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University of Kerbala
College of Pharmacy

Effect of Genetic Polymorphism of Organic Cation Transporter-1 on the Therapeutic Response to Metformin in Women with Polycystic Ovary syndrome in Iraq

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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2021 AD 1442 AH

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صدق الله العلي العظيم

من سورة فاطر الآية 28

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We certify that this thesis (***Effect of Genetic Polymorphism of Organic Cation Transporter -1 on the Therapeutic Response of Metformin in Women with Polycystic Ovary syndrome in Iraq)*** was prepared under our supervision at the Department of Pharmacology and Toxicology in College of Pharmacy / University of Kerbala, as a partial requirement for the degree of Master of Science in Pharmacology and Toxicology

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Dedication

To the Soul of My grandmother, Allah Almighty bless her

My great parents, who never stop giving
themselves in countless ways

My Sisters who stands by me
when things look bleak,

And finally to all women who dream to be a mother

I dedicate this thesis

Acknowledgment

First, I am grateful to God for the good health and wellbeing that were necessary to complete this thesis.

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I wish to express my thanks to all participants in this study and to all others who helped me in any way to complete this thesis.

Baraa

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List of Abbreviations	
Abbreviations	Key
ACC	Acetyl-coA carboxylase
ACTH	Adrenocorticotrophic hormone
AE-PCOS	Androgen excess –polycystic ovary syndrome society criteria 2006
AMP	Adenosine monophosphate
AMPK	Activated mitogen protein kinase
ATP	Adenosine triphosphate
BMI	Body mass index
CE	Cholesterol esterase
CHOD	Cholesterol oxidase
DHEAS	Dehydroepiandrosterone sulfate
ECLIA	Electrochemiluminescence
ELISA	Enzyme linked immunosorbent assay
FAI	Free androgen index
FSG	Fasting serum glucose
FSH	Follicle-stimulating hormone
FSI	Fasting serum insulin
Glut4	Glucose transporter 4
GnRH	Gonadotropin-releasing hormone
GWAS	Genome wide association study
HA	Hyperandrogenism
HbA1c	Glycosylated hemoglobin

HDL	High density lipoprotein
HOMA	Homeostasis model assessment
IGF	Insulin-like growth factor
IGFBP-1	Insulin-like growth factor binding protein 1
IR	Insulin resistance
IVF	In vitro fertilization
LDL	Low-density lipoprotein
LH	Luteinizing hormone
MAPK	Mitogen activated protein kinase
MATE	Multidrug and toxic compound extrusion
NIH	National institutes of health
OCT1,OCT2,OCT3	Organic cation transporter 1-3
PCOM	Polycystic ovary morphology
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
POD	Peroxidase
SHBG	Sex hormone binding-globulin
SLC22A	Solute carrier transporter 22A
SNP	Single nucleotide polymorphism
TC	Total cholesterol
TG	Triglyceride
TM	Trans membrane
TSH	Thyroid stimulating hormone
UV	Ultraviolet

List of Normal Laboratory Value	
Parameters	**Normal value**
FSH	2.9-12 m.lu/ml
LH	1.5-8 m.lu/ml
TSH	0.27-4.2 ulU/ml
Prolactin	6-29.9 ng/ml
Total Testosterone	0.06-0.82 ng/ml
SHBG	15 - 120 nmol/L
FSG	70-120 mg/dl
Fasting Insulin	2 - 25µIU/ml
HBA1c	4.4-6.5 %
TG	0-149 mg/dl
LDL	0-100 mg/dl
HDL	40-60 mg/dl
Cholesterol	50-199mg/dl

Abstract

Background:

Polycystic ovary syndrome (PCOS) is a highly prevalent endocrine-metabolic disorder that implies severe consequences to female health, including infertility. Although its exact etiology remains elusive, it is known to feature several hormonal disturbances, including hyperandrogenemia, insulin resistance (IR), and hyperinsulinemia. Insulin appears to disrupt all components of the hypothalamus-hypophysis-ovary axis and ovarian tissue.

The determinants of the variability in the clinical response to metformin as insulin sensitizer drug in women with PCOS are multifactorial. Organic cation transporter 1 (OCT1) plays a crucial role in the hepatic uptake of metformin. Several OCT1polymorphism in recent studies showed a significant effect on metformin response.

Aims :

The aims of current study were to correlate between polymorphisms in OCT1 with the variability in the response to metformin in Iraqi women with PCOS ; and find out the effect of OCT1 polymorphism on incidence of PCOS.

Subjects and Methods:

This prospective case-control study was done at Kerbala teaching hospital for obstetrics and gynecology, department of infertility as well as private clinic. Two hundred twenty two PCOS patients and 106 healthy control aged 20-40 years were enrolled in the study. The consultant

gynecologist diagnosed PCOS patients according to Rotterdam criteria and prescribed metformin 500 mg tablet twice daily for 3 months. Hormonal and biochemical tests and genetic study were performed for all patients and healthy control participants at the beginning of the study and after 3 months of patients flow up. Hormonal and biochemical tests were done in order to determine metformin response.

Results:

The results demonstrated that the alleles frequencies of OCT1 (R61C and 420del) were similar in PCOS and control groups. Concerning metformin response most patients with reference (wild type) and heterozygous alleles of OCT1 (R61C, and 420del) showed statistically significant hormonal and metabolic response to metformin, while patients with mutant alleles showed less or statistically non-significant response.

***Conclusions*:**

Genetic variation in OCT1 may be associated with heterogeneity in the response to metformin in Iraqi women with PCOS; while it was not associated with incidence of PCOS.

Chapter One
Introduction

Chapter One

Introduction

1.1 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) was first described by Stein and Leventhal in 1935 when they noted an association between the presence of bilateral polycystic ovaries and signs of amenorrhea, oligomenorrhea, hirsutism, and obesity [(1)]. This condition is also named as Schlerocystic Ovaries, Multicystic ovaries, Stein Leventhal Syndrome [(2)].

PCOS is one of the most common endocrine disorders in women of reproductive age. The main features include menstrual irregularity, oligo-anovulation, infertility, as well as hirsutism, acne and polycystic ovarian morphology on ultrasonographic imaging [(3)]. The aetiological causation behind PCOS is yet to be precisely defined, but it is evident that familiar genetic predisposing factors interact with environmental stimuli both in utero and in pre-pubertal life [(4)].

The main implicating pathophysiological features include insulin resistance and primary ovarian dysfunction, which consequently contribute to both the dysregulation of the reproductive system and the increased likelihood of developing systemic conditions, such as obesity, type 2 diabetes mellitus[(5)], cardiovascular disease [(6)], and neuropsychological disorders[(7)].

1.2 Clinical Presentation

The clinical presentation of PCOS varies widely, it may present with amenorrhea, infertility, features of hyperandrogenemia (HA), signs of metabolic disturbances like insulin resistance, and dyslipidemia[8]. Women with PCOS often seek care for menstrual disturbances, clinical manifestations of hyperandrogenism, and infertility. Menstrual disturbances commonly observed in PCOS include oligomenorrhea, amenorrhea, and prolonged erratic menstrual bleeding[9]. However, 30% of women with PCOS will have normal menses. Approximately 85%–90% of women with oligomenorrhea have PCOS [10].

Hirsutism is a common clinical presentation of hyperandrogenism occurring in up to 70% of women with PCOS[11]. Infertility affects 40% of women with PCOS[12]. PCOS is the most common cause of anovulatory infertility. Approximately 90%–95% of anovulatory women presenting to infertility clinics have PCOS.

Women with PCOS have a normal number of primordial follicles, primary and secondary follicles are significantly increased. However, due to derangements in factors involved in normal follicular development, follicular growth becomes arrested (follicles reach a diameter of 4–8 mm), and because a dominant follicle does not develop so the ovulation does not ensue[12,13]. Also, spontaneous abortion occurs more frequently in PCOS with incidences ranging from 42%–73% [14,15].

1.3 Diagnosis

When PCOS is suspected, a complete medical history, physical examination, blood tests, and a pelvic ultrasound should be performed; the medical history and physical examination will provide information about unexplained weight gain, menstrual cycle abnormalities, male pattern hair growth, skin changes, and elevated blood pressure.

Three sets of diagnostic criteria for the polycystic ovary syndrome in women have been developed[16]. Each set involves different combination of hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphologic features[17], while conditions such as insulin resistance and obesity which considered intrinsic to PCOS, none of them were included in guidelines [18]. Each guideline requires ruling out any pathological condition that might explain the hyperandrogenism or the menstrual irregularity[19]. The diagnostic criteria are showed in the table (1-1).

Table (1-1) Diagnostic criteria of polycystic ovary syndrome

National Institute of health criteria NIH 1992 (2 criteria)	Both of the following: 1-chronic anovulation, documented by oligo-or amenorrhea. 2-clinical and/or biochemical signs of hyperandrogenism [20]
Rotterdam criteria 2004 (2 out of 3 criteria)	At least two of the following: 1- chronic anovulation, determined by oligomenorrhea or amenorrhea 2- clinical and/or biochemical manifestations of hyperandrogenism 3- polycystic ovaries (by ultrasonography) [21]
Androgen Excess –PCOS Society criteria AE-PCOS 2006 (2 criteria)	1- Clinical and/or biochemical characteristics of hyperandrogenism 2- abnormal ovarian function (polycystic ovarian morphology and/or oligo ovulation/anovulation [22]

1.4 Epidemiology

The worldwide prevalence of PCOS in women of reproductive age is (4%-12%) and a higher prevalence of PCOS is among first degree relatives [23]. In fact, according to Rotterdam diagnostic criteria PCOS prevalence in adolescents varies between a minimum of (3%) and a maximum of (26%) in Lebanon [24]. PCOS prevalence rates in USA for underweight, normal weight, overweight, mildly obese, moderately obese, and severely obese women are (8.2%, 9.8%, 9.9%, 5.2%, 12.4%, 11.5%), respectively [23]. Although the prevalence of PCOS is similar in all countries, ethnic factors may impact the phenotypic manifestation of the syndrome, accordingly, the prevalence of PCOS in Caucasian women varies from 4.7% in Alabama to 6.5% in Spain and 6.8% in Greece [25]. In Iraq the prevalence of PCOS in females (20-40y) was 14%, 63% of them had hirsutism, 94% had menstrual disturbances and 90% had ultrasonic features of polycystic ovary [26].

1.5 Etiology and Pathophysiology

The etiology of PCOS is largely unknown, however, several etiological factors have been proposed including genetic causes, androgen programming, as well as environmental and metabolic factors can contribute in the pathogenesis of this syndrome [27].

1.5.1 Genetic Factors

Polycystic ovary syndrome is a heterogeneous disorder with unidentified etiology and it is known to be inherited genetically with the autosomal dominant manner and 50% of chances are documented of

inheritance from mother to daughter [28]. The cytochrome P450 group of enzymes play a vital role in the process of steroid synthesis, and thus any alterations in the genes encoding for them can be associated with PCOS [29]. Indeed, it was noted that in patients with PCOS the expression of CYP11A1 gene is upregulated in the theca cells, and thus androgen production is elevated. This gene polymorphism was also linked to obesity and lower FSH levels [30]. Moreover, the most common allelic alteration in the CYP17 gene associated with PCOS was demonstrated to enhance the PCOS phenotype [31]. Furthermore, in PCOS the CYP19 gene is down regulated leading to reduced aromatase activity, which consequently contributes to androgen excess [32]. In addition, the androgen receptor gene was shown to be less expressed in PCOS patients, thereby reducing the available receptors for androgens and this will translate into lower uptake and greater free circulating androgens [33].

1.5.2 Environmental Factors

The main environmental factors include environmental toxins like phthalate , diet (such as vegan and keto diet) and nutrition (amount of proteins, carbs and lipids in meals) , socioeconomic status, and geography (related to ethnicity) can induce PCOS[34] . Excess fetal exposure to maternal androgens is thought to contribute to inducing the PCOS phenotype in offspring/children, based on experimental data from animal studies as well as clinical material of pathological conditions in human populations (i.e., congenital adrenal hyperplasia)[35]. In humans, higher testosterone levels, which were elevated to male levels, have been found in the umbilical vein in female infants born to mothers with PCOS [36,37].

1.5.3 Neuroendocrine Factors

Hyperandrogenism and insulin resistance are the two most important factors that can explain the various symptoms of PCOS. There is evidence of increased androgen production and release by the theca cells of the ovaries in PCOS women [38], androgen hypersecretion and increased expression/efficacy of the key enzymes participating in the synthesis of androgens has been verified[39, 40].

The ovarian androgen excess is augmented by disordered feedback control of pulsatile gonadotropin-releasing hormone (GnRH) secretion in the hypothalamus, resulting in stimulated luteinizing hormone (LH) secretion from the anterior pituitary and a relative follicle-stimulating hormone (FSH) deficiency, which will favor androgen synthesis[38].

