Republic of Iraq
Ministry of Higher Education
And Scientific Research
University of Kerbala
Collage of Medicine

The effect of KISS1 gene polymorphism on development of polycystic ovary syndrome in Iraqi women

A Thesis

Submitted to the council of College of Medicine
Kerbala University in partial fulfillment of the requirements
for the degree of Master in Clinical Chemistry

By

Hiba Muayad Khudhair

B.Sc. Chemistry / University of Kerbala (2015)

Supervised by

.

Prof. Dr. Shatha Abdul Wadood Al-Shammaree
College of Science / University of Baghdad

Assist prof. Dr. Mousa Muhsin Al-Alaq
College of Medicine / University of Kerbala

2021 A.D. 1443 A.H.

بسم الله الرحمن الرحيم

(فَأَمَّا الزَّبَدُ فَيَذْهَبُ جُفَاءً وَأَمَّا مَا
يَنفَعُ النَّاسَ فَيَمْكُثُ فِي الْأَرْضِ
كَذَٰلِكَ يَضْرِبُ اللَّهُ الْأَمْثَالَ).

صدق الله العلي العظيم

سورة الرعد , الآية (17)

Supervisors Certification

We certify that the preparation of this M.Sc thesis titled:

(The effect of KISS1 gene polymorphism on development of polycystic ovary syndrome in Iraqi women)

Was presented by **Hiba moayad Khudhair**, and made under oure supervision at the department of Biochemistry / college of medicine / university of kerbala, as a partial requirements for the master degree in clinical chemistry

Professor

Dr. Shatha Abdul Wadood Al-Shammeree

Department of Chemistry

College of Science

University of Baghdad

Assistant professor

Mousa Muhsin Al-Alaq

Head of Obstetrics and Gynecology department /College of medicine

university of Kerbala

In the view of the available recommendation, we forward the present thesis for debate by the examining committee.

Asst. Prof. Dr. Rana Majeed Hameed

Head of Chemistry and Biochemistry Department

College of Medicine / University of Kerbala

Committee Certification

We, the examining committee, certify that we have read this dissertation and have examined the student (***Hiba Muayad Khudhair***) in its content and that in our opinion; it is adequate with "***Excellent***" as a thesis for the degree of (***Master in clinical Biochemistry***).

Assist professor

Dr. Rana Majeed Hameed

College of Medicine

University of Kerbala

(Member)

Assist professor

Dr. Hayder Ali muhammed

College of Veterinary Medicine

University of Kerbala

(Member)

Professor

Dr.Shatha Abdul Wadood Al-Shammaree

College of Science

University of Baghdad

(Supervisor/Member)

Assist professor

Dr. Mousa Muhsin Al-Alaq

College of Medicine

University of Kerbala

(Supervisor/Member)

Professor

Dr. Manal Kamel Rasheed

College of Medicine

University of Baghdad

(Chairman)

Approved By

College of Medicine / University of Karbala

Professor

Dr. Riyadh Dayhood AL-Zubaidi

Dean of the College of Medicine

University of Karbala

Dedication

To the one who will fill the earth with justice just as it was filled with injustice and tyranny..... ***The awaited Imam Mahdi (AJ)***

To the one who vowed her life to perform a message she made from the papers of patience, to the one who is priceless, the most wonderful woman in existence ……..…….. ***My Dear Mother***

To the one who harvested the thorns from my path to pave the path of knowledge for me, to the one I carry his name proudly the greatest father in the world ……..……..…..…… ***My Dear Father***

To who help and supported me ……..…… ***My Mother in Law***

To my soul mate, who was the best baptize in my scientific and research journey and spared no effort to help me …….…. ***My Dear Husband***

For the strong back and the spring from which I saturate..... ***My Brothers***

To whom my world bloomed with their arrival..... ***My Two daughters (Fatima, Ruqaia)***

Hiba

Acknowledgment

Foremost, I am very grateful to God for giving me the good health and ability to complete this thesis.

I would like to thank my research supervisor, ***Prof. Dr. Shatha Abdul Wadood Al-Shammeree*** who was generous in giving her time, encouragement, support, and advice continuous to throughout the work.

Also I would like to thank my supervisor, ***Assist Prof. Dr. Mousa Muhsin Al-Alaq*** for helping me in the diagnoses of patients and to obtain the samples.

My deep gratitude and appreciations to ***Asst Prof. Dr. Rana Majeed Hameed*** Head of Biochemistry Department/College of Medicine / University of Kerbala for her continuous support and guidance through the whole period of research.

I would like also to express my thanks to ***Assist Prof***. ***Dr. Hayder Ali muhammed*** for his continuous guidance regarding genetic part of the research.

Finally, I wish to express my thanks for all ***patients*** participating in this study and to everyone who helped me and supported me in any way to complete this research.

Thanks For All, Hiba

List of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviations	Meaning
ACTH	Adrenocorticotrophic hormone
AE-PCOS	Androgen Excess and PCOS Society
AES	Androgen Excess Society
AFC	Antral follicle count
ARC	Arcuate nuclei
ASRM	American Society for Reproductive Medicine
AVPV	Antero ventral preventricular
BMI	Body mass index
CDS	Coding Sequence
DAG	Diacylglycerol
DHEAS	Dehydroepiandrosterone sulphate
E2	Estradiol 2
ELISA	Enzyme Linked Immunosorbent Assay
EDTA-tube	Ethylene diaminetetra acetate tube
ER	Estradiol receptor
ERK1	Extracellular signal-regulated kinases 1
ERK2	Extracellular signal-regulated kinases 2
ESHRE	European Society for Human Reproduction and Embryology
FSH	Follicular stimulating hormone
FSHR	Follicular Stimulating Hormone Receptor
GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal
HPOA	Hypothalamic-pituitary-ovarian axis
iHH	hypogonadotropic hypogonadism
IP3	Inositol 1,4,5 trisphospate
IVF	In-vitro fertilization
Kp	Kisspeptin
LH	Luteinizing Hormone
LHR	Luteinizing Hormone Receptor
NIH	National Institutes of Health
OD	Optical Density
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction

PIP2	Phosphatidyl inositol 4,5-bisphosphate
PKC	protein kinase C
SHBG	Sex Hormone Binding-Globulin
SNPs	Single nucleotide polymorphisms
SP	signal peptide
TAC3	Tachykinin receptor
WHO	World Health Organization

Abstract

Background: Polycystic ovarian syndrome (PCOS) is a multifactorial illness that affects millions of women around the world. Kisspeptin is involved in the control of human reproduction bridging the gap between the sex steroid levels and feedback mechanisms that control the gonadotropin releasing hormone (GnRH) secretion; however, studies considering this peptide and infertility are limited. It's yet unclear what effect may play the genetic variants in the KISS1 gene in the development of PCOS.

Kisspeptin is a neuropeptide that stimulates the production of gonadotropin-releasing hormone (GnRH). It is required for the rise of Luteinizing Hormone (LH), Follicular stimulating hormone (FSH), and ovulation. This neuropeptide is necessary for normal reproductive function and the onset of puberty. Both GnRH and LH secretion are altered in women with polycystic ovarian syndrome (PCOS). This study aimed to study the genetic polymorphism of KISS1 gene and its effect on women with polycystic ovary syndrome development.

Subjects, Materials and Methods: The present work included a case control study for a group of 105 subjects (16 obese with PCOS, 19 overweight with PCOS, 35 normal weight with PCOS and 35 apparently healthy women as control group) were selected from the Infertility Department/ Gynaecology and Obstetrics Teaching Hospital in Karbala and from out clinic patients. Blood samples were collected from patients and control and each sample was divided into two parts used for genetic and serum analysis. Genetic polymorphism was performed for KISS1 gene and Enzyme Link Immunosorbent Assay system (ELISA) was

performed using sandwich method to measure the concentrations of serum Human kisspeptin 1as well as, hormonal analysis.

Results: the study's results indicated that primary infertility, irregular menstrual cycle, hirsutism, acne and hair loss were associated with both obese and non-obese PCOS groups. The Kisspeptin levels, LH, FSH, free Testosterone and prolactin were higher significantly in both obese and non-obese PCOS groups compared to control. Kisspeptin level was correlated positively with early Age (18-23years) in overweight PCOS group. The nonsynonymous SNP rs4889 (C/G) was found more frequently in the obese women with PCOS than in the overweight and normal weight women with PCOS and control groups. Allele C showed higher frequency in obese women with PCOS (31.2%) than overweight and normal weight women with PCOS (26.3%) and (28.5%) respectively, and control group (14.2%). The frequency of heterozygous allele (GC) was twice folds higher in PCOS cases than in controls. The homozygous genotypes (CC) were found just in PCOS with normal weight.

Conclusion: This study found the non-synonymous SNP rs4889 (C/G) more frequently in the obese women with PCOS than in the overweight and normal weight women with PCOS and control groups, and this SNP causes P81R substitution. And it has been observed that the allele frequency GG is statistically significant between the four groups (three of PCOS groups and control) in FSH, LH/FSH ratio, free testosterone and Kisspeptin1. While, the GC allele showed significant difference with LH, FSH, LH/FSH ratio between the four groups (three of PCOS groups and control). The significant difference was appeared in serum kisspeptin1 levels between PCOS groups as compared with control group. No significant correlations were found in other biochemical parameters.

Chapter One

Introduction

&

Literature Review

1.1. Introduction

polycystic ovarian syndrome (PCOS) is a serious gynecological condition that affects 3-10% of women of reproductive age [1]. It's a complicated endocrine illness marked by chronic anovulation, hyperandrogenism, and polycystic ovarian morphology, as well as primary clinical signs such as irregular or skipped periods and excessive hair growth on the face and body [2].

Despite extensive research and studies on PCOS, the underlying etiology of the condition remains a mystery. PCOS is caused by a combination of genetic, endocrine, metabolic, and environmental factors [3]. Biochemically, the increased production of luteinizing hormone (LH) and a normal or low amount of follicular stimulating hormone (FSH) by the anterior pituitary reveal ovarian dysfunction in PCOS [4].

It is widely acknowledged that genetic factors play a role in understanding the complex etiology of PCOS. In this regard, researchers have paid close attention to the genetic understanding of this disease in terms of hypothalamic-pituitary-gonadal (HPG) axis-related genetic variables [5]. Many previous studies have looked into HPG axis mutations that have been connected to the development of PCOS in adult females, including KISS1, G protein coupled receptor (GPR54), Gonadotropin-releasing hormone receptor GnRHR, luteinizing hormone receptor LHR, and follicular stimulating hormone receptor FSHR [6-8].

The KISS1 has emerged as one of the candidate genes contributing to a regulatory role in the female reproductive system, with an important part in HPG axis gonadotropin production [9]. Some single nucleotide polymorphisms (SNPs) in the KISS1 gene have been shown to impair the healthy functioning of the female reproductive system by disrupting the

HPG axis, and are thought to be important in the etiopathogenesis of PCOS [10].

Kisspeptins are peptides produced by the KISS1 gene that regulate the HPG axis. Kisspeptin works by binding to GPR54, a G protein-coupled receptor. Kisspeptin's main mechanism is to act directly on GnRH neurons, causing them to release GnRH into the portal circulation, which then stimulates LH and FSH, resulting in the release of androgens [11].

Determining the kisspeptin 1, LH, FSH levels, and KISS1 gene polymorphism in PCOS affected women may be useful in determining the multifactorial etiology of PCOS [12].

1.2. Literature Review

1.2.1. Polycystic ovary syndrome

The PCOS is a common endocrine and metabolic disorder, which is characterized by chronic anovulation, polycystic ovaries and hyperandrogenism [1], and it is one of the most common causes of female infertility, it affects 3-10% of women of reproductive age [13].

Although the pathogenesis of PCOS is still unclear, the disturbance of the hypothalamic-pituitary-gonadal (HPG) axis, usually characterized by multiple hormonal imbalances, featuring elevated LH/ FSH ratios, is strongly suspected to be associated with the development of PCOS in about 35-90% of patients. However, the mechanism involved with the dysregulated HPG axis in PCOS has not been well-illustrated yet [14-17].

The PCOS is a complex, heterogeneous disorder of uncertain etiology, but there is strong evidence that it can, to a large degree, be classified as a genetic disease [18].

The PCOS is grouped into three sorts:-

1. Patients with hyperandrogenism and prolonged anovulation but normal ovaries were diagnosed with classic PCOS.
2. Patients with polycystic ovaries and elevated LH levels should be included.
3. High level of LH and LH/FSH ratio but patient have normogenic phenotype, normal free androgen index, normal insulin sensitivity and normal BMI [19].

1.2.2. History

The PCOS (also named the Stein-Leventhal syndrome) was popularized by the two Americans whose names have been connected to the situation for a very long time. They described their treatment of anovulation utilizing wedge resection with striking achievement [20].

1.2.3. Prevalence of PCOS

The prevalence of PCOS in general population has not been definitively determined and appears to vary significantly among populations that have been studied [21]. Studies have shown that the prevalence of PCOS varies depending on the diagnostic criteria utilized [22].

According to the prevalence related to the population being assessed, because of ethnic differences in the clinical and biochemical features of PCOS [23]. Usually the reported prevalence range is between 3-10% in different countries, depending on the recruitment method, study population, criteria used for its definition and the method used to characterize each criterion[1, 24].

