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Association of Genetic Polymorphism of Insulin Receptor Substrate- 1 with Therapeutic Response of Metformin in Women with Polycystic Ovary Syndrome in Iraq

A Thesis

Submitted to the Council of college of Pharmacy as Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology and Toxicology

By

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ وَنُرِيدُ أَنْ نَمُنَّ عَلَى الَّذِينَ اسْتُضْعِفُوا فِي الْأَرْضِ

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DEDICATION

To the one who I carry his name proudly, the one who has help me to reach the goals that I want in my life, the greatest father in the world

To the meaning of love and compassion who her prayer is the secret of my success, my mother the angle of my life

To my soul mate Baraa

To my lovely daughter Ruqaia

To everyone who stood by my side to complete this thesis

Last but not the least, I would like to thank my family: my parents for giving birth to me at the first place and supporting me spiritually throughout my life.

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List of Abbreviations	
Abbreviations	***Full-Text***
ACTH	Adrenocorticotropic hormone
AES	Androgen Excess Society
ARMS-PCR	Amplification Refractory Mutation System Polymerase Chain Reaction
CE	Cholesterol esterase
CHOD	Cholesterol oxidase
EDTA	Ethylene diamine tetra acetate
ELISA	Enzyme Linked Immuno-Sorbent Assay
FOXO	Forkhead transcription factors O
FSH	Follicle-stimulating hormone
GLUT4	Glucose transporter 4
GnRH	Gonadotropin releasing hormone
Grb2	Growth factor receptor bound protein 2
GWAS	Genome wide association studies
HI	Hyperinsulinemia
HOMA	Homeostasis model assessment
HPOA	Hypothalamic-pituitary-ovarian axis
HRP	Horseradish peroxidase
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
ISD	Insulin sensitizing drugs
LH	luteinizing hormone

NIH/NICHD	The National Institutes of Health/National Institute of Child Health and Human Disease
PCOM	polycystic ovary morphology
PCOS	Polycystic ovary syndrome
PDK	Phosphoinositide-dependent protein kinases
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B also known as Akt
PKC	Protein kinase C
PTB	Phosphotyrosine binding
Rotterdam ESHRE/ASRM	The European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine
SHBG	Sex hormone binding-globulin
SHP2	Src homology containing protein tyrosine phosphatase 2
SNPs	Single nucleotide polymorphisms
STK11	Serine/threonine kinase 11
TTAB	Tetradecyl trimethylammonium bromide

List of Normal Laboratory Value	
Parameters	***Normal value***
luteinizing hormone (LH)	1.5-8 m.lu /ml
Follicle-stimulating hormone (FSH)	2.9-12 m.lu/ml
Thyroid stimulating hormone (TSH)	0.27-4.2 ulU/ml
Prolactin	6-29.9 ng/ml
Total Testosterone	0.06-0.82 ng/ml
Sex hormone binding-globulin (SHBG)	15 - 120 nmol/L
Fasting Serum Glucose (FSG)	70-120 mg/dl
Fasting Insulin	2-25 µIU/ml
Glycosylated Hemoglobin (HBA1c)	4.1- 6.5 %
Triglyceride (TG)	0-149 mg/dl
Low Density Lipoprotein (LDL)	0-100 mg/dl
High Density Lipoprotein (HDL)	40-60 mg/dl
Cholesterol	50-199mg/dl

Abstract

Background **:**

Polycystic ovary syndrome (PCOS) is highly prevalent hormonal disorder among reproductive-aged women. Its clinical manifestations are heterogeneous, the syndrome diagnosed according to Rotterdam criteria that depend on hyperandrogenism (clinical and/or biochemical), ovulatory dysfunction and / or polycystic ovarian morphology. Polycystic ovary syndrome is associated with hyperinsulinemia and insulin resistance that affected by insulin receptor substrate 1 (IRS1) protein, this protein is an important intermediate in insulin signaling and plays a key role in maintaining the basic function of the cell, so any polymorphism in IRS1 genes acts as a competitive inhibitor of the insulin receptor. IRS1 polymorphism may be associated with susceptibility to insulin resistance, poly cystic ovary syndrome and variable effect of metformin response in polycystic ovary syndrome

Objectives **:**

The present study was designed to evaluate the possible association of insulin receptor substrate 1 (IRS1) genetic polymorphisms (rs2943641 and rs1801123) with susceptibility to polycystic ovary syndrome. Also to evaluate the association of IRS1 genetic polymorphisms (rs2943641 and rs1801123) with the therapeutic response to metformin (after 12 weeks of 500 mg twice daily metformin treatment) for women with polycystic ovary syndrome in Iraq.

Subjects and Methods :

This research was done at the infertility department in Kerbela teaching hospital of gynecology and obstetrics and outpatient clinic from Jul

2019 to April 2020.The study included three hundred twenty newly diagnosed polycystic ovarian syndrome female patients and 104 apparently healthy control women their age range from 20 to 40 years. In this study, blood sample was collected from each participant after overnight fasting in day two of menstrual cycle or in any time if there is amenorrhea present, by vein puncture. Collected blood sample was divided into two parts, first part was kept in the ethylene diamine tetra acetate (EDTA) tube for HbA1c% and DNA extraction, the second part was kept in a gel tube to isolate serum, for hormonal (such as luteinizing hormone (LH), Follicle-stimulating hormone (FSH), LH/FSH Ratio , Thyroid stimulating hormone (TSH), Prolactin , Testosterone, Sex hormone binding-globulin (SHBG) and Free Androgen Index FAI) and other biochemical tests (such as Fasting Serum Glucose (FSG), Fasting Insulin (FI), homeostasis model assessment for insulin resistance (HOMA-IR) , Glycosylated Hemoglobin (HbA1c), Triglyceride (TG), Low Density Lipoprotein (LDL) , High Density Lipoprotein (HDL), and Cholesterol). Blood samples were collected from all participants and also after 12 weeks of treatment to follow up and determine the changes in the studied parameters in patients group only.

Results :

There was statistically significant differences in hormonal and metabolic parameters between the control group and poly cystic ovary syndrome patients group. IRS1 polymorphism (rs2943641 and rs1801123) don’t have a role in the pathogenesis of the poly cystic ovary syndrome in Iraqi women .

Conclusion :

This study, do not support that the IRS1 polymorphism (rs2943641 and rs1801123) are associated with increased susceptibility to polycystic ovary syndrome in Iraqi women but they can be the cause of therapeutic failure to metformin response in Iraqi females diagnosed with PCOS.

Chapter one

Introduction

1. 1 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) was named based on the pathological appearance of the ovary in women with menstrual irregularities and hyperandrogenism, thus its name alludes to polycystic ovary morphology (PCOM) as a prominent feature of the disorder[(1)]. Polycystic ovary syndrome also known as Stein-Leventhal syndrome, is a complex endocrine and metabolic disorder affecting the reproductive aged women[(2)].

This disorder is characterized by multiple hormonal imbalances, reflecting on a clinical presentation dominated by manifestations of hyperandrogenism[(3)], severe menstrual disturbance (amenorrhea or oligomenorrhea) , obesity [(4)] and ovarian dysfunction that continues to be the main feature which make this syndrome and cause anovulation [(5)] . Among many abnormalities , infertility is one of the most alarming associated morbidities , as it currently affects approximately 48.5 million women aged 20–44 years [(6)].

1.2 Clinical and Biochemical Presentation

The clinical manifestations of PCOS are heterogeneous , although some of the clinical symptoms and presentations are dependent on age , but ovarian failure and hyperandrogenemia are common characteristics at any age [(7)].The most common feature of polycystic ovary syndrome include biochemical and clinical hyperandrogenism of ovarian origin and to an lesser extent adrenal origin ; this evident in about 60–80% of PCOS patients ,

resulting in the main features of the syndrome [8] as oligomenorrhea or amenorrhea, menstrual irregularities , PCOM [9] , infertility, hirsutism, weight gain, central obesity, and acanthosis nigricans [10]. Hormonally, it can present as elevated luteinizing hormone (LH) level with normal or slightly decreased level of follicle-stimulating hormone (FSH), hyperinsulinemia (HI), low levels of sex hormone binding-globulin (SHBG) [11]. Dyslipidemia, dysglycemia, and insulin resistance that increase the risks of type II diabetes mellitus in PCOS patients [12].

1.3 Diagnostic Criteria

Although up to 70% of women with PCOS may be undiagnosed, because optimal diagnosis is often hindered due to its apparent similarities with many pathologies [13]. Many sets of diagnostic criteria of PCOS have been proposed [14] as shown in table (1-1) But Rotterdam criteria is the most one used[15, 16].

Table (1-1): The diagnostic criteria for polycystic ovary syndrome.

(NIH/NICHD)1990 [17] The National Institutes of Health/National Institute of Child Health and Human Disease	Requires the simultaneous presence of: 1. Hyperandrogenism (clinical and/or biochemical). 2. Ovarian dysfunction.
Rotterdam (ESHRE/ASRM)/2003 [18]. The European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine.	Requires the presence of at least two criteria : 1. Hyperandrogenism (clinical and/or biochemical) 2. Ovulatory dysfunction 3. Polycystic ovarian morphology.
Androgen Excess Society (AES/2006) [2]	Requires the presence of hyperandrogenism (clinical and/or biochemical) and either: 1. Ovulatory dysfunction 2. Polycystic ovarian morphology.
Androgen Excess and PCOS Society/2009 [19]	Requires the simultaneous presence of: 1. Hyperandrogenism (clinical and/or biochemical) 2. Ovarian dysfunction (ovulatory dysfunction and/or polycystic ovarian morphology.

1.4 Prevalence

The prevalence of PCOS in general population has not been definitively determined and appears to vary considerably among populations that have been studied [20]. Recently, several studies have demonstrated that the prevalence of PCOS varies depending on the diagnostic criteria used [21] . According to National Institutes of Health (NIH) criteria , PCOS is a common endocrinopathy affecting 6%–8% of women of reproductive age [22] . Many studies consistently report that the prevalence estimates using the Rotterdam criteria are two to three times greater than those obtained using the NIH criteria, PCOS prevalence with the implementation of the Rotterdam criteria increased to 15– 25% , while the use of Androgen Excess Society (AES) recommendations put PCOS prevalence at about 10– 15%[23] . Several studies have been performed to determine the prevalence of polycystic ovaries depending on ultrasound alone and have found 20 -30% of women have PCOS [20, 24] . In Iraq, The prevalence of PCOS in females (20-40y) was 14%[25].

1.5 Etiology and Pathophysiology

To date , the etiology and pathophysiology of PCOS remains unclear , substantial evidence suggests it is a multifactorial condition , where interactions between environmental, genetic, endocrine and metabolic factors act in consonance towards a common result [15] as shown in Figure (1-1)