Enhanced production of androgens will impair follicular development and increase the degree of follicular atresia leading to an elevated number of small follicles and enlarged stroma of the ovary. The clinical consequences of hyperandrogenism are the typical polycystic ovarian morphology figure (1-1), anovulation causing menstrual disorders, reduced fertility, hirsutism, and acne vulgaris [38, 41, 42].

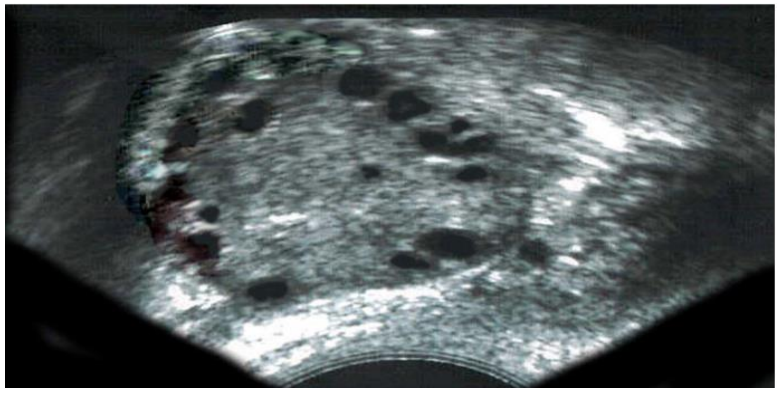

Figure (1-1) Ultrasound picture of a typical enlarged polycystic ovary with an increased number of small follicles [43].

The adrenal cortex synthesizes three major androgens; dehydroepiandrosterone sulfate (DHEAS), androstenedione, and testosterone, this is the other major site of female androgen production besides the ovaries. DHEAS is almost exclusively (97-99%) produced by the adrenal cortex and androstenedione is produced in both the adrenal gland and the ovaries[44], 25% of testosterone is synthesized by the adrenal gland, 25% in the ovary and the remaining part being produced through peripheral conversion from androstenedione in the liver, adipose tissue, and skin[44].

Women with PCOS also have an increased occurrence of insulin resistance, independent of obesity, leading to secondary hyperinsulinemia. The molecular mechanism seems to be a post-receptor defect in insulin signaling due to increased insulin receptor substrate-1 serine phosphorylation that selectively affects metabolic pathways in muscle, fat tissue, and in the ovary[45].

Hypersecretion of insulin directly or synergistically with LH stimulates androgen production from the ovarian theca cells. Furthermore, insulin inhibits the hepatic synthesis of sex hormone-binding globulin (SHBG) and insulin-like growth factor-binding protein 1 (IGFBP-1) and thereby increases free and bioavailable testosterone and insulin-like growth factor (IGF) concentration in the ovary. Thus, hyperinsulinemia contributes to hyperandrogenism and ovarian dysfunction in women with PCOS (Figure 1-2). Insulin resistance may cause metabolic symptoms including abdominal obesity that predisposes to type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease [46-48].

Testosterone may in turn induce insulin resistance by facilitating catecholamine-stimulated lipolysis in visceral fat tissue, thus exposing the liver to a high flux of free fatty acids, which could result in hepatic insulin

resistance [49]. Furthermore, increased testosterone could cause insulin resistance by inducing decreased capillary density in peripheral muscle tissue, as well as visceral fat accumulation demonstrated by testosterone treatment in women [50].

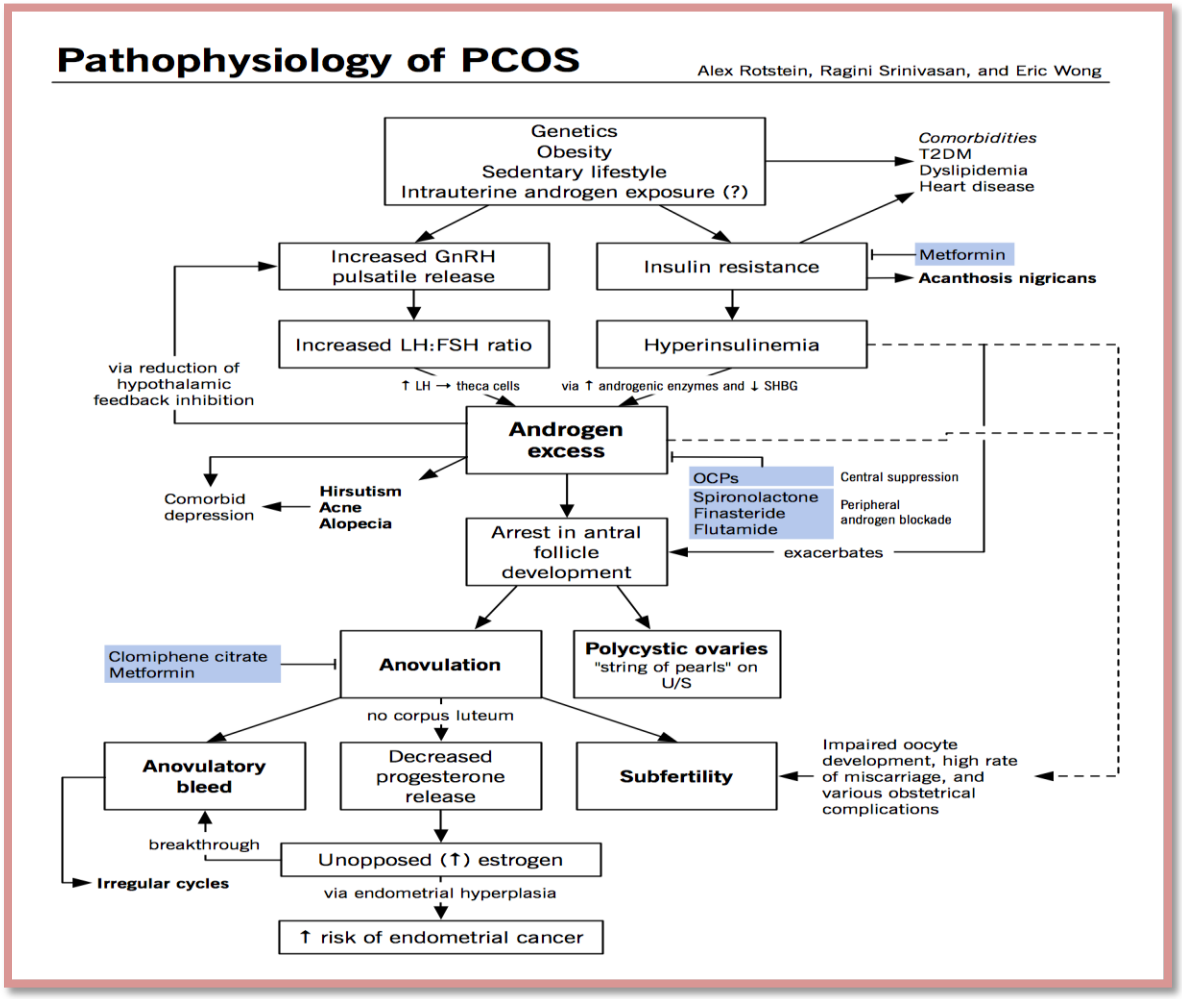

Figure (1-2) Pathophysiology of polycystic ovary syndrome[51].

1.6 Consequences

Polycystic ovary syndrome can cause many abnormalities throughout the life of affected women including impaired fertility, and even if pregnancy is achieved, women with PCOS have a greater risk of

pregnancy related complications such as gestational diabetes, preeclampsia, increased risk of miscarriage and premature delivery [52]. In addition, outcome of *in vitro* fertilization (IVF) treatment for patients who diagnosed with infertility and PCOS has been characterized by lower fertilization rate, impaired embryo cleavage , lower implantation rates of embryo, and a higher miscarriage rate [53, 54]. PCOS increase risk for cardiovascular diseases [55, 56], metabolic syndrome [54], dyslipidemia [54], type 2 diabetes [57] and mood disorders including depression [58].

1.7 Treatment

Importantly, no universal treatment for PCOS is available. Management of women with PCOS depends on the symptoms, these could be ovulatory dysfunction-related infertility, menstrual disorders, or androgen-related symptoms[59]. Treatment goals include alleviation of symptoms, restoration of fertility, and prevention of long-term complications. There are several therapeutic interventions available for the treatment of PCOS, including lifestyle modification, surgery, and pharmacologic therapy [60], as showed in figure (1-3).

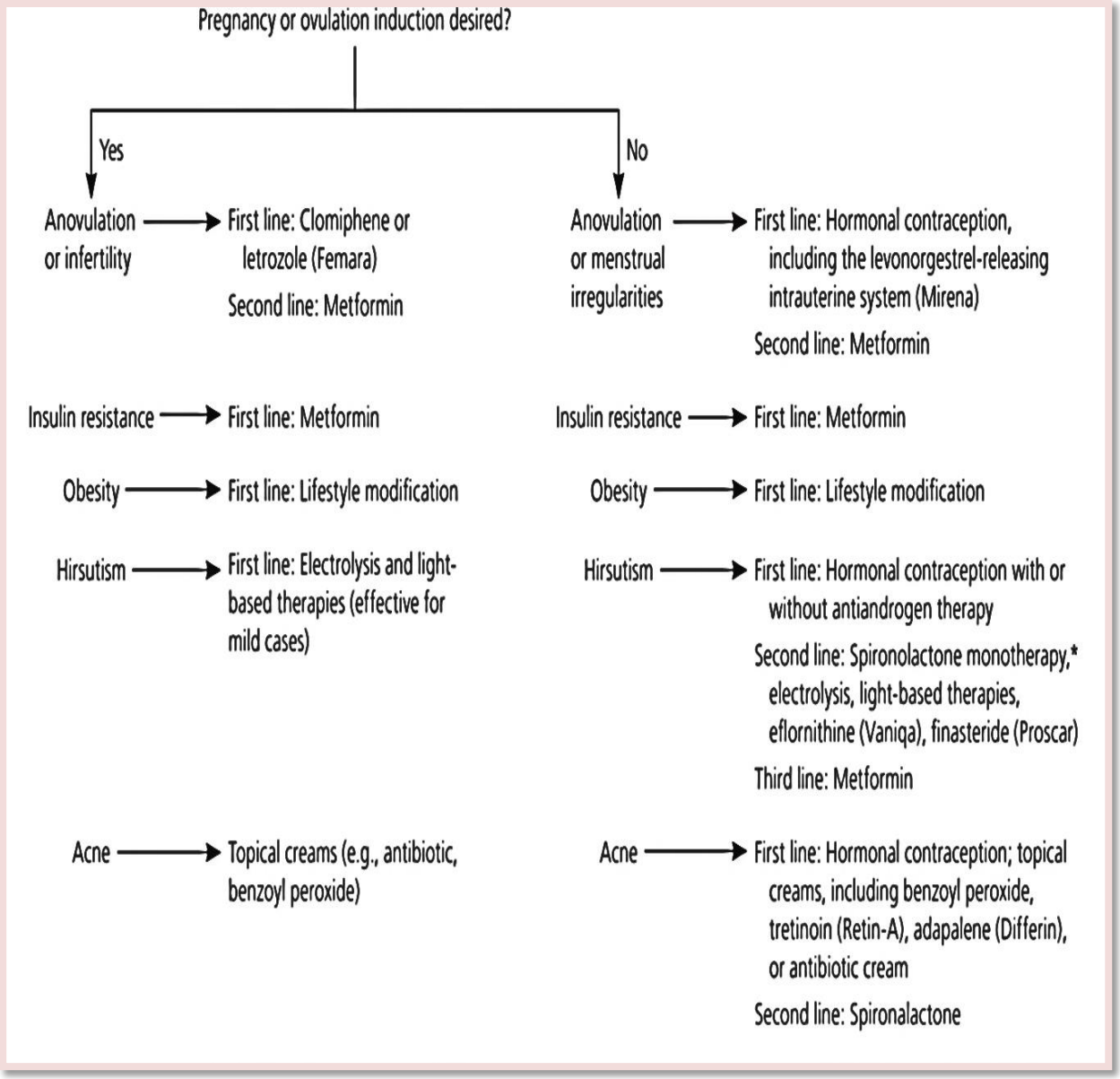

Figure (1-3) Management of polycystic ovary syndrome [61]

1.7.1 Metformin

Metformin is the most widely used drug for reproductive abnormalities associated with insulin resistance and also the oldest insulin sensitizer in the therapeutic management of type 2 diabetes mellitus[62]. Metformin is a stable hydrophilic biguanide compound that is highly polar,

positively charged with low molecular weight, and has pleiotropic actions[63]. Metformin distributes in many tissues including muscle, liver, pancreas, adipose tissue, hypothalamus, pituitary, and the gonads. Despite low lipid solubility, some subcellular studies in rat liver showed that metformin is mainly localized in the cytosol [64]and studies in mice showed that metformin may accumulate in certain tissues at higher concentrations than in plasma [65]. The passive diffusion of metformin into cells is limited [66],and it mainly transported via the organic cation transporters (OCT1, OCT2, OCT3) or multidrug and toxic compound extrusion transporters (MATE1, MATE2) which can internalize metformin in the gut, hepatocytes, renal tubular epithelial cells and reproductive tissues[67].