According to National Institutes of Health (NIH) criteria, PCOS is affecting 6%–8% of women of reproductive age [25]. Many studies permanently report that the prevalence estimates using the Rotterdam criteria are two to three times greater than 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 about 10– 15% [26].

Many studies have been performed to determine the prevalence of polycystic ovaries depending on ultrasound alone and have discovered 20 -30% of women have PCOS [26, 27]. The prevalence of PCOS in Iraqi females (20-40y) was 14% [28]

1.2.4. Etiology and Pathophysiology of PCOS

Although the etiology and pathophysiology of PCOS are yet unknown, evidence suggests that it is a complex condition in which interactions between environmental, genetic, endocrine, and metabolic factors work together to produce a shared outcome[29]. Nobody knows why these hormonal issues arise; some speculated that the problem could stem from the ovary, a region of the brain that controls hormonal production, or other glands in the body. Insulin resistance could possibly be to blame for these alterations [30]. The following signs are linked to abnormal hormone levels:

- High levels of LH stimulate ovulation, but if levels are too high, the ovaries' regular functioning may be interrupted [31].
- Insulin is a hormone that regulates blood glucose levels. Having PCOS; the body may not respond to insulin (insulin resistance), resulting in a higher glucose level. The body creates significantly more insulin to try to keep glucose levels from rising. With glucose intolerance and hyperandrogenism, increased insulin levels can cause weight gain, irregular periods, reproductive issues, and elevated testosterone levels [32].

While the actual cause of PCOS is unknown, it is thought to be a complex disorder with a genetic component. As with type 2 diabetes, approximately (20–40) percent of first-degree female relatives of women

with this condition acquire PCOS, even if it is never diagnosed [33]. The following are some of the most prevalent theories for elucidating the pathophysiology of PCOS

1.2.4.1. Neuroendocrine Disorder

The PCOS can be caused by defect brain neuronal pathways that control the hypothalamic-pituitary-ovarian axis (HPOA) [33]. An increase in the frequency and amplitude of gonadotropin releasing hormone (GnRH) release, which is represented by gonadotropin hormone production, was one of the most apparent neuroendocrine anomalies [34]. GnRH production from the hypothalamus varies in frequency and amplitude throughout the menstrual cycle, and regulate the synthesis and secretion of pituitary LH and FSH [35].

Women with PCOS showed unusual patterns gonadotropin secretion patterns, which are characterized by increased LH pulse frequency and amplitude along with normal or low FSH production, resulting in an elevated LH/FSH ratio [36]. Follicular maturation is slowed when FSH levels are low for a long time. The immature follicles may persist as cysts (resulting in a polycystic appearance), preventing ovulation. Because ovulation does not occur, the progesterone hormone is not produced, resulting in infertility, and a woman's menstrual cycle is erratic or absent without progesterone (oligominoreia or amenorrhea) [37, 38].

The ovaries also produce androgens, which are masculine hormones that prevent ovulation. An increase in serum androgens can cause the ovary's regular activity to be disrupted, as well as the menstrual cycle to be disrupted and follicle growth to be hampered [39]. Furthermore, decreased hepatic Sex Hormone Binding-Globulin (SHBG) synthesis by

the liver may cause an increase in blood free testosterone levels, making the syndrome worse [40].

1.2.4.2. Hyperandrogenemia

A) Ovarian steroidogenesis

Steroidogenesis in the ovary is a coordinated series of enzyme reactions that convert cholesterol, a C27 steroid, to three different forms of carbon-reduced steroid hormones: progestins (C21), androgen (C19), and estrogen (C18). Cholesterol is primarily generated from serum lipoproteins for steroidogenesis [41].

The total number of theca cells and their steroidogenic capacity are the two main elements that influence the total quantity of androgen released by the ovary, both of which are disrupted in women with PCOS. Theca interna hypertrophy is seen in many of the follicles in the PCOS ovary resulting in a higher number of steroidogenic cells [42]. A common feature of ovarian theca cells from women with PCOS is increased androgen production and secretion [43]. The increased number of antral follicles harboring thecal cells that hyper-secrete androgens causes most women with PCOS to have elevated androgen levels [44].

The vast amounts of androstenedione released by the theca cells are converted into testosterone and estrogen in peripheral tissues like adipose tissue, skin, and liver. In fact, the peripheral metabolism of androstenedione accounts for about half of the circulating testosterone in women [45].

B) Adrenal androgen production

Ovarian hyperandrogenism is also caused by androgen excess in the adrenal cortex, abdominal subcutaneous adipose depots, and other extra-ovarian sources. As a result, they show signs of androgen excess (hirsutism, acne, and central adiposity) [46, 47].

Female androgen production also takes place in the adrenal cortex. The adrenal gland uses the same steroidogenic pathway as the ovary, but is regulated by adrenocorticotrophic hormone (ACTH) rather than LH. The presence of high levels of adrenal androgens such as dehydroepiandrosterone sulfate (DHEAS) in women with PCOS suggests that their hyperandrogenism has an adrenal component [48]. The mechanisms of adrenal hyperandrogenism in PCOS, on the other hand, are unknown.

Hypersecretion of adrenocortical products, both basally and in response to ACTH stimulation, appears to be the source of adrenal androgen excess in women with PCOS, rather than dysfunctions of the hypothalamic-pituitary-adrenal (HPA) axis. A range of intricate biochemical pathways contribute to the elevated ovarian androgens production found in PCOS [48].

1.2.4.3. Insulin Resistance and Hyperinsulinemia

Insulin resistance and hyperinsulinemia are frequent in polycystic ovarian syndrome [49]. Insulin is involved in the pathophysiology of androgen excess in PCOS in both direct and indirect ways. Despite peripheral insulin resistance in women with PCOS; ovarian steroidogenesis appears to be insulin hypersensitive [50]. *In vivo*, prolonged hyperinsulinemia had a deleterious impact on oocyte

developmental competence. Insulin acted in the ovary in a similar way to gonadotropin. Insulin's effect on steroidogenesis *in vivo* appears to be dependent on glucose absorption [51].

Insulin increases the frequency of LH pulses, implying a physiological relationship between insulin and HPO axis activity [52].

1.2.4.4. Environmental Effect

The growth in PCOS prevalence in populations with a reasonably steady gene pool suggests that environmental variables are becoming increasingly important [53].

Environmental pollutants such as phthalate, diet (such as vegan and keto diets), nutrition (amount of proteins, carbs, and lipids in meals), socioeconomic level, and geography (associated to ethnicity) are all variables that might cause PCOS [54]. Excess maternal androgens during fetal life can have a direct impact on the child's development of PCOS women [54].

1.2.4.5. Genetic Effect

Previous research has suggested that genetic factors play a significant role in PCOS causation [39]. However, the route of inheritance for PCOS is unknown, and current research suggests that it may be a complicated feature [55]. This implies that a number of genes interact with environmental factors to cause the phenotype. Biochemical parameters, such as fasting insulin levels or hyperandrogenism, on the other hand, appear to be highly heritable, implying that some clinical signs, symptoms, or biochemical parameters of PCOS could be passed down

through the generations as Mendelian autosomal dominant or X-linked traits [56].

Multiple family investigations have shown a familial foundation for PCOS, with an increased frequency of hyperandrogenism, metabolic abnormalities, and PCO morphology in female relatives of affected women [57]. Furthermore, male relatives of women with PCOS are more likely to develop insulin resistance and other metabolic problems, implying that PCOS-related traits can be handed down to sons as well as daughters [58]. Genetic factors may possibly play a role in the etiology of PCOS, according to research on the prevalence of PCOS in twins [59]. Despite numerous association studies, no one gene has been identified as a significant contributor in the pathophysiology of PCOS, and the manner of PCOS inheritance is unclear [56].

1.2.5. Diagnostic criteria of PCOS

The Rotterdam criteria is a compromise between those of the National Institutes of Health (NIH) and the Androgen Excess and PCOS Society (AE-PCOS), and is the most widely utilized [60]. The diagnostic criteria for the three sets are as shown in the Table (1-1):

Table (1-1): The Diagnostic Criteria for Polycystic Ovary Syndrome.

Institute or Society	Diagnostic criteria
National Institute of health (NIH) 1990	Requires the presence of: 1. Chronic anovulation. 2. Clinical and/or biochemical signs of hyperandrogenism [61].
The European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine. Rotterdam (ESHRE/ASRM) 2003	Requires the presence of at least two of: 1. Oligo- and/or anovulation. 2. Clinical and/or biochemical signs of hyperandrogenism. 3. Polycystic ovaries [62].
Androgen Excess and PCOS Society (AE-PCOS) 2006	Requires the presence of: 1. Oligo-anovulation and/or polycystic ovarian morphology (Ovarian dysfunction). 2. Clinical and/or biochemical signs of hyperandrogenism [63].

1.2.6. Signs and Symptoms

The PCOS is a complicated condition with a significant impact on one's quality of life and include two kinds of features:

1.2.6.1. Clinical features of PCOS

Clinical anovulation or oligovulation and infertility is a common symptom of PCOS, as is irregular or no menstrual cycle, oligomenorrhea (fewer than nine menstrual cycles in a year) or amenorrhea (no menstrual cycle for three or more consecutive months), or any sort of menstrual disease [64]. However, 30% of women with PCOS will have regular periods. PCOS affects approximately 85–90 percent of women with oligomenorrhea [65]. The number of primordial follicles in women with PCOS is normal, but the number of main and secondary follicles is greatly increased. However, follicular growth is halted (follicles attain a diameter of 4–8 mm) due to disruptions in components involved in normal follicular development, and ovulation does not occur because a dominant follicle does not emerge [66].

Another common symptoms of PCOS are weight gain, abdominal and subcutaneous fat, hirsutism (facial and body hair), male-pattern alopecia (hair loss), acne, clitoromegaly (enlargement of the clitoris), deep voice, seborrhea (oily skin), insulin sensitivity, and the metabolic syndrome are all symptoms of androgen excess [67]. These symptoms develop because cysts create androgens, which enable females to express male-like characteristics; hence PCOS causes hyperandrogenism to emerge [68]. In addition, spontaneous abortion is more common in those with PCOS, with rates ranging from 42 percent to 73 percent [69]. Another symptoms of the PCOS patients were fatigue, sugar cravings, blurred vision, frequent urination, delayed healing, tingling sensation, anxiety and depression episodes and mood swing [70].

1.2.6.2. Biochemical features of PCOS

1.2.6.2.1. Hyperandrogenism

The presence of clinical and/or biochemical androgen excess is determined through PCOS clinical phenotyping (hyperandrogenism). The appearance of hirsutism, acne, and in certain cases alopecia is the most common clinical symptoms of androgen excess. While some studies have discovered that the majority of PCOS patients had hyperandrogenemia, [71] others have not [72].

The free testosterone (free androgen) index and testosterone testing are regarded to be sensitive ways of detecting hyperandrogenemia [73].

Biosynthesis of androgens

Besides the ovaries, the adrenal cortex synthesizes all three primary androgens: dehydroepiandrosterone sulfate (DHEAS), androstenedione, and testosterone, and is the other major location of female androgen synthesis. DHEAS is almost entirely (97-99%) generated by the adrenal cortex, and androstenedione is produced by both the adrenal gland and the ovaries, whereas the adrenal gland produces just 25% of testosterone, 25% is created in the ovary, with the rest coming through peripheral conversion of androstenedione in the liver, adipose tissue, and skin [30]. Around 60-80 percent of PCOS women have elevated testosterone levels in their blood [74].

Androgen is created in the ovary by the theca interna layer of the ovarian follicle, whereas adrenal androgens are produced by the zona fasciculata of the adrenal cortex. Excess androgen from the ovary and/or adrenal glands starts early in life, or even prenatally, creates a vicious cycle in which hyperandrogenism causes neuroendocrine abnormalities,

ovarian dysfunction, belly fat, and insulin resistance, all of which boost androgen production even more (Figure 1-1) [75].

