly associated with hyperandrogenism, IR, and obesity, which are the hallmarks of PCOS, as obesity is associated with insulin resistance and correlated with decreased SHBG which caused an increase in free testosterone level and FAI, consequently in present study 75 patients (31.91%) were recorded with low SHBG, this associated with certain other complications among women with PCOS including androgenic alopecia and dyslipidemia, accordingly, the total testosterone and SHBG concentrations are interdependent and ratio between them represent FAI. Mean SHBG level before and after treatment was (41.85± 22.63 ,51.91± 21.62) nmol/L respectively, increase in the mean value was significant (p value 0.001).

The free androgen index (FAI) is a ratio used to determine abnormal androgen status in humans. It is the total testosterone level divided by the sex hormone binding globulin (SHBG) level, multiplying by 100. In present study it was elevated in 69 patients (29.36%). The mean FAI level before and after treatment was (8.053± 10.39, 3.636± 7.823), respectively, this dropping in mean value was significant p value 0.002, due to an increase in SHBG concentration with decrease testosterone level. Improvement in FAI which occurred in the study was reported among the both patients with and without insulin resistance.

Estradiol level below 60 pg/ml in the patients at the follicular phase was reported in 137 patients (58.3%). Before treatment the level was (57.88± 49.27) pg/ml and after treatment was (68.1± 47.45) pg/ml, the increment was significant at p value 0.004.

3.4.2 Effect of Metformin on Metabolic Parameters

Comparison of metabolic parameters among the patients in current study was illustrated in table (3-9).

Table (3-9). Comparison of Metabolic Parameters (baseline and post metformin treatment)

Metabolic parameters	baseline. (n. =235)	POST. (n.=235)	P Value
FBS(mg/dl),	**96.2±13.18**	**92.62±13.16**	**0.03**
F.INSULIN(mIu/L)	**20.21±14.42**	**18.76±11.54**	**0.05**
HOMA IR	**4.74±3,71**	**4.11±2.63**	**0.008**
HbA1c%	**4.947±0.703**	**4.562±0.665**	**0.006**
TG(mg/dl),	**124.38±45.58**	**111.28±37.08**	**0.005**
LDL(mg/dl),	**91.76±26.14**	**83.37±21.67**	**0.002**
HDL(mg/dl),	**44.69±9.42**	**43.37±21.67**	**0.01**
TC(mg/dl),	**157.74±40.54**	**145.48±43**	**0.003**

Metabolic characteristics of patients before and after treatment. Paired t-test, $p<0.05$ statistically significant, SD=standard deviation, FBS fasting blood sugar, HbA1c glycated hemoglobin TG = triglycerides, TC = total cholesterol, HDL-C = high density lipoprotein- cholesterol, LDL-C = low density lipoprotein-cholesterol.

Level of serum fasting blood sugar (FBS) among all the patients were enrolled in this study was within normal range, mean FBS level before treatment was (96.2± 13.18) mg/dL and after treatment was (92.62±13.16) mg/dL, (p value 0.03), 88 patients (37.5%) were revealed improvement in glucose level, moreover, those dropping was occurred in both obese and non-obese patients.

High fasting insulin level or hyperinsulinemia in present study was revealed in 99 patients (42.2%), recent findings regarding a clear association between serum insulin and total testosterone. After metformin therapy a significant falling in F. insulin was reported, mean F.Insulin level before treatment was (20.21± 14.21) mlU/L and after treatment (18.76± 11.54) mlU/L, P value 0.05. Both groups with insulin-resistant and non-resistant one at baseline level were showed a lowering in insulin level upon a period of therapy, but more improvement was recorded within insulin resistance one, consequently the BMI was reduced considerably after treatment in both groups.

Insulin resistance in present study was calculated by using homeostasis model assessment for IR (HOMA-IR). The mean HOMA -IR level before and after treatment was (4.741± 3.721 ,4.11± 2.639), respectively, p value 0.008. Besides, the finding of present study revealed that insulin resistance (IR) more frequently observed in obese PCOS patients. HbA1c is a commonly used marker for chronic glycemia, where it reflects the average blood glucose levels over (2–3) months period. Its level unaffected by diet, exercise, insulin therapy and other drugs (152), so HbA1c has been suggested as a screening tool by the ADA (American Diabetes Association) due to its advantages over FG, mixed meal tolerance test (MMTT) and oral glycemic tolerance test (OGTT). Ordinarily, it is appropriate to screen for prediabetes in PCOS patients (153). Before treatment mean HbA1c level was (4.947±0.703) % and after treatment was (4.562± 0.665) %. Statistical analysis showed that the decrement in the mean value was significant, p value 0.006.

Dyslipidemia is the most common metabolic abnormality in PCOS and it raise the risk of arterial diseases 2-folds higher relative to non PCOS women. Patients enrolled in this study characteristically have decreased high-density lipoprotein HDL-C in 81 patients (34.5%), while triglycerides level was increased in 150 patients (63.8%), LDL-C and TC were increase in 82(34.9%), 51(21.7%), respectively. Mean levels of lipid profile before treatment were (124.38± 45.88,91.76±26.14 ,44.64±9.24, 157.74±40.45) mg/dL for TG, LDL-C, HDL-C, TC respectively and after treatment were (111.28±37.68, 83±21.76, 46.37±8.24 ,145.48±43.1) mg/dL respectively. Statistical analysis showed that decrease in the mean values was significant, accordingly metformin treatment was improved the lipid profile in patients with PCOS.

3.5 Comparison of Clinical, Endocrinal and Metabolic Characteristics between the non-Overweight /Obese and Overweight /Obese Patients.

Studied patients were classified according to body mass index (BMI) into overweight/obese (BMI ≥25 kg/m2) and non-overweight/obese patients (BMI<25 kg/m2. Mean age of overweight\ obese and second group were (26.1±5.21), (27± 3.32) respectively with a range of (18 – 40) years old, 179 patients (76.17%) with BMI ≥25 kg/m2 were recorded as an overweight\ obese group compare to 56 patients (23.83%) were considered as a second group. Accordingly, after the period of treatment the numbers decreased to 162 patients (68.94%) in overweight \obese group and increase to 73 patients (31.02%) in the other group. At baseline assessment, the prevalence of insulin resistance, dyslipidemia, hirsutism and alopecia in non - overweight/obese group was 42.34%, 3.66%, 69.76%, 21.39%, respectively, in comparison with 81.93%, 33.7%, 79.88% and 29.6% in overweigh\ obese group, menstrual irregularity was reported among all the patients in both groups. Present study was reported that overweight \obese PCOS patients were recorded more improvement in menstrual cycle regularity and clinical features of hyperandrogenism which are hirsutism and alopecia than non-overweight/obese after a period of treatment table (3-10).

Table (3-10). Comparison of clinical, endocrinal and metabolic parameters between non body weight patients (BMI<25 kg/m2) and overweight\ obese patients (BMI≥25 kg/m2)

DATA	BMI <25 (kg/m2)		BMI≥ 25 (kg/m2)		baseline and post changes in data in both groups		P value
	Baseline	Post	Baseline	Post	<25 (kg/m2)	≥25 (kg/m2)	
BMI(kg/m2)	23.13 ± 2.81	22.84 ± 3.17	32.23 ± 3.69	30.65 ± 3.67	- 1 ± 0.05	-2.05 ± 0.03	0.02
FSH(mIu/ml)	5.74 ± 1.98	7.02 ± 2.57	5.74 ± 2.01	6.16 ± 2.41	2.35 ± 0.03	1.03 ± 0.04	0.01
LH(mIu/ml)	9.24 ±6.47	8.48 ± 4.68	10.89 ± 5.62	8.55 ± 5.01	-0.52 ± 0.01	-2.59 ± 0.03	0.007
LH/FSH	1.41 ± 0.89	1.33 ± 0.87	1.8± 0.12	1.5 ± 1.02	- 0.24 ± 0.04	-0.32 ± 0.05	0.21
TSH(µlU /Ml)	2.01± 0.95	2.19 ± 0.92	2.8± 0.99	2.14 ± 0.85	-0.13 ± 0.02	-0.51 ± 0.05	0.18
Prolactin(ng/ml)	23.08 ± 13.4	19.41 ± 9.96	24.55 ± 13.84	20.13 ± 11.38	-3.67 ± 0.02	-4.42± 0.03	0.06
T.testosterone(ng/ml)	0.55 ± 0.32	0.38 ± 0.25	0.59 ± 0.37	0.51 ± 0.36	-0.24 ± 0.02	- 0.12 ± 0.04	0.08
SHBG(nmol/L)	48.44 ± 25.31	51.84 ± 23.82	37.96 ± 21.12	51.2 ± 20.54	3.55 ± 0.03	14.11 ± 0.054	0.003
FAI	8.76 ± 1.2	3.44 ± 6.07	9.64 ± 1.597	3.76 ± 8.69	-5.53 ± 0.03	-5.84 ± 0.02	0.09
Estradiol(pg/ml)	59.97 ± 56.14	73.48 ± 49.2	56.81 ± 45.15	64.27 ± 45.8	10.26 ± 0.02	6.69 ± 0.05	0.03
FBS(mg/dl)	94.44 ± 12.46	88.86 ± 13.76	97.08 ± 13.38	94.73 ± 12.11	-5.01 ± 0.07	-3.1 ± 0.01	0.01
F.Insulin level(mIu/L)	15.37 ± 13.3	14.78 ± 7.54	22.76 ± 14.43	19.4 ± 12.47	-1.9 ± 0.05	-3.2 ± 0.02	0.006
HOMA IR	3.67 ± 3.59	3.51 ± 1.8	5.55 ± 3.7	4.16 ± 3.17	-0.6 ± 0.05	-1.056 ± 0.04	0.008
HbA1C%	4.12±0.32	4.01±0.42	4.86±0.38	4.43±0.13	-0.2 ± 0.36	- 0.4± 0.28	0.05
TG(mg/dl)	119.18 ± 55.59	102.44 ± 40.53	130.5 ± 46.31	119.05 ± 37.22	-7.03 ± 0.04	-9.37 ± 0.05	0.003
LDL(mg/dl)	92.75 ± 33.28	84.36 ± 28.1	95.74 ± 31.71	85.31 ± 23.86	-22 ± 0.05	-44 ± 0.01	0.001
HDL(mg/dl)	45.14 ± 8.42	46.1 ± 7.75	44.22 ± 10.73	46.8 ± 9.67	1 ± 0.03	2.41 ± 0.02	0.004
T.Cholestrol(mg/dl)	141.52+- 46.61	135.96+- 43.87	175.59+- 42.24	153.6+- 44.99	-5.77 ± 0.01	-24 ± 0.05	0.001

Student t-test, p<0.05 statistically significant, SD=standard deviation, LH luteinizing hormone, FSH follicular stimulating hormone, TSH thyroid stimulating hormone, SHBG sex hormone binding globulin, FAI free androgen index, FBS fasting blood sugar, HbA1c glycated hemoglobin TG = triglycerides, TC = total cholesterol, HDL-C = high density lipoprotein- cholesterol, LDL-C = low density lipoprotein-cholesterol.