Its primary action is through its insulin-sensitizing effect in the liver resulting in a decrease of hepatic glucose output, mainly through inhibition of gluconeogenesis. This action is believed to occur via alterations in cellular energetics that involve inhibition of mitochondrial complex 1, resulting in lower ATP levels and consequently higher ratios of AMP/ATP and ADP/ATP. The increased levels of AMP and ADP result in the activation of AMP kinase (AMPK), which responsible for the beneficial effects of metformin on hepatic glucose output[68].

The main biological effects of AMPK are the phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), which plays a pivotal role in hepatic lipid metabolism [69]. It was observed that the stimulation of ACC phosphorylation by metformin induces reduction in triglyceride levels, which can be supported with increased fatty acid oxidation and/or decreased fatty acid synthesis [70].

1.7.1.1 Mechanisms of Metformin Action in Target Tissues Relevant to Polycystic Ovary Syndrome

Metformin acts on many tissues that have relevance to the metabolic and reproductive abnormalities in PCOS, namely the liver, skeletal muscle, adipose tissue, and ovary[71]. Reduction in hepatic glucose output is the principal action of metformin although its mechanism(s) has not been identified. Recent data indicate that lowered hepatic glucose output with metformin may result from inhibition of electron transport in mitochondrial respiratory Complex I[72], as well as antagonism of glucagon action in the liver[73]. Metformin suppresses lipolysis, modulates adipokine secretion, and decreases lipogenesis in adipose tissue[71].

In the ovary, metformin is presumed to exert both direct and indirect effects on androgen production. Many studies demonstrated a reduction in CYP17A1 activity in women with PCOS upon metformin treatment consequent to lowering of serum insulin levels [74]. Also, there is evidence that metformin directly inhibits ovarian steroidogenesis [75, 76]. Inhibition of mitochondrial Complex I has been implicated as one potential mechanism for this action[77]. Also metformin lowers androgen levels by an inhibitory effect on 3β-hydroxysteroid dehydrogenase [78].

1.8 Organic Cation Transporter

Organic cation transporters (OCTs) are a polyspecific, bi-directional, facilitative diffusional transporters that play major physiological roles in metabolite and drug clearance[79]. Organic cation transporters belong to the solute carrier family *(SLC22A).* In humans, OCT1 (SLC22A1) is

predominantly expressed in the hepatocytes [80], *OCT1* gene expression and protein levels are also detectable in adipose tissue[81], skeletal muscles, ovaries, and intestine[82]. OCT2 is expressed in proximal tubular cells of the kidney. OCT3 exhibits a broader tissue distribution and is found in astrocytes [83], blood-cerebrospinal fluid barrier in choroid plexus epithelial cells [84], as well as in the placenta, bronchial and intestinal epithelium[85]. OCTs play major roles in clearance for both endogenous and xenobiotic compounds.

OCT1 is predominantly expressed on the blood side (basolateral membrane) of hepatocytes. Although bi-directional, it typically behaves as an uptake transporter in vivo, extracting substrates from the blood into the hepatocyte, as the first step in the hepatic elimination of drug substrates[86]. OCT1 topology consists of 12 alpha-helical transmembranes (TM) domains with a large extracellular loop between TM1 and TM2 which is glycosylated [87] and a large intracellular loop between TM6 and TM7 which is involved in post-transcriptional regulation [88].

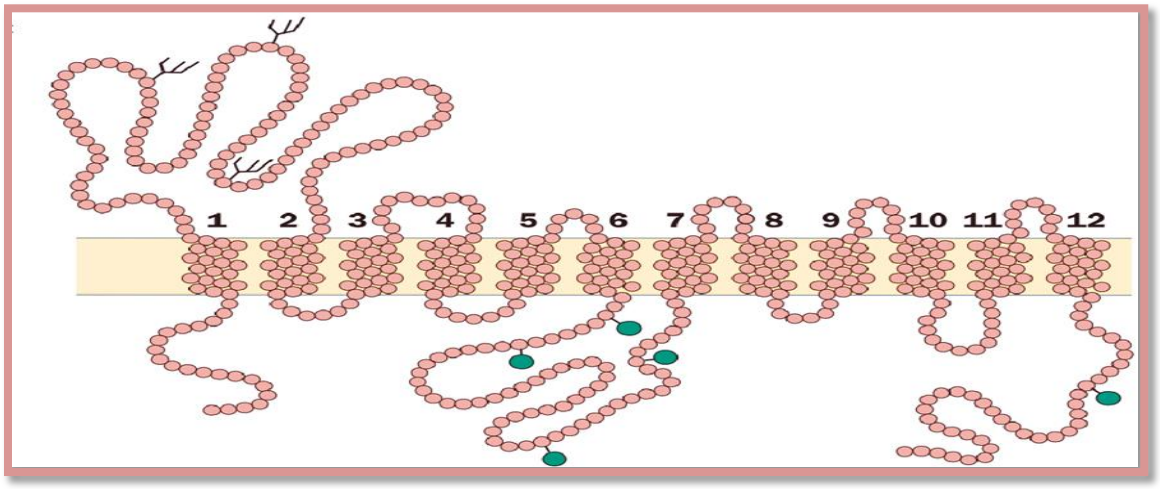

Figure (1-4) Predicted topology of human OCT1 with glycosylation sites on the large extracellular loop (ψ) and phosphorylation sites (green) [88]

OCT1 mediates transport of Type I organic cations (protonated molecules), such as dopamine and choline[88], as well as Type II cations (larger and bulkier molecules) such as metformin and quinidine. OCT1-mediated organic cation transport is electrogenic and sensitive to membrane potential[89]. The human *SLC22A1* gene encoding OCT1 consists of 11 exons, has been mapped to chromosome 6q26, and spans about 37kb[90].

1.9 Organic Cation Transporter1 Polymorphism and Metformin Response

There is significant variability in the clinical response to metformin treatment in PCOS. Studies revealed significant heterogeneity in its ability to reduce testosterone and insulin levels, regulate menses, and improve body weight [91]. This heterogeneity in response remained even after adjustment for many potential confounders, these findings suggest that unknown or unmeasured factor(s) impact the response to metformin therapy in PCOS[92]. In type 2 diabetes, the glycemic response to metformin is heritable, although the genetic contribution is probably a result of individual variants across the genome rather than a few loci . Similarly, genetic factors are likely to mediate the response to metformin in PCOS[93].

Polymorphisms in genes involved in metformin transport or action have been implicated in the heterogeneous response to metformin in type 2 diabetes [94, 95], and, to a lesser extent, in PCOS[96]. Polymorphism in genes encoding metformin transport proteins, such as OCT1, which is involved in the transport of metformin into hepatocytes [89] thought to play a role in metformin response [97]. Two small European studies, demonstrating that

polymorphisms in OCT1 may account for the variability of response to metformin in PCOS [98, 99]. OCT1 is highly polymorphic in ethnically diverse populations and mediate differences in transporter function [100]. Carriers of some of the variants in OCT1 display altered pharmacokinetic and pharmacodynamic properties of metformin compared with carriers of the reference allele [101,89].

Variant such as R61C position on chr6:160122116, which is a missense variant, the *181C>T* polymorphism at *rs122083571* consisting of an amino acid substitution (arginine to cysteine at position 61 *(Arg61Cys)*, is known to induce a robust substrate-wide loss of OCT1 activity, leading to decrease in OCT1-mediated uptake by more than 70% for all substrates including metformin[102]. The second variant of OCT1 is M420del position chr6:160139849, allele delATG, the *rs72552763* polymorphism constitutes a 3bp deletion at position 420 and is the most common functional OCT1 variant which can causes a robust decrease in metformin uptake (>60%). *Met420del* does not change OCT1 membrane localization and the exact mechanism how it affects OCT1 function remains unknown [103-106]. Many studies have identified genetic polymorphisms in the *SLC22A1* gene among different populations groups but there were still contradictory reports on the effects of OCT1 polymorphisms on metformin-related therapeutic responses [107].

1.10 Aims of Study

This study aimed to investigate

1- The effect of OCT1 polymorphisms on the therapeutic response of metformin in patients with polycystic ovary syndrome in Iraq.

2- The effect of OCT1 polymorphism on the pathogenesis of PCOS.

Chapter Two

Subjects, Materials and Methods

Chapter Two

Subjects, Materials and Methods

2.1 Subjects (Patients and Control)

A total of 320 patients aged 20-40 years were enrolled in this study during their visiting to Kerbala teaching hospital of obstetrics and gynecology and privet clinic seeking for medical treatment and advice concerning their cases. This study performed from July 2019 to April 2020. Only 222 patients continued the study, 65 patients refuse to complete the study due to quarantine applied because of Corona virus pandemic while 33 patients were excluded as they became pregnant during study time. All patients were diagnosed by consultant gynecologist according to Rotterdam criteria and treated according to practice guidelines. Also 106 healthy control aged 20-40 years were enrolled.

2.1.1 Patients Criteria

2.1.1.A Inclusion Criteria

Patients aged between 20 – 40 years, newly diagnosed with PCOS according to Rotterdam criteria.

2.1.1.B Exclusion Criteria

1-Patients with congenital adrenal hyperplasia, androgen-secreting tumors, cushing syndrome, hyperprolactinemia and thyroid dysfunction.

2- Patients suffering from any other diseases and patients taking any OCT1 substrate drugs.

3- Patients whom became pregnant during the study.

2.1.1. C Ethical and Scientific Approval

- The proposal of research was discussed and approved by the scientific and ethical committee in Collage of Pharmacy – Kerbala University.
- The agreement of general health director in Kerbala governorate was achieved
- All participant women were enrolled in this study after signing a written informed consent with full explanation of the aim of the study and requested to answer a specially designed questionnaire.

2.1.2 Study Design

In this prospective case control study 222 PCOS Iraqi female patients were included and 106 healthy females clinically without any disease were served as control group. From overnight fasted control participants at second day of menstruation, blood samples were collected for hormonal, biochemical and genetic study. A blood sample was also collected from each overnight fasted patient in day two of the menstrual cycle and any day in case of amenorrhea in some patients [(108)] for genetic, hormonal and biochemical tests before and after three months treatment with metformin 500 mg twice daily according to recent study [(109)].

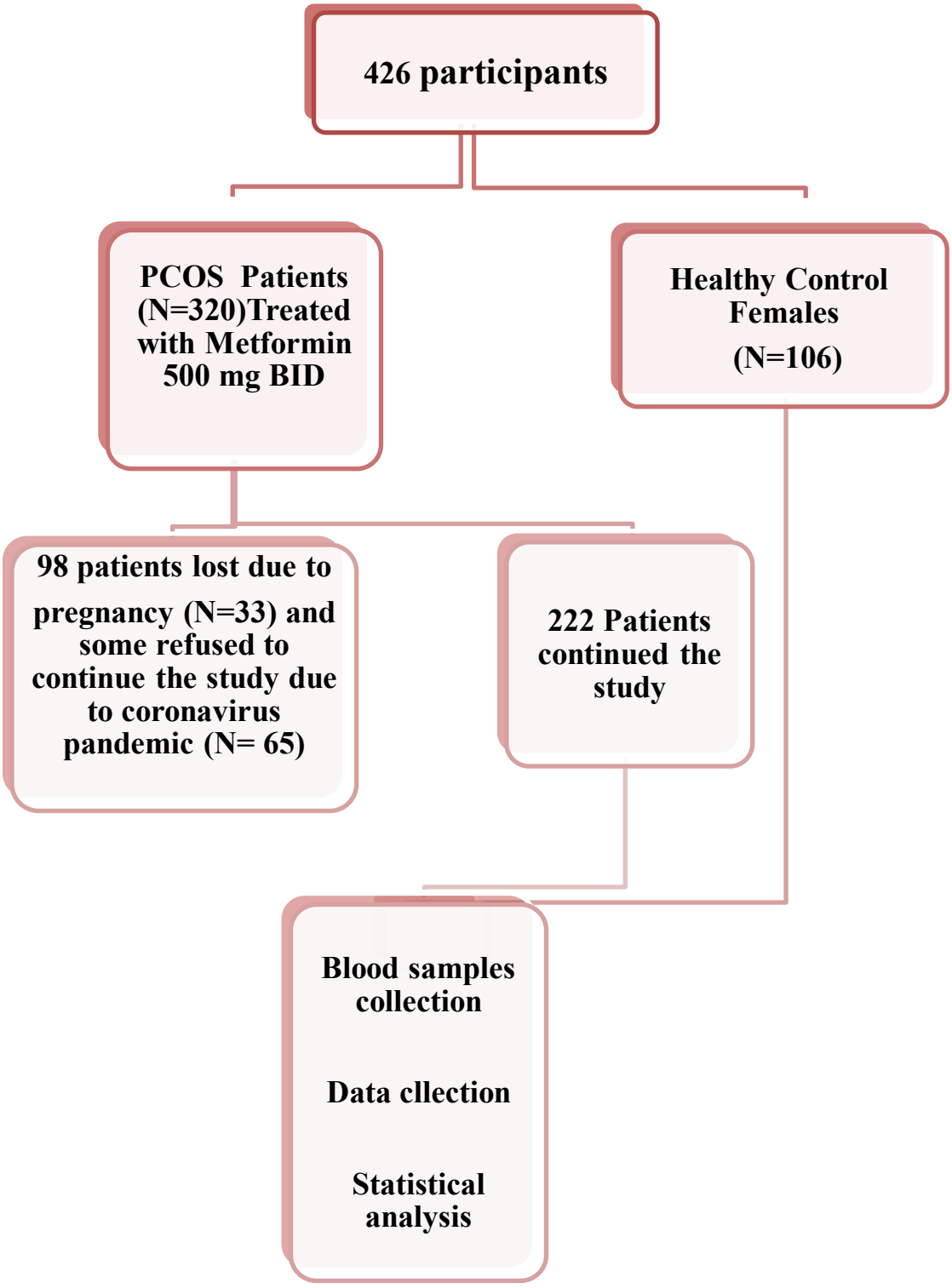

Figure (2-1): Flow chart of the study groups

2.2 Materials

2.2.1 Instruments and Equipment and Their Suppliers

All instruments used in this study are listed in table (2-1) accompanied with their manufacturing company

Table (2-1) Instruments and the manufacturing companies.