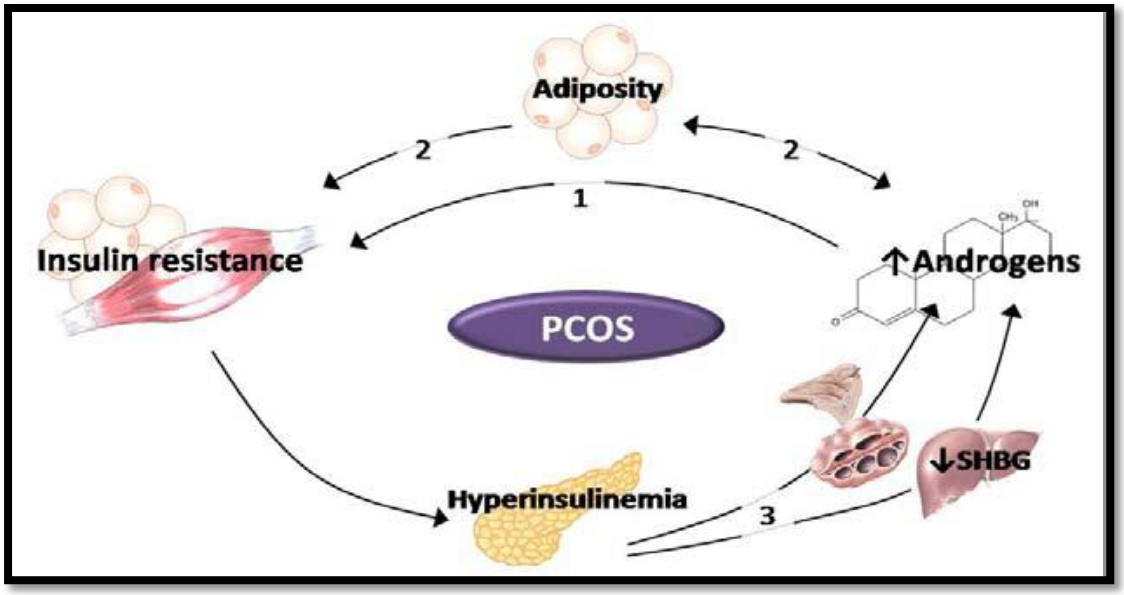


Figure (1-1): Hyperinsulinemia and hyperandrogenism, two characteristics of PCOS, are linked in a vicious cycle. 1) Insulin activity in adipocytes and skeletal muscle can be influenced directly by androgens. **2)** Insulin sensitivity can be influenced indirectly by androgens through their effects on lipid metabolism, adiposity/body fat distribution, and adipokine production. Adipokines, in turn, may have a direct impact on ovarian and adrenal function. **3)** Hyperinsulinemia, which is a compensatory response to insulin resistance, increases hyperandrogenism both directly and indirectly by inhibiting hepatic SHBG synthesis [75].

1.2.6.2.2. Hyperinsulinemia

Hyperinsulinism is a condition in which insulin expression is abnormally high, either clinically or biochemically. The link between insulin resistance, compensatory hyperinsulinemia, and hyperandrogenism has revealed new information about PCOS pathophysiology. Insulin resistance occurs when the glucose response to a

given amount of insulin is diminished. Insulin resistance, reduced hepatic insulin clearance, and/or enhanced pancreatic sensitivity are all possible causes. In women with PCOS, pancreatic-cell dysfunction has been documented, in which the basal secretion of insulin is enhanced but the postprandial response is inadequate. Even after losing weight, this flaw persists [76].

Women with PCOS (both obese and non-obese) are more insulin resistant and hyperinsulinemic than women with normal ovaries of the same age and weight. Thus, there appear to be variables that cause insulin resistance in women with PCOS that are independent of weight [77].

1.2.6.2.3. The Hypothalamic–Pituitary–Ovarian Axis

Pituitary gonadotrophin synthesis and secretion of FSH and LH are directly driven by hypothalamic GnRH, which is essential for reproductive function. During the menstrual cycle, the pituitary's sensitivity to GnRH fluctuates in tandem with changes in circulating estradiol (E2) concentrations [78]. FSH provides the initial stimulation for follicular maturation and stimulates the aromatase enzymes in granulosa cells, which helps to convert androgens to estrogens. LH, which promotes progesterone secretion in the luteal phase, is also important in the follicular phase, stimulating thecal androgen production (the substrate for E2 synthesis) and commencing oocyte maturation at midcycle [79].

As a result of increased amplitude and frequency of LH pulses, serum LH concentrations can be markedly raised in around 40-60% of PCOS women as compared to controls [72]. This high concentration has been linked to a lower chance of conception and a higher risk of miscarriage. Although an increase in serum LH concentration along by a

normal or moderate drop in (FSH) is exclusively seen in PCOS, a measurable increase in LH is not required to diagnose the condition [80].

1.2.6.2.4. Ovarian dysfunction

Oligomenorrhea, or abnormal bleeding, is a common early and dominating symptom of PCOS's anovulatory component [81]. PCOS causes chronic menstrual irregularity, which can present in a variety of ways. Anovulation is the most prevalent cause of irregular menstruation. Some women with PCOS experience long-term amenorrhea as a result of endometrial shrinkage. Others have normal periods at first, but then develop menstrual irregularities as a result of gaining weight [82].

The disruptions in folliculogenesis, the process by which small primordial follicles mature into giant preovulatory follicles and culminate in ovulation, cause ovulatory dysfunction in women with PCOS. Early follicular growth is increased, but when the follicles reach 2-9 mm in diameter (small antral follicles), a time during which the selection of a dominant follicle would typically take place, they stop growing [83].

1.2.7. Consequences of Polycystic ovary syndrome

Infertility is the one of the most common problems of PCOS, the World Health Organization (WHO) considers infertility to be a public health issue, and the agency defines infertility as the absence of conception after two years of regular intercourse without contraception, thus a process of evaluating possible reasons should begin [84].

The PCOS can lead to a variety of issues throughout a woman's life including decreased fertility, and even if pregnancy is obtained, women

with PCOS are more likely to experience pregnancy-related complications such as gestational diabetes, preeclampsia, a higher risk of miscarriage, and an earlier birth [85]. Furthermore, the outcome of IVF treatment for individuals with infertility and PCOS has been associated with a reduced fertilization rate, impaired embryo cleavage, poorer embryo implantation rates, and a higher miscarriage rate [86]. PCOS raises the risk of heart disease, metabolic syndrome, dyslipidemia, type 2 diabetes, and mood disorders, such as depression [87].

1.2.8. The history of kisspeptin discover

Kisspeptin's role in the reproductive system was found just recently in 2003; prior to that, it was well recognized for its capacity to inhibit tumors metastasis, which is why it's called Metastin. Kisspeptin was first discovered in 1996 as a tumor suppressor in melanoma patients [88]. Its receptor (GPR 54) was discovered four years later [89]. In 2003, two separate studies found that patients with hypogonadotropic hypogonadism (HH) had deletions and inactivating mutations in the GPR54 gene [90, 91]. These two researches were backed up by two more studies in 2006 and 2008, which verified kisspeptin's important involvement in reproduction [92, 93]. Furthermore, these findings cleared the path for additional research in several fields (Molecular Biology, Neuroanatomy, Physiology, and Pharmacology) to research the physiologic significance and mechanism of action of kisspeptin in reproduction, Table (1-2).

Table (1-2): The evolution of kisspeptin in the reproductive field from (2005 to 2011) [11].

Year of Study	The Discovery
2003	GPR54 inactivating mutations have been linked to HH in both humans and mice.
2004	KISS 1 mRNA-expressing neurons have been discovered in the arcuate nucleus (ARC) at the hypothalamic. Kisspeptins have been demonstrated to stimulate gonadotropin production in rodents. GnRH neuron are shown to express KISS mRNA receptor reported to induce GnRH secretion
2005	In the hypothalamus of rodents, two distinct populations of KISS 1 neurons have been identified: ARC and AVPV. KISS 1 neurons in the ARC are linked to negative feedback, while KISS 1 neurons in the AVPV are linked to positive feedback. Kisspeptins have been demonstrated to stimulate gonadotropin production in male.
2006	At puberty, there is changes in KISS1 neuron, increase in kisspeptin responsiveness and GPR54 signaling efficiency. During the preovulatory surge, KISS 1 neurons at the AVPV are activated. KISS 1 mRNA expression is increased by Leptin in the hypothalamus.

2007	Hormonal mechanisms for sexual differentiation of populations KISS 1 neurons are first exposed in rats. Kisspeptins have been found to stimulate gonadotropin secretion in females.
2008	The first activating mutation of GPR54 in humans has been discovered, with a putative relation to premature puberty. TAC3 inactivating mutations have been linked to HH in humans. Functional genomics examines the critical roles of KISS 1 neurons in negative and positive feedback.
2009	Kisspeptin antagonists of the first generation have been tried in a variety of mammalian species. ARC is thought to play a key role in the formation of GnRH pulses.
2010	Kisspeptin antagonists have been used to study the role of kisspeptins in puberty and the preovulatory surge. KISS 1 neuron mapping in the infundibulus and periventricular area of the human hypothalamus.
2011	Most of studies were on mouse models with selective ablation of KISS 1- and GPR54-expressing neurons were generated.

1.2.9. KISS1 Gene

The KISS 1 gene has three exons and two introns and is localized on chromosome 1q32. Only a portion of the second and third exons are translated into a 145-amino-acid precursor peptide, which is then cleaved into three kisspeptin forms of 54, 14, or 13 amino acids. The products from KISS1 are widely referred to "Kisspeptins" as the peptides that possess the highly conserved 10 amino acid RF-amide C terminus core sequence (kp-54, kp-14, kp-13 and kp-10) [97- 99]. The last two amino acids are arginine and phenylalanine that receive an amine group transferred from glycine at position 122 to residue 121 which is the C-terminal end of mature peptide amino acid [100]. The decapeptide kp-10 has the minimal length to completely stimulate the GPR54 and consequently increases phosphatidylinositol turnover. For this reason, it is considered the main peptide [101].

Following the identification of a single KISS 1 gene in eutherian mammals, researchers discovered up to three paralogous KISS genes in diverse vertebrates (*KISS 1*, *KISS 2*, and *KISS 3*). Each KISS gene encoding a polypeptide precursor, which is then processed to produce variations size of kisspeptin, as shown in Figure (1-2) [94].

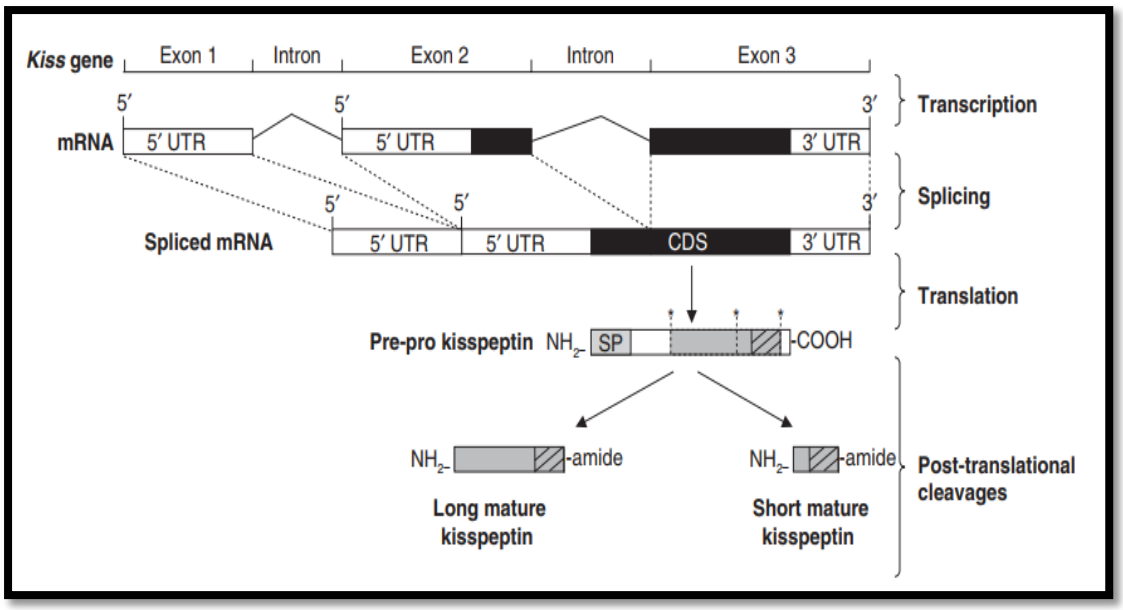


Figure (1-2): KISS1 Gene (transcription, translation, and post-translational processing). CDS: coding sequence; SP: signal peptide. The minimal bioactive sequence, composed by ten amino acids, Kp(10), is represented by a hatched square [94].

Mutations in the KISS1 gene, which is connected with the HPG axis, are thought to be a major genetic factor influencing PCOS risk [95]. It is widely known that the appropriate growth and maturation of the HPG axis is critical for the female reproductive system [96]. The KISS1 gene encoded kisspeptin-GPR54 pathway has a major effect in controlling the HPG axis by conveying signals to the GnRH neurons, which in turn releases GnRH into the portal circulation and increases the secretion of gonadotropins LH and FSH from the anterior pituitary gonadotropins. Furthermore, these two gonadotropins operate on the gonads and promote appropriate estrogen, testosterone, and progesterone release [11, 97].

KISS1 expression is negligible in prepubertal ovaries and abruptly increases at the time of preovulatory surge of gonadotropins [105]. As consequence, kisspeptin administration in immature rodents and primates was able to induce precocious activation of the gonadotropic axis and

precocious pubertal development [106]. Additionally, kisspeptin levels are higher in girls with central precocious puberty [107, 108]. In gonadal juvenile male monkeys, however, the continued kisspeptin administration decreases the LH levels, consequently leading to the assumption that kisspeptin secretion is pulsatile, and the continuous stimulation may induce receptor desensitization [109, 110].

The KISS1 gene-associated kisspeptin-GPR54 pathway is shown to be disrupted when genetic changes in the exonic region of the KISS1 gene occur, that leading to dysfunction in GnRH secretion, and eventually causes excess formation of androgens by the abnormal stimulatory effects of gonadotropin, LH, and encourages anovulatory hyperandrogenism associated with PCOS [98]. As a result, the implicated mechanism of KISS1 gene polymorphisms in the etiology of PCOS is thought to be linked to endocrine abnormalities. Only a few prior studies have looked into the role of KISS1 gene polymorphisms in the development of PCOS and related endocrine disorders in adult women. However, the findings of these investigations remain unconfirmed [10, 99]. As a result, more research is needed to confirm the link between KISS1 gene polymorphisms and PCOS and its related endocrine abnormalities [10].