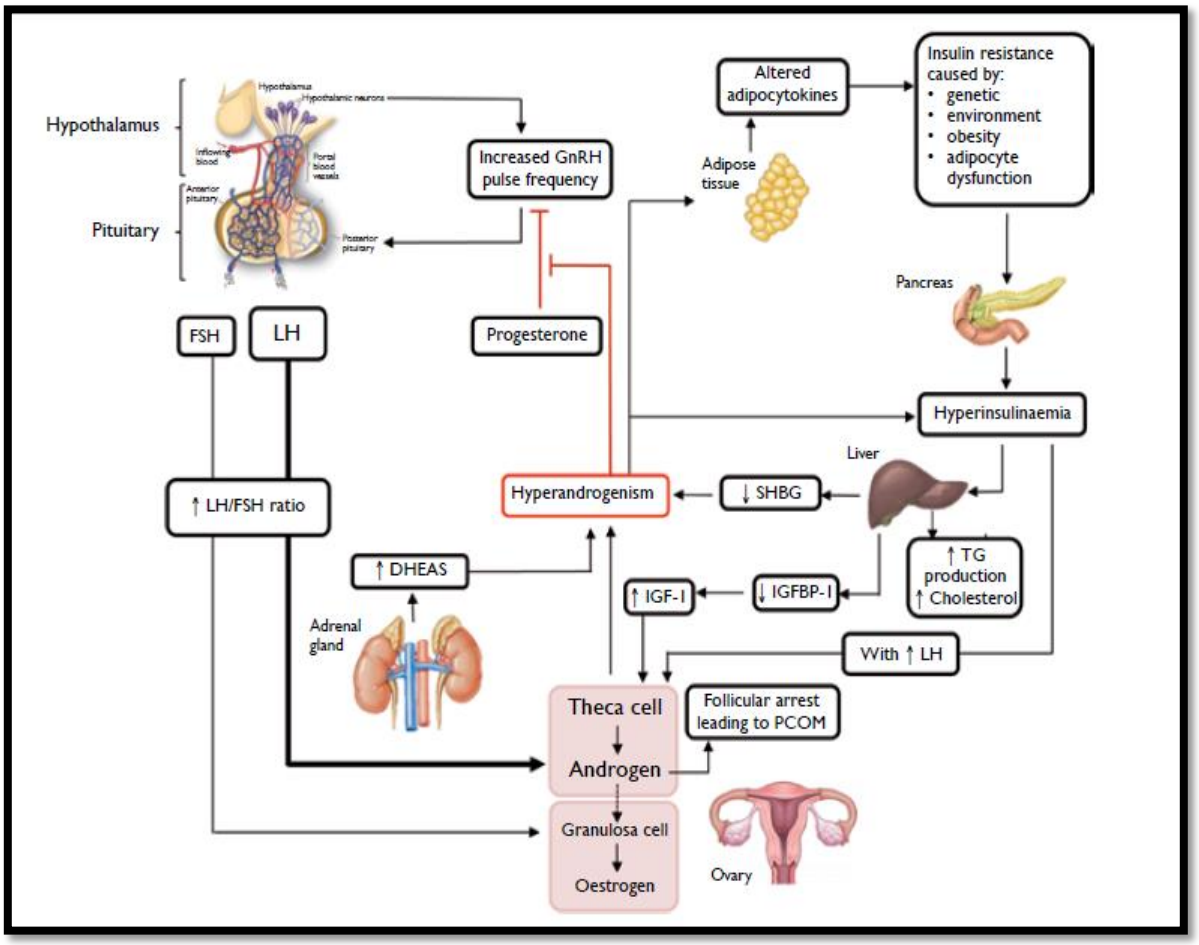

Figure (1-1): The pathophysiology of PCOS. [Abbreviations: DHEAS = dehydroepiandrosterone sulfate; FSH = follicle-stimulating hormone; GnRH = gonadotropin-releasing hormone; IGF-1 = insulin-like growth factor 1; IGFBP-1 = insulin-like growth factor–binding protein-1; LH = luteinizing hormone; PCOM = polycystic ovarian morphology; SHBG = sex hormone–binding globulin; TG = triglycerides] [(26)]

1.5.1 Environmental Factors

The rise in PCOS prevalence in populations where the gene pool has been relatively constant confirms that environmental factors are assuming an ever more important role [(27)] ; The development of obesity is linked to the

development of PCOS in susceptible individuals[28]; The modern living environment in developed countries is characterized by low daily energy expenditure and an abundant and inexpensive food supply, making positive energy balance common[29]; Exposure to excess maternal androgens during fetal life can directly influence the development of PCOS in the child. It remains possible, however, that maternal metabolic abnormalities during pregnancy have an effect, although there are no direct data to support this hypothesis [30] .

1.5.2 Genetic Factors

Recent genetic studies indicate that PCOS has a high heritability of approximately 80% [31] . Genome wide association studies (GWAS) have identified up to 19 distinct genetic loci in or near , known genes that are associated with PCOS [32] , Identifying single nucleotide polymorphisms (SNPs) that may contribute to the genetic basis of PCOS like Serine/threonine kinase 11 (STK11) SNPs and forkhead transcription factors O (FOXO) SNPs [32] . Studies of PCOS patients complicated with acute hyperglycemic attack discovered the possibility of genetic polymorphism that influences insulin receptor substrate 1 (IRS1) [33]. The IRS1 is an important intermediate in insulin signaling and plays a key role in maintaining the basic function of the cell , For this reason any polymorphism in IRS1 genes acts as a competitive inhibitor of the insulin receptor[34]. This mean, mutations in IRS1 gene may associated with PCOS and susceptibility to insulin resistance[35].

1.5.3 Endocrine Factors

Polycystic ovary syndrome may occur due to impaired neuronal pathways in the brain that control the hypothalamic-pituitary-ovarian axis (HPOA)[16]. The prominent neuroendocrine abnormalities involved an elevation of frequency and amplitude of gonadotropin releasing hormone (GnRH) release, which is reflected by gonadotropins hormones secretion [36]. The exact mechanisms for this abnormalities are not well understood [37] but there are many hypotheses have been suggested, including:

1) hyperinsulinemia act as a co-factor to stimulate adrenocorticotropic hormone(ACTH) mediated androgen production in the adrenal glands [26] . 2) low levels of serum progesterone that is followed by anovulation in PCOS conditions , which eventually removed the influence of negative feedback by progesterone on GnRH release [38], and 3) hyperandrogenism that changes the setting up of neuronal circuits for negative feedback of steroid hormones[39].

Women with PCOS showed high concentrations of luteinizing hormone (LH) and low concentrations of follicle-stimulating hormone (FSH) , respectively ,the 2/1 to 3/1 ratios usually were expressed for abnormal gonadotropin release [40] . The decreases in FSH for a long time leads to decrease in follicular maturation. The immature follicles may remain as cysts (giving polycystic morphology), subsequently, ovulation does not occur. As the ovulation does not occur the progesterone hormone is not made, causing subfertility [41-43] and without progesterone, a woman's menstrual cycle is sporadic or missing (oligominoreia or amenorrhea). Furthermore, the ovaries make male hormones androgens , which additionally counteract ovulation [41].

The increase in serum androgens level can disrupt the normal activity of the ovary, interfere with menstrual cycle [39] and impairs follicle growth [42] . Furthermore, the elevation of serum free testosterone levels may occur through decreased hepatic Sex Hormone Binding-Globulin (SHBG) production by the liver, that make the syndrome worse [43] .

1.5.4 Metabolic Factors

Polycystic ovary syndrome is commonly associated with insulin resistance and hyperinsulinemia [44]. Prolonged hyperinsulinemia in vivo had a negative impact on the developmental competence of oocytes [45, 46] . Insulin had gonadotrophic like actions in the ovary [47] and this actions of insulin are mediated via interactions with its receptors [48].

There are several lines of evidence strongly suggesting that insulin–FSH, insulin–LH and FSH–LH cross-talk takes place and it has biochemical and physiological consequences in the follicle. The in vivo effect of insulin on steroidogenesis appears to require the uptake of glucose[49] . There is considerable published data reporting that glucose is present in follicular fluid from humans and has important role in folliculogenesis, these data imply that the hormonal environment of the follicle can affect glucose uptake and that the ovary has reproductive mechanisms to regulate glucose uptake by ovarian cells [50] . Finally, insulin increased the expression of LH receptors [51] and stimulated androgen secretion by human theca cells [52-54].

In fact, insulin resistance may actually be at the root of PCOS women 's and playing a role in causing the condition in the first place, as well as exacerbating its symptoms [55] . 50% to 70% of women with PCOS have

insulin resistance that defined as the inability of insulin 1) to efficiently stimulate glucose uptake in the muscle and adipose tissue and 2) to inhibit hepatic glucose production. This is independent on obesity [26] but exacerbated in the presence of obesity [56] and glucose intolerance [26, 56] . leads to the potential for pre-diabetes and type two diabetes [57].

Although the full molecular mechanisms underlying insulin resistance in PCOS remain unclear , but decreased in insulin sensitivity , insulin receptor abundance , insulin receptor binding , or post binding defect in insulin signaling action may play a role [34] .

1.6 Management of Polycystic Ovary Syndrome

The management of women with polycystic ovary syndrome varies according to the main symptoms and primary problem experienced by the patients. The particular needs of the patient may change according to different stages of the life course , from adolescence through to reproductive age [26] .

1.6.1 Non Pharmacologic Management

1.6.1.1 Weight Loss and Diet regulation

Up to 88% of people with PCOS are overweight or obese [58] . Some of PCOS signs and symptoms were exacerbated by obesity, such as menstrual irregularity, reduced fertility rates, hyperandrogenemia and insulin resistance [59-61] . So successful weight reduction is the most effective method for restoring normal ovulation and menstruation [62] . Resistance exercise can decrease waist circumference and serum insulin levels [63]. It has been recommended that a diet in which a significant part of total carbohydrates is obtained from fruit , vegetables and whole grain sources may be helpful [64] .

1.6.1.2 Surgery

Laparoscopic ovarian surgery for the induction of ovulation in women with polycystic ovary syndrome provides a single-treatment option with a good rate of unifollicular ovulation , thereby minimizing the need for extensive ultrasound monitoring because of a low risk of multiple pregnancy [65] .

1.6.2 Pharmacological Management

Treatment goals include alleviation of symptoms, restoration of fertility, lowering insulin levels, treat hirsutism or acne, restore regular menstruation, prevent endometrial hyperplasia, endometrial cancer and long-term complications. But prior to the initiation of therapeutic interventions [66, 67] , general interventions that help to reduce weight or insulin resistance can be beneficial for all these aims , because they are believed to be the underlying causes [68] **.** There are a number of therapeutic interventions available for the treatment of PCOS shown in table (1-2).

Table (1- 2): Drugs used for the treatment of polycystic ovary syndrome [69].

Drugs	Place in therapy
Oral contraceptives	Contraception, androgen excess, regulation of cycle and protection of endometrium
Clomiphene	Ovulation induction
Gonadotropins	Ovulation induction
Pioglitazone, Rosiglitazone	Hyperinsulinemia, anovulation and androgen excess
Spironolactone	Hirsutuism, acne
Metformin	Hyperinsulinemia, anovulation and androgen excess

At an ovarian level, it is thought that hyperinsulinemia has direct and indirect actions on folliculogenesis and intra-ovarian gonadotropin-driven granulosa and thecal cell steroidogenesis[70]. Reducing insulin resistance by

improving insulin sensitivity through medications such as metformin have been an obvious approach [71] .

1.6.2.1 Metformin

Metformin is an oral anti-hyperglycaemic biguanide drug that improves systemic hyperglycaemia by reducing hepatic glucose production , increase glucose uptake by enhancing peripheral insulin sensitivity of tissues [72], reduces hyperinsulinaemia [73] and suppresses the excessive ovarian production of androgens in women with PCOS [72] as shown in figure (1-2).

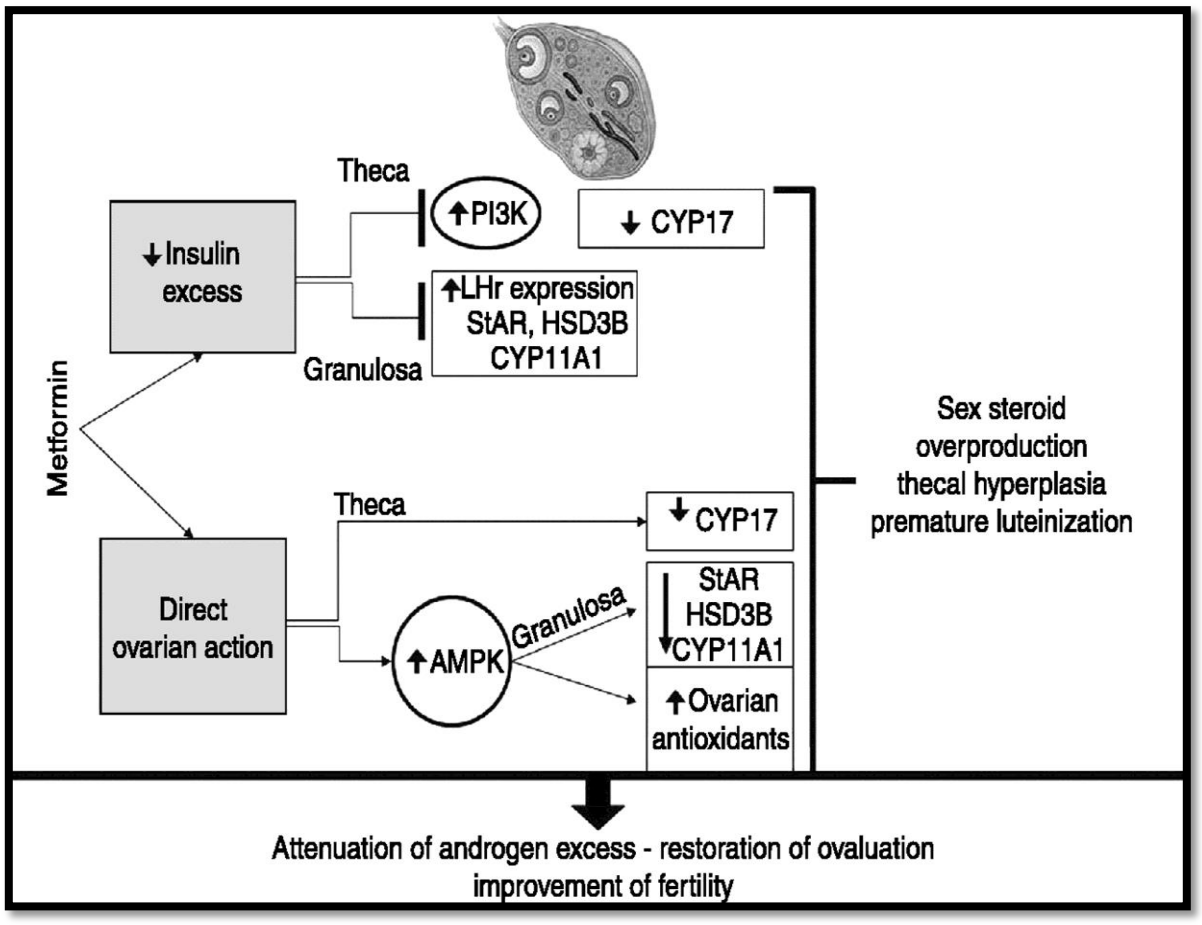

Figure (1-2) : Direct and indirect effects of metformin in polycystic ovary syndrome[72].