The comparison between clinical, endocrinal, and metabolic parameters, LH pulses frequency, TSH and prolactin were higher in over weight/obese compare to second group. Increase of body weight and fat in women was associated with several abnormalities of sex hormone balance and less level of SHBG was detected in overweight/obese women this associated with insulin resistance which caused an increase in free testosterone level, so FAI was higher in this group.

Levels of lipid profile were correlated with BMI as showed previoslly in table (3-7) resulted from increasing the release of free fatty acids (FFAs) from adipose tissue which could be improved by treatment with metformin. Metformin restored both LH spontaneous episodic secretion and ovarian function more in overweight/obese group. Moreover, improvement in TSH level more remarked among overweight/obese patients.

3.6 Incidence of Side Effects Among the Patients

Side effects like gastrointestinal complaints (nausea, diarrhea, flatulence, bloating, anorexia, metallic taste and abdominal pain) were recorded by many of patients through the follow period of the study, but none of these women discontinued the medication due to these side effects.

3.7 Result of Genotypes Analysis

3.7.1 Results of Amplification Reactions among the Genotypes of STK11 rs741765 and rs8111699

Allele-Specific PCR was used to assay genotypes of STK11 rs741765 and rs8111699 SNPs for rapid screening of polymorphism in the patients. Amplification products were obtained to have a size of 177 bp for rs741765 and 693 bp for rs8111699. PCR product was electrophoresed and directly visualized with agarose gel which colored with ethidium bromide under UV light.

The results of amplifications were analyzed and three genotypes were obtained for each SNP: for STK11 rs741765, (CC) genotype (homozygous wild type) represented in samples (three and five), (CT) (heterozygous type) in samples (one, four, and seven) and (TT) (mutant type) represented in samples (two, six and eight), figure (3-3).

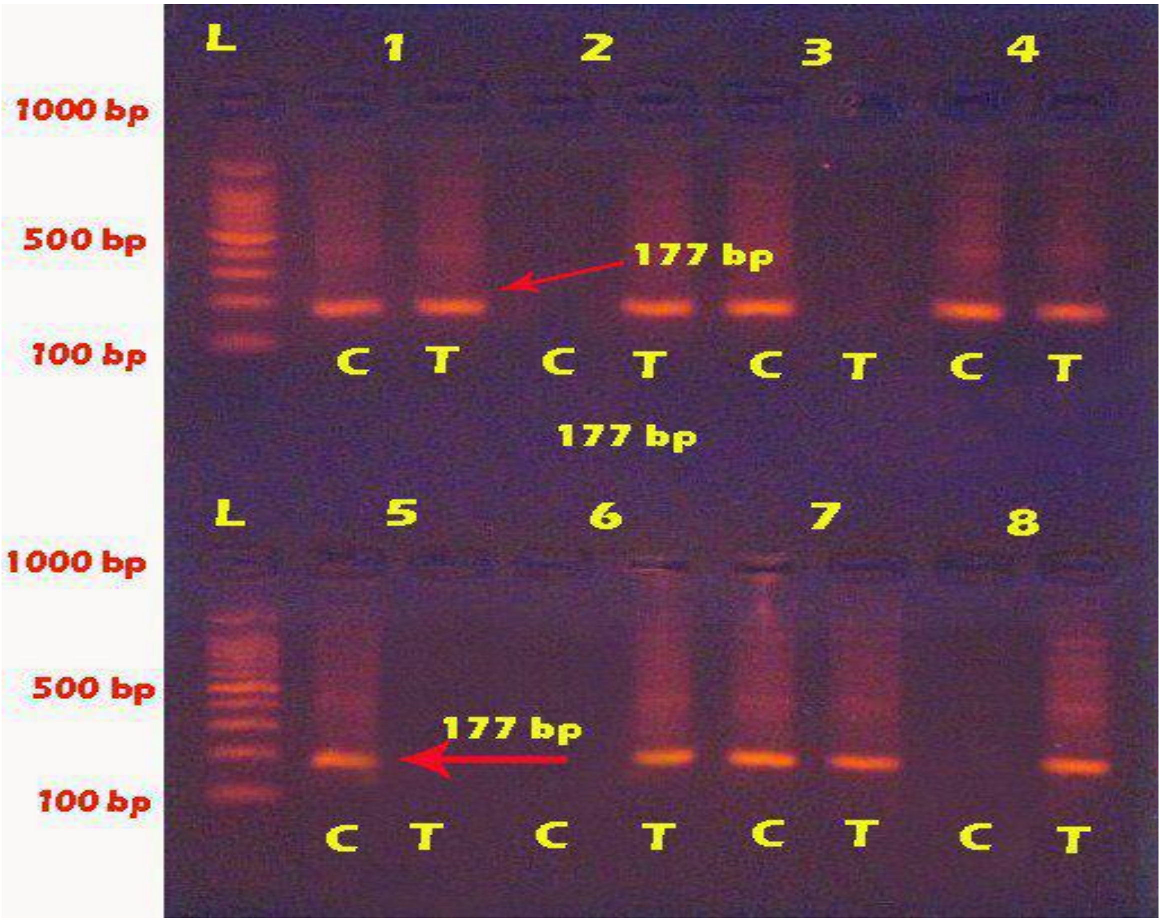


Figure (3-3). Result of amplification reactions of STK11 rs741765 genotypes by Allele Specific PCR

In case of STK11 rs8111699 SNP, (CC) genotype (homozygous wild type) in samples (three, five and six), (CG) (heterozygous type) in samples (one, four, and eight) finally (GG) (mutant type) samples (two and seven), figure (3-4).

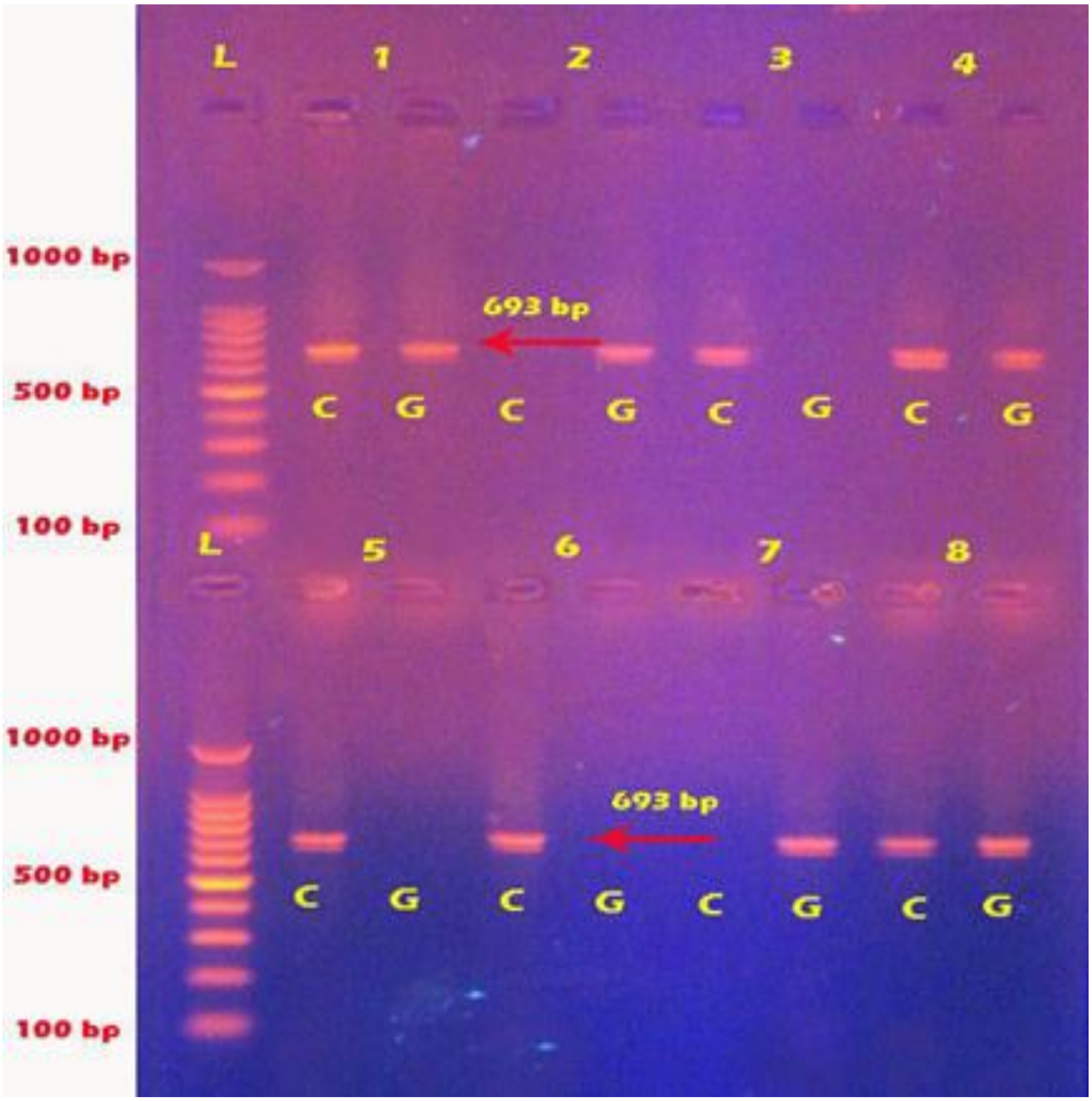
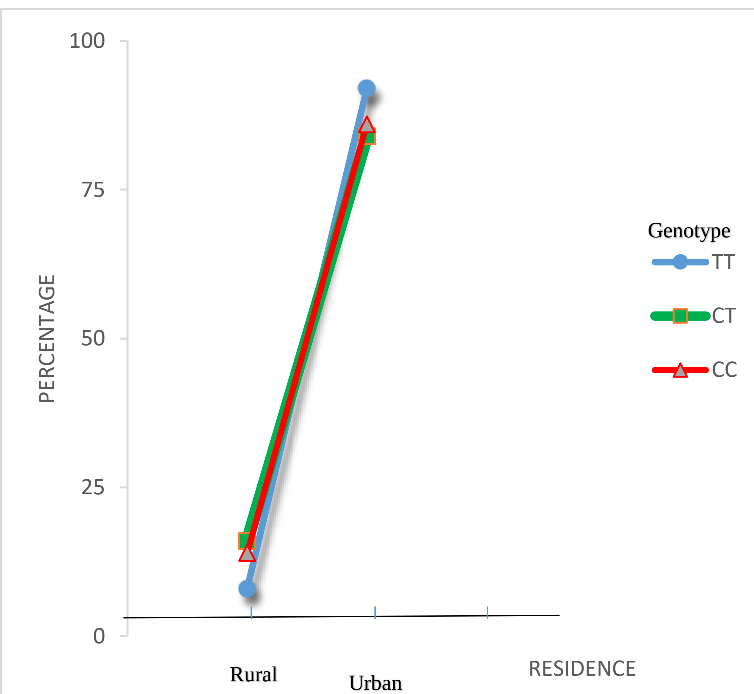