1.2.10. Kisspeptin

It is the natural ligand of the family of G protein-associated receptors (GPCR) and is involved in the regulation of the hypothalamic-pituitary-gonadal axis. This receptor is found in abundance in the human placenta, pituitary, ovary, pancreas, and medulla spinalis [100]. Kisspeptin activates GnRH neurons directly by binding to the kisspeptin receptor (GPR54), to release GnRH into portal circulation; this stimulates the gonadotrophin of the anterior pituitary to secrete LH and FSH. These hormones control the secretion of estrogen, testosterone, and progesterone via acting on the gonads [114], as shown in Figure (1-3).

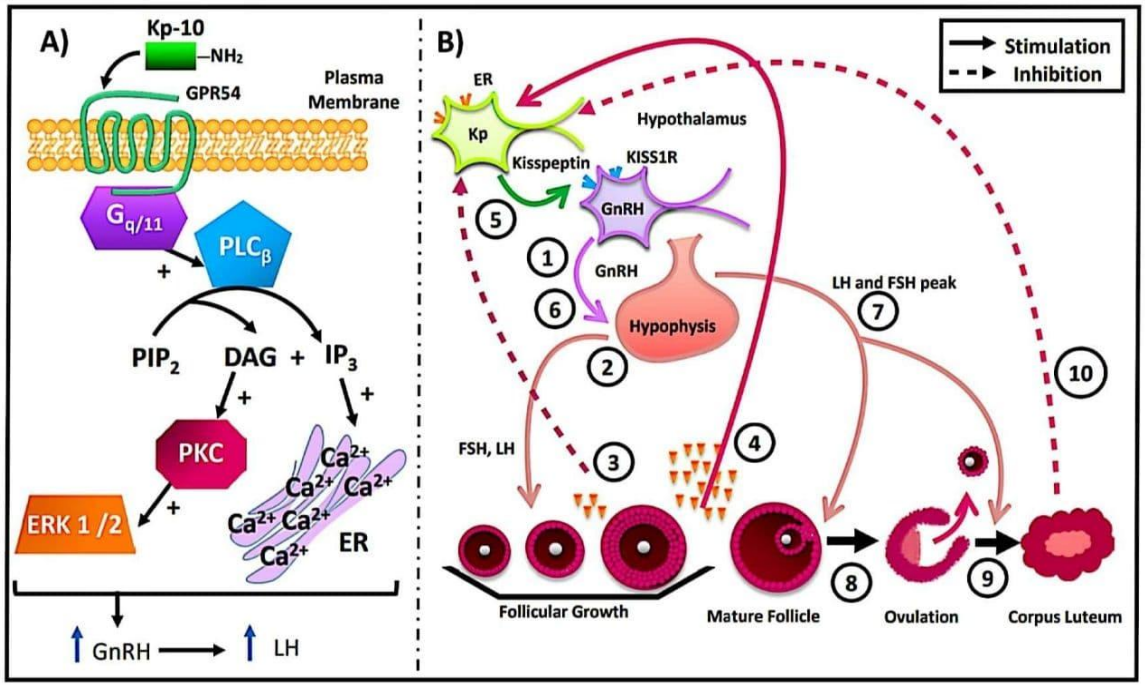


Figure (1-3): Cellular Signaling of kisspeptin/GPR54 and the physiologic role in menstrual cycle. A)) The intracellular signaling and GPR54. When kisspeptin stimulates its receptor, the GPR54 is coupled to Gq/11 protein; the Gq/11 activates phospholipase C (PLCβ) that hydrolyzes phosphatidylionositol 4,5-bisphosphate (PIP2) producing second messengers: inositol 1,4,5 trisphospate (IP3) and diacylglycerol (DAG). The (IP3) stimulates the endoplasmic reticulum (ER) to mobilize calcium (Ca^{2+}). The Increasing of intracellular (Ca^{2+}) levels activate calcium-dependent signaling pathways in GnRH neurons. The (DAG) activates calcium- dependent protein kinase C (PKC) that activates extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). B)) The role of kisspeptin in LH pulse. (1) GnRH stimulates the hypophysis, (2) Hypophysis release FSH and LH, (3) That stimulates the follicular growth and begin to produce estradiol (The estradiol suppresses hypothalamic–pituitary–gonadal axis). (4) In the late follicular phase when main follicle achieves around (12mm), the estradiol level increase and stimulates the kisspeptin (Kp) neurons which have an estradiol receptor (ER) releasing the kisspeptin. (5) kisspeptin (Kp) neurons stimulate the GnRH neurons, (6) That induces the LH release, (7) Ovulation, (8) and corpus the luteum development. (10) The Corpus luteum produces progesterone, estradiol and inhibin (A) that inhibit the hypothalamic–pituitary–gonadal axis [115].

Kisspeptin has therefore been proven to control LH secretion during ovulation promotion. Changes in the KISS 1 and GPR54 genes may affect serum kisspeptin levels and thus gonadotropin secretion [101]. Kisspeptin may affect the hypothalamic-pituitary gonadal axis by elevating LH levels and follicle growth in ovarian tissue.

In the nervous system, kisspeptin neurons were connected to GnRH neurons and experiments showed that central or peripheral administration of kisspeptin exerts a potent stimulatory effect on gonadotrophins secretion [117]. It was noted that low doses of intracerebroventricular kisspeptin markedly increased LH and FSH secretion [118]. This occurs through the activation of GnRH neurons, which express GPR54 [119]. Taking into account the participation of kisspeptins in folliculogenesis pharmacokinetic studies was developed. Kp-54 and kp-10 have had the similar activities when added to cell culture. However, kp-54 had a longer onset and more duration of action than kp-10 in the same molar concentration in rodents [120], and humans [121, 122].

There is evidence that kisspeptin levels increase each trimester during pregnancy [123] and it could reach a 200-fold higher level in the third trimester when compared with non-pregnant women [124]. Kisspeptin levels below 1630 pmol/L during the first trimester were considered a miscarriage biomarker, since women who suffer miscarriages show kisspeptin levels 60% lower than unaffected pregnancies [125].

In intracytoplasmic sperm injection (ICSI) treatments, kisspeptin levels on the day of human chorionic gonadotropin (hCG) administration were correlated with pregnancy rate. Kisspeptin was higher in the pregnant group with fetal heart beat than the non-pregnant group [126,127].

Kisspeptin appears to be a promising marker for PCOS studies. Several researches have yielded inconsistent outcomes regarding to serum kisspeptin levels in PCOS patients [102-104].

1.2.11. Kisspeptin and the Reproductive Axis

In the hypothalamus, kisspeptin neurons are found in two areas: the antero ventral preventricular (AVPA) and arcuate nuclei (ARC), both the (AVPA) and (ARC) KISS1 populations are regulated differently by testosterone and estrogen (E2) [105]. It has been discovered that GnRH neurons do not express the steroid receptor, for both negative and positive feedback control, while KISS1 neurons express sex steroid receptors. In female rats, AVPV KISS1 neurons are considered to mediate the positive feedback that triggers LH surge and subsequent ovulation [106].

Steroids, on the other hand, inhibit KISS 1 expression in the ARC nucleus. This suggests that these neurons are involved in gonadotropin secretion negative feedback control [98], as shown in Figure (1-4). KISS 1mRNA expression rose in the AVPA region during ovulation and LH surge, but decreased in the ARC region [19].

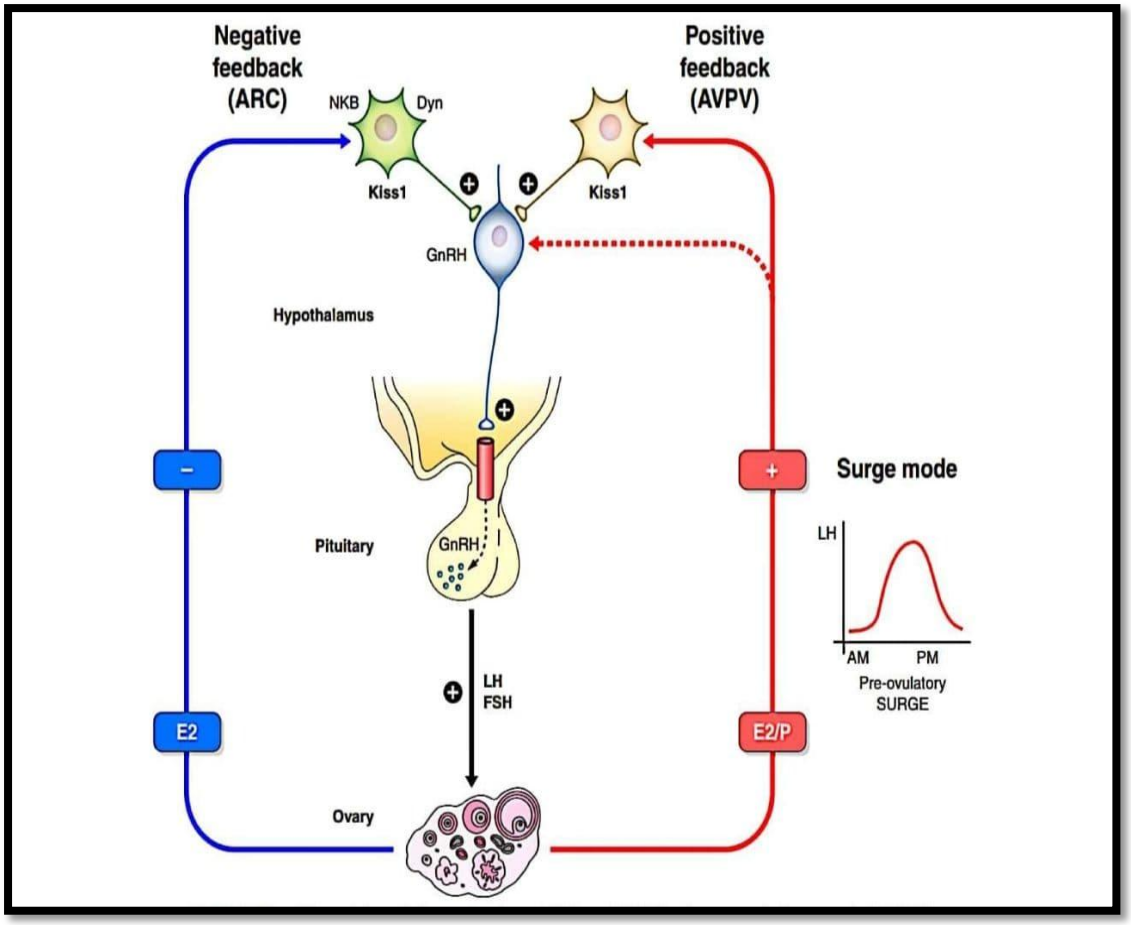


Figure (1-4): The Differential regulations and actions of (ARC) & (AVPV) KISS1 neurons in the control of GnRH in Rodent [11].

Human studies have shown Kisspeptin, like steroid hormones, has a fluctuating serum level over the menstrual cycle, with the amount in the follicular phase being lower than the preovulatory period, implying that kisspeptin stimulates LH secretion and improves ovulation [107, 108], Figure (1-5). Despite these findings, there are inter-subject differences in kisspeptin serum levels. This variety could be owing to kisspeptins episodic release, which could be due to its short half-life, or the fact that

serum kisspeptin comes from multiple sources as Kisspeptin gene has been found in a variety of peripheral organs (ovary, testes, uterus, and placenta) [109-111].

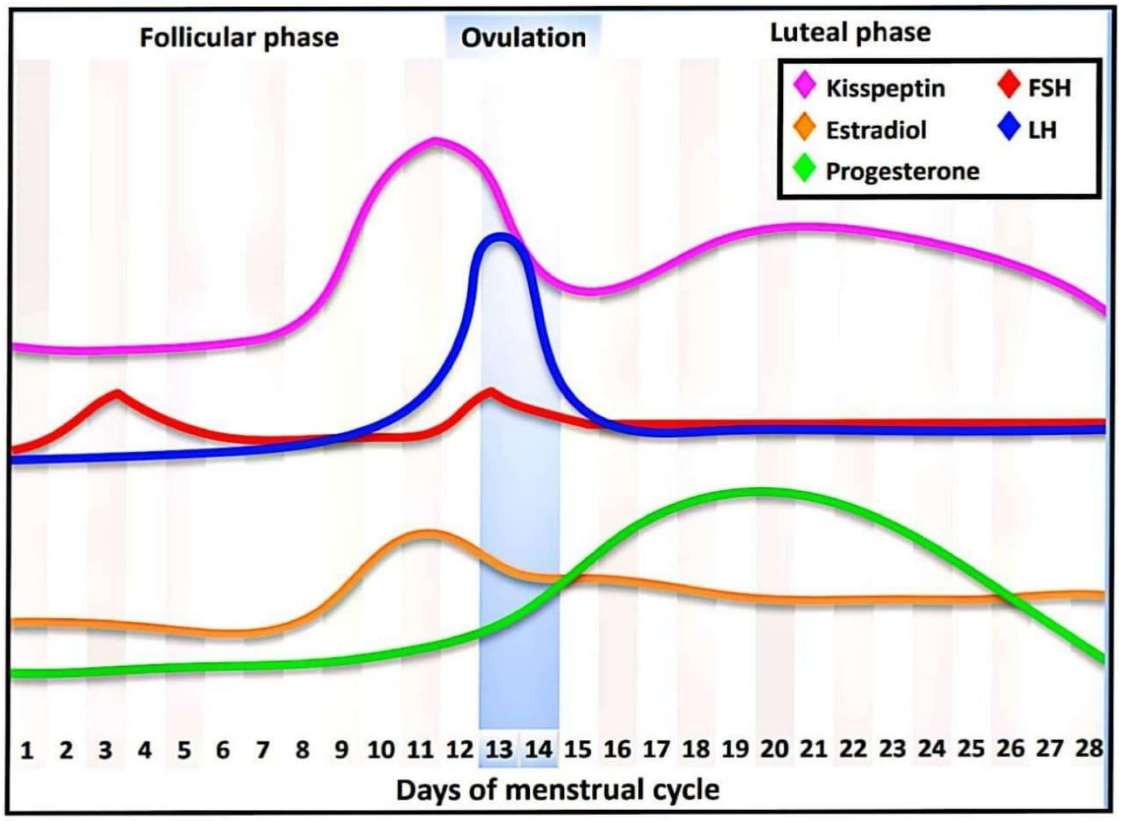


Figure (1-5) : Illustrates that serum kisspeptin levels rise before ovulation and then drop after ovulation [105].