1.6.2.2 Mechanisms of Action in Polycystic Ovary Syndrome

Metformin has insulin-lowering effects by improving insulin sensitivity and insulin-mediated glucose disposal[74] , transport in the skeletal muscle cell [75] and ovary [76]. In human granulosa cells, metformin interacted with the insulin receptor to activate insulin receptor substrate 1 (IRS1) leading to an increased insulin stimulated translocation of glucose transporter 4 (GLUT4) to the plasma membrane via PI3K activation of Akt[77], improve insulin signaling and sensitivity [75], which in turn , can decrease circulating androgen levels [74] . This ability of metformin to correct abnormal insulin signaling in ovarian cells could be one of its mechanisms of action in PCOS [78]. The overall metabolic actions of metformin on cells include systemic increases in insulin sensitivity , insulin receptor number and affinity in skeletal muscle and adipose cells [79] making it become one of the key drugs and plays a critical role in the treatment of PCOs because women with PCOs are at an increased risk for insulin resistance [80].

1.7 Insulin Receptor Substrate 1

Insulin receptor substrate 1 (IRS1) is encoded by Insulin receptor substrate (IRS) gene, the entire gene is about 68. 4 Kilo Base (kb) and contains 2 exons. IRS gene Location: 2q36.3 DNA/RNA[81]. The cDNA contains 8743 bp [82].

The Insulin Receptor Substrate (IRS) proteins are a family of cytoplasmic adaptor proteins that were first identified for their role in insulin signaling[83]. These proteins are characterized by the presence of a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain. The PH domain contributes to protein-protein binding and facilitates the recruitment of IRS proteins by cell membrane receptors. The PTB domain is bind to phosphatidylinositol 3-kinase (PI3K), growth factor receptor bound protein 2(Grb2) and Src homology containing protein tyrosine phosphatase 2 (SHP2) , as shown in figure (1-3).

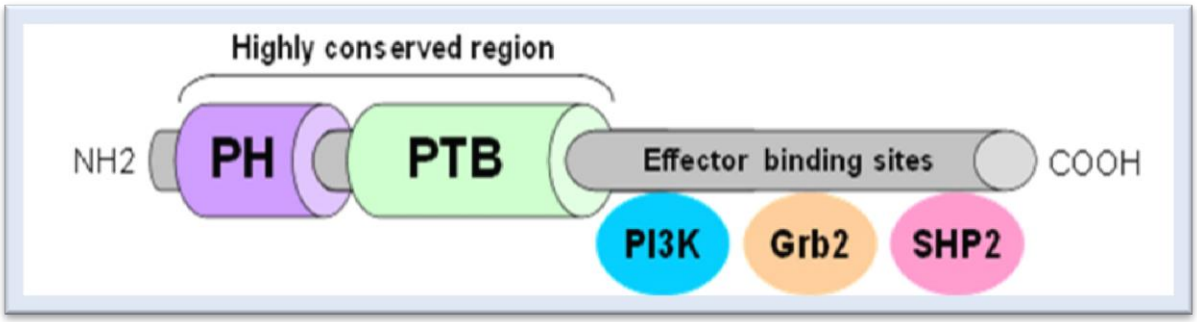

Figure (1.3): Schematic structure of IRS1. Interaction domains of IRS1: pleckstrin homology (PH) domain (purple), phosphotyrosine binding (PTB) domain (green) and effector binding sites (including PI3K, Grb2 and SHP2[84].

Insulin Receptor Substrate 1 (IRS1) is first identified as a signaling intermediate of the insulin signaling pathway, with important role in

this pathway. The majority of the studies characterize the insulin signaling pathways that control glucose transport in muscle , fat cells and liver, but there are evidences that these pathways also present within the ovary, and are important for glucose metabolism and normal folliculogenesis[85, 86].

The insulin receptor is a heterotetrameric protein that consists of two extracellular α subunits and two transmembrane β subunits connected by disulfide bridges [87-89]. Upon binding of insulin to the extracellular α subunit, conformational changes occur that results in autophosphorylation of the β subunit [90, 91]. At normal condition, the first step after activation of the insulin receptor is tyrosine phosphorylation of a cytoplasmic protein insulin receptor substrate 1 (IRS1)[34].

IRS1 is a docking protein that needs to become phosphorylated in order to bind and activate the enzyme phosphatidylinositol 3-kinase (PI3K) , a necessary step for the initiation of several effects of insulin such as glucose transport[91]. The PI3K protein then phosphorylates membrane phospholipids (phosphatidylinositol 4,5-bisphosphate PIP2 converted to PIP3) leading to activation of the 3-phosphoinositide-dependent protein kinases (PDK)[92]. These kinases activate the typical protein kinase B (PKB) that also known as Akt and atypical protein kinase C (PKC), both of these pathways stimulate the translocation of glucose transporter4 (GLUT4) to the cell surface[93] .Upon successful activation of IR / IRS1 signaling pathways, translocation of Glut-4 to the cellular membrane occurs, allowing the control of glucose uptake in target tissues, hence regulating glucose metabolism [93] .

1.7.1 The Association between Insulin receptor substrate 1 Polymorphism and Metformin response

Variability in drug response with respect to efficacy, tolerability, and safety, is a major issue for most drugs[94]. There is significant variability in the clinical response to metformin in PCOS women [95]. Pharmacogenetic studies identify genetic variations and specific genes single nucleotide polymorphisms that have a role in metformin response [96]. The role of genetic factors in predicting response variability to metformin has been the subject of many investigations, multiple studies have reported the associations between genomic variations of metformin transporters and its pharmacokinetics and response, but a few have explored the role of pharmacodynamics genes/variants in drug efficacy[97].

Polymorphisms in genes involved in metformin transport or action have been implicated in the heterogeneous response to metformin in type 2 diabetes [98] and, to a lesser extent, in PCOS[99]. It has been suggested that metformin can induce phosphorylation of insulin receptor β-subunit and IRS1 proteins[100]. Insulin receptor substrate-1 (IRS1) may modulate the response to metformin treatment in women with polycystic ovary syndrome[101]. Depending on this, IRS1 polymorphism may also modulate the response to metformin[100].

Metformin had differential effects on fasting insulin levels and homeostasis model assessment (HOMA) indices on the basis of IRS1 genotype, Also, the positive effects of metformin on reproductive parameters such as decreasing hyperandrogenism and inducing ovulation are attributed to its insulin-sensitizing actions [102], with accumulating evidences of a direct effect of metformin on ovarian steroidogenesis [103, 104].

1.8 Aims of the Study

The present study was designed to evaluate:

- The association of insulin receptor substrate 1 (IRS1) genetic polymorphisms (rs2943641 and rs1801123) with susceptibility to polycystic ovary syndrome in Iraq.

- The possible association of IRS1 genetic polymorphisms (rs2943641 and rs1801123) with the therapeutic response to metformin for women with polycystic ovary syndrome in Iraq.

Chapter Two

Subjects, Materials and Methods

2.1 Subjects (Patients and Control)

One hundred four healthy women as control and three hundred twenty newly diagnosed patients with polycystic ovary syndrome (their age range from 20 to 40 years) participated in this study, patients had attended the infertility department in Kerbala teaching hospital of gynecology and obstetrics and outpatient clinic from Jul 2019 to April 2020 seeking for medical treatment. Those patients selected by a consultant gynecologist and diagnosed as PCOS according to Rotterdam criteria. All participant women were enrolled in this study after signing a written informed consent and requested to answer a specially designed questionnaire. 105 patients were lost due to pregnancy (N=33) and some refuse to continue the study due to coronavirus pandemic (N= 72).

2.1.1 Patients Criteria

2.1.1.A Inclusion Criteria

Patients aged between (20 – 40) years, newly diagnosed with PCOS

2.1.1.B Exclusion Criteria

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

2- Patients whom became pregnant during the study.

3- Patients taking any drugs or supplements.

2.1.2 Study Design

This prospective case control study done on participants (two hundred fifteen PCOS patients and one hundred four healthy control). From each overnight fasted participants blood sample was collected for hormonal, biochemical and genetic study in day two of the menstrual cycle and any day in case of amenorrhea in some patients[105] . And only from patients, blood sample was collected again after 12 weeks of 500 mg twice daily of metformin treatment [106]. 105patients lost due to pregnancy (N=33) and some refuse to continue the study due to coronavirus[107] pandemic (N= 72).