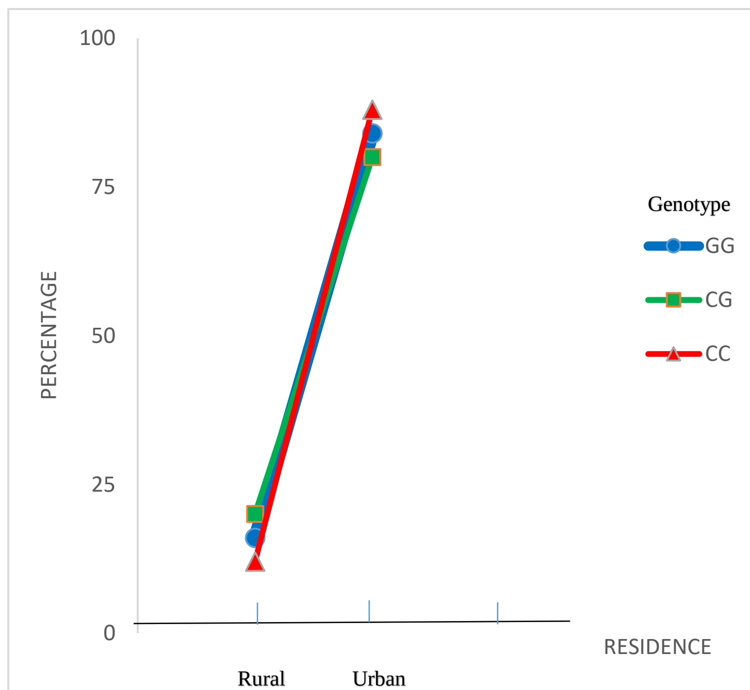
Figure (3-4). Result of amplification reactions of STK11 rs8111699 genotypes by Allele Specific PCR

3.7.2 Assessment of Demographic Parameters in STK11 rs741765 and rs8111699 Genotypes.

The relationship between STK11 rs741765 and rs8111699 genotypes and demographic features were analyzed in current study. Residence study was shown a highest percentage in both SNPs genotypes was from urban population, due to elevated number of patients from urban population in comperison with rural one, among genotypes of each residence the distribution was very closly in both SNPs, and very slight difference in percentages among the same residence gave no evidence to predict genotypes distribution, figure (3-5).



rs741765

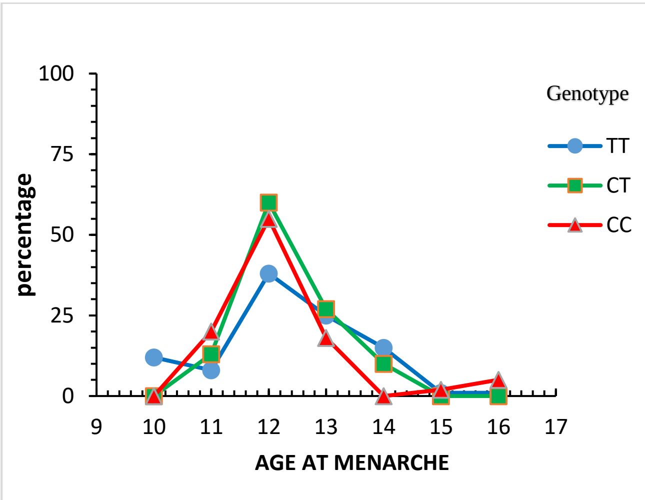


rs8111699

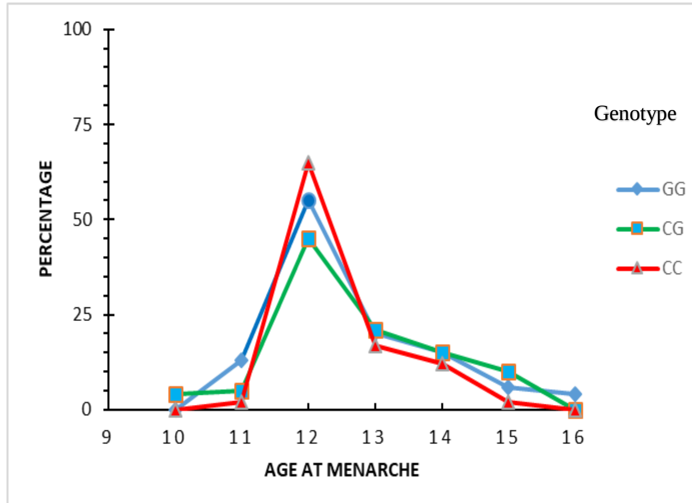
Figure (3-5) Residence distribution among the genotypes.

(Percentage in the figure is indicated to the percentage of the patients with specific genotype)

The distribution curves of age at menarche among the genotypes of each SNP were approximately similar, with maximum percentage at age 12 years old in both SNPs, figure (3-6)



rs741765

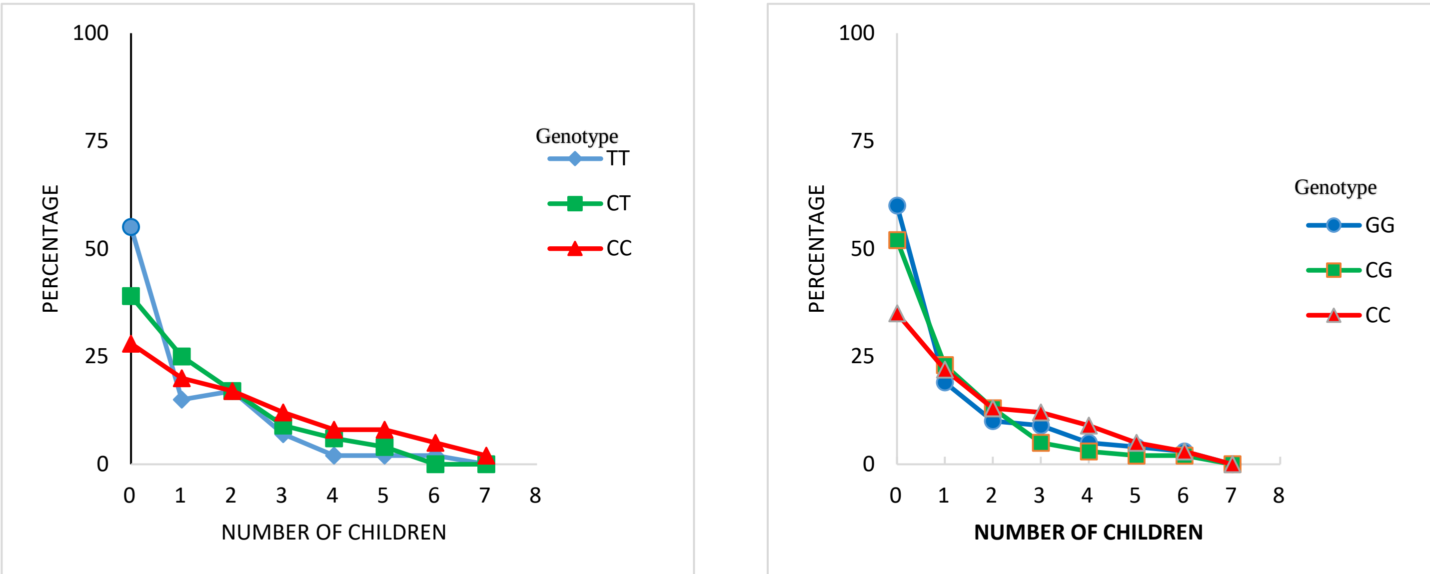


rs8111699

Figure (3-6) Relationship between genotypes and age at menarche.

(Percentage in the figure is indicated to the percentage of the patients with specific genotype)

Infertilility was common among all genotypes in present study, and the curves were very closly with decreasing in the percentage of patients by increasing the number of children. Mutant allele carriers, T allele in rs741765 SNP and G in rs8111699 SNP, were showed slightly higher infertility percent than C allele carriers, conversely, the same mutant alleles carriers were showed a good improvement upon metformin treatment compared with C allele carriers, where no or slightly improvement was recorded, figure (3-7).



rs741765 ***rs8111699***

Figure (3-7). Relationship between genotypes and the number of children.

(Percentage in the figure is indicated to the percentage of the patients with specific genotype)

3.7.3 Assessment of Clinical Parameters in STK11 rs741765 and rs8111699 Genotypes.

The improvement in clinical parameters after a period of therapy differ according genotypes in both SNPs. In STK11 rs741765 there is significant variation in menses regularity among the three genotype (X2= 178, Df= 5 $P< 0.01$), with more percentage of patients with regular cycle reported among the mutant genotype (TT) after a period of treatment compare with no or very slight improvement in wild type (CC), consequently, the C allele in the STK11 gene was associated with a significantly decreased chance of menses regularity in polycystic ovary syndrome patients treated with metformin, table (3- 11).

Table (3-11) comparison of clinical parameters among STK11 rs741765 genotypes

Clinical parameters		Patients genotypes							P value
		CC n. 82		CT n. 109		TT n. 44		Chi-square	
		Baseline	Post	baseline	Post	Baseline	Post		
Regularity	Present	0	6	0	52	0	27	X2= 178 Df= 5	P< 0.01
	No Present	82	76	109	57	44	17		
Hirsutism	Present	77	75	95	75	36	18	X2= 70.77 Df= 5	P< 0.01
	No Present	5	7	14	34	8	26		
Alopecia	Present	73	69	85	69	29	15	X2= 75.63 Df= 5	P< 0.01
	No Present	9	13	24	40	15	29		

From all the patients there was (91.96%) in present study, were suffering from hirsutism as consequence of hyperandrogenism and insulin resistance, moreover, there was a significant difference in improvement among the three genotypes (X2= 178, Df= 5 P< 0.01) with clear improvement in patients with mutant genotypes (TT) while there was very slight improvement with wild one.

Significant improvement (X2= 75.63, Df= 5, P< 0.01) from the baseline elevated percentage of alopecia (81.86%) was noticed among the patients after metformin treatment and mostly among mutant genotypes carriers compare with heterozygous and wild types.

Change in BMI among the three genotypes was also adjusted. In wild genotype (CC) the change between baseline and post treatment value was non- significant at value (0.067), while in heterozygous (CT) and mutant genotype (TT) it was significant at p values (0.047, 0.042), respectively, with more improvement among mutant allele carriers, figure (3-8).