1.2.12. Kisspeptin and Polycystic ovary syndrome

Kisspeptin stimulates LH secretion by triggering GnRH for inducing ovulation. Although ovarian kisspeptin expression has been observed in a variety of species, including humans, the specific mechanism through which kisspeptin is involved in ovulation is still unknown. Ovulatory dysfunction, hyperandrogenism, and metabolic changes are all symptoms of PCOS, a neuroendocrine condition [19].

Because LH and GnRH levels have been shown to be high in PCOS patients, it was hypothesized that kisspeptin levels would be high as well. Many studies have been done on the function of kisspeptin in the pathogenesis of PCOS, and the same studies have found that PCOS patients have higher levels of kisspeptin than normal women [103, 112-114]. While other studies found no differences in kisspeptin levels [102, 104, 115].

Although the precise mechanism for kisspeptin's role in the etiology of PCOS is still unknown, it has been demonstrated that metabolic problems in PCOS may lead to kisspeptin level changes. Any fluctuation in serum kisspeptin levels could be due to the varied nature of the genetic etiology of PCOS. Five single nucleotide polymorphisms (SNPs) were discovered in the GPR 54 gene, whereas two SNPs were found in the KISS1 gene. There was no significant difference in frequency between PCOS patients and controls for any of the detected SNPs [99].

1.2.13. Aims of the study

- To study the genetic polymorphism of KISS1 gene and its effect on women with polycystic ovary syndrome development.
- To explore the structure-activity relationship through measurement of serum kisspeptin levels in healthy and polycystic ovary syndrome women.
- To explore the association of body mass index with KISS1 phenotype on polycystic ovary syndrome.

Chapter Two

Subjects, Materials & Methods

2.1. Subjects

2.1.1. Study Design

The present work included a case control study for a group of (105) subjects: 16 obese, 19 over weight, and 35 normal weight women who have PCOS and 35 apparently healthy subjects serve as control. The study was conducted from February 2021 to June 2021, with age ranged between (18-40) years.

Patients with PCOS were selected from the Infertility Department/ Gynecology and Obstetrics Teaching Hospital and out clinic patients in Kerbala Province. History of patients, clinical and biochemical features, also weights and heights were taken from each patient. The socio-demographic aspects of the patients were collected through the self-reported technique (questionnaire) including age, residency, BMI, education, social status, type and duration of infertility, and having any current chronic diseases. All patients were diagnosed as PCOS by consultant gynecologist according to Rotterdam criteria. Biochemical parameters were measured to all patients participated in this study in the follicular phase (2-5) days of cycle.

2.1.2 Patients Criteria

A) Inclusion Criteria:

All patients were subjected to the clinical examination, and laboratory investigations. The diagnosis of the PCOS in women was according to:

1. The Rotterdam criteria two of three of: hyperandrogenism, menstrual disturbance (involved amenorrhea which is marked by

an absence of menstrual cycle for more than 6 months and oligomenorrhea which defined as a delay in the menses of > 35 days to 6 months) or ultrasound polycystic ovary morphology.

2. Age of women between (18-40) years.

B) Exclusion Criteria:

The females who excluded from this study:

1. Patients who were less than eighteen years old because incomplete reproductive function.
2. Patients who were more than forty years old because menopause and menstrual irregularities.
3. Thyroid dysfunction because hypothyroidism cause abnormal menstrual cycle.
4. Patients with congenital adrenal hyperplasia or adrenal tumors
5. Patients with androgen-secreting tumors (Androgen secreting).
6. Patients with diabetes, impaired kidney function, Cushing syndrome, or hyperprolactinemia.

2.1.3. Study Variables

Dependent Variable:

Serum Kisspeptin1, LH, FSH, Prolactin, Testosterone, Gene polymorphism of KISS1.

Independent Variable:

The weight, height, occupation, residency, social status, type of infertility and its duration, hirsutism, acne and hair loss.

2.1.4. Ethical and Scientific Approval

The proposal of the research was discussed and approved by the scientific and ethical committee in Collage of Medicine – Kerbala University. Approval was also taken from Kerbala Health Directorate and Administration of Gynecological and Obstetric Teaching Hospital. In addition, consent was taken from each patient to obtain a sample of blood after explaining the nature and purpose of study.

2.1.5. Data and samples Collection

2.1.5.1. Data collection

A structured questionnaire was specifically designed to obtained information which helps to select the patient according to the study criteria. Clinical manifestations were determined by consultation of Gynecologist / Infertility Department.

At the time when the blood samples were obtained socio-demographic aspects of the subjects (patients and control) were also included in the questionnaire such as: name, age, address, BMI, education, social state (married or not), numbers of kids, menstrual regularity, infertility (primary or secondary), hirsutism, acne, hair loss and other chronic disease. Patients were divided according to BMI into three groups: obese patients with BMI $\geq$ 30 kg/m^2, overweight patients with BMI $\geq$ 25 kg/m^2 and normal weight patients with BMI < 25 kg/m^2 [116]. Furthermore biochemical parameters were being measured for all patients and control which were: FSH, LH, free testosterone, Prolactin and serum kisspeptin levels.

2.1.5.2. Blood Collection and Storage

Blood samples were obtained from all subjects by vein puncture using 5 ml disposable syringes. Five ml of blood were withdrawn from all women that participated in this study in follicular phase.

From withdrawn blood sample of each patient; (2ml) were placed in EDTA-tube used for genetic study, and the remaining 3ml were placed in gel tube, left to clot, then serum were collected after centrifugation at 4000 rpm for 10 minutes. Serum samples were aliquot into two Eppendroff. One Eppendroff was used for measurement the following markers: FSH, LH, Prolactin, free testosterone levels. The second Eppendroff was stored in the freezer and used for measurement of serum kisspeptin using ELISA technique.

2.2. Materials

2.2.1. Apparatus

Various apparatus were used in this study as listed in Table (2-1) A and B.

Table (2-1) A: The apparatus used in serum analysis study.

No.	Apparatus	Company / country
1	Oven	Binder / Germany
2	Bench centrifuge	Hettichi / Germany
3	Cobas e 411	ROCHE / Germany
4	Micropipettes	Eppendorf / Germany

Table (2-1) B: The apparatus used in genetic analysis study.

No.	Apparatus	Company / country
1	Autoclave	Hirayama / Germany
2	Sensitive balance	Sartorius / Germany
3	Vortex mixer	Cyan / Belgium
4	Electrophoresis equipment	Biometra / Germany
5	Gradient PCR thermal cycler	Biometra / Germany
6	U.V. Transilliuminator	Cleaver / USA
7	High speed Centrifuge	Mikro 200R Hettich / Germany
8	Water bath	Memmert / Germany
9	Minispin Centrifuge	Eppendorf / Germany
10	Nanodrop spectrophotometer	Bio drop / England

2.2.2. Chemicals and biological materials

Various chemicals, Kits and biological materials were used in the study as listed in Table (2-2) A and B.

Table (2-2) A: The Kits used in serum analysis study.

No.	Chemicals	Company / country
1	Elecsys Free Testosterone Kit	Roche / Germany
2	Elecsys FSH Kit	Roche / Germany
3	Elecsys LH Kit	Roche / Germany
4	Elecsys Prolactin Kit	Roche / Germany
5	Human Kisspeptin1 Elisa Kit	Bioassay Technology / China

Table (2-2) B: The chemicals, biological materials and Kits used in serum analysis study.

No.	Chemicals	Company / country
1	Absolute ethanol	Phamacia / Sweden
2	Agarose	Biobasic / Canada
3	DNA ladder	Bioneer / Korea
4	Ethedium bromide	Sigma /USA
5	Free nuclease D.W	Promega /USA
6	Genomic DNA Extraction Kit	Bioneer / Korea
7	Green master mix	Promega /USA
8	Loading dye	Biobasic / Canada
9	Primers	Bioneer / Korea
10	Tris Borate EDTA (TBE)10X	Biobasic / Canada

2.3. Methods

2.3.1 Determination of Body mass index (BMI)

Body Mass Index (BMI) is a simple index of weight for height that is commonly used to classify underweight, overweight, and obesity. It is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2). According to WHO criteria, overweight was defined as a BMI > 25 kg/m^2 and being obese as >30 kg/m^2 [116].

2.3.2 Serum Analysis

2.3.2.1 Determination of Serum Follicular Stimulating Hormone Concentration

The quantitative determination of FSH in human serum was done using a kit from Elecsys com. and Cobas e 411 analyzer depending on method of electro chemiluminescence immunoassay "ECLIA" [117].

Procedure

The total duration of assay is 18 minutes by using sandwich principle:

- First incubation of 40 µL of sample, a biotinylated monoclonal FSH-specific antibody, and a monoclonal FSH-specific antibody labeled with a ruthenium complex form a sandwich complex were mixed.
- Second incubation was after addition of streptavidin-coated microparticles; the complex becomes bound to the solid phase *via* interaction of biotin and streptavidin.
- The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell/ProCell M. Application of a voltage to the electrode then

induces chemiluminescent emission which is measured by a photomultiplier.

- Results were determined *via* a calibration curve which is done by the instrument specifically generated by 2-point calibration and a master curve provided *via* the reagent barcode or e-barcode [118].

Reagents - Working Solutions

Table (2-3): Reagents of the kit for determination of serum FSH concentration.

Reagents	Number and volume	Description
M Streptavidin-coated microparticles (transparent cap)	1 bottle, 6.5 mL	Streptavidin-coated microparticles 0.72 mg/Ml
R1 Anti-FSH-Ab~biotin (gray cap)	1 bottle, 10 mL	Biotinylated monoclonal anti-FSH antibody 0.5 mg/L, MES buffer 50 mmol/L.
R2 Anti-FSH-Ab~Ru(bpy) (black cap)	1 bottle, 10 mL	Monoclonal anti-FSH antibody (mouse) labeled with ruthenium complex 0.8 mg/L, MES buffer 50 mmol/L; pH 6.0

Handling of Reagents

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

Storage and Stability of the Kit's Reagents

- Store at 2-8 °C without freezing.
- The Elecsys reagents ' kit were stored upright in order to ensure complete availability of the micro particles during automatic mixing prior to use.

Calculations

The analyzer calculates the concentration of FSH for each sample automatically either in mIU/mL or in IU/L.

2.3.2.2. Determination of Serum Luteinizing Hormone Concentration

The quantitative determination of LH in human serum was done by a kit from Elecsys and Cobas e 411 analyzer device depending on method of electro chemiluminescence immunoassay "ECLIA"[119] .

Procedure

The total duration of assay is 18 minutes by using sandwich principle**.**

- ❖ First incubation of 20 µL of sample, a biotinylated monoclonal LH-specific antibody, and a monoclonal LH-specific antibody labeled with a ruthenium complex form a sandwich complex were mixed.
- ❖ Second incubation is after addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase *via* interaction of biotin and streptavidin.

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

- Results were determined *via* a calibration curve which is done by the instrument specifically generated by 2-point calibration and a master curve provided *via* the reagent barcode or e-barcode [118].

Reagents - Working Solutions

Table (2-4): Reagents for the determination of serum luteinizing hormone concentration.

Reagents	Number and volume	Description
M Streptavidin-coated microparticles (transparent cap)	1 bottle, 6.5 mL	Streptavidin-coated microparticles 0.72 mg/mL
R1 Anti-LH-Ab~biotin (gray cap)	1 bottle, 10 mL	Biotinylated monoclonal anti-LH antibody (mouse) 2.0 mg/L; TRIS buffer 50 mmol/L
R2 Anti-LH-Ab~Ru(bpy) (black cap)	1 bottle, 10 mL	Monoclonal anti-LH antibody (mouse) labeled with ruthenium complex 0.3 mg/L; TRIS buffer 50 mmol/L

Handling of Reagents

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

Storage and Stability of the Kit's Reagents

- Store at 2-8 °C without freezing.
- The Elecsys reagents ' kit were stored upright in order to ensure complete availability of the micro particles during automatic mixing prior to use.