Equipment	Company	Country
Centrifuge	SIGMA	Germany
Cobas e 411	Roche	Germany
Digital camera	Canon	England
Distillator	GFL	Germany
Gel documentation system	Techin me	England
Hood	LabTech	Korea
Micropipettes	SLAMED	Japan
PCR machine	TECHINE	UK
Sensitive balance	AND	Taiwan
UV- Transilluminator	Syngene	England
Vortex mixer	HumanTwist	Germany
Water bath	LabTech	Korea

2.2.2 Chemicals, Kits, Drug and Their Suppliers

All chemicals and kits used in this study are listed in table (2-2) accompanied with the producing company

Table (2-2) Chemicals and kits and their producing companies.

	Chemicals and Kits	Company	Country
Chemicals	Agarose powder	CONDA	Spain
	Ethanol 90%	SDI	Iraq
	Ethedium Bromide	Intron	Korea
	Isopropanol	SDI	Iraq
	Metformin 500mg tab	Merck	France
	Nuclease free water	Intron	Korea
Hormonal Kits	FSH kit	Roche	Germany
	LH kit	Roche	Germany
	Prolactin kit	Roche	Germany
	SHBG kit	Roche	Germany
	Testosterone kit	Roche	Germany
	TSH kit	Roche	Germany

Biochemical Kits	Cholesterol kit	Roche	Germany
	Fasting serum glucose kit	Roche	Germany
	Glycosylated Hemoglobin kit	Roche	Germany
	HDL kit	Roche	Germany
	Insulin kit	Drug International	USA
	LDL kit	Roche	Germany
	Triglyceride kit	Roche	Germany
Kits For Genetic Study	DNA extraction kit (G-DEX IIb)	Intron	Korea
	PCR mastermix kit	Genome	Korea
	DNA ladder marker	Genome	Korea
	Primers OCT1 rs12208357 c>t OCT 1 rs72552763 ATGAT>AT	Bioneer	Korea

2.3 Methods

2.3.1 Samples Collection

After overnight fasting,7 ml blood samples were collected from all patients and healthy control by vein puncture, before starting metformin treatment and then after three months of patients follow up to determine the changes in the studied parameters. Collected blood samples were divided into three parts, first part was kept in the EDTA tube for DNA extraction, the second part was kept in an EDTA tube for HbA1c% and the last part was kept in a gel tube to isolate serum for hormonal and other biochemical tests.

2.3.2 Hormonal and Biochemical Assays

2.3.2.1 Determination of Glycemic Indices

2.3.2.1.A Measurement of Fasting Serum Glucose (FSG)

Glucose level estimated by UV, Enzymatic reference method with hexokinase in which Hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate by ATP. Glucose-6-phosphate dehydrogenase oxidizes glucose-6- phosphate in the presence of NADP to gluconate-6-phosphate. No other carbohydrate is oxidized. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and is measured photometrically [(110)].

2.3.2.1.B Measurement of Fasting Serum Insulin (FSI)

Serum insulin Level was determined using a ready-made kit for this purpose, the insulin ELISA kit is a solid phase - enzyme- linked

immunosorbent assay based on the sandwich principle. The micro-titer wells were coated with monoclonal antibody directed towards a unique antigenic site on human insulin molecule. An aliquot of patients serum samples containing endogenous insulin was incubated in the coated well with enzyme conjugate, which is an anti-insulin antibody conjugated with biotin. After incubation the unbound conjugate was washed off. During the second incubation step streptavidin-peroxidase enzyme complex binds to biotin anti-insulin antibody. The mount of bound horseradish peroxidase (HRP) complex is proportional to the concentration of insulin in the sample. Having added substrate solution, the intensity of color developed is proportional to the concentration of insulin in the patient sample with running proper standards of insulin. Absorbance was measured spectrophotometrically at 450nm. The results were expressed as μ.IU/ml [(111)].

2.3.2.1.C Estimation of Insulin Resistance

Insulin resistance was determined using the homeostasis model assessment (HOMA) which is calculated from fasting serum glucose (FSG) and fasting serum insulin (FSI) using the following formula.

HOMA = FSI(μIU/ml) * FSG (mg/dl) /405 [(112)]

2.3.2.1.D Measurement of Glycosylated Hemoglobin (HbA1c)

The blood specimen that was kept in the EDTA tube hemolyzed automatically on the cobas integra 400+ analyzer with cobas integra hemolyzing reagent gen.2. This method used tetradecyltrimethylammonium bromide (TTAB) as the detergent in the hemolyzing reagent to eliminate

interference from leukocytes (TTAB does not lyse leukocytes). Sample pre-treatment to remove labile HbA1c was not necessary. All hemoglobin variants which were glycated at the beta-chain N-terminus and which have antibody recognizable regions identical to that of HbA1c were measured by this assay. Glycohemoglobin (HbA1c) in the sample reacts with an anti-HbA1c antibody to form soluble antigen-antibody complexes. Since the specific HbAq2c antibody site is present only once on the HbA1c molecule, the complex formation does not take place (polyhapten reagent). The polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be measured turbidmetrica [113].

2.3.2.2 Measurement of Serum Follicle Stimulating Hormone (FSH) , Luteinizing Hormone(LH) level and (LH/FSH Ratio Calculation)

Immunoassay for the *in vitro* quantitative determination of FSH and LH in human serum was used in this study. The electrochemiluminescence immunoassay "ECLIA" is intended for use on elecsys and cobas e immunoassay analyzers which employs two different monoclonal antibodies specifically directed against these hormones to form a sandwich complex. The microparticles are magnetically captured onto the surface of the electrode. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier [114, 115].

2.3.2.3 Measurement of Serum Prolactin

Immunoassay for the *in vitro* quantitative determination of prolactin in human serum was used in this study. The

electrochemiluminescence immunoassay "ECLIA" is intended for use on elecsys and cobas e immunoassay analyzers which employs two different monoclonal antibodies specifically directed against prolactin hormone to form a sandwich complex. The microparticles are magnetically captured onto the surface of the electrode. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier [116].

2.3.2.4 Measurement of Serum Sex Hormone Binding Globulin (SHBG)

The SHBG was measured by specific ELISA kit using the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to human SHBG. Samples (or Standards) and biotinylated detection antibody specific for human SHBG were added to the micro ELISA plate wells. Human SHBG would combine with the specific antibody, then Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contain human SHBG, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 ±2 nm. The OD value is proportional to the concentration of human SHBG.

2.3.2.5 Measurement of Serum Testosterone

Immunoassay for the *in vitro* quantitative determination of testosterone in human serum was used in this study. The electrochemiluminescence immunoassay "ECLIA" is intended for use on elecsys and cobas e immunoassay analyzers which 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 complex) for the binding sites on the biotinylated antibody. Application of a voltage to the electrode induces chemiluminescent emission which was measured by a photomultiplier [117].

2.3.2.6 Estimation of Free Androgen Index (FAI)

Free Androgen Index was determined from total testosterone level and sex hormone-binding globulin (SHBG) by using a specific formula.

Free androgen index (FAI) = Total testosterone (nmol/L)*100/ sex hormone binding globulin (SHBG) (nmol/L)[118]

2.3.2.7 Measurement of Serum Thyroid Stimulating Hormone (TSH)

Immunoassay for the *in vitro* quantitative determination of TSH in human serum was used in this study. The electrochemiluminescence immunoassay “ECLIA” was intended for the use on Elecsys and cobas e immunoassay analyzers and the principle was the same as described for prolactin hormone [119].

2.3.2.8 Determination of Lipid Profile

2.3.2.8. A Measurement of Total Cholesterol (TC)

In vitro quantitative enzymatic colorimetric method was used for the determination of total cholesterol in serum on cobas integra systems. Cholesterol esters are cleaved by the action of cholesterol esterase (CE) to yield free cholesterol and fatty acids. Cholesterol oxidase (CHOD) then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase (POD), the hydrogen peroxide formed effects the oxidative coupling of phenol and 4-aminoantipyrine (4-AAP) to form a red quinone-imine dye.

$$\text{Cholesterol esters} + H_2O \xrightarrow{CE} \text{cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CHOD} \text{cholest-4-en-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AAP} + \text{phenol} \xrightarrow{POD} \text{quinone-imine dye} + 4\,H_2O$$

The color intensity of the dye formed is directly proportional to the cholesterol concentration. It was determined by measuring the increase in absorbance at 512 nm [120].

2.3.2.8. B Measurement of Serum Triglyceride (TG)

In vitro quantitative enzymatic colorimetric method was used for determination of triglyceride in serum on cobas integra systems [121]

$$\text{triglycerides} + 3\,H_2O \xrightarrow{\text{Lipoprotein lipase}} \text{glycerol} + 3\,\text{RCOOH}$$

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{Glycero Kinase}} \text{glycerol-3-phosphate} + \text{ADP}$$

glycerol-3-phosphate + O2 $\xrightarrow{\text{Glycerol-3-Phosphate Oxidase}}$ dihydroxyacetone phosphate + H2O2

H2O2 + 4-aminophenazone + 4-chlorophenol $\xrightarrow{\text{peroxidase}}$ 4-(p-benzoquinone-monoimino)-phenazone + 2 H2O + HCl

2.3.2.8. C Measurement of Serum High Density Lipoprotein (HDL)

In vitro quantitative enzymatic colorimetric method was used for the determination of HDL in serum on cobas integra systems. In the presence of magnesium ions and dextran sulfate, water-soluble complexes with LDL, VLDL, and chylomicrons are formed which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-cholesterol was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approximately 40 %). Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide [122].

HDL-cholesterol esters + H2O $\xrightarrow{\text{PEG-cholesterol esterase}}$ HDL-cholesterol + RCOOH

HDL-cholesterol + O2 $\xrightarrow{\text{PEG-cholesterol oxidase}}$ Δ4-cholestenone + H2O2

2 H2O2 + 4-aminoantipyrine + HSDAa) + H+ + H2O $\xrightarrow{\text{peroxidase}}$ purple blue pigment ([a) Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline]) + 5 H2O

The color intensity of the blue quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It was determined by measuring the increase in absorbance at 583 nm.

2.3.2.8.D Measurement of Serum Low Density Lipoprotein (LDL)

Cholesterol esters and free cholesterol in LDL are measured based on a cholesterol enzymatic method using cholesterol esterase and cholesterol oxidase in the presence of surfactants which selectively solubilize the only LDL. The enzyme reactions to the lipoproteins other than LDL are inhibited by surfactants and a sugar compound. Cholesterol in HDL, VLDL, and chylomicron is not determined.

LDL-cholesterol esters + $H_2O \xrightarrow{\text{detergent}}$ cholesterol + free fatty acids

(selective micellary solubilization)

Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase.

LDL-cholesterol + $O_2 \xrightarrow{\text{cholesterol oxidase}}$ Δ4-cholestenone + H_2O_2

In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide.