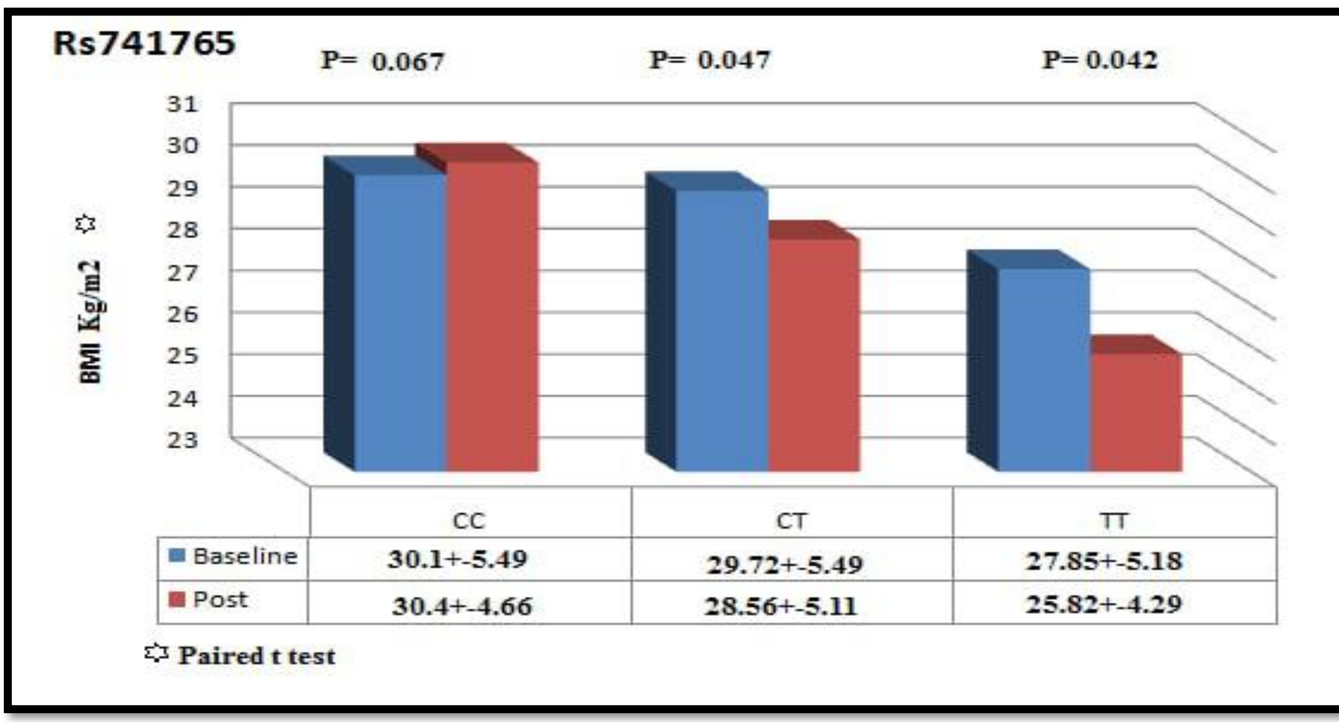


Figure (3-8) Baseline and after treatment levels of BMI among STK11 rs741765 genotypes

In STK11 rs8111699 SNP improvement in clinical parameters also differ significantly according to the genotypes and mutant allele carriers were recorded good improvement in menses regularity (X2= 187.88, Df= 5, P< 0.01), also in others clinical symptoms like; hirsutism (X2= 130.79, Df= 5, P< 0.01) and alopecia (X2= 13.35, Df= 5, P< 0.01), table (3-12).

Table (3-12) comparison of clinical parameters among STK11 rs8111699 genotypes

Clinical parameters		Patients genotypes						Chi-square	P value
		CC n. 90		CG n. 111		GG n. 34			
		Baseline	Post	Baseline	Post	Baseline	Post		
Regularity	Present	0	19	0	57	0	27	X2= 187.88 Df= 5	P< 0.01
	No Present	90	71	111	54	34	7		
Hirsutism	Present	86	81	79	42	28	9	X2= 130.79 Df= 5	P< 0.01
	No Present	4	9	32	69	6	25		
Alopecia	Present	73	71	73	54	22	14	X2= 13.35 Df= 5	P< 0.01
	No Present	27	19	38	57	12	20		

The change in BMI among the three genotypes was also differ. In wild genotype (CC) the change between baseline and post treatment value was non- significant at p value (0.065), while in heterozygous (CG) and mutant genotype (GG) it was significant at p values (0.047, 0.041), respectively, figure (3-9).

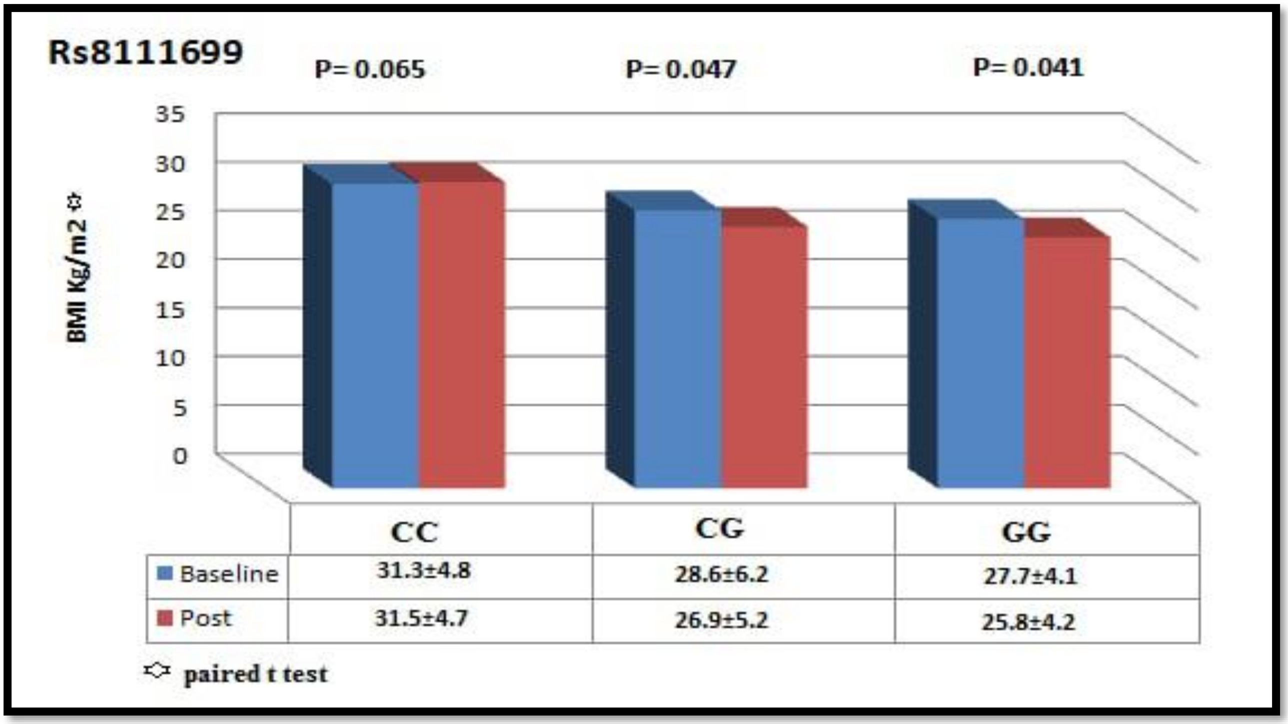


Figure (3-9) Baseline and after treatment levels of BMI among STK11 rs8111699 genotypes

3.7.4 Analysis of *STK11* rs741765 and rs8111699 Polymorphism on Metformin Efficacy with Comparison of Biochemical Parameters.

Pronounced differences in the efficacy of metformin was found in this study according to the STK11 rs741765 and rs8111699 genotypes, as reflected by a robust level of the main endocrine and metabolic parameters before and after three month of metformin therapy in each genotype in both SNP.

3.7.4.1 Analysis of *STK11* rs741765 and rs8111699 Polymorphism on Metformin Efficacy with Comparison of Endocrinal Parameters

Regarding to metformin efficacy with endocrine profile such as changes LH, FSH, LH/FSH ratio, FAI, and others endocrinal markers that measured in this study, there is clear variation in improvement among the three genotypes.

STK11 rs741765 SNP genotypes improvement was as the following: for, TT homozygotes (n. = 44) had robust metabolic improvements, CT heterozygotes (n. =109) had intermediate responses, and CC homozygotes (n. = 82) had almost no response, and the change in this parameters between the baseline and after treatment was non-significant among wild genotype carriers(CC) except for LH/FSH and FAI, while among the heterozygous genotype carriers(CT), the change in this parameters was significant except for TSH and total testosterone. In mutant allele carriers(TT), all the parameters were change significantly, table (3- 13).

Table (3-13) Endocrinal variables before and after metformin therapy according to STK11 rs741765 genotype in the studied patients.

Endocrinal data		Genotypes (n.235)					
		CC n.82	P value	CT n.109	P value	TT n.44	P value
FSH(mIu/ml)	Baseline	5.93± 1.93	0.058	5.73± 1.99	0.038	5.16 ± 2.13	0.027
	After 3 month	5.51± 1.53		6.36± 2.53		7.01 ± 2.87	
LH(mIu/ml)	Baseline	9.1± 4.84	0.052	10.48± 6.24	0.025	8.99 ± 5.68	0.021
	After 3 month	8.67± 4.87		8.5± 4.95		6.69 ± 4.45	
LH/FSH	Baseline	1.64± 0.9	0.047	1.91± 1.06	0.041	1.78 ± 0.81	0.024
	After 3 month	1.59± 0.9		1.36± 0.73		1.06 ± 0.64	
TSH(µlU /Ml)	Baseline	2.25± 0.87	0.92	2.3± 1	0.062	2.52 ± 0.96	0.044
	After 3 month	2.25± 0.79		2.41± 2.54		2.13 ± 0.9	
Prolactin (ng/ml)	Baseline	26.12± 14.3	0.062	23.86± 12.82	0.049	22.04 ± 12.3	0.031
	After 3 month	25.91± 10.63		20.34± 10.99		17.48 ± 10.1	
T.testosterone (ng/ml)	Baseline	0.65± 0.36	0.072	0.55± 0.34	0.057	0.72 ± 0.4	0.027
	After 3 month	0.71± 0.39		0.4± 0.27		0.32 ± 0.23	
SHBG (nmol/L)	Baseline	50.48± 19.82	0.052	41.43± 23.64	0.037	41.26 ± 18.9	0.038
	After 3 month	46.63± 19.77		50.62± 22.55		57.74 ± 17.8	
FAI	Baseline	6.92± 9.21	0.048	8.82± 14.22	0.028	6.1 ± 3.35	0.006
	After 3 month	5.26± 6.61		3.34± 6.28		1.95 ± 1.73	
Estradiol(pg/ml)	Baseline	49.87± 29.44	0.16	61.23± 56.64	0.033	58.79 ± 58.0	0.045
	After 3 month	49.77± 29.07		71.39± 47.6		78.01 ± 62.5	

Paired t-test, p<0.05 statistically significant, SD=standard deviation, LH luteinizing hormone, FSH follicular stimulating hormone, TSH thyroid stimulating hormone, SHBG sex hormone binding globulin, FAI free androgen index.

In rs8111699 SNP, GG homozygotes (n. = 34) had robust metabolic improvements, CG heterozygotes (n. = 111) had intermediate responses, and CC homozygotes (n. =90) had almost no response. The change in this parameters among wild genotype carriers (CC) was non-significant except for TSH and SHBG, it was significant. But among the heterozygous genotype carriers(CG) the change in parameters was significant except for FSH and LH, while in mutant allele carriers (GG) except LH/FSH ratio, the change in endocrinal parameters was significant, table (3-14).