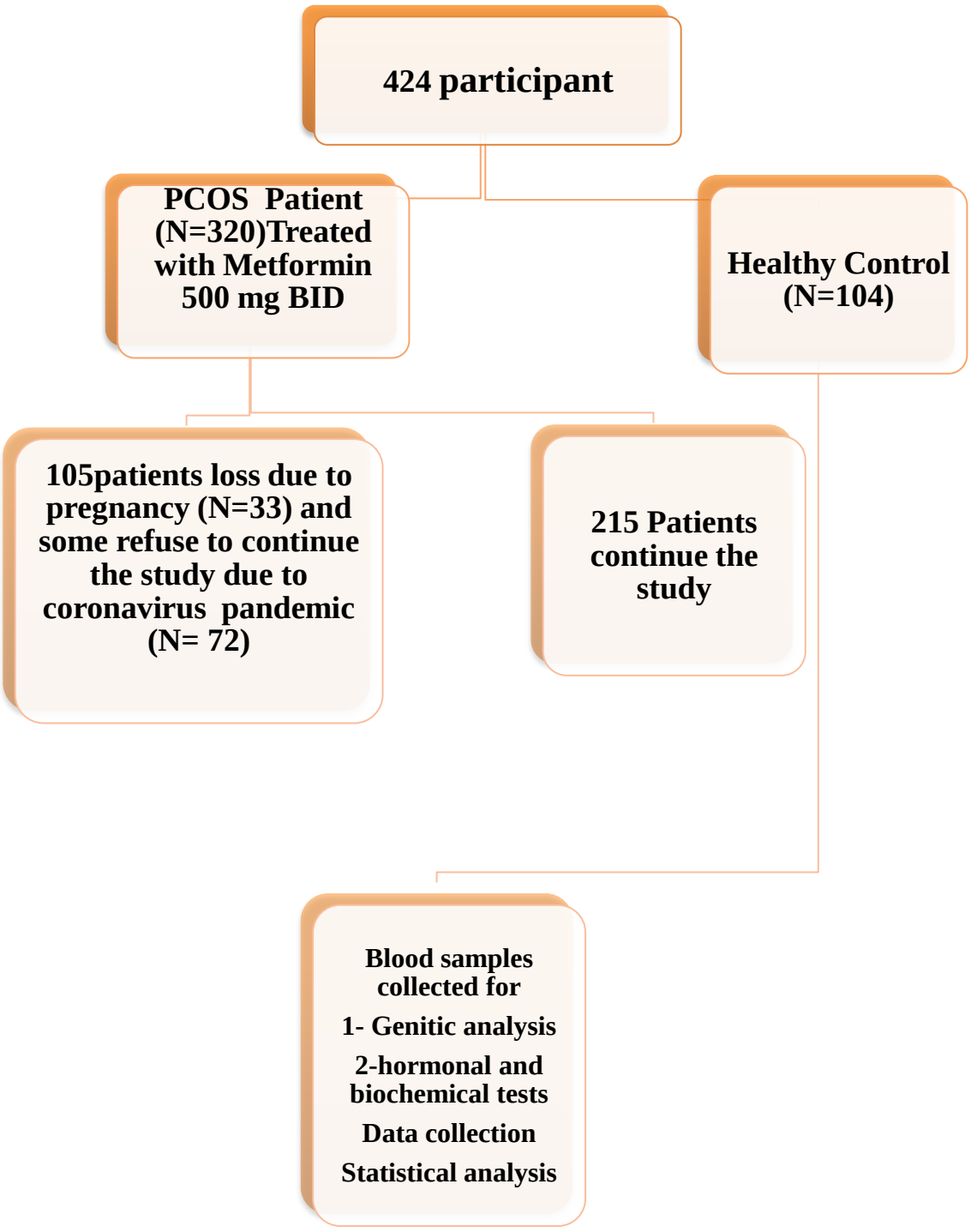

Figure (2-1): Flow chart of the study groups

2.2 Materials

2.2.1 Instruments, Equipment and Their Suppliers

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

Table (2-1) Instruments and the manufacturing companies

Equipments	Company	Country
Centrifuge	SIGMA	Germany
Cobas e 411	Roche	Germany
Digital camera	Canon	England
Distillator	GFL	Germany
Electrophoresis apparatus	Techinme	England
Hood	LabTech	Korea
Micropipettes	SLAMED	Japan
Sensitive balance	AND	Taiwan
UV- Trans illuminator	Syngene	England
Vortex mixer	HumanTwist	Germany
Water bath	LabTech	Korea
PCR machine (Thermo cycler)	Techne	UK

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 and country.

Table (2-2): Chemicals and Kits and their producing companies

	Chemicals and Kits	Company	Country
Chemicals	Agarose	CONDA	Spain
	Ethanol	SDI	Iraq
	Ethedium Bromide	Intron	Korea
	Isopropanol	SDI	Iraq
	Metformin 500mg tab	Merck	France
Hormonal Kits	FSH kit	Roche	Germany
	LH kit	Roche	Germany
	Prolactin kit	Roche	Germany
	TSH kit	Roche	Germany
	SHBG Kit	Demediteec Diagnostics Gmbh	Germany
	Testosterone kit	Roche	Germany
Biochemical Kits	Cholesterol Level kit	Roche	Germany
	Fasting Serum Glucose kit	Roche	Germany
	Glycosylated Hemoglobin kit	Roche	Germany
	High Density Lipoprotein kit	Roche	Germany
	Insulin kit	DRG International Inc	USA
	Low Density Lipoprotein kit	Roche	Germany
	Triglyceride kit	Roche	Germany
Kits For Genetic Study	DNA Extraction Kit from blood (G-DEX IIb)	Intron	Korea
	PCR Mastermix Kit	Genome	Korea
	DNA ladder Marker (100 bp)	Genome	Korea
	Primers for detection of: IRS1 rs12943641 T > C IRS 1 rs1801123 T > C	Bioneer	Korea

2.3 Methods

2.3.1 Samples Collection

Collected blood sample was divided into two parts, first part was kept in the ethylene diamine tetra acetate (EDTA) tube for HbA1c% and DNA extraction, the second part was kept in a gel tube to isolate serum, for hormonal and other biochemical tests. Blood samples were collected from all participants by vein puncture (after overnight fasting), and then after 12 weeks of treatment to follow up and determine the changes in the studied parameters only in patients group.

2.3.2 Hormonal and Biochemical Assay Methods

2.3.2.1 Determination of Glycemic Indices

2.3.2.1.A Estimation of Fasting Serum Glucose Level

Glucose level estimated by 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[108].

2.3.2.1.B Estimation of Fasting Serum Insulin Level

Serum insulin was determined by using a ready-made insulin ELISA kit. ELISA kit is a solid phase - enzyme- linked immunosorbent assay based on the sandwich principle. An aliquot of patients Samples containing endogenous insulin is incubated in the coat well with enzyme conjugate

(which is an anti-insulin antibody) that conjugated with biotin. During the second incubation step streptavidin-peroxidase enzyme complex binds to biotin anti-insulin antibody. The mount of bound peroxidase complex is proportional to the concentration of insulin in the sample. 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[109].

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 insulin (FSI) and fasting serum glucose (FSG) using the following formula.

HOMA = FSI(µIU/ml) * FSG (mg/dl) /405 [110]

2.3.2.1.D Estimation of Glycosylated Hemoglobin Level (HbA1c)

The blood specimen in the EDTA tube was hemolyzed automatically on the Cobas Integra 400+ analyzer with cobas integra hemolyzing reagent gen.2. This method uses tetradecyltrimethylammonium bromide (TTAB) as the detergent in the hemolyzing reagent to eliminate interference from leukocytes (TTAB does not lyse leukocytes). Glycohemoglobin (HbA1c) in the sample reacts with an anti-HbA1cd antibody to form soluble antigen-antibody complexes which can be measured [111].

2.3.2.2 Estimation 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 is measured by a photomultiplier[112, 113].

2.3.2.3 Estimation of Serum Prolactin Level

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 on the surface of the electrode. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier[114].

2.3.2.4 Estimation of Serum Sex Hormone Binding Globulin (SHBG)

Sex hormone binding-globulin (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 are added to the micro ELISA plate wells. Human SHBG would combine with the specific antibody. Then Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human SHBG, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectro-photometrically at a wavelength of 450 ±2 nm. The OD value is proportional to the concentration of Human SHBG. The concentration of Human SHBG in the samples can be calculated by comparing the OD of the samples to the standard curve [(115)] .

2.3.2.5 Estimation of Serum Testosterone Level

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 analyzer 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 competes with the added testosterone derivative (that labeled with a ruthenium complex) for the binding sites on the antibody. Application of a voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier[116].

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)*[117]

2.3.2.7 Estimation 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" is intended for use on Elecsys and cobas e immunoassay analyzers and the principle is the same as described for prolactin hormone[118].

2.3.2.8 Determination of Lipid Profile

2.3.2.8.A Estimation of Cholesterol Level

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[119].

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

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

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

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

2.3.2.8.B Estimation of Serum Triglyceride (TG) Level

In vitro quantitative enzymatic colorimetric method was used for determination of Triglyceride in serum on cobas integra systems[120].

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

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

glycerol-3-phosphate + O_2 $\xrightarrow{\text{Glycerol-3-Phosphate Oxidase}}$ dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-aminophenazone + 4-chlorophenol $\xrightarrow{\text{peroxidase}}$

4-(p-benzoquinone-monoimino)-phenazone + 2 H2O + HCl

2.3.2.8.C Estimation of Serum High Density Lipoprotein (HDL) Level

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 is 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[121].

HDL-cholesterol esters + H_2O $\xrightarrow{\text{PEG-cholesterol esterase}}$ HDL-cholesterol + RCOOH

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

2 H_2O_2 + 4-aminoantipyrine + HSDAa) + H+ + H_2O $\xrightarrow{\text{peroxidase}}$ purple blue pigment ([a) Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline]) + 5 H_2O

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

2.3.2.8.D Estimation of Serum Low Density Lipoprotein (LDL) Level

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[(122)].

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 photometer.

2.3.2.9 Measurement of Body Mass Index

Body Mass Index (BMI) is a value obtained from the weight and height of an individual. The BMI is described as the body weight divided by the square of the body height and is globally expressed in units of kg/m^2, resulting from the mass in kilograms and height in meters[(123)].

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

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

G-DEX IIb kit was used for DNA extraction from Intron Company. In epindorf tube, (1ml) blood sample was centrifuged (2000rpm for 1 min) then suction of buffy coat done by micropipette and transferred to another epindorf. RBC lysis solution (900 µl) was added to this epindorf and pipetting up and down carefully then infinity like movement was done for well mixing, left for 5 min. After that, the solution centrifuged (10000rpm for 1 min). Supernatant must be thrown except 100 µl remained in the bottom with pellet in epindorf. Vortex for 1 min in order to dissolve the pellet, after this step, 300µl of cell lysis solution was added and pipetting up and down for good mixing was done , centrifuged again for 2 min and 100 µl protein precipitate (PPT) buffer was added then vortex for 20 sec and centrifuged (16000rpm for 5 min) to precipitate protein in the solution, from which, 300 µl of supernatant that contain DNA were kept in other epindorf tube and added to it 300 µl isopropanol and mixed by infinity like movement then centrifuged (16000rpm for 1 min), white pellet was appeared. Isopropanol was discarded, and (1ml) of ethanol was added and centrifuged, ethanol was discarded and the epindorf left it in air for several min, after this drying step (200µl) of DNA rehydration solution was added to epindorph and then heating in water bath (56 ˚C for half hour), This dissolved DNA solution was stable and suitable for ARMS-PCR. This procedures were depends on Molecular cloning book for Sambrook.

2.3.3.2 Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR)

2.3.3.2 A. Primer Preparation

Insulin receptor substrate 1(IRS1) primers that designed by **Asst. Prof. Dr. Hassan Mahmoud Mousa Abo Almaali by using primer BLAST software** and purchased from Bioneer, Korea as lyophilized product of different picomols concentrations, were dissolved in specific volumes of nuclease free water to obtain a concentration of 100 pmol/ µl stock solution. From which, diluted work solution was prepared by adding 10µl of each stock solution primer to 90 µl of nuclease free water. This work solution were kept at -20 ˚C until further use. Table (2-3) illustrates the primers used to amplify the IRS1 gene polymorphism.

Table (2-3): Primers sequences of IRS1 rs2943641 Alleles: T>C and rs1801123 Alleles: T>C

Primers		**Sequence**	Product size (bp)
Primers sequences of IRS1 rs2943641 Alleles T > C	O-F	TGGTTCTGTAACTGGGTG	537
	O-R	AGTTGAAGTAGCCATCTTTC	537
	Allele T	ATCAGGGCTAATAGTTAGAAG A	387
	Allele C	GTTGGAAATGAGAGGAACC	190
Primers sequences of IRS 1 rs1801123 Alleles T > C	O-F	GGTAGGCCTGCAAATGCTA	700
	O-R	CTCCGACTGCTACTACGGC	700
	Allele T	CTCTATGCTGCAACAGCA	543
	Allele C	GTGGAAGAGGAAGAATCATCC	195

O-F: outer forward

O-R : outer revers

2.3.3.2 B. Optimization of Polymerase Chain Reaction Conditions

After several trials, optimization of PCR reaction was done to detect the best annealing temperature and numbers of amplification cycles, that was appropriate for the ARMS-PCR reaction. The components of PCR reaction for all the amplified fragments and the optimized PCR programs are stated in Tables (2-4), (2-5) and (2-6) respectively.

2.3.3.2 C. Running the Polymerase Chain Reaction

The PCR reaction was done by mixing PCR components with DNA solution and using the optimized PCR programs as shown in Tables (2-4), (2-5) and (2-6) respectively.

Table (2-4): PCR mix reaction for genotyping Of IRS 1 rs2943641 Alleles: T>C and rs1801123 Alleles: T>C

Component	**Volume (µl)**
Outer Forward primer	1.25
Inner Forward primer	1.25
Reverse primer allele T	1.25
Reverse primer allele C	1.25
DNA template	5
Deionized water	5
Master mix	10

Table (2-5): PCR Condition for genotyping of IRS 1 gene rs2943641 Alleles: T>C

Steps	Temperatures/c	Time /second	Cycle
Denature template	94	3minutes	1
Initial denaturation	94	35	30
Annealing	56	45	
Extension	72	55	
Final extension	72	5minutes	1

Table (2-6): PCR Condition for genotyping of IRS 1 genotyping rs1801123 Alleles: T>C

Steps	Temperatures/c	Time /second	Cycle
Denature template	94	3 minutes	1
Initial denaturation	94	30	30
Annealing	53	45	
Extension	72	55	
Final extension	72	5 minutes	1

2.3.3.3 Agarose Gel Electrophoresis

Agarose gel was prepared by dissolving 1.5 g of agarose powder in 100 ml of 1x Tris-borate EDTA (TBE) buffer (pH = 8) in microwave for several minutes, the solution was cooled to 50˚C and two microliters of ethidium bromide dye were added for it. Then comb was fixed at one end of the tray for making wells, used for loading the samples, i. e. DNA or PCR product samples. After that the solution was poured gently into the tray, and allowed to solidify at room temperature for 30 min. Then the comb was removed gently from the tray and fixed in an electrophoresis chamber. This chamber was filled with a TBE buffer. Finally, five microliters from each DNA sample was transferred to solidified agarose half a microliter of loading dye was added to it. 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. This procedures were depends on Molecular cloning book for Sambrook.