Calculations

The analyzer calculates the concentration of LH for each sample automatically either in mIU/mL or in IU/L.

2.3.2.3 Determination of Serum Prolactin Concentration

The quantitative determination of prolactin in human serum was done by a kit from Elecsys and Cobas e 411 analyzer device depending on method of electro chemiluminescence immunoassay "ECLIA" [120].

Procedure

The total duration of assay is 18 minutes by using Sandwich principle**.**

- ❖ First incubation was mixing of 10 µL of sample and a biotinylated monoclonal prolactin specific antibody to form the first complex.
- ❖ Second incubation was after addition of a monoclonal prolactin-specific antibody labeled with a ruthenium complex and streptavidin-coated microparticles; a sandwich complex is formed

and becomes bound to the solid phase *via* interaction of biotin and streptavidin.

- The reaction mixture was aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results were determined *via* a calibration curve which is done by the instrument specifically generated by 2-point calibration and a master curve provided *via* the reagent barcode or e-barcode [121].

Reagents - Working Solutions

Table (2-5): Reagents of determination of serum Prolactin concentration.

Reagents	Number and volume	Description
M Streptavidin-coated microparticles (transparent cap)	1 bottle, 6.5 mL	Streptavidin-coated microparticles 0.72 mg/mL.
R1 Anti-prolactin-Ab~biotin (gray cap)	1 bottle, 10 mL	Biotinylated monoclonal anti-prolactin antibody (mouse) 0.7 mg/L; phosphate buffer 50 mmol/L.
R2 Anti-prolactin-Ab~Ru(bpy) (black cap)	1 bottle, 10 mL	Monoclonal anti-prolactin antibody (mouse) labeled with ruthenium complex 0.35 mg/L; phosphate buffer 50 mmol/L.

Calculations

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

ng/mL x 21.2 = μIU/mL (mIU/L).

2.3.2.4 Determination of serum Testosterone concentration

The quantitative determination of testosterone hormone in human serum was done by a kit from Elecsys and Cobas e 411 analyzer depending on method of electro chemiluminescence immunoassay "ECLIA"

Procedure

The total duration of assay is 18 minutes by using sandwich principle:

- First incubation of 20 μL of sample was made with a biotinylated monoclonal testosterone‑specific antibody. The binding sites of the labeled antibody become occupied by the sample analyte (depending on its concentration).
- Second incubation was made after addition of streptavidin-coated microparticles and a testosterone derivate labeled with a ruthenium complex, the complex becomes bound to the solid phase *via* interaction of biotin and streptavidin.
- The reaction mixture was aspirated into the measuring cell where the micro particles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell/ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier [122]

- ❖ Results were determined *via* a calibration curve which was done by the instrument specifically generated by 2point calibration and a master curve provided *via* the reagent barcode or e-barcode [123].

Reagents - Working Solutions

Table (2-6): Reagents for the determination of serum testosterone hormone concentration.

Reagents	Number and volume	Description
M Streptavidin-coated microparticles (transparent cap)	1 bottle, 6.5 mL	Streptavidin-coated microparticles 0.72 mg/mL, preservative.
R1 Anti- Testosterone - Ab~biotin (gray cap)	1 bottle, 10 mL	Biotinylated monoclonal anti-testosterone antibody (sheep) 40 ng/mL; releasing reagent 2-bromoestradiol; MES buffer 50 mmol/L, pH 6.0; preservative
R2 Testosterone-peptide~Ru(bpy) (black cap)	1 bottle, 9 mL	Testosterone derivative, labeled with ruthenium complex 1.5 ng/mL; MES buffer 50 mmol/L, pH 6.0; preservative.

2.3.2.5 Determination of Serum Human Kisspeptin1

Enzyme Linked Immunosorbent Assay (ELISA) was performed using sandwich method for the accurate quantitative detection of human KISS-1 in serum.

Procedure

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA).

- The plate has been pre-coated with human KISS-1antibody.
- KISS-1 present in the sample was added and binds to antibodies coated on the wells.
- And then, biotinylated human KISS-1 Antibody was added which binds the KISS-1 in the sample.
- Then, Streptavidin-HRP was added and binds to the Biotinylated KISS-1antibody.
- After incubation, unbound Streptavidin-HRP was washed away during a washing step.
- Substrate solution was then added and the color develops in proportion to the amount of human KISS-1.
- The reaction was terminated by addition of acidic stop solution and the absorbance was measured at 450 nm.

Reagents:

The ELISA kit applied for quantitative determination of human KISS-1 in serum, list of reagents are shown in the table below:

Table (2-7): Reagents and equipment for the determination of Kisspeptin1 concentration.

Components	Quantity
Standard Solution (1600ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 x 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated human KISS-1Antibody	1ml x1
User Instruction	1
Plate Sealer	2piecs
Zipper bag	1piece

Samples and Reagents Preparation

Reagents

All reagents were brought and were prepared freshly at room temperature before use. Stock solutions were prepared based on the procedure of the manufacturer.

The Standard Curve

A 120µl of the standard (1600ng/L) was reconstituted with 120µl of standard diluent to generate an 800ng/L standard stock solution. The standard had been allowed to sit for 15 min with gentle agitation prior to making dilutions. Duplicate standard points was prepared by serially diluting the standard stock solution (800ng/L) 1:2 with standard diluent to produce 400ng/L, 200ng/L, 100ng/L and 50ng/L solutions. Standard

diluent solution was served as the zero concentration (0ng/L). Any remaining solution was frozen at -20°C and used within one month. Dilution of standard solutions suggested is as follows in Table (2-8) and Fig. (2- 1):

Table (2-8): The concentrations for dilution of standard solutions.

Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
1600ng/L	800ng/L	400ng/L	200ng/L	100ng/L	50ng/L

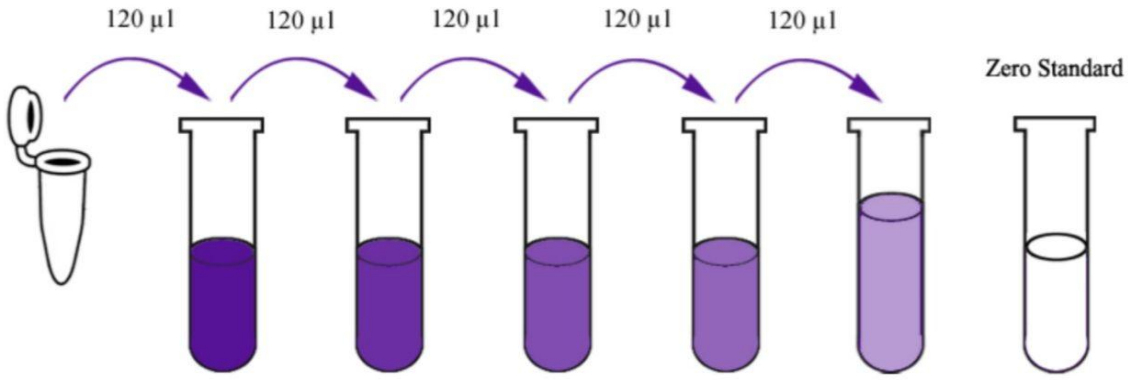


Figure (2-1): Dilution steps for preparation of standard solutions for human kisspeptin1 determination.

Wash Buffer

A 20ml of Wash Buffer Concentrate was diluted 25x with deionized or distilled water to yield 500 ml of 1x Wash Buffer. Mixing gently is necessary if crystals are formed until complete dissolving.

The Standard Curve

A standard curve for human Kisspeptin 1determination is generated to calculate the concentration of each sample from the equation of the curve.

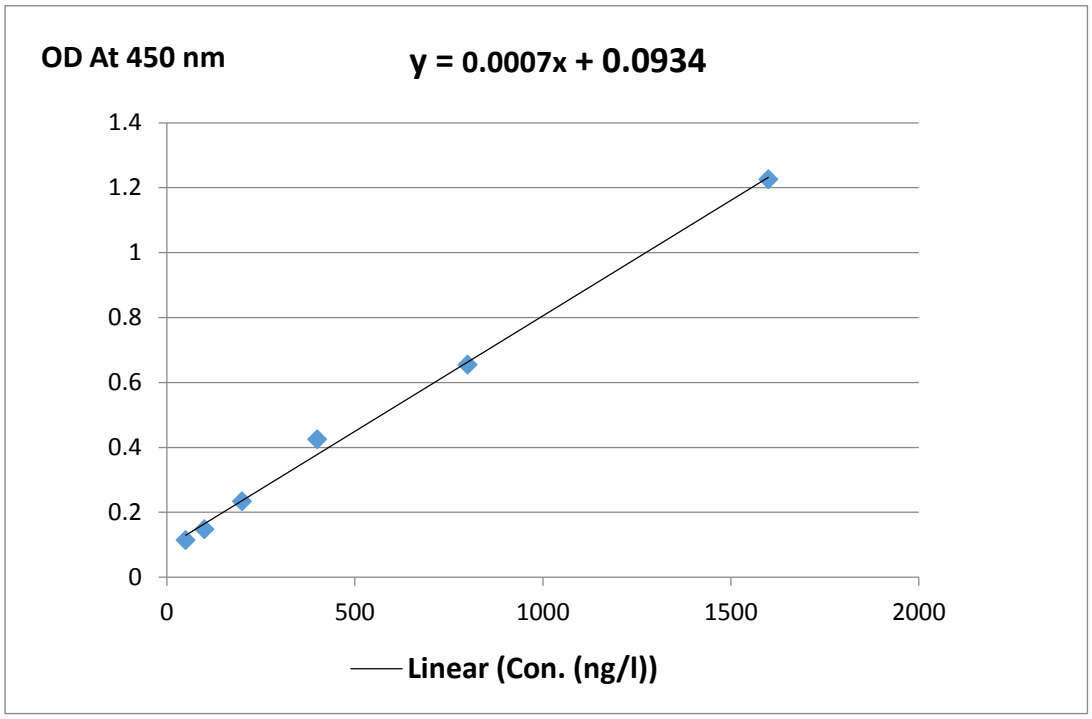


Figure (2-2): Calibration curve of human Kisspeptin1.

Assay Procedure:

1. All reagents, standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use. The assay was performed at room temperature.

2. The number of strips required for the assay was determined. The strips were inserted in the frames for use. The unused strips were stored at 2-8°C.

3. A 50µl of standard was added to standard well. The antibody was not added to standard well because the standard solution contains biotinylated antibody.

4. A 40µl of sample was added to sample wells then, a 10µl of anti-KISS-1 antibody was added to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells (Not to blank control well) and were mixed well. The plate was covered with a sealer and incubated 60 min at 37°C.

5. The sealer was removed and the plate was washed 5 times with wash buffer. The wells were soaked with at least 0.35 ml wash buffer for 30 to 60 sec for each wash. For automated washing; all wells were aspirated and washed 5 times with washed buffer, the wells was overfilled with wash buffer. The plate was blotted onto paper towels or other absorbent material.

6. A 50µl of substrate solution A was added to each well and then 50µl of substrate solution B was added to each well. The plate was covered with a new sealer and incubated for 10 min at 37°C in the dark.

7. A 50µl of Stop Solution was added to each well, the blue color changed into yellow immediately.

8. The optical density (OD value) was determined for each well immediately using a microplate reader set at 450 nm within 10 min after adding the stop solution.

2.3.3. Genetic Analysis

A- Extraction of Genomic DNA

Description of kit

The AddPrep Genomic DNA Extraction Kit offer simple and rapid method for isolating genomic DNA from blood up to 200 µl of a blood sample, and is suitable to use with whole blood treated with either citrate or EDTA.

Kit's Components

Table (2-9): The solutions and materials of the kit

Solutions and Materials	size
Binding solution	25ml
Washing 1 solution	30 ml
Washing 2 solution	12 ml
Elution solution	25 ml
Spin column	100
Proteinase K (20 mg/ml)	1.2 ml × 2 tubes

Preparation of Solutions

1. Ethanol (22.5 ml and48 ml) was added to Washing 1 and Washing 2 solution respectively before used.
2. Lysis, Binding and Washing 1 Solutions were checked for any precipitation, and any precipitant was dissolved by warming at 50°C.

Extraction Procedure

1. A 20 µl of Proteinase K solution (20 mg/ml) was added to a 1.5 ml micro-centrifuge tube.
2. A 200 µl of whole blood sample was transferred to the 1.5 ml micro-centrifuge tube containing Proteinase K solution.
3. A 200 µl of Binding Solution was added to the sample tube, and was mixed well by pulse-vortex for 15 sec.
4. The mixture was incubated at 56℃ for 10 min. Longer incubation times have no effect on yield or quality of the purified DNA.
5. A 200 µl of absolute ethanol was added and mixed well by pulse-vortex for 15 sec. After this step, briefly spinning down to get the drops clinging under the lid.
6. Carefully, the lysate was transferred into the upper reservoir of the spin column with 2.0ml collection tube without wetted the rim.
7. The mixture was centrifugated at 13,000 rpm for 1 min. Poured off the flow-through and assembled the spin column with the 2.0 ml collection tube.
8. A 500 µl of Washing 1 Solution was added to the spin column with collection tube and centrifuged at 13,000 rpm for 1 min. Poured off the flow-through and assemble the spin column with the 2.0 ml collection tube.
9. A 500 µl of Washing 2 Solution was added to the spin column with collection tube and centrifuged at 13,000 rpm for 1 min. Poured off the flow-through and assemble the spin column with the 2.0 ml collection tube.
10. The spin column was dried by additional centrifugation at 13,000 rpm for 1 min to remove the residual ethanol in the spin column.