Table (3-14) Endocrinal variables before and after metformin therapy according to STK11 rs8111699 genotype in the studied patients.

Endocrinal data		Genotypes (n.235)					
		CC n.90	P value	CG n.111	P value	GG n.34	P value
FSH(mIu/ml)	Baseline	6± 2.01	0.086	5.54± 1.54	0.068	5.67± 2.04	0.026
	After 3 month	6.39± 2.58		5.04± 2.04		7.14± 3.04	
LH(mIu/ml)	Baseline	9.38± 5.15	0.056	8.99± 4.91	0.059	10.14± 6.23	0.037
	After 3 month	8.09± 4.68		8.84± 5.38		6.67± 4.97	
LH/FSH	Baseline	1.68± 0.91	0.055	1.63± 0.89	0.021	1.86± 1.02	0.057
	After 3 month	1.29± 0.68		1.79± 0.79		1.04± 0.67	
TSH(µlU /Ml)	Baseline	2.37± 0.97	0.045	2.36± 0.85	0.042	2.5± 0.88	0.047
	After 3 month	2.49± 2.49		2.51± 1.01		2.08± 0.93	
Prolactin (ng/ml)	Baseline	26.3± 16.7	0.918	25.68± 12.23	0.029	24.46± 12.1	0.036
	After 3 month	20.7± 9.93		20.35± 10.77		16.06± 10.0	
T.testosterone (ng/ml)	Baseline	0.61± 0.39	0.07	0.63± 0.35	0.037	0.68± 0.36	0.026
	After 3 month	0.68± 0.41		0.41± 0.27		0.29± 0.22	
SHBG (nmol/L)	Baseline	49.32± 21.4	0.05	45.27± 21.16	0.017	40.28± 22.2	0.037
	After 3 month	49.49± 20.6		43.06± 19.94		58.98± 17.4	
FAI	Baseline	6.59± 8.23	0.056	7.36± 9.71	0.008	7.83± 9.04	0.019
	After 3 month	5.71± 3.28		2.84± 3.88		1.67± 1.28	
Estradiol(pg/ml)	Baseline	49.18± 31.1	0.058	56.38± 27.95	0.013	59.03± 64.19	0.008
	After 3 month	47.22± 55.4		68.41± 41.5		73.07± 50.7	

Paired t-test, $p<0.05$ statistically significant, SD=standard deviation, LH luteinizing hormone, FSH follicular stimulating hormone, TSH thyroid stimulating hormone, SHBG sex hormone binding globulin, FAI free androgen index.

So regarding to clinical and endocrinal parameters mutant allele carriers for each SNP TT and GG were improved well than wild allele one, predicted that there was a significant influence of STK*11* rs741765 and rs8111699 SNPs on metformin efficacy, accordingly the efficacy of metformin was increased as following TT>CT>CC for rs741765 and GG>CG>CC for rs8111699.

3.7.4.2 Analysis of *STK11* rs741765 and rs8111699 Polymorphism on Metformin Efficacy with Comparison of Metabolic Parameters

Clear variation regarding metabolic parameters upon metformin treatment among the different alleles were examined, such differences were found for 3 –months changes in FBS, insulin resistance, SHBG, lipid profile and others markers that measured in this study. In wild genotype carriers (CC) were showed non- significant change in metabolic parameters upon a period of therapy. In heterozygous genotype carriers (CT) the change was significant for; FBS, TG, LDL and T. cholesterol. In contrast to mutant allele carriers(TT), change was significant in all parameters except HbA1c, so good improvement was recorded among mutant allele carriers (T) in rs741765 SNP, table (3-15).

Table (3-15) Metabolic variables before and after metformin therapy according to STK11 rs741765 genotype in the studied patients.

Metabolic parameters		Genotypes No.235					
		CC n.82	P value	CT n.109	P value	TT n.44	P value
FBS(mg/dL)	Baseline	99.83± 11.44	0.61	95.98± 13.78	0.48	91 ± 13.96	0.026
	After 3 month	97.83± 11.92		90.33± 15.68		88.81 ± 10.4	
F.Insulin (mIu/L)	Baseline	24.35± 13.8	0.23	19.63± 14.11	0.52	21.11 ± 16.6	0.022
	After 3 month	24.5± 13.58		17.96± 10.43		16.15 ± 10.0	
HOMA IR	Baseline	6.01± 3.62	0.65	4.73± 3.64	0.71	4.46±3.43	0.049
	After 3 month	5.78± 3.4		4.04± 2.61		2.15 ± 0.93	
HbA1C%	Baseline	4.82± 0.58	0.15	4.9± 0.73	0.062	5.21 ± 0.732	0.053
	After 3 month	4.62± 0.63		4.77± 0.61		4.51± 0.76	
TG(mg/dL)	Baseline	118.87± 43.2	0.63	124.25± 44.19	0.044	123.3 ± 37.4	0.021
	After 3 month	116.66± 37.4		111.32± 39.14		103.7 ± 33.2	
LDL(mg/dL)	Baseline	101.9± 104.4	0.058	91.79± 30.36	0.041	96.87 ± 34.0	0.039
	After 3 month	85.09± 25.86		83.9± 25.99		80.72 ± 22.4	
HDL(mg/dL)	Baseline	47.85± 9.51	0.16	44.6± 9.56	0.057	43.81 ± 11.7	0.033
	After 3 month	47.4± 10.13		45.93± 9.26		47.81 ± 11.0	
T.Cholestrol Mg/dl	Baseline	170.65± 45.2	0.64	156.7± 38.03	0.047	152.4 ± 52.3	0.028
	After 3 month	167.5± 42.33		142.3± 40.43		132.4 ± 52.0	

Paired t-test, $p<0.05$ statistically significant, SD=standard deviation, FBS fasting blood sugar, HOMA-IR homeostatic model assessment of –IR HbA1c glycated hemoglobin TG = triglycerides, TC = total cholesterol, HDL-C = high density lipoprotein-cholesterol, LDL-C = low density lipoprotein-cholesterol.

In STK11 rs8111699 the improvement in metabolic parameters was also differ according to the genotypes and the variation in metabolic parameters. In wild allele carriers(CC) the change in all metabolic parameters was non – significant except for LDL, it was significant. While in heterozygous genotype carriers (CG) the change was non-significant only in HbA1c and HDL. Mutant allele carriers (GG) were showed significant change in this parameters except for HbA1c, table (3-16).

Table (3-16) Metabolic variables before and after metformin therapy according to STK11 rs8111699 genotype in the studied patients.

Metabolic parameters		Genotypes No.235					
		CC n. 90	P value	CG n.111	P value	GG n.34	P value
FBS(mg/dL)	Baseline	95.36± 11.8	0.59	97.99± 12.93	0.044	99.05± 11.8	0.024
	After 3 month	89.77± 15.4		90.36± 13.24		87.88± 11.5	
F.Insulin (mIu/L)	Baseline	18.31± 13.2	0.28	20.41± 13.89	0.047	25.69± 13.9	0.029
	After 3 month	22.37± 16.8		16.63± 9.88		15.52± 8.64	
HOMA IR	Baseline	4.98± 3.3	0.61	5.49± 3.61	0.031	6.21± 3.64	0.038
	After 3 month	5.15± 4.23		2.63± 1.13		3.62± 2.38	
HbA1C%	Baseline	4.58± 0.66	0.61	4.82± 0.61	0.073	4.89± 0.727	0.058
	After 3 month	5.37± 0.727		4.5± 0.64		4.46± 0.88	
TG(mg/dL)	Baseline	119.1± 37.0	0.58	120.74± 41.5	0.044	125.4± 41.5	0.025
	After 3 month	127.2± 40.6		109.63± 37.8		106.1± 35.8	
LDL(mg/dL)	Baseline	85.78± 25.9	0.042	100.28± 98.3	0.033	92.89± 33.5	0.032
	After 3 month	95.69± 25.9		83.26± 25.84		81.24± 22.2	
HDL(mg/dL)	Baseline	47.18± 9.62	0.19	45.62± 9.75	0.062	44.99± 9.8	0.046
	After 3 month	45.76± 8.6		43.17± 11.81		48.41± 10.9	
T.Cholestrol Mg/dl	Baseline	162.3± 46.0	0.68	166.86± 45	0.038	159.6± 39.5	0.035
	After 3 month	150.61± 53.8		145.14± 38.2		132.87± 58.	

Paired t-test, p<0.05 statistically significant, SD=standard deviation, FBS fasting blood sugar, HOMA-IR homeostatic model assessment of –IR, HbA1c glycated hemoglobin TG = triglycerides, TC = total cholesterol, HDL-C = high density lipoprotein-cholesterol, LDL-C = low density lipoprotein-cholesterol.

The comparisons were also adjusted for differences in baseline levels also, STK11 rs8111699 SNP was related with baseline insulin sensitivity by comparing baseline levels of FBS, F. insulin and HOMA IR and HbA1C among the three genotypes and revealed that the mutant (GG) genotype was associated with elevated baseline insulin level, with significant p values ware recorded except for HbA1C, table (3-17).

Table (3-17) Baseline comparison of metabolic parameters in STK11rs8111699

Data	Baseline level			
	CC	CG	GG	P-Value
n.	90	111	34	
FBS(mg/dL)	95.36± 11.87	97.99± 12.93	99.05± 11.86	0.05
F.Insulin level(mIu/L)	18.31± 13.25	20.41± 13.89	25.69± 13.97	0.008
HOMA IR	4.98± 3.3	5.49± 3.61	6.21± 3.64	0.02
HbA1C%	4.58± 0.66	4.82± 0.61	4.89± 0.727	0.23

Anova test was used, $p<0.05$ statistically significant, SD=standard deviation, FBS fasting blood sugar, HbA1c glycated hemoglobin, HOMA-IR homeostatic model assessment of –IR.

Bearing in mind that STK11 may be constitutively active, we postulate that carrying the G allele in the STK11 rs8111699 SNP can result in lower STK11 activity and therefore lower efficiency of AMPK phosphorylation by STK11. Conversely, the same G allele in the STK11 rs8111699 SNP was associated with more improvement in endocrine, metabolic parameters after treatment, accordingly, this SNP have a role in both baseline insulin sensitivity and metformin efficacy, predicted that STK11 rs8111699 SNP may thus play a broader role in PCOS by modulating the baseline condition as well as by the response to treatment. Similar result on metformin efficacy was noticed in T allele carriers of rs741765 upon metformin treatment, this effects were nearly absent in homozygous (CC) genotype carrier patients in both SNPs.

Chapter Four

Discussion

4. Discussion

Polycystic ovary syndrome is the most common endocrine disorder among infertile women (154) and a recent study was identified a wide variation among PCOS patients, moreover, it is complicated by epigenetic and environmental factors.