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.

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.

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.

To assess the predictability of PCOS, logistic analysis of both SNPs was applied, this yielded odds ratio (OR). Also the 95% confidence interval 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

3.1 The Demographic, Hormonal and Metabolic Parameters of the Control group and Polycystic Ovary Syndrome Patients group

A total of 215 PCOS women and 104 healthy women (control group) were enrolled in this study. Data presented in table (3-1) revealed that there were no significant differences between PCOS and control groups in their age, marital status, education, and employment status, (P-value > 0.05). Furthermore, hirsutism and alopecia had been reported in PCOS women only and none among control group. The mean number of children was significantly lower (1.2 ± 1.1) among PCOS women than controls, (2.0 ± 1.3), (P-value <0.001). Abortion was significantly more frequent among PCOS women than controls, 31.1%vs.15.5% respectively, (P. value =0.021). Regarding BMI, PCOS patients had significantly higher BMI (P-value <0.001) than healthy control subjects.

Table (3-1): Socio-demographic data of the control group and polycystic ovary syndrome group

Variables	Control	PCOS	p-value
Number	104	215	-
Age (y)	28.0 ± 5.9	27.3 ± 5.1	0.273 [NS]
BMI (kg/m^2)	27.7 ± 4.2	31.4 ± 4.1	**<0.001 [S]**
Hirsutism, n (%)	0 (0.0%)	199 (92.6%)	**<0.001 [S]**
Alopecia, n (%)	0 (0.0%)	199 (92.6%)	**<0.001 [S]**
Married, n (%)	87 (83.6%)	189 (87.9%)	0.950 [NS]
No of children	2.0 ± 1.3	1.2 ± 1.1	**<0.001 [S]**
Abortion, n (%)	9 (15.5%)	52 (31.1%)	**0.021 [S]**
Employed, n (%)	19 (18.3%)	32 (14.9%)	0.465 [NS]
Education, n (%)			0.907 [NS]
Illiterate	4 (3.8%)	7 (3.3%)	
Primary	45 (43.3%)	102 (47.4%)	
Secondary	32 (30.8%)	60 (27.9%)	
College	23 (22.1%)	46 (21.4%)	

Results are presented as mean ± SD

n= number of subjects

(p< 0.05) considered significantly different

[S] significant

[NS] not significant

As shown in the table (3-2), The serum LH level was significantly lower in the control group than PCOS group (P=0.02), while serum FSH and AHBG level was conversely different, it was higher in the control group than PCOs group (P=0.001 and <0.001 respectively), with a significant increase in the LH/FSH ratio in PCOS group compared to control group (p<0.001), there were a significant increase in TSH (p= 0.045) , testosterone and FAI levels in PCOS group compared to controls (p <0.001 for both).

Table (3-2): Hormonal parameters of healthy control group and polycystic ovary syndrome patients group

Variables	**Control**	**PCOS**	**p-value**
Number	104	215	-
LH (mIU/L)	7.9 ± 2.1	9.8 ± 5.3	**0.020 [S]**
FSH (mIU/L)	6.5 ± 1.7	5.8 ± 2.0	**0.001 [S]**
LH/FSH ratio	1.3 ± 0.3	1.8 ± 0.9	**<0.001 [S]**
TSH (uIU/L)	2.0 ± 0.6	2.3 ± 0.9	**0.045 [S]**
Prolactin (ng/mL)	20.4 ± 5.1	23.6 ± 13.4	0.173 [NS]
Testosterone (ng/ml)	0.75 ± 0.2	17.75 ± 0.4	**<0.001 [S]**
SHBG nmol/L	84.6 ± 6.3	53.3 ± 24.8	**<0.001 [S]**
FAI	0.3 ± 0.2	7.1 ± 17.9	**<0.001 [S]**

Results are presented as mean ± SD
(p< 0.05) considered significantly different, [S] significant,[NS] not significant

However, data presented in the table (3-3), showed that serum insulin level and HOMA-IR were significantly higher in the PCOS patients group when compared with the healthy control group (P<0.001), but with no significant differences in FSG level and HbA1c between PCOS group and control group (p = 0.908 and 0.227 respectively).

Table (3-3): Glycemic parameters of the healthy control group and polycystic ovary syndrome patient group

Variables	**Control**	**PCOS**	**p-value**
Number	104	215	-
FSG (mg/dL)	98.4 ± 10.7	98.1 ± 12.7	0.908 [NS]
Fasting Insulin (µIU/ml)	20.0 ± 2.3	23.2 ± 1.0	**<0.001 [S]**
HOMA-IR	4.9 ± 0.6	5.7 ± 0.2	**<0.001 [S]**
HbA1c (%)	5.0 ± 2.6	5.1 ± 4.0	0.227 [NS]
Results are presented as mean ± SD (p< 0.05) considered significantly different, [S] significant, [NS] not significant			

Regarding lipid profile, the data illustrated in the table (3-4), showed a significant increase in triglyceride, LDL and cholesterol levels in the PCOS patients group when compared with the control group ($p<0.001$), while the HDL level was significantly reduced ($p = 0.002$) in PCOS patients.

Table (3-4): Lipid Profile of control and polycystic ovary syndrome patients group

Variables	Control	PCOS	p-value
Number	104	215	-
Triglyceride (mg/dL)	75.7 ± 19.4	127.8 ± 44.0	**<0.001 [S]**
LDL (mg/dL)	51.5 ± 16.1	97.8 ± 67.0	**<0.001 [S]**
HDL (mg/dL)	49.1 ± 9.0	46.1 ± 10.6	**0.002 [S]**
Cholesterol (mg/dL)	118.6 ± 29.4	163.0 ± 42.6	**<0.001 [S]**
Results are presented as mean ± SD **(p< 0.05) considered significantly different** **[S] significant**			

3.2 Genetic Analysis to Assess the Association of Insulin Receptor Substrate 1 Polymorphism with Polycystic Ovary Syndrome Pathogenicity

The analyses were conducted to assess the association between the SNP1 rs2943641 [TT (Wild type), TC (heterozygous type), and CC (mutated type)], and SNP2 rs1801123 [TT (Wild type), TC (heterozygous type), and CC (mutated type)] with the pathogenesis of PCOS according to

logistic regression and figures (3.1and 3.2) . This survey demonstrated that there was no significant association between different alleles for both SNPs with the pathogenesis of PCOS as shown in tables (3-5 and 3-6).

Table (3-5): Distribution of SNP1 rs2943641 and SNP2 rs1801123 in the healthy control group and polycystic ovary syndrome group

Variables	Control	PCOS	p-value
Number	104	215	-
SNP1 (rs2943641)			
TT (Wild type)	24 (23.1%)	65(30.2%)	0.368
TC (Heterozygotes)	68 (65.4%)	124 (57.7%)	
CC (Mutant)	12 (11.5%)	26 (12.1%)	
SNP2 (rs1801123)			
TT (Wild type)	30 (28.8%)	58 (27.0%)	0.248
TC (Heterozygotes)	68 (65.4%)	152 (70.7%)	
CC (Mutant)	6 (5.8%)	5 (2.3%)	
Chi-Square test was applied to detect allelic frequency			

Data presented in table (3-6) showed that the odds ratio was not significantly different in both studied SNPs

Table (3-6): Logistic analysis of SNP1 rs2943641 and SNP2 rs1801123 to predict polycystic ovary syndrome pathogenesis

Variables	OR (95%CI)	p-value
SNP1 (rs2943641)		
TT (Wild type)	1.0	-
TC (Heterozygotes)	0.67 (0.39 – 1.17)	0.161
CC (Mutant)	0.80 (0.35 – 1.83)	0.598
SNP2 (rs1801123)		
TT (Wild type)	1.0	
TC (Heterozygotes)	1.16 (0.68 – 1.96)	0.588
CC (Mutant)	0.43 (0.12 – 1.53)	0.193

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

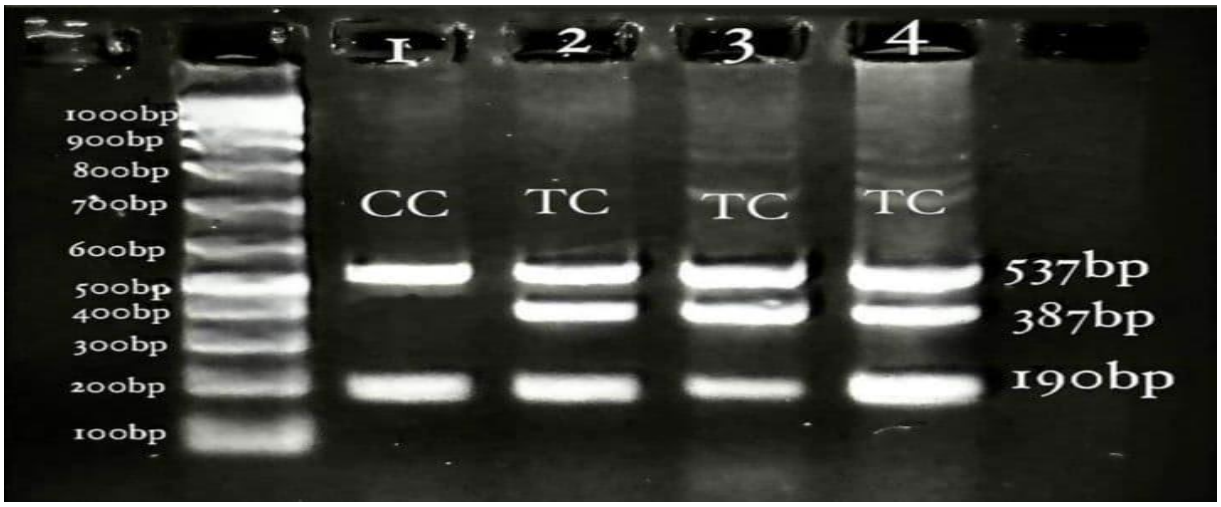

Figure (3-1): ARMS-PCR amplification of IRS1 gene T> C showing the outer primer 537 bp in size, T allele is 387 bp in size while C allele is 190 bp in size

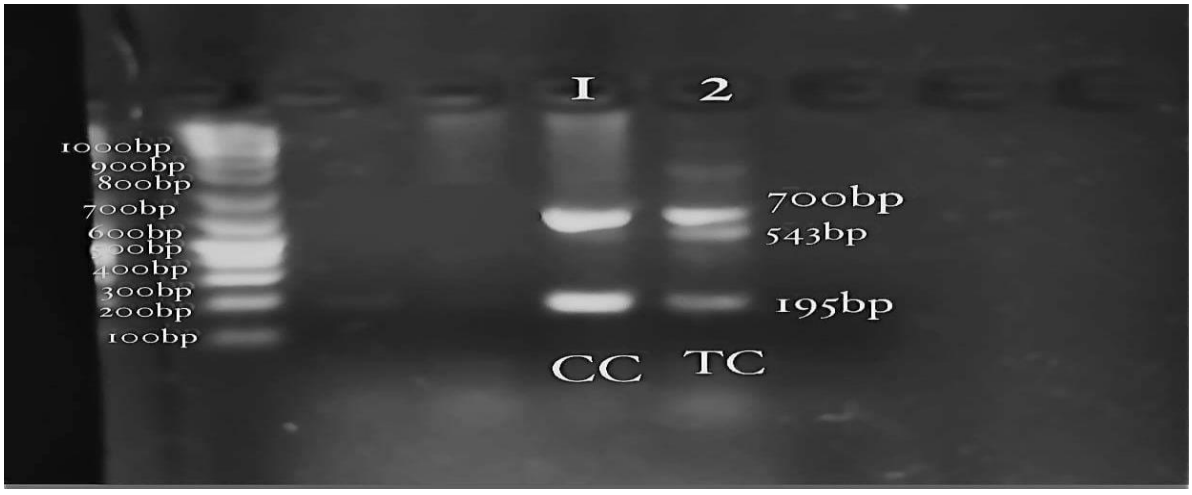

Figure (3-2): ARMS-PCR amplification of IRS1 gene T> C showing the outer primer 700 bp in size, T allele is 543 bp in size while C allele is 195 bp in size

3.3 Assessment of Hormonal and Metabolic Parameters in Polycystic Ovary Syndrome Women before and after 12 Weeks of Metformin Treatment According to SNP 1 rs2943641

After 12 weeks of metformin treatment, the changes in hormonal level are demonstrated in (Table 3-7), where LH level was significantly reduced after treatment in women with TT and TC alleles (P-value =0.023 and 0.002 respectively) but no significant reduction in those with CC allele (P. value > 0.05). FSH was not significantly changed after treatment, neither in TT, TC nor in CC alleles (P>0.05). The LH/FSH ratio was significantly reduced after treatment in women with TT and TC alleles, (P=0.001 and >0.001, respectively), while in women with CC allele no significant changes were observed during the treatment period (P>0.05).Testosterone was significantly reduced in women with TT (P=0.033) and TC, (P<0.001), while not in those with CC, (P>0.05). Regarding SHBG it was only significantly elevated after treatment in women with TT, (P-value =0.013). FAI was significantly reduced in PCOS women with TT and TC, (P<0.001), but not in those with CC, (P>0.05).