2 H_2O_2 + 4-aminoantipyrine + EMSEa) + H_2O + $H^+ \xrightarrow{\text{Peroxidase}}$ red purple pigment ([a] N-ethyl-N-(3-methylphenyl)-N-succinylethylenediamine) + 5 H_2O

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-aminoantipyrine and EMSE to form a red-purple dye. The color intensity of this dye is directly proportional to the cholesterol concentration and is measured by photometer [123].

2.3.2.9 Determination of Body Mass Index

Body Mass Index (BMI) is a value obtained from the weight and height of an individual. The BMI was described as the body weight divided by the square of the body height and is globally expressed in units of kg/m2, resulting from the mass in kilograms and height in meters [124].

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

Normal weight falls between BMI values of 18.5-24.9, overweight between 25-30 and obese above 30.

2.3.3 Genetic Analysis

2.3.3.1 Extraction of Genomic DNA from Blood Sample

DNA extraction kit from Intron offer simple DNA extraction method for high molecular weight genomic DNA suitable for storage and immediate application.

- (1ml) blood sample in Eppendorf tube was centrifuged (2000rpm -1 min) then buffy coat collected by micropipette and transferred to another tube.
- 900 µl of RBC lysis solution was added and the solution mixed well then inverted several times and incubated 5 min then the solution centrifuged (10000rpm -1min).
- Supernatant poured off except 100 µl remained with pellet which was vortex in order to be dissolved. 300µl of cell lysis solution was added then the tube refrigerated for 5 min.
- 100µl of PPT buffer was added and mixed by vortex 20sec then the mixture centrifuged (16000rpm -5min).Protein precipitated in this step while DNA remain in the supernatant.
- 300 µl of supernatant transferred to another tube and 300 µl isopropanol was added and the tube inverted several times and centrifuged (16000-1min).
- After discarding supernatant, 1ml Ethanol added then centrifuged and supernatant discarded.
- After drying in air for few minutes, 200 µl of DNA rehydration solution was added and the tube kept in water bath 56 C for ½ hours. After that DNA dissolved in the solution and it ready to use.

2.3.3.2 Determination of Concentration and Purity of Genomic DNA

DNA concentration was measured using Nano-spectrophotometer (NanoDrop) by placing 1 µl of the DNA sample on the highly sensitive micro-detector. The purity was detected by noticing the ratio of optical density at 260/280.

2.3.3.3 Allele Specific Polymerase Chain Reaction

2.3.3.3.A Primers Preparation

Polymerase Chain Reaction (PCR) was performed by using specific primers to amplify OCT1 gene rs12208357 and rs72552763. The primers of this study were designed by **Asst. Prof. Dr. Hassan Mahmood Mousa Abo Almaali** using primer-blast software and purchased from Bioneer, Korea as lyophilized product of different picomols concentrations. Lyophilized forward and revers primers were dissolved in specific volumes of nuclease free water to get a concentration of 100 pmol/ µl (stock solution). To prepare 10pmol/ µl of working solution of each primer, 10µl of each primer (stock solution) was diluted with 90 µl of nuclease free water. The primers were kept at -20 C until further use. Table (2-3) illustrates the primers used to amplify the gene alleles.

Table (2-3) Primers sequences of OCT1 rs12208357 and rs72552763

Primers		Sequence	Product size (bp)
Primers sequences of OCT 1 (R61C) rs12208357 Alleles C>T	Forward	CAGATGGCCACGTGCATTCTTC	-
	Allele C R1	AGGGCTCCAGCCACAGCG	407
	Allele R2	CAGGGCTCCAGCCACAGCA	407
Primers sequences of OCT 1 (420del) rs72552763 Alleles: ATGAT>AT	Un-delet	GCAGCCTGCCTCGTCATG	100
	DEL-ATG	GCAGCCTGCCTCGTCATT	100
	o-r	AGTCACAACACTTTCCCCACA	-

2.3.3.3.B Optimization of Polymerase Chain Reaction Conditions

Optimization of PCR was done by several attempt to detect the best annealing temperature, the best concentration of both DNA and primers, and the best number of amplification cycles. The components of PCR for all the amplified fragments and the optimized PCR programs are stated in Table (2-4), Table (2-5) and Table (2-6) respectively.

2.3.3.3.C Running the Polymerase Chain Reaction

The PCR was prepared by mixing reaction components with the optimized concentrations and using the optimized PCR programs as shown in the tables below.

Table (2-4) PCR mix reaction for genotyping Of OCT 1 rs12208357 Alleles: C>T and rs72552763 Alleles: ATGAT>AT

Component	**Volume (µl)**
Forward primer	1.25
Reverse primer	1.25
Reverse primer	1.25
DNA template	5
Deionized water	7.5
Master mix	10

Table (2-5) PCR Condition for genotyping of OCT 1 gene (R61C)
rs12208357 Alleles: C>T

Steps	Temperatures/c	Time /second	Cycle
Denature template	94	3minutes	1
Initial denaturation	94	30 Sec	30
Annealing	63	40 Sec	
Extension	72	55 Sec	
Final extension	72	5minutes	1

Table (2-6) PCR condition for genotyping of OCT 1 gene (420del)
rs72552763 Variant type: delin. Alleles: ATGAT>AT.

Steps	Temperatures/c	Time /second	Cycle
Denature template	94	3 minutes	1
Initial denaturation	95	30 Sec	30
Annealing	58	45 Sec	
Extension	72	55 Sec	
Final extension	72	5 minutes	1

2.3.3.4 Agarose Gel Electrophoresis

• Agarose gel was prepared by dissolving 1.5 g of agarose powder in 100 ml of 1x TBE buffer (pH 8) in microwave.

• The solution was cooled to 50˚C.

• Two microliters of ethidium bromide solution were added.

• The comb was fixed at one end of the tray for making wells used for loading the samples, i. e. DNA or PCR product samples.

• The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min.

• The comb was removed gently from the tray.

• The tray was fixed in an electrophoresis chamber. The chamber was filled with a TBE buffer.

• Five microliters of each DNA sample was transferred to an Eppendorf tube; half a microliter of loading buffer was added to the tube; the mixture was loaded into the wells in agarose gel.

• PCR products were directly loaded into the wells without mixing with the loading dye.

• The voltage of the electrophoresis apparatus was fixed to ensure an electrical field adjusted with 5 v.cm-1 for distance between cathode and anode.

• At the end of the run, ultraviolet trans-illuminator was used at 320-336 nm for bands detection.

• The gel was photographed using a digital camera.

2.4 Statistical Analysis

Data of study participants were transferred into computerized database, revised for errors or inconsistency and then managed, processed and analyzed using the statistical package for social sciences (SPSS) version 25, IBM, US. All continuous (scale) variables were examined for normal statistical distribution using Kolmogorov Smirnov test and histogram. Descriptive statistics for nominal (categorical) variables presented as frequency (number of participants) and proportion (percentage). Scale variables presented in mean, standard deviation (SD).

Scale variables like age, BMI and number of children follow the statistical normal distribution, parametric tests were applied. To compare the studied parameters between studied groups, Student t test for two samples was applied. While other parameters did not follow the statistical normal distribution, non-parametric tests were applied. To compare the studied parameters between both studied groups, Mann-Whitney U test for two independent samples was applied. To compare the studied parameters within PCOS group, before and after treatment, Wilcoxon Signed Ranks Test was applied.

Test for Hardy-weinberg equilibrium in controls and allelic or genotypic association in cases versus control were evaluated by Chi – square (x^2) test. This analysis was performed for all genotypes in this study using Hardy-weinberg equilibrium online calculator and P value less than 0.05 was regarded as significant.

Chi square test used to assess the significance of comparison of nominal /categorical variables including, number of abortions, employment and education level between both studied groups.

To assess the predictability of PCOS, logistic analysis of R61C and 420del was applied, this yielded odds ratio (OR) also the 95% confidence interval of the OR was calculated which is good estimator for the significance of the OR; when the value of "one" included within interval, this is an indicator that the OR is not significant.

All statistical procedures and tests were applied under a level of significance (P- value) of less than 0.05 to be considered as significant difference or correlation.

Chapter Three

Results

Chapter Three

Results

3.1 Socio-demographic Data

Socio-demographic data for 222 PCOS patients and 106 healthy women (control group) demonstrated in the table (3-1). The age range was between 20-40 years with a mean ± SD of 28.1± 6.4 for control and 27.6 ± 5.1 for patients, there was non-significant difference in age between the two groups . BMI was 27.8± 4.7 for control and 31.4 ± 4.9 for patients; this result showed that BMI of PCOS patients was significantly higher than those of healthy control women. One hundred eighty six (83%) patients had alopecia and 206 (92%) of them had hirsutism. Abortion percentage was higher in PCOS patients 27.7% than in control women 15%. Regarding the education status there was non-significant difference between the two groups.

Table (3-1) Assessment of socio-demographic data between PCOS group and healthy control group

Variables	Control	PCOS	p-value
Number	106	222	-
Age (y)	28.1 ± 6.4	27.6 ± 5.1	0.451 [NS]
BMI (kg/m^2)	27.8 ± 4.7	31.4 ± 4.9	<0.001 [S]
Hirsutism, n (%)	0 (0.0%)	206 (92.8%)	<0.001 [S]
Alopecia, n (%)	0 (0.0%)	186 (83.8%)	<0.001 [S]
Married, n (%)	89 (84.0%)	195 (87.8%)	0.429 [NS]
Abortion, n (%)	9 (15.0%)	54 (27.7%)	0.046 [S]
Employed, n (%)	41 (38.7%)	73 (32.9%)	0.364 [NS]
Education, n (%)			0.945 [NS]
Illiterate	5 (4.7%)	9 (4.1%)	
Primary	46 (43.4%)	104 (46.8%)	
Secondary	32 (30.2%)	63 (28.4%)	
College	23 (21.7%)	46 (20.7%)	

Results are presented as mean±SD , n= number of subjects , (p< 0.05) considered significantly different, [S] significant ,[NS] non-significant

3.2 Hormonal and Metabolic Parameters for Control Group and Polycystic Ovary Syndrome Group

3.2.1 Hormonal Profile of Healthy Control Group and Polycystic Ovary Syndrome Patients Group

As shown in table (3-2), serum FSH level was significantly lower in the PCOS group than in the control group (P = 0.002). In contrast, serum LH level and LH/FSH ratio was significantly higher in PCOS patients than control (P = 0.011, 0.001) respectively. Thyroid-stimulating hormone (TSH) and testosterone were significantly higher in PCOS patients (P = 0.010, <0.001) respectively while SHBG was significantly lower in PCOS patients (P<0.001) compared to control.

Table (3-2) Hormonal parameters of a control group and polycystic ovary syndrome group

Variables	Control	PCOS	p-value
Number	106	222	-
LH (m.IU/L)	7.8 ± 2.1	9.9 ± 5.4	0.011 [S]
FSH (m.IU/L)	6.4 ± 1.7	5.8 ± 2.0	0.002 [S]
LH/FSH ratio	1.3 ± 0.3	1.8 ± 0.9	<0.001 [S]
TSH (uIU/L)	1.9 ± 0.6	2.2 ± 0.9	0.010 [S]
Prolactin (ng/mL)	18.3 ± 5.0	23.6 ± 13.5	<0.001 [S]
Testosterone (ng/ml)	0.3 ± 0.2	0.6 ± 0.4	<0.001 [S]
SHBG (nmol/L)	85.2 ± 6.9	53.2 ± 24.7	<0.001 [S]
FAI	0.77 ± 0.2	5.72 ± 2.5	<0.001 [S]
Results are presented as mean±SD **(p< 0.05) considered significantly different** **[S] significant** **[NS] non-significant**			

3.2.2 Glycemic Parameters of Healthy Control Group and Polycystic Ovary Syndrome Patients Group

Data in the table (3-3) showed a significant difference in insulin level and HOMA-IR between the two groups, there were a significant increase in insulin level and HOMA-IR value of PCOS group (P <0.001) compared to control, although there was no significant difference in FSG and HbA1c between two groups.

Table (3-3) Glycemic parameters of control group and polycystic ovary syndrome group

Variables	Control	PCOS	p-value
Number	106	222	-
FSG (mg/dL)	98.6 ± 10.9	98.4 ± 12.8	0.864[NS]
Insulin (µIU/ml)	18.49 ± 19.6	23.06 ± 13.8	<0.001 [S]
HOMA-IR	4.54 ± 5.1	5.65 ± 3.6	<0.001 [S]
HbA1c (%)	5.1 ± 3.9	5.0 ± 2.6	0.060[NS]

Results are presented as mean±SD
(p< 0.05) considered significantly different
[S] significant
[NS] non-significant

3.2.3 Lipid Profile of Healthy Control Group and Polycystic Ovary Syndrome Patients Group

The data in table (3-4) indicated that PCOS patients had significantly higher levels of TG, LDL, and cholesterol (P<0.001) compared to controls, while HDL levels was significantly lower in the PCOS group than control (P =0.009).