4.1 Analysis of demographic parameters results

Several demographic and biochemical parameters were analyzed among them and revealed a significant variation. In present study PCOS reported to be more prevalent in age groups less than 30 years old, the same finding was recorded (155), who reported that 73% of Iraqi PCOS women were young because in Iraq diagnosis of PCOS often associated with infertility and mostly start in the first year after the marriage and the majority of patients in current study were married, even though some symptoms may start at menarche age. Higher proportion was reported from urban population in comparison to rural counterparts, this result agree with(156) , it is likely that prevalence of PCOS varies significantly between urban and rural regions owing to different environments, lifestyles, incidence of obesity and dietary variation. Factors that may be play a role in this variations are: BMI, physical activity, family history, type of diet vegetarian or non-vegetarian and stress factor, also may be behind those variations was the location of collecting of the samples was in the city which allow easy arrival to this site compare to rural population.

Majority of patients with age at menarche in the 12 years old, (157) have opinion differ from current study and showed that the age at menarche is larger in obese patients, while in present study revealed that no correlation with obesity.

The syndrome is represented the most but treatable cause of infertility in women with reproductive age (158), and high percentage of infertility in this study may explain by, in addition to the genetic factors, the Iraqi environment suffers from pollution like vehicle emissions, power generators, small fires especially from oil, gas refineries, and war-induced pollution that might causes women infertile and increase infertility in females since the hormonal system in females is more sensitive to environmental variations. Moreover, Iraqi society suffered from many psychological difficulties that affect women's behavior leading to disturbance in the female hormonal system which might lead to PCOS, this in agreement with(159).

Hormonal imbalance interferes with growth and release of eggs from the ovaries which resulted in secondary infertility and relative infertility of these women lead to increase the interval between pregnancies and reduces the number of children in women with PCOS, this compatible with(160). Repeated miscarriages among PCOS patients in present study was prevalence, (161) reported that women with PCOS suffering from repeated miscarriage three times higher than normal due to hormonal disturbance. Progesterone synthesis deficiency of luteal phase and/or action is the major cause of spontaneous abortion due to elevation of both LH and insulin concentration.

Increase in progesterone concentration was found to be increased in the metformin-treated PCOS patients because it decrease both LH and insulin concentrations and result in minify the probability of abortion (162), study (163) was showed that elevated plasminogen-activator inhibitor-1(PAI-1) levels in PCOS have been found to be a risk factor for early spontaneous abortion (PAI-1 is a 52 kDa glycoprotein that inhibits plasmin formation during plasminogen activation and fibrinolysis and is the most potent inhibitor of fibrinolysis).

Metformin has been shown to reduce the risk of abortion in women with PCOS at high risk of pregnancy and neonatal complications by increasing some factors needed for implantation with pregnancy safekeeping, like insulin-growth factor-binding protein-1(IGFBP-1) and glycodelin levels that known as human placental protein-14 (PP-14). By contrast, metformin reduces factors increasing the risk of abortion, such as endometrial androgen receptor expression, (PAI-1), plasma ET-1 (endothelin-1). Most of these effects are probably mediated by the metformin-induced improvement in insulin sensitivity.

4.2 Analysis of clinical parameters results

Present study reported that none of the PCOS patients had normal menstrual cycles, this result in agreement with(164). Hormonal imbalance result in high levels of androgens and excess insulin can disrupt the monthly cycle of ovulation and menstruation(165), as well as, physiological link between irregular menses and anovulation relates to the persistence of estrogen production that arising primarily from extra glandular conversion of androgens to estrogens. Accordingly, irregular bleeding is a consequence of excessive endometrial proliferation due to unopposed chronic estrogen secretion. Restoration of regular menstrual cycle with metformin was reported in elevated percent among the patients in present study, this agree with(166) and (167), those previously were indicated that the percentage of regularity improvement by metformin not exceed 60% in PCOS patients.

Hirsutism and alopecia are the clinical features of hyperandrogenism of the syndrome, ordinarily hirsutism and alopecia may be occur simultaneously due to androgen production and increased FAI in patients with PCOS, resulted from an increased activity of androgens in the pilosebaceous units through action of 5-α reductase, an enzyme that transforms testosterone into the more active dihydrotestosterone(168), present results consistent with finding of (164). Insulin has direct and indirect influence on increasing serum androgen levels, so it has an important role in development of hirsutism and alopecia, nevertheless direct insulin effect by stimulation both ovarian and adrenal androgen secretion that can block the hair follicles causing what is called Androgenic alopecia (AGA). Indirect effect by decreasing SHBG, this effect increase FAI(169). Each affected hair becomes thinner in diameter, shorter in length and lighter in color, prevalence of hirsutism in PCOS patients in previous studies was (40 – 92 %) in European and American females, it is even more common in darker skin types and rare in Japanese and oriental females. Almost 10% of Caucasian females feature symptoms of hirsutism (170). Opinion of the present study boils down to that, the increase frequency of hirsutism and alopecia in obese patients compared with normal one which associated with increasing levels of androgens to peripheral tissues. Metformin may reduce hirsutism through amelioration of their hyperandrogenemia by reducing circulating insulin levels.

Obesity was correlated with insulin resistance (IR), this consistent with finding of (171), insulin acting through a raising production of androgens and resulted in weight gain typically in the abdomen. At ovarian level, insulin stimulates ovarian steroidogenesis by interacting with insulin and insulin growth factor type I receptors, accordingly in granulosa, thecal and stromal cells it decreases insulin growth factor binding protein-1(IGFBP-1) that regulates ovarian and cyst formation with adrenal steroidogenesis, furthermore insulin increases 17-hydroxylase and 17,20 lyase activity as well as stimulates the expression of 3-hydroxysteroid dehydrogenase in granulosa cells. Upregulates 17α-hydroxylase activity via PI3K, as the activity of this enzyme being responsible for synthesizing androgen precursors (172). In present study obesity was worsened and BMI was increased in about (4.3%), which might have been due to genetic factors and decreased physical activity during treatment period.

Diagnostic criteria of PCOS do not include being overweight, where obesity is a risk factor of PCOS, this result in agreement with(173). Metformin reduces BMI by decreasing insulin resistance and modulating the level of various peptides involved in controlling appetite like ghrelin, neuropeptide YY and adipokines, via hypothalamic adenosine 5- monophosphate-activated kinase (AMP Kinase) (89). Study of (174) incompatible with present result had showed that BMI did not change significantly after metformin

Body mass index is associated with dyslipidemia in patients with PCOS, this correlation due to increase BMI correlated with insulin resistance result in increased fatty acid synthesis through fatty acid oxidation because of decreasing the activity of AMPK that act by phosphorylation or inactivation of Acetyl-CoA-Carboxylase (ACC)(89).(137) was reported that PCOS and weight both of them are independent cardiovascular (CV) risk factors and Endothelin-1(ET-1)(well-known marker of abnormal vascular reactivity) was normal in lean control and PCOS patients and increased in overweight group.

4.3 Analysis of endocrinal parameters results

Hormonal assessment was shown significant variation in present study, elevated LH value was reported among high percent of the patients mostly in overweight /obese patients, due to insulin resistance that exaggerated GnRH pulsatile, this compatible with finding of(175). The exaggerated GnRH induced LH response is reduced with metformin by reduction in serum androgen concentrations, and hyperinsulinemia that considered to be a cause of altered pituitary sensitivity to gonadotrophin releasing hormone which leads to over secretion of LH, this in agreement with(176), accordingly PCOS women may therefore had less hypothalamic and pituitary androgen exposure, as its role is either direct or indirect actions on pituitary gland, theca cell compartment or both (177), direct effect of metformin on ovarian steroidogenesis is independent of its effects on insulin sensitivity(178). (174), showed that the decreasing in LH hormone was non significant after metformin therapy, whereas (179) reported that LH level remain unaffected upon treatment with metformin.

Low level of FSH can be explained by the increase of the conversion of androstenedione in adipose tissue which additionally stimulates LH and inhibits FSH, lead to hormonal disturbance (180), this finding compatible with opinion of(181). Increase FSH by metformin resulting from insulin sensitization, which suppress activity of LH and increasing FSH level leading to follicular development and ovulation. Another study showed that there is no significant change in FSH level after treatment with metformin and reduction in LH level only remained significant suspected that pituitary LH secretion was more sensitive to metformin than FSH secretion (182), while (183) was showed that the metformin markedly reduced FSH level, this effect was exerted by inhibition of basal and ligand-induced up-regulation of follicular stimulating hormone receptors (FSHR) expression by metformin (184). Elevated LH/FSH ratio presumably due to high levels of LH and reduced production of FSH(161) and the significant improvement of this ratio upon metformin was recorded, this result was consistent with finding of(181). (185) demonstrated that LH value or LH/FSH ratio is not helpful as a diagnostic criteria for PCOS, because LH levels may vary dramatically over short periods of time , it is probably ill-advised to consider the diagnosis based on the level of LH in individual patients.

Reducing effect of metformin to TSH including; an increase in number and sensitivity of TSH and thyroid receptors, sensitization of cells in anterior pituitary to the effects of thyroxine, augmentation of the central hypothalamic dopaminergic response in TSH secretion and/or a direct effect of metformin on TSH secretion through the hypothalamic AMP Kinase (186), in present study metformin suppressive effect on TSH more reported in overweight women, this the same opinion of (187). Prolactin secretion by the anterior pituitary is primarily under the inhibitory control of dopamine which secreted into the portal circulation, it has been suggested that hyperprolactinemia seen in some patients with PCOS implies a defect in hypothalamic dopaminergic activity, as well as dopamine infusion lowers LH secretion and decreased dopamine activity in the hypothalamus, in addition, estrogen secretion in PCOS is chronic and unopposed by progesterone. Chronic exposure to estrogen stimulates lactotrope growth and prolactin production, which probably accounts for the occasional increased levels observed in PCOS. Hyperprolactinemia also is associated with increased adrenal production of dehydroepiandrosterone sulfate (DHEAS) is a male sex hormone, but in general the elevated adrenal androgen production seen in patients with PCOS does not correlate with hyperprolactinemia(188).

Metformin action on prolactin correlated with the degree of a reduction in HOMA-IR, and there is signal pathways regulating prolactin production and release, and signal pathways affected by insulin may overlap to some extent, study of (189) was showed that metformin penetrate blood–brain barrier and its content in the pituitary is higher than in any other brain structure, so the pituitary is an important target for metformin action and that the prolactin lowering effect resulted from its action at the level of pituitary lactotropes (184) was revealed that there is no effect of metformin on prolactin level in PCOS patients, this discrepancy may be explained by difference in study populations and therapy period.

Elevated total testosterone level in present study was consistent with results of (190), while incompatible with(191) who revealed that no alteration was noted for testosterone in PCOS patients. present finding reported that there was a clear association existing between serum insulin and total testosterone demonstrated that increased level of insulin will lead to increases the endocrinological and morphological response of the ovaries and women with PCOS usually exhibit increased expression of cytochrome P450-C17α (CYP17). Cytochrome P450-C17 α, which is a key enzyme in androgen synthesis, it is related to increase levels of insulin which results in increased activity of 17α-hydroxylase and 17,20-lyase in ovarian theca cells. This dysregulation of CYP 17 enhances the production of 17α hydroxyprogesterone, androstenedione and testosterone(192) , as consequence high level of total testosterone is produced, decreases in ovarian CYP450 C17α activity with total testosterone have been improved following metformin therapy, secondary result of the decrease in insulin secretion(95), another study showed that metformin directly inhibit ovarian androgen production in human ovarian cells independent of changes in weight, metabolic parameters, or insulin sensitivity (192), this direct action of metformin on ovarian steroid secretion could secondarily induce an improvement of obesity, insulin action, and hyperinsulinemia(193), while (194) was showed that testosterone dropping by metformin is not significant.