Table (3-7): Hormonal parameters in polycystic ovary syndrome women before and after Treatment According to SNP 1 rs2943641

Parameter	SNP1 Alleles	Before Treatment	After Treatment	P. value
		Mean ± SD	Mean ± SD	
LH (mIU/L)	TT(Wild)	9.4 ± 4.6	8.3 ± 4.1	**0.023 [S]**
	TC (Hetro)	9.5 ± 5.2	8.2 ± 4.8	**0.002 [S]**
	CC (Mutant)	9.4 ± 5.9	10.0 ± 6.3	0.361 [NS]
FSH (mIU/L)	TT (Wild)	5.7 ± 1.6	6.5 ± 2.2	0.092 [NS]
	TC (Hetro)	5.7 ± 2.0	6.3 ± 2.4	0.074 [NS]
	CC (Mutant)	5.9 ± 2.8	6.4 ± 2.5	0.300 [NS]
LH / FSH ratio	TT (Wild)	1.7 ± 0.8	1.4 ± 0.9	**0.001 [S]**
	TC (Hetro)	1.7 ± 1.0	1.4 ± 0.7	**< 0.001 [S]**
	CC (Mutant)	1.6 ± 0.8	1.7 ± 1.1	0.657 [NS]
Testosterone (ng/ml)	TT (Wild)	0.6 ± 0.4	0.5 ± 0.3	**0.033 [S]**
	TC (Hetro)	0.7 ± 0.4	0.5 ± 0.3	**< 0.001 [S]**
	CC (Mutant)	0.6 ± 0.5	0.7 ± 0.4	0.151 [NS]
SHBG nmol/L	TT (Wild)	52.1 ± 27.8	59.1 ± 23.0	**0.013 [S]**
	TC (Hetro)	52.1 ± 24.5	54.8 ± 21.7	0.051 [NS]
	CC (Mutant)	64.3 ± 18.2	59.0 ± 19.5	0.086 [NS]
FAI	TT (Wild)	16.75 ± 10.4	5 ± 2.8	**< 0.001 [S]**
	TC (Hetro)	13.5 ± 8.3	4.25 ± 2.7	**< 0.001 [S]**
	CC (Mutant)	6.7 ± 4.3	8.05 ± 6.7	0.751 [NS]

Results are presented as mean ± SD (p< 0.05) considered significantly different
[S] significant [NS] not significant

Data presented in table (3-8) showed significant reduction in BMI of TT and TC alleles (P<0.001) but not in those with CC allele . Assessment of changes in FSG, HbA1c, Fasting insulin, and HOMA IR in PCOS women after treatment according to SNP 1 are shown in (Table 3-8), FSG and HbA1c,were significantly lowered in TT and TC group (P<0.05) while not in the CC group (P>0.05). Fasting insulin and HOMA- IR were significantly reduced only in the TT group (P=0.003 and 0.002, respectively).

Table (3-8): Glycemic parameters in polycystic ovary syndrome before and after treatment according to SNP 1 rs2943641

Parameter	SNP1 Alleles	Before treatment	After treatment	P. value
		Mean ± SD	Mean ± SD	
BMI	TT (Wild)	31.0 ± 4.1	28.6 ± 4.8	**<0.001 [S]**
	TC(Hetro)	31.6 ± 4.1	28.8 ± 4.4	**<0.001 [S]**
	CC (Mutant)	31.8 ± 4.5	30.1 ± 4.3	0.127[NS]
FSG (mg/dL)	TT (Wild)	98.0 ± 14.0	92.8 ± 15.9	**0.018 [S]**
	TC (Hetro)	98.8 ± 11.7	95.0 ± 12.3	**<0.001 [S]**
	CC (Mutant)	96.0 ± 16.5	98.9 ± 11.7	0.354 [NS]
HbA1c (%)	TT (Wild)	6.3 ± 8.0	4.6 ± 0.7	**0.001 [S]**
	TC (Hetro)	4.8 ± 0.8	4.5 ± 0.7	**<0.001 [S]**
	CC (Mutant)	4.7 ± 0.8	4.7 ± 0.7	0.611 [NS]
Fasting Insulin (µIU/ml)	TT (Wild)	25.2 ± 17.2	19.3 ± 9.9	**0.003 [S]**
	TC (Hetro)	21.4 ± 12.9	20.5 ± 11.8	0.073 [NS]
	CC (Mutant)	25.6 ± 14.2	25.4 ± 10.0	0.889 [NS]
HOMA IR	TT (Wild)	6.2 ± 4.3	4.5 ± 2.6	**0.002 [S]**
	TC (Hetro)	5.3 ± 3.4	4.9 ± 3.0	0.058 [NS]
	CC (Mutant)	6.1 ± 3.4	6.1 ± 2.4	0.97 [NS]

Results are presented as mean ± SD

(p< 0.05) considered significantly different, [S] significant , [NS] not significant

Data presented in table (3-9) demonstrated the results of the lipid profile of the participants PCOS women before and after treatment according to the SNP1 which revealed that triglycerides and LDL levels were significantly reduced after treatment in TT and TC groups, (P<0.05), but not in those with CC, (P>0.05), while the HDL level was significantly elevated only in the TC group, (P<0.05) and not in TT and CC groups, total cholesterol level was significantly decreased after treatment in TT and TC groups (P<0.05) but not in the CC group, (P>0.05).

Table (3.9): Lipid profile of polycystic ovary women before and after treatment according to SNP1rs2943641

Parameter	SNP1 Alleles	Before treatment Mean ± SD	After treatment Mean ± SD	P. value
TG (mg/dL)	TT (Wild)	135.8 ±47.2	119.1 ± 36.2	**<0.001 [S]**
	TC (Hetro)	125.2 ± 44.9	114.2 ± 38.4	**0.001 [S]**
	CC(Mutant)	125.4 ± 41.1	117.2 ± 41.3	0.778 [NS]
LDL (mg/dL)	TT (Wild)	115.6 ± 113.7	91.0 ± 23.3	**0.001 [S]**
	TC (Hetro)	93.5 ± 31.1	83.4 ± 23.7	**<0.001 [S]**
	CC(Mutant)	91.8 ± 30.2	94.4 ± 23.4	0.248 [NS]
HDL (mg/dL)	TT (Wild)	45.0 ± 9.5	47.2 ± 9.1	0.144 [NS]
	TC (Hetro)	46.2 ± 10.7	47.7 ± 9.5	**0.021 [S]**
	CC(Mutant)	45.8 ± 11.8	42.1 13.1	0.286 [NS]
Cholesterol (mg/dL)	TT (Wild)	172.5 ± 45.4	153.9 ± 48.8	**0.001 [S]**
	TC (Hetro)	160.5 ± 44.7	149.6 ±39.5	**<0.001 [S]**
	CC(Mutant)	159.8 ±48.7	171.6 ±34.1	0.107 [NS]

Results are presented as mean ± SD , (p< 0.05) considered significantly different
[S] significant , [NS] not significant

3.4 Assessment of Hormonal and Metabolic Parameters in Polycystic Ovary Syndrome Women before and after 12 Weeks of Treatment with Metformin According to SNP2 rs1801123

The hormonal changes after metformin treatment according to the SNP2 were demonstrated in (Table 3-10), where LH level was significantly reduced after treatment in women with TT and TC, alleles (P-value <0.05), FSH was significantly increased after treatment only in the TT allele, (P<0.05). The LH/FSH ratio was significantly reduced after treatment in women with TT and TC alleles, (P<0.001 and =0.002, respectively), while there was no significant difference in women with CC allele, (P>0.05).

Testosterone was significantly reduced in women with TT, (P<0.001), and TC (P=0.008), while not in those with CC, (P>0.05). SHBG was only significantly elevated after treatment in women with TT, (P. value =0.018). FAI was significantly reduced in PCOS women with TT and TC, (P =0.004 and <0.001 respectively), but not in those with CC allele, (P>0.05)

Table (3-10): Hormonal parameters of polycystic ovary syndrome women before and after metformin treatment according to SNP 2 rs1801123

Parameter	SNP2 Alleles	Before treatment	After treatment	P. value
		Mean ± SD	Mean ± SD	
LH (mIU/L)	TT (Wild)	8.3 ± 4.1	7.7 ± 3.9	**0.014 [S]**
	TC (Hetro)	8.7 ± 5.2	8.2 ± 4.8	**0.018 [S]**
	CC (Mutant)	8.5 ± 3.2	10.0 ± 6.3	0.754 [NS]
FSH (mIU/L)	TT (Wild)	6.5 ± 2.2	6.9 ± 2.4	**0.002 [S]**
	TC (Hetro)	6.2 ± 2.2	6.3 ± 2.4	0.575 [NS]
	CC (Mutant)	6.4 ± 2.5	6.5 ± 2.9	0.077 [NS]
LH / FSH ratio	TT (Wild)	1.4 ± 0.9	1.2 ± 0.5	**<0.001 [S]**
	TC (Hetro)	1.5 ± 0.9	1.4 ± 0.7	**0.002 [S]**
	CC (Mutant)	1.5 ± 0.7	1.7 ± 1.1	0.084 [NS]
Testosterone (ng/ml)	TT (Wild)	0.6 ± 0.4	0.5 ± 0.3	**<0.001 [S]**
	TC (Hetro)	0.7 ± 0.4	0.5 ± 0.3	**0.008 [S]**
	CC (Mutant)	0.6 ± 0.5	0.7 ± 0.4	0.209 [NS]
SHBG nmol/L	TT (Wild)	52.3 ± 21.6	59.1 ± 23.0	**0.018 [S]**
	TC (Hetro)	54.8 ± 21.7	58. ± 21.7	0.072 [NS]
	CC (Mutant)	46.9 ± 21.6	50.0 ± 19.5	0.136 [NS]
FAI	TT (Wild)	14 ± 8.6	5 ± 3.7	**0.004 [S]**
	TC (Hetro)	13.5 ± 8.9	5.1 ± 3.0	**<0.001 [S]**
	CC (Mutant)	13.75 ± 6.1	11.25 ± 6.6	0.506 [NS]

Results are presented as mean ± SD
(p< 0.05) considered significantly different, [S] significant , [NS] not significant

Data presented in table (3-11) showed significant reduction in BMI of TT and TC alleles (P<0.001) but not in those with CC allele. Assessment of changes in FSG, HbA1c, insulin level, and HOMA- IR in PCOS women after metformin treatment according to SNP 2 was also demonstrated in (Table 3-11), FSG was significantly lowered only in the TT

allele (P<0.001) while not in TC or CC alleles (P>0.05). HbA1c was significantly reduced in TT and TC groups, (P<0.001). Fasting insulin and HOMA- IR were significantly reduced only in the TT group, (P<0.001).