Table (3-4) Lipid profile parameters of the control group and polycystic ovary syndrome group

Variables	Control	PCOS	p-value
Number	106	222	-
Triglyceride (mg/dL)	76.1 ± 19.5	127.8 ± 43.5	<0.001 [S]
LDL (mg/dL)	51.2 ± 15.2	97.6 ± 66.2	<0.001 [S]
HDL (mg/dL)	49.0 ± 8.9	45.9 ± 10.6	0.009 [S]
Total Cholesterol (mg/dL)	119.2 ± 29.6	162.8 ± 42.5	<0.001 [S]

Results are presented as mean±SD
(p< 0.05) considered significantly different
[S] significant

3.3 Genetic Analysis

Analyses were conducted to assess the association between the OCT1 polymorphism R61C (rs12208357) and 420del (rs72552763) with the pathophysiology of PCOS.

- R61C (rs12208357) [**CC(Reference allele),CT (heterozygous type)**, and **TT (mutant type)**] as shown in figure (3-1)
- 420del (rs72552763) [**ATG-ATG (Reference allele)**, **ATG-Del (heterozygous type)**, and **Del-Del (mutant type)**] as shown in figure (3-2). Results in the table (3-5) showed that the frequencies of the polymorphisms of OCT1 were not significantly different between the control and PCOS ($p>0.05$).

Table (3-5) Alleles frequencies of R61C and 420del in the study groups

SNP	Alleles	Control	PCOS	p-value
R61C (rs12208357)	TT (Mutant)	25 (23.6%)	49 (22.1%)	0.842
	CT (Heterozygous)	35 (33.0%)	69 (31.1%)	
	CC (Reference)	46 (43.4%)	104 (46.8%)	
420del (rs72552763)	Del-Del (Mutant)	25 (23.6%)	46 (20.7%)	0.757
	ATG-Del (Heterozygous)	38 (35.8%)	88 (39.6%)	
	ATG-ATG(Reference)	43 (40.6%)	88 (39.6%)	

As illustrated in table (3-6) odds ratio was not significantly different between PCOS group and healthy control group.

Table (3-6) Logistic analysis of R61C and 420del to predict polycystic ovary syndrome

	Variables	OR (95%CI)	p-value
R61C (rs12208357)	TT	0.87 (0.48 – 1.57)	0.637
	CT	0.87 (0.51 – 1.49)	0.616
	CC	1.0	-
420del (rs72552763)	Del-Del	0.90 (0.49 – 1.65)	0.732
	ATG-Del	1.13 (0.67 – 1.92)	0.646
	ATG-ATG	1.0	-

OR: odds ratio
CI: confidence interval
(p< 0.05) considered significantly different

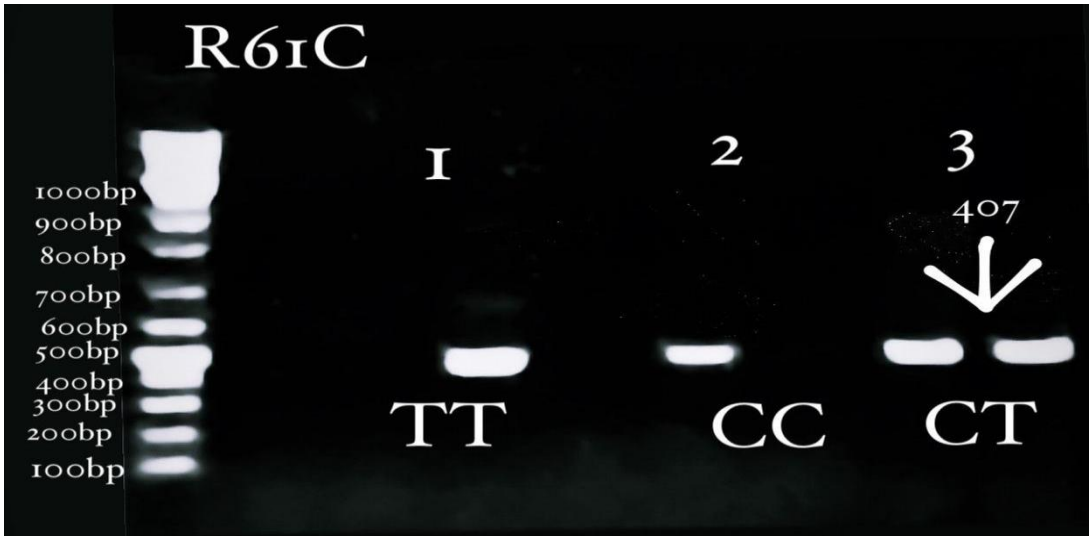

Figure (3-1) PCR amplification of OCT1 gene C>T showing the C and T alleles (both alleles are 407bp in size)

Figure (3-2) PCR amplification of OCT1 gene: delin. Alleles: ATGAT>AT showing the ATG and Del alleles (both alleles are 100bp in size)

3.4Effects of Organic Cation Transporter1 Polymorphism R61C(rs12208357) on Hormonal and Metabolic Response to Metformin

3.4.1 Effect of R61C (rs12208357) on Hormonal Profile After Three Months of Metformin Treatment

It is clear from Table (3-7) that the metformin therapy was modulated the hormonal disturbance in PCOS women after 3 months of treatment, as shown that LH level was significantly reduced after three months of treatment with metformin in women with CC and CT alleles, (P-value <0.001) but there was no significant reduction in those with TT, (P-value > 0.05). FSH was significantly increased after treatment only in women with CC allele, (P<0.001), while not in those with CT and TT (P>0.05). The LH/FSH ratio was significantly reduced after treatment in women with CC and CT, (P<0.001), while there was no significant difference in women with TT, (P>0.05).

Testosterone was significantly reduced after treatment, (P<0.001) in women with CC and CT alleles while not in those with TT, (P>0.05). SHBG was significantly elevated after treatment in women with CC and CT (P-value < 0.001), but the difference in mean SHBG before and after treatment was statistically insignificant in women with TT, (P>0.05). FAI was significantly reduced in all PCOS women at all three alleles; CC, CT, and TT, (P-value <0.001).

Table (3-7) Hormonal parameters in polycystic ovary syndrome women before and after treatment with metformin according to R61C

Variables	Allele	Before	After	P. value
LH (m.IU/L)	CC	9.6 ± 5.1	8.3±4.5	<0.001 [S]
	CT	9.8 ± 6.1	8.1±4.8	<0.001 [S]
	TT	10.5 ± 5.2	10.0±5.8	0.771 [NS]
FSH (m.IU/L)	CC	5.7 ± 1.9	6.5±2.3	<0.001 [S]
	CT	5.6 ± 2.0	6.0±2.3	0.100 [NS]
	TT	6.1 ± 2.1	6.5±2.4	0.374 [NS]
LH/FSH Ratio	CC	1.8 ± 0.9	1.4±0.7	<0.001 [S]
	CT	1.8 ± 1.0	1.4±0.8	<0.001 [S]
	TT	1.8 ± 0.9	1.7±1.2	0.485 [NS]
Testosterone (ng/ml)	CC	0.60 ± 0.38	0.49±0.29	<0.001 [S]
	CT	0.62 ± 0.35	0.49±0.32	<0.001 [S]
	TT	0.69 ± 0.44	0.64±0.41	0.541 [NS]
SHBG (nmol/L)	CC	53.6 ± 24.3	58.1±22.1	<0.001 [S]
	CT	48.2 ± 22.7	53.5±20.8	<0.001 [S]
	TT	59.2 ± 27.4	58.1±23.5	0.111 [NS]
FAI	CC	6.75± 2.4	4.1± 2.59	<0.001 [S]
	CT	8.5 ± 2.8	4.75 ± 3.8	<0.001 [S]
	TT	8.25 ±2.6	5.75 ± 2.1	< 0.001 [S]

Results are presented as mean±SD
(p< 0.05) considered significantly different
[S] significant,[NS] non-significant

3.4.2 Effect of R61C(rs12208357) on Glycemic Parameters After Three Months of Metformin Treatment

Data in table (3-8) showed significant reduction in body mass index after three months of metformin treatment for all three alleles, however the reduction in CC and CT alleles($P< 0.001$) was higher than TT alleles ($P= 0.021$). The changes in FSG, HbA1c, Insulin level, and HOMA-IR in PCOS women after treatment were shown in table (3-8), FSG and HbA1c were significantly lowered in CC and CT alleles after treatment with metformin ($P<0.001$) but not in the TT allele ($P>0.05$). The insulin level and HOMA-IR were significantly reduced in the CC and CT groups ($P=0.001$).

Table (3-8) Glycemic parameters in the polycystic ovary syndrome women before and after treatment with metformin according to R61C

Parameter	Allele	Before	After	P. value
BMI (kg/m^2)	CC	31.2 ± 4.7	28.5 ± 3.6	< 0.001[S]
	CT	31.8 ± 4.5	28.4 ± 3.9	< 0.001[S]
	TT	31.1 ± 4.6	28.9± 5.2	0.021 [S]
FSG (mg/dL)	CC	97.1 ± 13.0	93.2 ± 14.7	0.001 [S]
	CT	98.8 ± 12.6	94.6 ± 11.7	<0.001 [S]
	TT	100.3 ± 12.4	99.7 ± 12.7	0.576 [NS]
Insulin (µIU/ml)	CC	22.6 ± 14.5	20.2 ± 11.3	<0.001 [S]
	CT	23.9 ± 15.1	20.9 ± 11.5	<0.001 [S]
	TT	22.8 ± 10.6	23.3 ± 10.4	0.332 [NS]
HOMA-IR	CC	5.4 ± 3.5	4.7 ± 2.8	<0.001 [S]
	CT	5.9 ± 4.0	5.0 ± 2.9	<0.001 [S]
	TT	5.7 ± 2.7	5.8 ± 2.7	0.446 [NS]
HbA1c (%)	CC	4.9 ± 0.7	4.5 ± 0.7	<0.001 [S]
	CT	4.9 ± 0.7	4.5 ± 0.6	<0.001 [S]
	TT	5.6 ± 5.4	5.5 ± 0.6	0.894 [NS]

Results are presented as mean±SD
($p< 0.05$) considered significantly different
[S] significant, [NS]: non-significant

3.4.3 Effect of R61C(rs12208357) on Lipid Profile After Three Months of Metformin Treatment

Table (3-9) demonstrated the results of lipid profile for the participants PCOS women before and after treatment with metformin which indicated that triglycerides levels were significantly reduced after treatment in all women with CC, CT, and TT alleles, ($P<0.001$). LDL level was significantly reduced while HDL level was significantly increased in women with CC and CT alleles, ($P<0.05$), but no significant changes in LDL and HDL levels in women with TT, ($P>0.05$). The total cholesterol level was significantly lower after treatment in all subgroups with CC, CT, and TT, ($P<0.05$).

Table (3-9) Lipid profiles of polycystic ovary syndrome women before and after treatment with metformin according to R61C

Parameter	Allele	Before	After	P. value
TG (mg/dL)	CC	127.2 ± 43.1	114.2 ± 35.5	<0.001 [S]
	CT	132.5 ± 45.4	118.8 ± 36.9	<0.001 [S]
	TT	122.4 ± 41.8	120.8 ± 40.9	0.037 [S]
LDL (mg/dL)	CC	99.8 ± 90.3	83.0 ± 20.4	<0.001 [S]
	CT	97.8 ± 35.2	88.4 ± 25.2	<0.001 [S]
	TT	92.5 ± 29.1	91.7 ± 27.2	0.996[NS]
HDL (mg/dL)	CC	45.8 ± 10.3	47.8 9.2	0.003 [S]
	CT	45.6 ± 11.2	47.0 ± 10.3	0.002 [S]
	TT	46.4 ± 10.5	44.3 ± 11.6	0.339 [NS]
Total Cholesterol (mg/dL)	CC	161. 0 ± 42.3	148.8 ± 44.8	<0.001 [S]
	CT	166.5 ± 43.2	151.2 ± 41.7	<0.001 [S]
	TT	164.3 ± 41.9	161.3 ± 42.4	0.001 [S]
Results are presented as mean±SD, (p< 0.05) considered significantly different, [S] significant, [NS]: non-significant				

3.5 Effects of Organic Cation Transporter 1 Polymorphism 420del (rs72552763) on Hormonal and Metabolic Response to Metformin

3.5.1 Effect of 420del (rs72552763) on Hormonal Profile After Three Months of Metformin Treatment

The hormonal parameters of PCOS women before and after treatment according to 420del were summarized in (Table 3-10) where LH, and the LH/FSH ratio were significantly decreased after treatment in women with ATG-ATG and ATG-Del, ($P<0.05$), but no significant differences were found in these parameters among PCOS women with Del-Del, ($P>0.05$).