Sex hormone-binding globulin (SHBG), is a (93.4-kDa) glycated homo-dimeric plasma transport glycoprotein produced by hepatocytes, SHBG receptors (RSHBG) are expressed in sex steroid-dependent cells and tissues such as ovaries, endometrium, prostate, colon, hypothalamus, breast, placenta, liver, epididymis, immune cells, and cardio myocytes. Its binds and controls the levels of sex-hormones within the circulation by control of their respective metabolic clearance rates (37). Reduced SHBG level is commonly companied with hyperandrogenism, IR, and obesity. Testosterone and SHBG concentrations are interdependent and ratio between them represent FAI.

Metformin administration in PCOS patients could indirectly contribute to increase SHBG concentration, by improving insulin sensitization, weight loss, and carbohydrate metabolism. SHBG concentration is related to the decrease in BMI by the changes in hepatic metabolism resulting in decreased fat metabolism and improved protein synthesis including SHBG which are consequences of weight loss, as a result symptoms of androgenization reduce, this result compatible with results of(195). FAI is an indicator for hyperandrogenism, which play a major role in the development of severe endocrine and metabolic disturbances which associated with the syndrome, elevated level of FAI in present study was conforming with finding of (196), accordingly suppressed SHBG in the presence of high or normal total testosterone leading to an increase in levels of androgen with a consequent increase in peripheral androgen activity and glucose intolerance among women with PCOS, dropping effect of metformin to FAI in present study was consistent with (194). Improvement in FAI that occurred in studied patients not related to presence or absence of insulin resistance and the reduction in hyperandrogenaemia could secondarily induce an improvement of central obesity and insulin action this opinion compatible with finding of (197).

Decrease level of estradiol (E2) among the patients due to granulosa cells generate very little estrogen because there is a lack of mature follicle development (low FSH), that result in less activation of aromatase enzyme which is a key enzyme in granulosa cells of the ovary for estrogen synthesis(198).

4.4 Analysis of metabolic parameters results

Metabolic parameters were also showed a significant variation among the patients, to assess insulin resistance, the homeostatic model assessment index (HOMA-IR) was used, hyperinsulinemia reported among the most patients this finding was in agreement with the result reported by(199), that showed (50–60%) of PCOS patients have insulin resistance, because insulin acts by stimulation of ovarian androgen production and enhancing serum luteinizing hormone pulses and activity that enhances ovarian cytochrome P45017α activity in PCOS patients as evidenced by identification of insulin receptors in human pituitary tissue (200), its effect on muscles and adipose tissues through increasing plasma FFA, as well as increased levels of plasma IGF-1 potentiate the activity of LH concomitant with suppression of sex hormone binding globulin synthesis from the liver. After metformin therapy, serum hormone status documented a marked fall in fasting serum insulin. Metformin exerts its effect by promoting peripheral glucose utilization and reduce CYP17α activity that decreasing the ovarian androgen production and this occur in both insulin-resistant and non-resistant patients, this result was in line with the finding of (194) and disagree with (201) who showed that metformin has good effects on patients with insulin resistance only and may support the idea that metformin improves insulin sensitivity through weight loss. (202) was showed that improvement of insulin sensitivity has been observed without a significant change in BMI and improvement in insulin sensitivity was matched by a reduction in ovarian hyperandrogenism and these effects appeared to be independent of weight loss.

Lowering effect of metformin on fasting glucose and HbA1c done by inhibition of hepatic glucose production, in peripheral tissue it increases glucose uptake and utilization into muscle tissue by its role in increasing GLUT 4 receptors expression (203), as well as decreases intestinal glucose uptake and increases insulin sensitivity in peripheral tissues, in addition metformin has antilipolytic effects by restricting FFA efflux from adipose via improving of insuline sensitivity and glucose uptake(204).

Dyslipidemia resulted from hyperinsulinemia, androgen excess and obesity, all this factors may contribute to the abnormalities of lipid metabolism (205). Increases plasma FFA and insulin concentration stimulated the synthesis and secretion of VLDL in the liver resulting in hypertriglyceridemia, which in turn enhances post-prandial accumulation of lipoproteins (LDL-C, VLDL) in plasma with lowering of HDL-C (206). Insulin resistance play a key role in development of endothelial damage, inducing disturbances of subcellular signaling pathways common to both insulin action and nitric oxide production and it could also act by increasing (ET-1) levels (137).

Hyperandrogenism decrease the activity of plasma hepatic lipase, an enzyme that is remarkably sex steroid sensitive , so effect on HDL-C expression in PCOS (207). Beside, in granulosa cells Apo lipoprotein A-1 (Apo A-I) major structural protein component of HDL-C particles was reduced, this may influence the expression of steroidogenic enzymes and the production of the steroid hormone progesterone and inhibition of LDL oxidation, and removal of toxic phospholipids (208).

Metformin treatment improves lipid profile in patients with PCOS and this finding was reported also by Hoffman and Ehrmann.,2008(209), by significantly increased HDL- C and reduced the levels of LDL-C, TC and TG, it acts by suppression of acetyl CoA carboxylase activity, an important rate-controlling enzyme for the synthesis of malonyl-CoA, a critical precursor in the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation, therefore lead to decrease fatty acid synthesis, with increased mitochondrial fatty acid oxidation mainly through the role of metformin in AMPK-signaling pathway, accordingly, it regulates the partitioning of fatty acids between oxidative and biosynthetic pathways(83) , ordinarily, it reduce the lipid uptake or synthesis in the intestine and in the hepatocytes, consequently the decreased in the releasing of (FFAs) from adipose tissue could also partly explain the improvement of lipid profile during metformin treatment, at least in obese patients (210). Inhibition of FFAs releasing (i.e. lipolysis) from adipose tissue will decrease the competition between serum glucose and FFAs as energy substrates in peripheral tissues might result in an improvement of glucose oxidation and consequently insulin sensitivity and hyperinsulinemia, as summary metformin improved dyslipidemia either directly through its action on fatty acid metabolism in the liver or indirectly by improving hyperinsulinemia. Current study revealed that increasing dyslipidemia mostly correlated with age this finding was the same result revealed by (211).

4.5 Analysis the results of the comparison of clinical, endocrinal and metabolic parameters between non overweight\obese and overweight\obese patients

Elevated LH value mostly in overweight/obese was recorded compare with non-overweight/obese at baseline level, this compatible with finding of(175), and disagree with (212), that showed non-obese and obese PCOS patients do not represent distinct pathophysiological subsets of this disorder, so did not modify mean LH concentrations, nevertheless LH pulse frequency or amplitude and LH level improvement after therapy occurred in both groups but clearer with obese women, this result disagree with (213) which showed that improvement occurs within non-obese PCOS patients only, consequently in present study the dropping in prolactin level revealed in patients with BMI >25 more than lean body weight due to more improvement in insulin sensitivity(2) reported that weight loss within (5–7%) leads to more regular periods of menstrual cycle and improvement in infertility, moreover it prevents type 2 diabetes, circulatory diseases and endometrial cancer. However, current findings disagree with (214), who suggested that the impact of obesity in PCOS is not reflected in discernible changes in gonadotropin release or in the gonadal steroid feedback environment , as well as insulin does not have a major role in the perpetuation of PCOS, since obese and non-obese PCOS women had similar reproductive hormone levels despite significantly different degrees of hyperinsulinemia.

Present studies reported that obese PCOS patients have more severe hyperandrogenism and related clinical features as hirsutism and alopecia than normal weight one, because of excess adipose tissue in obese patients creates the paradox of having both excess androgens (which are responsible for hirsutism and alopecia) and estrogens (which effect GnRH pulsatile), that result in high insulin level and metabolic disorder(48) . (215) was showed that metformin increased insulin sensitivity and improved other parameters in obese women while non-obese women did not benefit from metformin, but (216) illustrated that metformin lowering effect to BMI was depend on the dose where more response occurs with high dose.

4.6 Analysis of genetic results

4.6.1 Analysis the results of demographic parameters with STK11 rs741765 and rs8111699 genotypes

Genetic analysis of STK11 rs741765 and rs8111699 SNPs revealed a significant variation among the genotypes, present study was showed that highest percentage in both SNPs genotypes were from urban population, due to elevated number of the patients from urban population in current study in comperison with rural one, in addition their was slight differ in percentages among the genotypes in the same residence which gave no evidance to predict genotypes distribution. Maximum percentage of age at menarche was 12 years old in both SNPs genotypes. Mutant allele carrieres (T ,G) were showed slightly higher infertility percent than C allele at baseline levels, this may be due to impairing the CAMP signaling pathway and lower efficiency of AMPK phosphorylation by STK11at baseline levels among mutant alleles carriers,

4.6.2 Analysis the results of clinical parameters with STK11 rs741765 and rs8111699 genotypes

Mutant allele carriers were showed significant improvement of menses regularity, hirsutism and alopecia after a period of therapy. This mutant alleles in STK11 gene are increase metformin efficacy with the likelihood of AMPK phosphorylation by

STK11 at threonine 172 subunit with subsequence mechanism that improve insulin resistance, FAI, and ovulation in PCOS patients(217), compared with wild type carriers where no or slightly improvement was recorded among them, so hirsutism and alopecia improved well in mutant genotypes (TT and GG) in comparison with other genotypes.

4.6.3 Analysis the results of endocrinal and metabolic parameters with STK11 rs741765 and rs8111699 genotypes

Pharmacological mechanism of metformin is by increasing utilization efficiency of plasma glucose by enhancing a body's sensitivity of insulin in mutant allele carrier by activation of AMPK that represent an important regulator of glucose and lipid metabolism and finally more improvement, this mechanism interrupted with wild CC genotype that showed no response to metformin. Present results compatible with (112), who studied ovulation rate in STK11 rs8111699 variants and found a stepwise increase in ovulation rate from C/C to C/G to G/G genotype in patients with PCOS treated with metformin.

STK11 and AMPK are not direct targets of metformin , but they are necessary for metformin actions (218). The STK11-AMPK signaling pathway is one of the key regulators of insulin sensitivity and glucose homeostasis, playing a critical role in metabolic processes such as fatty acid synthesis and gluconeogenesis not only by allosteric mechanism via CAMP, but also through phosphorylation of a key threonine residue (Thr172) on the catalytic subunit, the latter is catalyzed by STK11 and metformin is frequently used in research as an AMPK agonist which activate a necessary element in cell metabolism that is required for maintaining energy homeostasis(99). It has been proposed that STK11 is constitutively active and that metformin exerts its effect on PCOS patients through a modification in AMPK, rendering it a better substrate for STK11, which can phosphorylate within the activation loop of the α-subunit (219) , so STK11 gene is required for metformin efficacy by modulation of insulin sensitivity.