Table (3-11): Glycemic parameters in polycystic ovary syndrome women before and after metformin treatment according to SNP 2 rs1801123

Parameter	SNP2 Alleles	Before treatment	After treatment	P. value
		Mean ± SD	Mean ± SD	
BMI	TT (Wild)	31.3 ± 4.6	28.8 ± 4.3	**<0.001 [S]**
	TC (Hetro)	31.5 ± 3.9	28.9 ± 4.5	**<0.001 [S]**
	CC (Mutant)	31.3 ± 6.1	28.7 ± 5.9	0.158[NS]
FSG (mg/dL)	TT (Wild)	94.1± 11.4	92.8 ±15.9	**<0.001 [S]**
	TC (Hetro)	95.1 ± 14.2	95.0 ± 12.3	0.004 [NS]
	CC (Mutant)	94.2 ± 11.4	98.9 ± 11.7	0.875 [NS]
HbA1c (%)	TT (Wild)	4.9 ± 0.7	4.5 ± 0.5	**<0.001 [S]**
	TC (Hetro)	4.8 ± 0.8	4.6 ± 0.7	**<0.001 [S]**
	CC (Mutant)	4.9 ± 0.9	4.8 ± 0.8	0.814 [NS]
Fasting Insulin µIU/ml	TT (Wild)	19.3 ±9.9	16.6 ± 9.9	**<0.001 [S]**
	TC (Hetro)	21.7 ± 11.3	20.5 ± 11.8	0.14 [NS]
	CC (Mutant)	25.7±11.6	25.4 ± 10.0	0.937 [NS]
HOMA- IR	TT (Wild)	4.5 ± 2.6	3.9 ± 2.5	**<0.001 [S]**
	TC (Hetro)	5.2 ± 2.9	4.9 ±3.0	0.111 [NS]
	CC (Mutant)	6.1 ± 3.0	6.1 ±2.4	0.814 [NS]

Results are presented as mean ± SD , (p< 0.05) considered significantly different, [S] significant , [NS] not significant

Data presented in table (3-12) showed the comparison of lipid profile across the SNP2 TT, TC, and CC subgroups. The triglyceride level was significantly reduced after treatment in all SNP2 subgroups, (TT, TC, and

CC), (P<0.05), while there was no significant difference in HDL level in all SNP2 subgroups, (P>0.05). Total cholesterol and LDL levels were significantly reduced after treatment in TT and TC alleles (P<0.05) but not in the CC allele, (P>0.05).

Table (3-12): Lipid profile of polycystic ovary syndrome women before and after metformin treatment according to SNP2 rs1801123

Parameter	SNP2 Alleles	Before treatment	After treatment	P. value
		Mean ± SD	Mean ± SD	
TG (mg/dL)	TT (Wild)	129.0 ±54.1	115.3 ±49.5	**0.002 [S]**
	TC (Hetro)	128.2 ± 41.5	117.6 ±34.3	**<0.001 [S]**
	CC (Mutant)	127.4 ± 55.2	98.9 ±26.1	**0.041[S]**
LDL (mg/dL)	TT (Wild)	102.1 ±32.7	86.8 ±25.4	**<0.001[S]**
	TC (Hetro)	99.9 ±76.8	87.6 ±23.7	**0.001[S]**
	CC (Mutant)	88.0 ±32.9	80.0 ±19.9	0.724[NS]
HDL (mg/dL)	TT (Wild)	45.0 ±10.8	46.7 ±10.1	0.173[NS]
	TC (Hetro)	46.1 ±10.5	46.8 ±10.1	0.087[NS]
	CC (Mutant)	45.2 ±9.0	48.0 ±9.4	0.638[NS]
Cholesterol (mg/dL)	TT (Wild)	164.4 ±37.3	140.7 ±37	**<0.001[S]**
	TC (Hetro)	163.8 ±47.3	157. ±42.86	**0.003[S]**
	CC (Mutant)	163.9 ±56.5	153.5 ±47.6	0.433[NS]
Results are presented as mean ± SD **(p< 0.05) considered significantly different, [S] significant, [NS] not significant**				

Chapter Four

Discussion

4.1 Demographic data

Polycystic Ovary Syndrome (PCOS) is the most common endocrine disorder amongst women of reproductive age, whose etiology remains unclear, and is marked by irregular menses, high androgens, and insulin resistance[(124)].

Table (3-1) demonstrates that, there is a significant increase in BMI for PCOs group (BMI considered as anthropometric indices for assessing obesity and insulin resistance [(125)]). Currently, most data appear to suggest that the development of obesity in PCOS patient's families are primarily made by genetic factors, although the degree and overall prevalence of obesity in the PCOS patients may exhibit, to a significant extent, the surrounding environment, unhealthy lifestyle[(126)] and insulin resistance. Our results are compatible with many studies[(127)]. It has also been reported that women with PCOS sequelae may suffer a greater risk of spontaneous abortion[(127)], Because of the close link between PCOS and obesity, and the association of obesity with poor pregnancy outcomes [(128)]. our results showed that the percentage of Abortion was significantly higher, but number of children was significantly lower in PCOs patient.

The findings of the current study confirm that, the incidence of spontaneous abortion increases with increasing BMI [(129)] and obesity [(127)].

Also, hirsutism and alopecia had been reported in PCOS women only, (92.6% and p-value <0.001 for both), This is because of high serum androgen levels, These results are compatible with studies reported by Liou et al.[130].

4.2 Hormonal, Glycemic parameters and Lipid Profile in Control Group and Polycystic Ovary Syndrome Patients

4.2.1 Hormonal Parameters

The diagnosis of PCOS in this study depends on Rotterdam criteria that are confirmed by the results obtained from analysis data related to the measured hormones [131]. In this study, table (3-2) showed that the serum LH, LH/FSH ratio, testosterone, FAI, and TSH in PCOS patients were significantly higher than control, with no significant difference in prolactin level between the study groups. While serum FSH and SHBG, were significantly lower in PCOS patients, in contrast, to control groups.

The increase of LH secretion is due to decreased sensitivity of the GnRH pulse generator to feedback inhibition by ovarian steroids and a steroid-permissive milieu results in a persistently rapid GnRH pulse frequency and perturbations in gonadotropin secretion that prefer the secretion of LH over FSH [132], Marshall *et al.* indicated that PCOS is characterized by exaggerated LH responses to exogenous GnRH which leads to increase LH pulses and an increase of LH/FSH ratio, whereas serum FSH levels are relatively low[133] .

The FSH plays a significant role in the monitoring of follicle development, decreases secretion of FSH in PCOS result in gonadal dysfunction, this can also result from high prolactin level which tends to suppress the ovulatory

cycle by impairing the secretion of FSH[134] , Lewy *et al*. confirmed that the increase in LH, LH/FSH ratio, total testosterone and decreases SHBG levels leading to increased FAI [136 ,135] .

In this study, there was an increase in the TSH level in the PCOs group, 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, these effects through undefined mechanisms leads to decreased deiodinase-2 activity at the pituitary level resulting in relative T3 deficiency and an increase in TSH levels, the raised TSH levels act on adipocytes to increase their proliferation[137] , El-Hafez *et al* found that euthyroid, insulin-resistant -PCOS women had higher TSH levels compared with euthyroid, non- insulin resistant -PCOS women[138] .

4.2.2 Glycemic Parameters

The PCOS patients had the highest fasting insulin and HbA1c than the control group as shown in table (3-3), insulin resistance has an essential role in PCOs which is documented by the highest and significant HOMA-IR in the PCOS patients group. Our results were compatible with that confirmed by Hafsa Majid et al. [139].

Obesity is responsible for insulin resistance and hyperinsulinemia in women with PCOS[140]. It has been hypothesized that enlargement of adipose tissue mass raises the availability of several metabolites (i.e., free fatty acids, lactate) during the lipolysis, which can influence the metabolism and the secretion of insulin as well as its peripheral action by interfering with glucose uptake in target tissues [141]. Moreover, the

study by Stepto *et al.* shows that hyperinsulinemia is present in 85% of patients with PCOS women [142], and these women have a compensatory increase in insulin levels due to a decrease in insulin sensitivity in adipose tissue [143].

4.2.3 Lipid Profile

In the present study, lipid abnormalities are found in women affected by PCOS as shown 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 control ($p < 0.001$) except HDL which was lower in PCOS ($p = 0.002$). A recent study showed that mild hypercholesterolemia, low levels of (HDL) are predictive for the occurrence of metabolic syndrome (MS) [144], high triglyceride (TG), and low-density lipoprotein (LDL) are frequently encountered in such women [145, 146]. Results of studies by Dunaif et al. [147] Roa et al. [148] 2009 in Venezuela, Talbott et al., [149] Orio et al. [150] in Italy, Legro et al., and [151] Erel et al. in Turkey [152] are consistent with our results. A study found that the implementation of a high-fat diet in pre-pubertal rats induced metabolic and ovarian alterations that were frequently present in PCOS women, suggesting a potential impact of hyperlipidemia on the hormonal profile [153]. Shaman et al. observed phenotype-specific differences in lipid profiles based on androgen levels [154], suggesting that androgens play an important role in hyperlipidemia [155].

4.3 The Correlation between Insulin Receptor Substrate 1 Polymorphism and Polycystic Ovary Syndrome Pathogenicity

The exact cause of PCOS is unknown, but several studies suggest a strong genetic component that is affected by gestational environment and lifestyle factors, or both[156]. Thus, numerous genetic variations have been related to the presence of PCOS in different populations[157]. In the present study, we investigated the possible association between the single nucleotide polymorphisms (SNPs) (rs2943641 and rs1801123) of the IRS1 gene and susceptibility to PCOS in Iraqi women, (TT wild type, TC heterozygotes, and CC mutated form for the two SNPs). The frequencies of SNP1 rs2943641 and SNP2 rs1801123 variant observed in our study were not significantly different between PCOS and healthy control women (12.1% vs. 11.5% , P= 0.368 and 2.3% vs. 5.8%, P= 0.248 respectively) as shown in tables (3.5 and 3.6).

Our data demonstrates that the IRS-1 polymorphism (rs2943641 and rs1801123) is not associated with increased susceptibility to PCOS in Iraqi populations. However, we cannot exclude the possibility that other genetic polymorphisms of the IRS1 family are associated with PCOS and might be clinically useful as markers to assess the disease risk, as polymorphism of Gly972Arg that could play a contributory role in the pathophysiology and risk of PCOS [158]. But C allele of rs2943641 is associated with increased hyperinsulinemia and impaired insulin sensitivity[159]. While there is no available data about rs1801123 in PCOS patients and our study may consider one of the first studies in this regard.

4.4 The Influences of Inulin Receptor Substrate-1 Polymorphism on the Effects of Metformin on Hormonal and Metabolic Profile in Women with Polycystic Ovary Syndrome

Pharmacogenetics is the study of the contribution of inheritance to variation in drug response, a variation that can range from a loss of the desired therapeutic effect at one end of the spectrum to an adverse drug reaction at the other [160, 161]. Some of the individual differences that underlie the variation in response to metformin are likely genetic [162]. Several pharmacogenetic studies revealed that variants in genes related to the pharmacokinetics and pharmacodynamics of metformin were associated with its variable effect [163].