11. The spin column was transferred to the new 1.5 ml micro-centrifuge tube.
12. A 130µl of Elution Solution was added to the spin column with micro-centrifuge tube, and left to stand for at least 1 min.
13. The genomic DNA was eluted by centrifugation at 13,000 rpm for 1 min.
14. The genomic DNA was preserved in freezing.

B- Determination the Concentration and Purity of the Extracted DNA

The concentration and purity of the extracted DNA were measured by absorbance using spectrophotometric method and Nanodrop instrument.

Principle

- The reading of absorbance was done at 260 nm and at 280 nm.
- At 260 nm, the DNA strongly absorbs light; however at 280 nm, the protein absorbs light most strongly.
- The purity of DNA was measured by the (A260/A280) ratio. The A260/A280 ratio in the range1.8-2.0 is commonly accepted and it indicates a high quality DNA sample [124].

Procedure

- Nanodrop is highly sensitive micro detector. This micro detector was cleaned up by 1 µL of nuclease free water as blank. The micro detector was cleaned up from blank. After that 1 µL of DNA sample was placed on the micro detector of nanodrop. This process was applied for each sample.

- The purity was detected by reading the ratio of optical density at (260/280).
- Reading of the concentration and A260/A280 ratio of DNA was documented for each sample from the instrument.

C- Amplification of DNA by using polymerase chain reaction Technique

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a target DNA template to produce specific DNA fragments *in vitro*. The PCR has become one of the most valuable techniques currently used in bioscience, diagnostics and forensic science. In order to amplify the target gene (KISS 1), the PCR was used with a specific sequence of primer pairs to amplified the required region of the DNA strand to generate several million copies. The mechanism of PCR include three steps cycled about (25-45) times [125] and these steps are:

- **1st step (Denaturation):** The initial step denatures the target DNA by heating it for about (94-95) °C or higher for 15 sec to 2 min. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermo-stable DNA polymerase.
- **2nd step (Annealing):** In this step of a cycle, the temperature reduced to approximately (55–65) °C. At this temperature, the oligonucleotide primers formed stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase. This step lasted approximately (15–60) sec.
- **3rd step (Extension**): Finally, the synthesis of new DNA was done that is complementary to the template strand as the reaction

temperature is raised to the optimum for the DNA polymerase, this temperature is in the range of (72–74) °C and this occurs in the presence of the Taq DNA polymerase because of its ability to operate efficiently at high temperatures without denaturation. The extension step lasts approximately (1-2) min and the next cycle begins with a return to 94°C for denaturation**.**

Primers

A short sequence of nucleotides consisting from (18-22) bases that provides a starting point for DNA synthesis. In a PCR reaction, the experimenter determines the region of DNA that will be copied, or amplified, by the chosen primers [126].

Primer Design

Polymerase chain reaction was performed using a specific primer pairs designed for KISS 1 gene. The primers was designed based on https://www.ncbi.nlm.nih.gov/ websites database, by using Primer-Blast program [127]. Table (2-8) illustrates the sequence of the primers (forward and reverse) used to amplify the KISS1 gene.

Table (2-10): Specific primers sequence for PCR of KISS1 gene.

Primers	Sequence	Length (bp)	Tm (˚C)	Product size
Forward primer	AGTTCCAGTTGTAGTTCGGCAG	22	57.06	300 bp
Reverse primer	CATCTTTCTGTGCCCTCTGTCCTA	24	58.95	

The preparation of stock primer and working solutions

The primers were shipped in a lyophilized state; the units of lyophilized primer are familiar as a mass in picomoles. The lyophilized forward primer and lyophilized reverse primer were dissolved and the following steps were used for reconstitution and dilution:

1. The tubes of lyophilized forward and reverse primers were centrifuged at 10000 x g for 5-10 min before opening the cap.
2. To prepare stock primer specific amount of nuclease free water (sterile dH_20) was added to the lyophilized forward and reverse primers according to the manufacture to obtain a 100 pmol/µl (Master stock).
3. The forward and reverse primers were re-suspended equally by vortex mixer.
4. To prepare working solution, in a 0.5 ml eppendorf tube, 10µl of each stock solution primer was diluted with 90 µl of nuclease free water (sterile dH_20) and then mixed by mini spin centrifuge.
5. The tubes of master stock and working solutions were kept at -20 ˚C until further use.

The solutions of GoTaq® G2 Green Master Mix

The solutions are premixed ready-to-use in the amplifications by PCR and that contain the following:

1. DNA Polymerase

2. Deoxy ribonucleotide triphosphate (dNTPs).

3. Magnesium chloride ($MgCl_2$)

4. Reaction buffers

At optimal concentrations and for efficient amplification of a wide range of DNA templates; the reactions assembled with GoTaq® G2 Green Master Mix have sufficient density for direct loading onto agarose gels. GoTaq® G2 DNA Polymerase exhibits (5′→3′) exonuclease activity [128].

The components of PCR reaction

To amplify the target gene KISS1; PCR technique was used with a specific primers (Forward and Reverse). The final PCR reactions volume was 25µl .The components of reaction was added in the PCR tubes and the volumes was completed to 25 µl by using deionized water under sterile conditions as shown in the table below.

Table (2-11): The components of PCR reaction for detection of KISS1 gene.

Component	Volume(µl)
Green Master Mix	13 µl
Forward	1.5 µl
Reverse	1.5 µl
Template DNA	4 µl
Deionized water	5 µl

Running Program of the PCR

Several experiments were made to optimize conditions for PCR reaction of KISS1 gene to detect about the best annealing temperature, numbers of amplification cycles and concentration of both primers and

DNA. The program of optimized PCR was illustrated in Table (2-10) and the total time for this program was (1h: 24min: 30sec)

Table (2-12): PCR program for detection of KISS1 gene

No.	Step	Temperature	Time	Cycle
1	Initial Denaturation	95 °C	2 min.	1
2	Denaturation	95 °C	30 sec.	31
3	Annealing	61.1 °C	30 sec.	
4	Extension	72 °C	35 sec.	
5	Final Extension	72 °C	5 min.	1
6	Hold Phase	12 °C	_	1

D- Agarose Gel Electrophorese

Agarose gel electrophoresis is a technique used to identify, separate and purify of DNA fragments based on their size (varying sizes ranging from 10 bp to 1000 bp), charge and shape [129]. The molecules travel through the gel in different directions or at different speeds, allowing them to be separated from one another. It is an efficient way to separate DNA fragments and to make sure of the presence of PCR amplification [130]. The type of agarose, presence of ethidium bromide in the gel and electrophoresis buffer, molecular size of the DNA, concentration of agarose, DNA conformation, and the applied voltage all these factors affecting on the migration rate of DNA during agarose gel electrophoresis [131].

Procedures

1- The Preparation of Tris Borate EDTA buffer

The contents of 1X TBE buffer (Tris borate EDTA) was:

- Tris base (108)g/l
- boric acid (55g/l)
- 0.5M EDTA (pH 8.0) 40 ml/l

And the percentage of 1X TBE buffer dilution was 1:10 (one volume of 10X TBE buffer with 9 volume of deionized water).

2- Preparation of the Agarose Gel and DNA Electrophoresis

1. To prepare (2%) agarose gel; 2g were weighted and added to 100 ml of 1X TBE solution in a beaker with gentle mixing .
2. The solution was heated in microwave with gentle stirring for mixing to avoid bubbles for several min until the solution became clear and pure.
3. The solution was left to cool down after that (3 µl) of Ethidium Bromide dye solution was added.
4. In the gel chamber, the comb was lay down about 1 inch from one ending of the tray for making wells, that was used for samples, then the agarose solution was poured gently into the tray and has been allowed to solidify at room temperature for 30 min approximately.
5. After the solution was solidified completely, the comb was removed gently from the gel tray and then (5µl) from the produced samples of PCR was directly loaded to each well in agarose gel except the last well which was loaded by (5µl) DNA ladder (100bp

plus marker) with great careful to avoid damages of the wells and cross contamination with neighboring wells.

6. The plates of gel tray were placed in a horizontal electrophoresis chamber filled with 1X TBE-buffer (the same solution that used for preparation of the gel agarose) that reached to flood the wells only.
7. The negative pole (cathode) was linked to the negative side of the unit and the positive pole (anode) to the positive side and Electrical power of the Electrophoresis system was turned on at 70 V, for 30-40 min that cause the traveling DNA from the negative pole (cathode) across the gel to positive pole (anode).
8. At the end of the electrophoresis run, the agarose gel was put in the UV transilluminator device and then was exposed to UV light to detect the bands, and then by using digital camera linked to PC the bands were visualized.

E- DNA sequencing

A 40 samples (volume of each sample was 20 µl) of PCR purified products for the KISS1 genes regions in patients and control groups (10 samples from each group) with the primers were sent to Macrogen corporation in Korea to determine the sequence of the nitrogen bases for KISS1 gene polymorphism, and it was read by using the SnapGene Viewer (3.5) software. Using the BLAST search tool, the nucleotide sequences were compared to information in the gene bank of the National Center for Biotechnology Information (NCBI) web site databases and SNPs were searched for.

2.4 Statistical analysis

All the data were analyze in Excel , the results were analyzed using PAST3 (Ver. 3) and arithmetic averages were measured on ANOVA Multidimensional scale, Tukey's post hoc analysis was used to determine significance values (P) ≤ 0.05 or ≤ 0.01. Correlation analysis was used to identify the linearly related values of both variables of PCOS with other parameters.

The frequencies of the KISS1 gene polymorphisms were expressed in numbers and percentages for homozygous wild, homozygous mutation and heterozygosity genotypes. χ^2 test was used to evaluate consistency of genotype distributions with PCOS.

Chapter Three

Results

&

Discussion

3. Results and Discussions

The result was represented as case-control study involving 105 women were 70 women have PCOS who attended Karbala Maternity and Pediatric Teaching Hospital, and 35 women were apparently healthy.

3.1. The distribution of study population according to demographic data.

The study groups were classified into four categories. The first group consisted of 35 women having PCOS who were diagnosed as overweight and obese. Thirty five normal weight women with PCOS made up the second group. The third group consisted of 35 apparently women who were fertile and had a regular menstrual cycle.

In women with PCOS, abdominal obesity can cause both local and systemic oxidative stress. The abdominal obesity increases the likelihood of PCOS-induced problems being more severe in the context of abdominal obesity. The increase of visceral fat appears to be a significant factor in the development of PCOS [132] .

Table (3-1): The distribution according to demographic data.

Characteristics	PCOS Obese	PCOS Overweight	PCOS Normal weight	control
Number	16	19	35	35
Age (years)	27 ±4.73	24.21 ±4.4	24.7 ±4.12	24.91 ±3.33
BMI (kg/m^2)	33.9 ±3.09	27.46 ±1.55	23.30 ±1.96	22.79 ±2.38
Residency/ Kerbala (number: percentage):				
Inside	12: (75%)	16: (84.21%)	24: (68.57%)	26: (74.28 %)
Outside	4: (18.75%)	3: (15.78%)	11: (31.42%)	9: (25.71%)
Occupation (number: percentage):				
Employed	3: (18.75%)	4: (21.05%)	3: (8.57%)	23: (65.71 %)
Student	4: (25%)	2: (10.52%)	10: (28.57%)	7: (20%)
House wife	9: (56.25%)	13: (68.42%)	22: (62.85%)	5: (14.28%)

The origin of PCOS begins in the birth and continues throughout the lifecycle, and environmental insults and lifestyle issues may affect vulnerable women, resulting in the occurrence of PCOS phenotypic characteristics. Diet appears to be one of the most important environmental determinants of PCOS occurrence [133].

Table (3-2): The distribution according to demographic data.

Characteristics	PCOS Obese (Number: percentage)	PCOS Overweight (Number: percentage)	PCOS Normal weight (Number: percentage)	control (Number: percentage)
Economic status				
High	2: (12.5%)	3: (15.78%)	4: (11.24 %)	3: (8.57 %)
Moderate	13: (81.85%)	16: (84.21%)	30: (85.71%)	32: (91.42%)
Low	1: (6.25%)	0	1 : (2.85 %)	0
Social status				
Married	15: (93.75%)	17: (89.47%)	25: (71.42 %)	20: (57.14%)
Unmarried	1: (6.25%)	2: (10.52%)	10: (28.57%)	15: (42.85 %)
Education				
Illiterate	0	1: (5.26%)	3: (8.57 %)	0
Primary	3: (18.75 %)	3: (15.78%)	4: (11.24 %)	1: (2.85 %)
secondary	6: (37.5%)	10: (52.63%)	12: (34.28%)	1: (2.85 %)
University	7: (43.75%)	5: (26.31%)	16: (45.71 %)	33: (94.82%)

Some lifestyle, occupational, and environmental factors may increase the development of PCOS or exacerbate the incidence and/or phenotypic indications of PCOS; however the cause-effect relationship between these factors and PCOS is currently unclear or inadequate. There are also insufficient or inconsistent studies on the effects of environmental, occupational, and lifestyle factors on PCOS. Furthermore, women are accidentally exposed to a variety of chemicals during their daily

activities, some of which may have estrogenic or anti-estrogenic, androgenic or anti-androgenic effects [134, 135] .