STK11 rs8111699 SNP reported to be associated with baseline insulin sensitivity, and carrying the G allele can result in lower STK11 activity and therefore lower efficiency of AMPK phosphorylation by STK11. AMPK pathway is a master regulator of glucose that increase utilization efficiency of plasma glucose by enhancing a body's sensibility of insulin and lipid metabolism with pleiotropic actions in the liver, skeletal muscle, pancreas, and brain (111), as consequence improvement in endocrine-metabolic profile and better response (220) revealed that a deletion of STK11 in the liver of adult mice resulted in hyperglycemia with increased gluconeogenic and lipogenic gene expression , in the other hand, studies on diabetic patients revealed that polymorphisms in the STK11 gene have been associated with different metabolic disorders(105). Results of present study consistent with (111), who studied the candidate genes polymorphism that may play an important role in PCOS improvement after period of treatment with metformin, this included *STK11*, estrogen receptor 1 (*ESR1*), CYP genes (*CYP2C9* and *CYP2D6*) and concluded that in *STK11* gene polymorphism was associated with a response to metformin in PCOS patients. The present study was the first study which investigate the relationship between STK11 rs741765 and rs8111699 genetic polymorphism and metformin therapeutic efficacy in Iraqi population specifically in Kerbala province.

Conclusions and Recommendations

Conclusions

1) Metformin treatment for three months in newly diagnosed polycystic ovary syndrome patients resulted in a significant improvement in clinical, metabolic and endocrinal parameters that measured in present study.
2) Polymorphism of Serine-threonine kinase11 gene (*STK11) C/T of rs741765 and C/G of rs8111699 SNPs* was associated with metformin efficacy and mutant alleles carriers in both SNPs (TT) and (GG); were showed a significant improvement in clinical, metabolic and endocrinal parameters that measured in present study.
3) Serine-threonine kinase11 gene (STK11) rs8111699 SNP was associated with baseline insulin sensitivity and carrying the G allele resulted in elevated insulin level compare with wild (C) allele carriers.
4) No strong association was found between genotypes variation with residence and age at menarche among the studied patients.
5) Strong association was found between body mass index and dyslipidemia among studied patients, with high prevalence of dyslipidemia was among obese patients.
6) A significant difference was found in response to treatment between overweight / obese patients and those non overweight / obese body one, mainly in metabolic parameters.

Recommendations

1) Relationship between metformin and the Serine-threonine kinase11(*STK11*) polymorphism will be better understanding regarding response in patients with PCOS after verification for a longer time.

2) Based on the results achieved, metformin drug could be used as a part the treatment of polycystic ovary syndrome and prescribed at a dose of 500mg twice daily for three months.

3) Serine-threonine kinase11 gene (STK11) rs8111699 (C/G) polymorphism can be used as a biomarker to indicate the insulin resistance in polycystic ovary patients.

Future Work

1) Benefiting from the genetic results of the current study on patients' women in Kerbala province to study other patients treated with metformin for Type 2 diabetes mellitus with fasting glucose impairment.

2) Trying to discover more details about the relation of Serine-threonine kinase11 (STK11) activity in pathogenicity mechanisms of polycystic ovary syndrome.

3) Study other SNPs of STK11 gene which effect CAMP signaling pathway.

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Appendices

Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Kerbala
College of Pharmacy
Scientific section

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الصيدلة
شعبة الشؤون العلمية

العدد : ع/6/ 2562
التاريخ : 7 / 11/ 2019

الى /دائرة صحة كربلاء/ قسم التدريب والتنمية البشريه / وحدة البحوث

م / اشراف بحوث

تحية طيبة

من مبدأ التعاون و لتعزيز المسيرة العلمية بين كليتنا و دائرتكم يرجى التفضل بالموافقة على بيان عدم ممانعتكم من اشراف الطبيبة الاستشارية (**د.حميدة هادي عبد الواحد**) على البحث الموسوم

(association of genetic polymorphism of LKB/STK11 with therapeutic response of metformin in women)

لطالبة الدراسات العليا / ماجستير الادوية و السموم في كليتنا (**منتهى رحيم حسين**) كمشرف ثاني .

شاكرين تعاونكم معنا خدمة للصالح العام... مع التقدير

أ.م.د احمد حقي اسماعيل
معاون العميد للشؤون العلمية
2019/ 11 /

ورة عنه إلى:
....
مكتب السيدالعميد المحترم ، للتفضل بالاطلاع مع التقدير.
مكتب معاون العميد للشؤون العلمية ، للتفضل بالاطلاع مع التقدير.
شعبة الشؤون العلميه / للحفظ مع الاوليات.
الصادرة .

Ministry of Higher Education
and Scientific Research
University of Karbala
College of Pharmacy
Department of Postgraduate Studies

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

Issue No.:
Date:

العدد: د.ع/ 6 / 2651
التاريخ: 17 / 11 / 2019

الى/رئاسة جامعة كربلاء/قسم الدراسات العليا
م/اقرار البحوث العلمية

تحية طيبة..

نود اعلامكم باجتماع اللجنة الاخلاقية السريرية والمشكلة حسب الامر الاداري المرقم (د/2317/8) في (2019/10/1) و المتضمن اقرار صلاحية اجراء وتنفيذ البحوث الخاصة بطلبة الدراسات العليا (الماجستير) في فرع الادوية والسموم.

مع التقدير..

المرفقات:-

-الامر الاداري انفا
محضر اللجنة الاخلاقية انفا-

أ.م.د. احمد حقي اسماعيل
معاون العميد الشؤون العلمية
2019 / 11 / 17

نسخة منه الى:
- مكتب السيد العميد ، للتفضل بالاطلاع .
- مكتب معاون العميد للشؤون العلمية .
- شعبة الدراسات العليا للحفظ مع الاوليات .
- الصادرة .

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العراق- محافظة كربلاء- مكتب بريد كربلاء- ص ب 1125

استمارة ملئ المعلومات الديموغرافية للمرضى

No.	Patient name	Address	Age	BMI	B.P	Hirs.	Alop.	Marital s.	No. of kids	times of abortion	Presence of other diseases	Smoking	Menstrual cycle		
													Regular	Irregular	frequency

استمارة مليء المعلومات الحيوية للمرضى

No.	Patient name	FSH	LH	TSH	Prol.	T.T	SHBG	F.A.I	Esdra.	F.G	F.I	HbA1C	TG	LDL	HDL	TC

الخلاصه

الاساس النظري: يعد مرض تكيس المبايض من أكثر اضطرابات المبيض شيوعًا وينتج بسبب ارتفاع هرمون الذكوره (الاندروجين) حيث تشمل اعراضه عدم انتظام الطمث او انعدامه او غزارته وكذلك زياده نمو الشعر في انحاء الجسم و حب الشباب والعقم .

الاهداف:

تهدف الدراسة الحالية إلى التحقق من ارتباط تعدد الأشكال الجينيه لجين LKB\ STK11 مع الاستجابه لعلاج المتفورمين بعد اعطاءه لمده ثلاثة اشهر للنساء المريضات بمتلازمه تكيس المبايض في مدينة كربلاء.

طريقه العمل:

اجريت هذه الدراسة الاستطلاعية لمئتين وخمسة وثلاثين مريضه عراقيه شخصوا لاول مره بالإصابة بمتلازمة تكيس المبايض بناءً على معايير روتردام من قبل الطبيبه الاختصاص في استشاريه العقم لمستشفى النسائيه والتوليد التعليمي في كربلاء / العراق.

بدأت الدراسة بتناول عقار الميتفورمين بجرعة 500 ملغم مرتين في اليوم و لمده ثلاثة أشهر. استبعدت من الدراسة النساء اللاتي يعانين من خلل وظيفي في الغدة الدرقية وتضخم الغدة الكظرية أو اللواتي يستخدمن الأدوية الخافضة لسكر الدم كبديل عن الميتفورمين او الأدوية التي تتداخل مع آلية عمل الميتفورمين.

سجلت المعلومات الديموغرافيه لجميع المرضى والتي شملت العنوان و العمر و مؤشر كتلة الجسم و قيمة ضغط الدم و التدخين و انتظام الدورة الشهرية و الحالة الزوجيه وعدد الأطفال وعدد مرات الاجهاض.

سحبت عينه الدم وريديا بمقدار (8 مل) من جميع المرضى ، حيث استعملت (5 مل) للاختبار الجيني وقياس الجلوكوز وHbA1C . وضع (3 مل) في أنبوب جل ، وطردت مركزيا بسرعة 3000 دورة في الدقيقة لمدة 10 دقائق حيث استخدمت في قياس المؤشرات التاليه:استراديول, الأنسولين ,LH, FSH, TG, LDL-c, SHBG, HDL-c, الكولسترول, TSH, البرولاكتين و التستوستيرون الكلي.

النتائج:

اظهرت الدراسه ان استعمال علاج المتفورمين بجرعه 500ملغم لمده ثلاثه اشهرادى الى تحسن واضح بنسبه عاليه من المريضات بمقارنه المؤشرات السريريه والحيويه للمريضات قبل وبعد العلاج.

وكما اظهرت نتائج الدراسه ان التغاير الجيني STK11rs741765 و STK11 rs811699 له دور فعال في مدى الاستجابه للمتفورمين.حيث ان فعاليه المتفورمين تزداد ضمن النسق الاتي بالنسبه للطفره STK11rs741765، TT> CT> CC اما بالنسبه للطفره STK11rs8111699 فأن الزياده بفعاليه المتفورمين كانت كالاتيGG> CG> CC ،وايضا سجلت الدراسة ان التغاير(GG) له علاقه بحساسيه الانسولين عند مقارنه المستويات مع بقيه المتغيرات الجينيه قبل بدء العلاج.

الاستنتاج:

استنتج من الدراسة أن تعدد الأشكال الجينيه (STK11 rs741765،rs811699) يرتبط بعمل المتفورمين ومن ثم الاستجابة له في المرضى المصابين بمتلازمة تكيس المبايض في مدينه كربلاء ، سجلت الدراسة الحالية ان نسب النساء اللواتي أستجبن للعلاج ازداد بزياده (T) أليلrs741765 وكذلك بالنسبه للطفره(G) اليل rs8111699كذلك اثبتت هذه الدراسه ان الاليل (G) rs8111699 مرتبط بحساسيه الانسولين.

وزاره التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الصيدلة

ارتباط تعدد الأشكال الجيني لجين الـSTK11
مع الاستجابة العلاجية للميتفورمين لدى النساء المصابات بمتلازمة تكيس المبايض

رساله مقدمة الى

مجلس كليه الصيدلة/ جامعه كربلاء

كجزء من متطلبات نيل درجه الماجستير في الادويه والسموم

من قبل

منتهى رحيم حسين

بكلوريوس صيدلة، جامعة الكوفة(2006)

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1442هجري **2020 ميلادي**