The Testosterone level was significantly reduced after treatment in women with ATG-ATG and ATG-Del alleles, ($P<0.05$), but no significant difference was reported in those with Del-Del, ($P>0.05$). There was a significant elevation in the level of SHBG after treatment in women with ATG-ATG and ATG-Del, (P. value < 0.001 and 0.004, respectively), but the difference in mean SHBG before and after treatment was statistically insignificant in women with Del-Del, ($P>0.05$). FAI was significantly reduced in all PCOS women at all three alleles; ATG-ATG, ATG-Del, and Del-Del, (P. value <0.001).

Table (3-10) Hormonal parameters of polycystic ovary syndrome women before and after three months of metformin treatment according to 420del

Variables	Allele	Before	After	P. value
LH (m.IU/L)	ATG-ATG	10.0± 5.2	8.3±4.6	<0.001 [S]
	ATG-Del	10.0±5.1	8.7±4.7	<0.001 [S]
	Del-Del	9.5±6.3	9.3±5.9	0.765[NS]
FSH (m.IU/L)	ATG-ATG	5.6 ± 2.0	6.2±1.9	<0.001 [S]
	ATG-Del	5.9±2.0	6.5±2.8	0.031 [S]
	Del-Del	5.9±1.9	6.2±2.2	0.128 [NS]
LH/FSH Ratio	ATG-ATG	1.9 ± 1.0	1.4±0.9	<0.001 [S]
	ATG-Del	1.8±0.8	1.5±0.8	<0.001 [S]
	Del-Del	1.6±0.9	1.5±0.8	0.225 [NS]
Testosterone (ng/ml)	ATG-ATG	0.65±0.4	0.52 ± 0.37	<0.001[S]
	ATG-Del	0.57± 0.3	0.46±0.30	<0.001 [S]
	Del-Del	0.71±0.4	0.65±0.39	0.167 [NS]
SHBG (nmol/L)	ATG-ATG	52.3±24.6	59.5 ± 22.5	<0.001 [S]
	ATG-Del	53.0±25.7	57.2 ± 22.0	0.004 [S]
	Del-Del	52.4±23.8	54.8 ± 21.7	0.775 [NS]
FAI	ATG-ATG	7.1±2.26	6.3 ± 6.11	<0.001 [S]
	ATG-Del	7.1 ±2.7	4.25 ± 3.4	<0.001 [S]
	Del-Del	7.5 ±2.5	4.75 ± 2.3	<0.001 [S]

Results are presented as mean±SD
(p< 0.05) considered significantly different
[S] significant, [NS]: non-significant

3.5.2 Effect of 420del (rs72552763) on Glycemic Profile After Three Months of Metformin Treatment

Data in table (3-11) showed significant reduction in body mass index after three months of metformin treatment for all three alleles, however the reduction in ATG-ATG and ATG-Del alleles($P< 0.001$) was higher than Del-Del alleles ($P=0.028$). Assessment of changes in FSG, HbA1c, Insulin level, and HOMA-IR in PCOS women after treatment according to 420del are demonstrated in (Table 3-11). FSG was significantly lowered in ATG-ATG group ($P<0.001$) and ATG-Del ($P<0.002$) while not in Del-Del groups ($P>0.05$). HbA1c, Insulin level, and HOMA-IR were significantly reduced in ATG-ATG and ATG-Del groups, ($P<0.001$).

Table (3-11) Glycemic parameters in polycystic ovary syndrome women before and after treatment with metformin

Variables	Allele	Before	After	p-value
BMI (kg/m^2)	ATG-ATG	31.4 ± 4.9	28.0 ± 3.6	< 0.001[S]
	ATG-Del	31.3 ± 4.7	28.8 ± 4.5	< 0.001[S]
	Del-Del	31.2 ± 4.7	29.1 ± 3.7	0.028 [S]
FSG (mg/dL)	ATG-ATG	96.5 ± 12.5	93.5 ± 10.7	<0.001 [S]
	ATG-Del	99.2 ± 12.5	94.6 ± 16.1	0.002 [S]
	Del-Del	100.3 ± 13.6	98.7 ± 12.8	0.126 [NS]
Insulin (µIU/ml)	ATG-ATG	23.2 ± 15.6	20.8 ± 11.8	<0.001 [S]
	ATG-Del	21.8 ± 13.7	19.8 ± 10.7	<0.001 [S]
	Del-Del	25.1 ± 10.6	24.0 ± 10.5	0.178 [NS]
HOMA-IR	ATG-ATG	5.6 ± 3.8	4.8 ± 2.9	<0.001 [S]
	ATG-Del	5.4 ± 3.5	4.8 ± 2.9	<0.001 [S]
	Del-Del	6.2 ± 2.8	5.9 ± 2.6	0.114 [NS]
HbA1c (%)	ATG-ATG	5.2 ± 4.0	4.5 ± 0.7	<0.001 [S]
	ATG-Del	4.9 ± 0.9	4.6 ± 0.6	<0.001 [S]
	Del-Del	4.8 ± 0.7	4.7 ± 0.7	0.268 [NS]

Results are presented as mean±SD
p< 0.05) considered significantly different
[S] significant, [NS]: non-significant

3.5.3 Effect of 420del (rs72552763) on lipid Profile After Three Months of Metformin Treatment

The lipid profile of the participant PCOS women before and after treatment according to the 420del was shown in (Table 3-12), which revealed that triglycerides levels were significantly reduced after treatment in women with ATG-ATG and ATG-Del alleles, ($P<0.001$ and $P=0.001$), but no significant difference was found in women with Del-Del. LDL level was significantly reduced while HDL level was significantly elevated in women with ATG-ATG and ATG-Del, ($P<0.05$), but non-significant changes in LDL and HDL levels in women with Del-Del, ($P>0.05$). Total cholesterol level was significantly lower after treatment in women with ATG-ATG and ATG-Del, alleles ($P<0.05$), with non-significant difference in women with Del-Del allele, ($P>0.05$).

Table (3-12) Lipid profile of polycystic ovary syndrome women before and after treatment with metformin according to 420del

Parameter	Allele	Before	After	P. value
Triglycerides (mg/dL)	ATG-ATG	126.4±41.9	115.1 ± 37	<0.001 [S]
	ATG-Del	134.6 ± 46.5	121.2 ± 39.7	<0.001 [S]
	Del-Del	117.6 ± 39.3	113.2 ± 32	0.817 [NS]
LDL (mg/dL)	ATG-ATG	91.3 ± 26.7	84.4 ± 21.5	<0.001 [S]
	ATG-Del	108.5 ± 99.3	89.8 ± 27.4	<0.001
	Del-Del	89.0 ± 29.8	84.8 ± 19.9	0.087 [NS]
HDL (mg/dL)	ATG-ATG	47.3 ± 11.2	49.4 ± 10.6	<0.001 [S]
	ATG-Del	44.5 ± 9.9	45.8 ± 7.8	0.013 [S]
	Del-Del	45.7 ± 10.5	43.6 ± 12.0	0.289 [NS]
Total Cholesterol (mg/dL)	ATG-ATG	162.4 ± 34.7	148.7 ± 40.3	<0.001 [S]
	ATG-Del	165.0 ± 47.1	154.4 ± 45.5	<0.001 [S]
	Del-Del	159.2 ± 47.3	158.4 ± 45.4	0.639 [NS]

Results are presented as mean±SD
p< 0.05) considered significantly different
[S] significant, [NS]: non-significant

Chapter Four

Discussion

Chapter Four

Discussion

4.1 Socio-demographic Data

Polycystic ovary syndrome (PCOS) is a complex endocrine condition that affects reproduction[70] and contributes to long-term metabolism disturbance, such as diabetes and heart disease [7]. High androgen level and insulin resistance result in metabolic and hormonal dysfunctions in PCOS patients [125], which lead to high comorbidity rates [126].

Table (3-1) demonstrates 222 PCOS patients and 106 healthy control enrolled in this study were at the reproductive age. Both groups in this study were overweight, BMI for PCOS patients was 31.4 ±4.9 and for healthy control 27.8 ±4.7. Barber *et al* 2006 . confirmed that weight gain and obesity occur in approximately (76%) of women with PCOS [127]. Alopecia and hirsutism were (92%) and (83%) in PCOS patients, PCOS can cause both alopecia and hirsutism as one of the most reliable results of hyperandrogenism associated with this syndrome [128].

4.2 Hormonal and Metabolic Variation between Control Group and Polycystic Ovary Syndrome Group

In the present study, serum LH and testosterone levels were significantly higher in PCOS patients, while FSH was significantly lower in patients in comparison with control participants. Hence the LH/FSH ratio was significantly elevated in PCOS patients as showed in the table (3-2).

These results compatible with that reported by Neoklis *et al* 2016 .,who found that the LH, LH/FSH ratio, total testosterone, and FAI were elevated in women with PCOS as compared to control with lower FSH level in PCOS women than control subjects[129].

Insulin resistance and hyperinsulinemia may play a part in the development of the typical increases in the amplitude and frequency of GnRH and LH pulse secretion in PCOS[130]. This effect may be mediated by the action of insulin on GnRH-secreting cells in the hypothalamus and potentiating GnRH gene transcription through the MAPK pathway. Increased GnRH synthesis and secretion lead to a subsequent elevation in LH level, this continuous stimulation would translate into the augmented synthesis of ovarian steroid hormones, particularly androgens [131].

Serum prolactin level was elevated in PCOS patients with the excluding of hyperprolactinemia during the diagnosis of PCOS. Prolactin is a circulating hormone released from the pituitary gland and is regulated by the suppression effect of dopamine which is secreted from the hypothalamus[132], one of the possible causes of PCOS is the abnormal hypothalamus-pituitary axis that leads to impaired suppression of prolactin[132, 133]. PCOS is characterized by increased inflammation with abdominal obesity [134]. Furthermore, the macrophage-derived from adipose tissue released prolactin in response to inflammation and high glucose concentration [135].

We found that the SHBG level was significantly lower in PCOS patients than healthy control while FAI was significantly higher. SHBG is the main transporter protein for testosterone that modulates its biological activity [136]. Polymorphism of the sex hormone-binding globulin (*SHBG*)

gene has been associated with low SHBG levels and increased risk for PCOS or hyperandrogenism[137]. Insulin inhibits the hepatic synthesis of SHBG and insulin-like growth factor-binding protein 1 (IGFBP-1) thereby increases free and bioavailable testosterone and insulin-like growth factor (IGF) concentration in the ovary [46], Cupisti et al. reported that obese PCOS women were associated with significantly increased FAI, and decreased SHBG [138], an observation that was also confirmed by Mueller *et al.* [139]. Both studies findings were compatible with the current study.

In this study, the TSH level was significantly higher in PCOS patients compared to the control group but still within the normal range. The link between thyroid function and PCOS is unclear however PCOS is associated with an increase in pro-inflammatory markers and an increase in insulin resistance[140] which through undefined mechanisms, leads to decreased deiodinase-2 activity at the pituitary level resulting in relative increase in TSH levels, the raised TSH levels act on adipocytes to increase their proliferation [141], El-Hafez *et al.2016* found that euthyroid, IR-PCOS women had higher TSH levels compared with euthyroid, non-IR-PCOS women[142].

Regarding the metabolic picture of PCOS patients, study data in the table (3-3) revealed elevated serum insulin levels in PCOS patients, but with no significant differences in fasting blood sugar and HbA1c between study groups. The HOMA-IR was significantly higher in PCOS patients than in healthy control, these results were compatible with those observed by Behboudi-Gandevani *et al 2016*. who found a significant increase in insulin level and HOMA-IR in obese PCOS women[143]. 50% to 90% of women diagnosed with PCOS have insulin resistance (IR) [144], hyperandrogenism

(HA) aggravate IR by reducing the sensitivity and expression level of glucose transporter protein-4 (Glut-4), inhibiting the degradation of insulin by the liver, and exacerbating central obesity[145]. Hyperinsulinemia develops as a compensatory response to IR in PCOS. However, theca cells remain insulin sensitive and the insulin interacts synergistically with LH and stimulates the androgen production in theca cells [146].

In the present study as illustrated in table (3-4) we found that the mean values of the lipid profile components were significantly higher in the PCOS group as compared to healthy women except for HDL which was lower in PCOS. The findings of our study were following Kader *et al* 2007. who suggested that this compromised metabolic profile in PCOS puts these women at higher cardiovascular risk[147], Kalra *et al* 2006, in their study found that insulin-resistant PCOS women had high triglycerides , total cholesterol and lower high-density lipoprotein compared to insulin-sensitive women, they concluded that insulin resistance is associated with dyslipidemia in women with PCOS [148]. The presence of an atherogenic lipid profile in women with PCOS was also confirmed by Valkenburg *et al* 2013 [149]. The effects of IR on lipid metabolism were well known. IR impairs the ability of insulin to suppress lipolysis increasing mobilization of free fatty acids from adipose stores with consequent increased hepatic delivery of free fatty acids, thus impairing insulin inhibition of hepatic very-low-density lipoprotein synthesis, and altering catabolism of very-low-density lipoprotein[150].