4.4.1 The Influences of Inulin Receptor Substrate-1 Polymorphism on the Effects of Metformin on Hormonal Parameters

Metformin was the first insulin-sensitizing drug (ISD) to be used in PCOS, Velazquez, and colleagues reported a significant improvement in menstrual regularity and reduction in circulating androgen levels as well as a significant reduction in body weight which confermed their findings [164]. Several effects have been reported related to metformin in PCOS patients including restoring ovulation, reducing weight, circulating androgen levels, the risk of miscarriage, and reducing the risk of gestational diabetes mellitus (GDM) [102]. ISD works in PCOS by reducing the circulating insulin and androgen level through direct effect on ovarian steroidogenesis[165].In this

study, It was found that there were significant changes in hormones that are demonstrated in tables (3-7 and 3-10), where, LH level was significantly reduced after treatment in women with TT and TC while not significant reduction in those with CC alleles in both SNPs. FSH was not significantly increased after treatment, neither in TT, TC nor CC according to SNP1, while there is a significant increase after treatment only in the TT group for SNP2. The LH/FSH ratio and testosterone were significantly reduced after treatment in women with TT and TC, and not significantly different than its baseline level in women with CC in both SNPs. However, FAI was significantly reduced in women with TT for both SNPs, and TC group for SNP 2, while, there is non-significant reduction in those with SNP1.For CC group, there is non- significant (increase for SNP1 and decrease for SNP2). Regarding SHBG significantly elevated after metformin treatment in women with TT allele group for the two SNPs. Our findings regarding intact IRS1 genotypes compatible with several studies that demonstrate the effect of metformin on androgen secretion and/or production at the ovarian level, these observations are consistent with those of Mansfield et al. who reported that, in vitro, the production of androgens by theca cells can be reduced by the addition of metformin [(165)]. Also, several studies found that the effect of metformin on androgen production has been controversial[(166)]. It has been suggested that metformin reduces hyperandrogenism through its effect on both the ovary and adrenal gland androgen production, reducing pituitary luteinizing hormone, and increases the production of sex hormone-binding globulin by the liver[(167)]. Harborne and colleagues, on the other hand, reported no significant changes in androgen or sex hormone-binding globulin levels in patients treated with

metformin and assigned the improvement in symptoms to the reduction of circulating insulin levels[167]. Reducing fasting insulin and insulin-stimulated glucose levels lead to a reduction in ovarian cytochrome P450c17α activity that responsible for steroidogenesis in PCOS patients[168]. The mutant alleles carrier group showed no significant reduction in hormonal parameters, this is may be due to IRS1 polymorphism that leads to hyperinsulinemia and subsequent hyperandrogenemia.

4.4.2 The Influences of Inulin Receptor Substrate-1 Polymorphism on the Effects of Metformin on Glycemic Parameters

It has been suggested that Insulin resistance is a common finding in patients with PCOS[169]. The reason for the insulin resistance that is seen in women with PCOS remains to be elucidated, but it is generally believed that it originates from post-receptor defects [170], as IRS1 polymorphisms.

In recent years, the recommended management strategy for long-term treatment of PCOS includes lifestyle modification and insulin sensitizers, this comprehensive intervention protocol results in better regularity of menses and fertility potential [171]. Although metformin has been used for 40 years, its mechanisms of action are not fully understood. The postulated mechanisms are reduced absorption of glucose from the gastrointestinal system, decreased endogenous gluconeogenesis from the liver, and increased peripheral glucose uptake. At the cellular level, there is growing evidence that metformin may augment tyrosine phosphorylation of insulin receptor β-subunit and IRS1

proteins. It may also augment insulin-dependent and non-dependent cellular glucose uptake through the family of glucose transporter proteins [172]. This made metformin has favorable metabolic outcomes in women with PCOS[173]. This study confirmed that metformin can affect body weight. It was found that patients with homo (TT) and hetero (TC) alleles showed significant reduction in BMI while mutant (CC) alleles showed not significant reduction for both SNPs. Metformin can reduce body weight by several mechanisms including phosphorylation of insulin receptor and IRS1 which leads to normal activation of insulin signaling (metabolic) pathway that end with translocation of GLUT4 and reduce hyperglycemia, also, enhance tissue sensitivity to insulin and reduce the hyperinsulinemia which is the main cause of central obesity in PCOS patients[174] . Patients with mutant allele had a serine phosphorylation rather than tyrosine phosphorylation of IRS1 which inhibit the insulin action[175] and lead to further insulin resistance[15] and obesity.

According to SNP 1 and SNP2 genotype, (tables 3-8 and 3-11), FSG was significantly lowered in TT and TC group of SNP1, but only in TT group in SNP2, while not in CC group for both SNPs, HbA1c was significantly lowered in TT and TC group of both SNPs. Insulin level and HOMA- IR were significantly reduced only in the TT group for both SNPs. Improvement in HbA1c and insulin levels in our study, indicating that metformin improves sensitivity of insulin by many mechanisms, such as inhibition of hepatic gluconeogenesis [72] and reducing the circulating insulin levels and this agreed with many studies [165]. Metformin had differential effects based on IRS genotype[101], variant IRS1 protein may not be able to propagate the signals that are transmitted from the tyrosine-phosphorylated

insulin receptor β-subunit, and thus may not be able to increase the glucose uptake into the target tissues. This may lead to hyperinsulinemia, insulin resistance that leads to hyperandrogenemia. The decrease in insulin resistance in the intact IRS1 group comparing to the non-significant change in the variant group made us think that the IRS1 polymorphisms may play an important role in this variable response to metformin therapy.

4.4.3 The Influences of Inulin Receptor Substrate-1 Polymorphism on the Effects of Metformin on Lipid Profile

The incidence of dyslipidemia in patients with polycystic ovary syndrome was twice more than other women. Several studies demonstrated that dyslipidemia mostly present as an increase in triglycerides(28.3%) and a decrease in HDL (57.6%) [(176)]. This was confirmed by Rocha et al. who found that the levels of serum lipids (cholesterol, LDL, triglycerides) in patients with polycystic ovary syndrome were higher, and the level of HDL was lower when compared with healthy women [(177)].

Tables(3-9 and 3-12), demonstrated the results of the assessment of lipid profile for the participant PCOS women before and after treatment according to the SNP1 and SNP2, which revealed that triglycerides, LDL, and cholesterol levels were significantly reduced after treatment in TT and TC groups, for both SNPs. However, the HDL level was significantly elevated only in the TC group of SNP1. Our results are in agreement with other studies that reported reduction in cholesterol [(178)] and TG with an increase of HDL[(177, 179)]. These finding suggest that, metformin therapy associated with an improvement in the lipid profile [(180)].

Metformin promotes a reduction in glucose production and improves insulin sensitivity, the latter being a consequence of changes in lipid metabolism[181]. The changes arise through inhibition of fatty acid (FA) synthesis. In this way, metformin prevents lipid storage and enhance FA oxidation in insulin-sensitive tissues[182]. Female with IRS1 mutant alleles showed diminish in metformin response because of higher insulin resistance.

Insulin resistance plays a pivotal role by stimulation of lipolysis and altered expression of lipoprotein lipase and hepatic lipase[183]. Resistance to the action of insulin on lipoprotein lipase in peripheral tissues may contribute to elevated TG and reduced level of HDL due to increase in the rate of apolipoprotein A1/HDL degradation, which exceeds the rate of its synthesis[184]. Till know there are no available data concerning the correlation between IRS1 polymorphism (rs2943641 and rs1801123) and the effect of metformin on hormonal and metabolic parameters in polycystic ovary syndrome women.

4.5 Conclusions

From the results of the present study it was concluded that the :

1- Genetic polymorphisms of IRS1(rs2943641 and rs1801123) are not associated with the pathogenesis of PCOS in Iraqi women.

2- IRS1 (rs2943641 and rs1801123) polymorphism can be the cause of therapeutic failure to metformin in Iraqi females diagnosed with PCOS.

4.6 Recommendations

1. Additional SNPs of the insulin receptor substrate 1 (IRS1) gene has to be studied to confirm the role of insulin receptor substrate 1 (IRS1) polymorphism towards PCOS and to determine the correlation of different response for metformin treatment in PCOS women.

2. Insulin receptor gene polymorphisms has to be studied to determine its effects on PCOS pathogenicity and variable metformin response in women with this syndrome.
3. Sequence of this gene is recommended.

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الخلاصة

المقدمة :

متلازمة تكيس المبايض : هي اضطرابات هرمونية شائعة بين النساء في عمر الإنجاب مابين ٢٠ إلى ٤٠ سنة. مظاهره السريرية متعددة ويتم تشخيصه اعتمادا على معايير روتردام التي تعتمد على توفر عاملين من ثلاثة :

١.فرط الاندروجين السريري أو البايوكيميائي

٢.الطمث الغير منتظم أو المنقطع

٣.شكل المبيض متعدد الكيسات

ترتبط هذه المتلازمة عادة بفرط ومقاومة الأنسولين وتتأثر ب بروتين ركيزة مستقبلات الأنسولين الذي له دور مهم في عمل الأنسولين والمحافظة على الوظيفة الأساسية للخلية لذلك تعدد الأشكال الجيني لقاعدة نتروجينيه مفردة لهذا البروتين يعمل ك مثبط تنافسي ل مستقبلات الأنسولين وممكن أن يؤدي إلى عدة أمور منها مقاومة الأنسولين ومتلازمة تكيس المبايض وأيضا اختلاف تأثير الاستجابة العلاجية لعقار المتفورمين لدى النساء العراقيات المصابات حديثا بمتلازمة تكيس المبايض.

الهدف من الدراسة:

صممت هذه الدراسة لتقييم العلاقة المحتملة بين تعدد الأشكال الجينية لقاعدة نيتروجينية واحد في بروتين ركيزة مستقبلات الأنسولين وعلاقتها ب امراضية متلازمة تكيس المبايض. وأيضا لتقييم علاقتها بالاستجابة العلاجية لعقار المتفورمين بعد ١٢ اسبوع من تناوله بجرعة ٥٠٠ ملغم مرتين باليوم في النساء العراقيات المصابات حديثا بمتلازمة تكيس المبايض

الاشخاص و طرق العمل :

تمت هذه الدراسة في مستشفى النسائية والتوليد التعليمي في كربلاء المقدسة وأيضا في العيادة الخاصة منذ حزيران ٢٠١٩ إلى نيسان ٢٠٢٠

تضمنت هذه الدراسة (٣٢٠ مريضة مشخصة حديثا بمتلازمة تكيس المبايض و ١٠٤ امرأة سليمة) أعمارهم بين ٢٠ و ٤٠ سنة

تم سحب الدم من كل مشاركة بعد صيامها ليلا في اليوم الثاني من الطمث او في اي يوم بغياب الطمث وتم تقسيم الدم المسحوب إلى قسمين :

القسم الأول تم وضعه في اي دي تي اي تيوب ل استخلاص ال دي أن أي وأيضا ل فحص الكلايكوسليتد هيموكلوبين

القسم الثاني تم وضعه في جل تيوب لعزل السيروم وإجراء الفحوصات الهرمونية والبايوكيميائية.
تم سحب الدم من المرضى فقط بعد ثلاثة أشهر من إعطاء عقار المتفورمين لمتابعة التغيرات في البراميترات المدروسة.

النتائج: في هذه الدراسة تم قياس التوزيع الجيني لتعدد الاشكال الجينية لركيزة مستقبلات الانسولين في النساء السليمات وايضا في مريضات متلازمة تكيس المبايض ودراسة تأثيره على استجابة المريضات بمتلازمة تكيس المبايض لعلاج المتفورمين. وجدنا دلالة إحصائية واضحة بين مجموعة المرضى و مجموعة الأصحاء من الناحية الهرمونية والاستقلابية المتمثلة بتحاليل السكر والدهون. وأيضا هنالك دلالة إحصائية واضحة بين مجموعة المرضى قبل اخذ علاج المتفورمين وبعد اخذة ب ١٢ اسبوع وذلك اعتمادا على التنميط الجيني لمستقبلات ركيزة الأنسولين. وفقا لهذه الدراسة وجدنا انه تعدد أشكال النيوكليوتيدات المفردة لركيزة مستقبلات الأنسولين ليس لها دور في التسبب في متلازمة تكيس المبايض في النساء العراقيات لكن المرضى الذين يعانون من تعدد الأشكال الجيني لركيزة مستقبلات الأنسولين يكونون عرضة لمقاومة الأنسولين وقد لا يكون لديهم استجابة لعقار المتفورمين.

الاستنتاج: بحثنا لا يدعم تأثير تعدد الأشكال الجيني لركيزة مستقبلات الأنسولين على أنه هو السبب في إحداث متلازمة تكيس المبايض في النساء العراقيات و لكنها السبب في فشل الاستجابة العلاجية ل عقار المتفورمين في النساء العراقيات المشخصات بهذه المتلازمة .

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الصيدلة

علاقة تعدد الاشكال الجيني لركيزة مستقبلات الانسولين (IRS1) مع الاستجابة العلاجية للمتفورمين لدى النساء المصابات بمتلازمة تكيس المبايض في العراق

رسالة مقدمة الى

كلية الصيدلة في جامعة كربلاء كجزء من متطلبات درجة الماجستير في الادوية و السموم

من قبل

زهراء فؤاد فاضل

(بكالوريوس صيدلة ٢٠١٤)

بإشراف

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