4.3 The Association of Organic Cation Transporter 1 Polymorphism with the Incidence of Polycystic Ovary Syndrome

Since 1968, studies have suggested an important genetic role contributing to the etiology of PCOS[151]. Genome-Wide Association Studies (GWAS) have identified several new risk loci and candidate genes for PCOS. Despite these findings, the association studies have explained less than 10% of heritability[152]. In this study as shown in table (3-5) and (3-6) the allele frequencies of R61C (*rs122083571* [CC (reference allele), CT (heterozygous type), and TT (mutated type)] and 420del (*rs72552763)* [ATG-ATG (reference allele), ATG-Del (heterozygous type), and Del-Del (mutated type)]) were similar in both PCOS patients and control group, this indicates that OCT1 polymorphism was not associated with PCOS pathophysiology and this agreed with a study by Hui Hua Chang *et al* 2019. who showed similar findings [153].

4.4 Influence of Organic Cation Transporter 1 Polymorphism on Metformin Response

Metformin was the first insulin-sensitizing drug to be used in PCOS [154]. Velazquez and colleagues reported a significant improvement in menstrual regularity and reduction in circulating androgen levels in PCOS patients treated with metformin[154]. Considerable inter-individual variability exists in response to metformin, both non-genetic and genetic factors were determinants of the metformin effect. [155] The majority of pharmacogenetic

studies performed with metformin have been focusing on the identification of gene variants related to metformin pharmacokinetics[156].

Pharmacogenetics differs from more classical genetic approaches in that there must be an interaction between gene and drug therapy, as opposed to a more straightforward association to disease. Drug-genome interactions can occur in several ways including

a-genetic variation in the direct molecular target of a drug class ,

b- class of genes are those involved in drug ADME absorption, distribution (such as *OCT1*), metabolism, and excretion. In general, ADME genes can affect the action of many drugs, independent of their intended molecular target. Shu *et al.* 2007[157] in their integrative pharmacogenomics study revealed the diversity of responders to a drug leads to personalized treatment regimens that ensure the administration of the right drug for the right person at the right time[158].

Organic cation transporter (OCT) proteins mediate the transport of organic cations across the cell membrane. Metformin has been demonstrated to be a substrate of liver-specific OCT1 [159], several studies indicated that the pharmacokinetic and pharmacodynamics profiles of metformin are mediated by the activity of OCT1 [160].

In this study, the follow up of PCOS cases with metformin for 3 months revealed a significant fall in serum LH, LH/FSH ratio, and testosterone in patients with reference alleles and heterozygous alleles for both R61C and 420del, while PCOS women with mutant alleles did not show significant reduction of these parameters. Metformin significantly increases serum FSH level in patients with reference alleles, while mutant alleles didn't show significant elevation for both R61C and 420del.

Although patients with heterozygous alleles of 420del showed a significant increase in the FSH level, they didn't show that effect in R61C. Concerning the SHBG level, metformin increases its concentration in patients with reference alleles and heterozygous alleles for both R61C and 420del while the mutant allele didn't show a significant response. These findings suggested that metformin lowers testosterone levels in women with PCOS**;** this effect may be attributed to a reduction in hyperinsulinemia because of enhanced insulin sensitivity [161, 162], and reduction in *CYP17* activity in women with PCOS upon metformin treatment consequent to lowering of serum insulin levels [161]. However, there was evidence that metformin directly inhibits ovarian steroidogenesis [163] [164]. Jolanta Nawrocka *et al*.2007 indicated that the metformin causes a statistically significant decrease in luteinizing hormone (LH) , the free androgen index (FAI), and significant increases in FSH, FSH/LH ratio and SHBG concentration [165].

In women with mutant alleles diminished metformin response may be due to reducing the transport of metformin via (OCT1) in the intestine leading to decrease metformin bioavailability and consequently diminished metformin effects on its main target tissue in PCOS like liver, skeletal muscles, and ovary, make the patients liable to gastrointestinal side effects due to high metformin concentration in gastrointestinal tract(GIT) [166].

This study revealed that metformin can affect body weight. All patients in this study (carrier of reference allele, heterozygous allele and mutant allele) for both R61C and 420del showed significant reduction in BMI after three months of 1000 mg metformin treatment. Metformin can reduce body weight by several mechanisms, Its primarily acts on the central nervous system to reduce appetite by attenuating hypothalamic AMPK

activity, which decreases orexigenic peptides, neuropeptide (NPY), and increases pro-opiomelanocortin (anorectic) expression, also metformin has an additional food-lowering effects by improving leptin and insulin sensitivity, increasing glucose-like polypeptide-1 levels, and affecting gut flora. Metformin also reduces ectopic lipid depots (i.e. liver and skeletal muscle) through increased fat oxidation and decreased lipid synthesis, which may be regulated to some extent by circadian clock genes.

Polymorphism in OCT1 gene (R61C and 420del) did not affect the ability of metformin for lowering the body weight, this may be due to metformin main action on body weight occur in brain, skeletal muscles, adipose tissue where metformin uptake accrue via passive diffusion and other transporters. Reduced body weight maybe due to lifestyle modification like low carbohydrate diet, avoidance of fatty meals and daily exercise.

Fasting blood sugar, insulin level, HbA1C, and HOMA-IR were significantly reduced in patients with reference alleles and heterozygous alleles but they did not in mutated alleles for both R61C and 420del. Metformin exerts pleiotropic actions in several tissues, primarily the liver, where it inhibits hepatic gluconeogenesis and glycogenolysis, through which the drug contributes to improving insulin sensitivity. The potential mechanisms for inhibiting the hepatic gluconeogenesis including direct inhibition of gluconeogenic enzymes (e.g. phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase), reduced hepatic uptake of substrates for gluconeogenesis, and increased phosphorylation of insulin receptor and insulin receptor substrates (IRS)-1 and -2 [167-169]. Other investigators have also demonstrated the inhibition of

mitochondrial respiration by metformin, may reduce the energy supply required for gluconeogenesis [170].

Patients with mutated alleles didn't have a significant response and this may be due to reduce or loss of OCT1 function and diminished hepatic uptake of metformin due to gene polymorphisms. Our study was compatible with Yan Shu *et al* 2007 who showed that the effects of metformin on glucose tolerance tests were significantly lower in individuals carrying reduced function polymorphisms of *OCT1*[157]. Sundelin *et al* 2017 in their study showed that hepatic distribution of metformin was significantly reduced after oral intake in carriers of *420del* and R61C variants in *SLC22A1* [171].

Dyslipidemia is common in PCOS patients, characterized by higher triglycerides (TG) and lower high-density lipoprotein (HDL). Dyslipidemia occurs independently of body mass index (BMI); however, there is a synergistic deleterious effect of obesity and insulin resistance in PCOS analogous to that seen in T2DM. Dyslipidemia in PCOS has multifactorial causation, insulin resistance plays a pivotal role by the stimulation of lipolysis and altered expression of lipoprotein lipase and hepatic lipase [6].

In this study after three months of metformin treatment, we found that TG, LDL, and cholesterol were significantly reduced in patients with reference alleles and heterozygous alleles, while mutant alleles showed less response in both R61C and 420del. The cholesterol level in patients with R61C mutant alleles, respond better than those with mutant alleles of 420del. HDL level significantly increases in patients with reference alleles and heterozygous alleles, while patients with mutant alleles didn’t respond,

altered OCT1 function as a result of polymorphism able to influence the intracellular metformin concentrations and therefore metformin action on lipogenic enzymes, particularly inhibition of acetyl-CoA carboxylase (ACC) activity via an AMPK-dependent pathway [70].

The results were in line with previous studies on PCOS, where metformin has been shown to improve the lipid profile, mainly by increasing serum HDL concentrations [172], Glueck *et al* 2003 reported a significant decrease in triglyceride levels[173], on the other hand, Rautio *et al* 2005 reported a non-significant change in serum cholesterol, triglycerides, and LDL-C levels [174].

4.5 Conclusions

These data provide a proof of concept, in a clinical setting that

- OCT1 polymorphism can be considered as one of the genetic factors responsible for heterogeneity in the response to metformin in Iraqi female suffering from PCOS.
- Carrier of reference allele and heterozygous alleles for both SNPs showed significant response after three months of metformin treatment
- Carrier of mutant alleles showed non-significant response after three months of metformin treatment
- Polymorphism of the OCT1 gene is not associated with the incidence of PCOS.

4.6 Recommendations and Future works

1- Further studies will be necessary to confirm the impact of genetic variants in other candidate genes associated with metformin disposition like OCT2 and MATEs

2- Studying other OCT1 SNPs in order to find out the effect of multiple OCT1 polymorphisms on metformin response in PCOS patients

3- Metformin plasma level should be determined in next study to proof the effect of transporter gene polymorphism on metformin bioavailability.

4-Larger scale studies enrolling more PCOS patients and healthy control participants from different Iraqi cities are required

4- In clinical setting, I recommend to develop genetic tests that could predict a person's response to metformin treatment and create personalized drugs with greater efficacy and safety.

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النتائج: وضحت النتائج تعدد الاليلات الجينية لناقل الكاتيون العضوي الاول كان متشابها في مجموعة النساء المريضات والنساء السليمات. في ما يتعلق بالاستجابة العلاجية لعقار المتفورمين ، معظم المريضات الحاملات للاليل السائد والاليل المتنحي اظهروا استجابة ذات دلالة احصائية واضحة ولكن المريضات الحاملات للاليل الطافر لم يظهروا استجابة علاجيه في

الاستنتاج: قد يترافق الاختلاف الجيني لناقل الكاتيون الاول مع عدم التجانس في استجابة المرضى للميتفورمين ، لكن ليس له علاقة بإمراضيه متلازمة تكيس المبايض.

الخلاصة

الخلفية : متلازمة تكيس المبايض هي اضطرابات ايضيه و هرمونيه شائعة جدا ولها عدة اضرار على صحه النساء المصابات واهمها العقم. بالرغم من عدم معرفة السبب الرئيسي لهذه المتلازمة الى الان لكن يعتقد ان الاسباب تكمن في اضطراب الهرمونات واهمها الهرمون الذكري الاندروجين وكذلك مقاومة الانسولين وارتفاع نسبة الانسولين في الدم. هرمون الانسولين يسبب تشويش في عمل الغده تحت المهاد والغدة النخامية وكذلك المبيض وذلك يؤدي الى خلل في الايض الخلوي وزيادة هرمون الاندروجين الذي يعتبر المسبب الرئيسي في الاعراض السريرية لدى النساء المصابات بمتلازمة تكيس المبايض. يعتبر المتفورمين من اهم العلاجات المستخدمة لهذه المتلازمة الا ان استجابة المريضات متباينة لعدة اسباب. ناقل الكاتيون العضوي يلعب دور مهم في ادخال المتفورمين الى خلايا الكبد. وجد في دراسات حديثة ان تعدد الاشكال الجينية لهذا الناقل تؤثر على استجابة المرضى للميتفورمين.

الهدف من الدراسة: الغرض من هذه الدراسة هو لمعرفة تأثير تعدد الاشكال الجينية لناقل الكاتيون العضوي الاول على الاستجابة العلاجية للميتفورمين لدى مريضات تكيس المبايض في العراق.

المرضى والطرق: هذه الدراسة مرتقبة تمت في مستشفى النسائية والتوليد التعليمي في كربلاء قسم العقم وكذلك في العيادة الخاصة لطبيبة استشارية اخصائية نسائية وتوليد وتم تشخيص المريضات من قبل الطبيبة اعتمادا على قواعد روتردام. تضمنت هذه الدراسة 222 مريضة بمتلازمه تكيس المبايض و 106 امرأة سليمة تتراوح اعمارهم بين 20-40. اعطي علاج المتفورمين بجرعة 500ملغ مرتان يوميا لمدة ثلاث اشهر. اخذت عينات دم من المريضات قبل وبعد ثلاثة اشهر من علاج المتفورمين لغرض قياس نسب الهرمونات ومستويات السكر والانسولين والدهون ولغرض اجراء الدراسة الجينية لمعرفة تعدد الاشكال الجينية للناقل الكاتيون العضوي الاول ودراسة تأثيره على استجابة المريضات بمتلازمة تكيس المبايض لعلاج المتفورمين.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الصيدلة

علاقة تعدد الأشكال الجيني لناقل الكاتيون العضوي الاول مع الاستجابة العلاجية للميتفورمين في النساء المصابات بمتلازمة تكيس المبايض في العراق

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

من قبل

براء حسين علي

(بكالوريوس صيدلة 2014)

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٢٠٢١ ميلادي

١٤٤٢ هجري