Result found the most obese, overweight and normal weight women with PCOS had finished secondary school, it was observed to be 6(37.5%), 10 (52.63%) and 12(34.28%) respectively. Women with little or no formal education and a history of menstrual abnormalities were the most likely to report female sexual dysfunction, according to the data. More research in women with PCOS is needed to educated about their health care by professionals and develop therapeutic approaches [136] .

Table (3-3): The distribution of demographic data.

Characteristics	PCOS (Obese) (Number: percentage)	PCOS (Overweight) (Number: percentage)	PCOS (Normal weight) (Number: percentage)	control (Number: percentage)
Infertility Type				
Primary	11: (68.75%)	17: (89.47%)	25: (71.42%)	-
Secondary	5: (31.25%)	2: (10.52%)	10: (28.57%)	
PCOS Duration				
≥ 1 year	7: (43.75%)	9: (47.36%)	13: (37.14%)	-
< 1year	9: (56.25%)	10: (52.63%)	22: (62.85%)	
Hirsutism				
Positive	10: (62.5%)	12: (63.15%)	26: (74.28 %)	2: (5.71%)
Negative	6: (37.5%)	7: (36.84%)	9: (25.71 %)	33: (94.28 %)
Acne				
Positive	4: (25%)	16: (84.12%)	16: (45.71 %)	1: (2.85 %)
Negative	12: (75%)	3: (15.78%)	19: (54.28%)	34: (97.14 %)
Hair loss				
Positive	12: (75%)	17: (89.47%)	31: (88.57%)	9: (25.71 %)
Negative	4: (25%)	2: (10.52%)	4: (11.24 %)	26: (74.28%)
Menstrual cycle				
Regular	6: (37.5%)	6: (31.57%)	5: (14.28%)	-
Irregular	10: (62.5%)	13: (68.42%)	30: (85.71%)	

There are two types of infertility: primary and secondary infertility. Primary infertility refers to couples who have not delivered after at least one year of sexual activity without using contraception. Secondary

infertility is a term that refers to couples who have been able to have children at least once but are now unable to do that. The result did not found any significant differences between women who have type 1infertility and women who have type 2 infertility. In most women with PCOS, ovulation issues are the primary cause of infertility and the ovulation may not occur as a result of an increase in testosterone production or as a result of immature follicles on the ovaries [137] .

Non-significant differences were found between PCOS duration more than one year or less than one year. A study refers to PCOS, which is typically defined as a cycle length greater than 35 days, affects approximately 85-90 percent of women who have oligomenorrhea [138] . The diagnostic criteria for PCOS, on the other hand, have changed over time. Currently, there are three clinical definitions of PCOS that are overlapping but not entirely consistent with one another. The irregularity and length of the menstrual cycle are characteristics of PCOS that are included in all three PCOS definitions [139].

Hirsutism, which is characterized by excessive hair growth in women, was shown to have the greatest impact on patients' quality of life , and it is generally characterized by the presence of terminal hair like that in male distribution, in women with PCOS being the most common etiology of hirsutism [140] .

Acne is the most common clinical manifestation of hyperandrogenism in PCOS women, according to researchers. The prevalence of acne in PCOS women ranges between 17 % and 83 %, with the median being 17 %. Acne is another indication of hyperandrogenism that is frequent. As a result, an adolescent female with moderate to severe acne should be evaluated for PCOS, according to the guidelines. Furthermore, the progression or persistence of acne into adulthood is

exceptional, and it should be brought to the attention of the public. However, it has also been demonstrated that hyperandrogenism may be associated with hypermasculinity [141] .

Through observation about hair loss in Table (3-3), found that most cases of women were of the type polycystic ovary with normal weight, where the current study recorded that 31 (88.57%) women suffer from baldness followed by 17(89.47%) and 12(75%) women of type PCOS with overweight and obese respectively, the study found only nine women (25.71%) with baldness from the control group.

Hair loss is one of the symptoms of PCOS that has been reported. People with PCOS frequently experience acne, thinning hair, and hair loss, as well as limp, lackluster hair that break easily and are dry and damaged. This is primarily due to the high levels of androgenic hormones present in the body of these individuals. Androgenic alopecia, also known as male pattern baldness, can be caused by a hormonal imbalance and manifest itself as the following characteristics: The frontal and parietal (side) areas of the scalp are the most commonly affected by hair loss. As a result, the hair in the parting area becomes noticeably thinner [142].

The hairline starts to recede a little bit, hair loss caused by PCOS can occur as root loss, where the entire hair including the follicle falls out, or as breakage because the hair is drier and more prone to damage from heat and brushing. This means that the scalp, particularly at the crown and hairline, may be more visible, or that there may be more frizz as a result of hair breakage [143].

3.2. The differences between biochemical parameters

The results of Kisspeptin1showed significant difference (P< 0.01) among PCOS for obese, overweight and normal weight against control group, as shown Table (3-4). On the other hand, our results related to LH levels were significantly (P< 0.05) higher among PCOS women more than control group. Kisspeptin is a neuropeptide that increases the release of the GnRH. It is required for the production of LH and the occurrence of ovulation. Women who have PCOS have altered amounts of the hormones GnRH and LH secretion, respectively [144] .

Table (3-4): The differences (Mean ± SD) of KISS1and hormonal disturbance in women with PCOS and control group.

Parameters	PCOS (Obese)	PCOS (Overweight)	PCOS (Normal weight)	Control	P value
Kisspeptin1 (ng/L)	396.32 ±137.26	419.16 ±149.66	370.85 ±86.25	323.60 ±52.25	< 0.01
LH (mIU/ml)	10.89 ±6.19	10.48 ±5.24	6.94 ±1.32	5.08 ±1.23	< 0.01
FSH (mIU/ml)	6.30 ±2.47	5.32 ±1.66	4.89 ±0.99	6.92 ±1.15	< 0.01
LH/FSH Ratio	1.68 ±0.33	1.94 ±0.68	1.419 ±0.488	0.735 ±0.134	< 0.01
Free Testosterone (ng/ml)	0.68 ±0.40	0.79 ±0.53	0.927 ±0.379	0.382 ±0.09	> 0.05
Prolactin (ng/ml)	22.36 ±4.70	19.40 ±8.9	22.854 ±9.919	17.594 ±3.481	> 0.05

The PCOS is the most common cause of ovulatory subfertility. Women who have this syndrome also have irregular menstrual cycles, excessive hair growth, acne, and are overweight. Women of normal weight, on the other hand, are more likely to be affected. LH levels are frequently elevated in PCOS women. The FSH level is usually in the lower range. A decrease in FSH levels of overweight and normal weight women with PCOS (5.32 ±1.66) and (4.89 ±0.99) when compared with that of control group (6.92 ±1.15). This could be connected to a decrease in the sensitivity of the GnRH pulse generator to steroid feedbacks as well as an increase in LH secretion, as previously studies [145] .

A significant difference in serum Testosterone levels and prolactin was observed between overweight/obese group and normal weight PCOS patients. Furthermore, a significant difference between overweight/obese and non- obese healthy women was obtained. On the other hand, their levels in normal weight PCOS patients were higher than those in obese PCOS women.

Some of studies demonstrated that anovulation with decrease in LH secretion were proposed as a result of reduced KISS 1 expression in testosterone-treated rats, however, rats treated with dihydrotestosterone had normal levels of the hormone LH [146].

In agreement with results, increase serum Kisspeptin level in PCOS patients were observed in several studies [103, 113, 147]. While , other studies didn’t find this variation [104, 115].

Bacopoulou 2017 stated that the increased serum kisspeptin level in patients with ovarian disorder may be due to strong output from hypothalamus in order to maintain menstruation [109].

Kisspeptin within the ovary has critical transient and spatial specificity, recommending that the kisspeptin/ KISS1R system performs different functions at multiplet physiological stages in the ovary [148]. Animal model with PCOS expressed high level of Kisspeptin m RNA in the ovarian tissue [149] . On the other hand, there is evidence of direct kisspeptin action on the ovary. In fact, there are kisspeptin receptors in the luteal granulosa cells of patients under ovarian stimulation protocol [150] ,Where the use of kisspeptin-54 as an oocyte maturation trigger augmented expression of genes involved in ovarian steroidogenesis in human GL cells including, FSH receptor (FSHR), LH/hCG receptor (LHCGR), steroid acute regulatory protein (STAR), aromatase, estrogen receptors alpha and beta (ESR1, ESR2), 3-beta-hydroxysteroid dehydrogenase type 2 (3BHSD2) and inhibin A (INHBA) [151] . Ovarian kisspeptin may also participate in PCOS pathogenesis but it's still unclear if it affects circulating Kisspeptin or not.

Koladzeijski 2018 [152] found that level of Kisspeptin was significantly higher in normal- weight women as compared to obese women; they also demonstrated that there were negative correlations between Kisspeptin & BMI.

3.3. The correlation between hormones levels in PCOS and control groups using linear regression (Pearson correlation) analysis

The correlation analysis of hormones levels and KISS1 is shown in Table (3-5) and (3-6). There were correlations between FSH and LH (high increase in FSH with high increase in LH) in the obese and overweight women with PCOS, where a positive significant correlation in PCOS patients (r=0.950) and (0.725) respectively was found.

Table (3-5): The correlation coefficients between kisspeptin and hormones levels in the obese women with PCOS.

	LH	FSH	F-Testosterone	Prolactin	Kisspeptin
Kisspeptin	0.12	0.17	-0.35	-0.25	1
Prolactin	0.23	0.21	0.05	1	
F-Testosterone	-0.13	0.03	1		
FSH	0.95	1			
LH	1				

Table (3-6): The correlation coefficients between kisspeptin and hormones levels in the overweight women with PCOS.

	LH	FSH	F-Testosterone	Prolactin	Kisspeptin
Kisspeptin	-0.23	-0.23	-0.02	0.23	1
Prolactin	-0.23	-0.18	-0.06	1	
F-Testosterone	-0.002	-0.10	1		
FSH	0.72	1			
LH	1				

No significant correlations were found in all hormones concentration of obese and overweight women with PCOS, the positive correlation between FSH and LH revealed to hyperandrogenism in PCOS patients and could be a useful marker for predicting PCOS risk [153]. A study revere to the PCOS patients with higher LH/FSH ratio are primarily caused by hypothalamic-pituitary dysfunction, while the PCOS patients with normal LH/FSH ratio are mainly caused by metabolic disorders [154]. We don't found any significant correlation in all hormones concentration in serum of women have PCOS with normal weight and control.

Table (3-7): The correlation coefficients between Kispeptin1 and hormones levels in the normal weight with PCOS.

	LH	FSH	F-Testosterone	Prolactin	Kisspeptin
Kisspeptin	-0.08	-0.16	-0.07	-0.007	1
Prolactin	-0.35	0.21	-0.02	1	
F-Testosterone	-0.14	-0.09	1		
FSH	0.33	1			
LH	1				

The reason for the absence of significant differences may be the size of the sample used, where the sample size for the groups was (16, 19, 35) and here is a relationship that occurs with women. It is worth noting that most cases of polycystic ovaries have overweight status.

Table (3-8): The correlation coefficients between kisspeptin1 and hormones levels in the control group.

	LH	FSH	F-Testosterone	Prolactin	Kisspeptin
Kisspeptin	0.23	0.35	0.48	0.01	1
Prolactin	0.05	-0.10	0.01	1	
F-Testosterone	0.23	0.30	1		
FSH	0.55	1			
LH	1				

3.4. Distribution of Kispepetin1 levels according to age among patients groups

Table (3-9) shows the distribution of Kisspeptin 1 level of the study samples among obese, overweight and normal weight women with PCOS respectively, according to age. There is no significant difference in KISS 1 levels between three age groups.

Table (3-9): Distribution of serum Kispeptin1 levels according to age in the obese, overweight and normal weight women with PCOS.

Patients groups	Age groups (years)	Number	Kisspeptin ng/L (Mean ± SD)	P value
Obese PCOS	18-23	5	453.968 ±171.049	> 0.05
	24-29	7	438.761 ±134.728	
	30-35	4	286.418 ±75.467	
Overweight PCOS	18-23	10	412.410 ±151.881	> 0.05
	24-29	7	381.405 ±77.795	
	30-35	2	535.123 ±235.43	
Normal weight PCOS	18-23	14	387.218 ±104.274	> 0.05
	24-29	16	346.998 ±55.782	
	30-35	5	377.253 ±88.945	

3.5 Polymorphism Genotyping

3.5.1 Estimation of DNA concentration and purity

DNA concentration and purity were presented in Table (3-10). DNA samples were found to be pure and the DNA concentrations ranged from 20- 98 µg/ml.

Table (3-10): The Purity and concentration of the extracted DNA.

	Mean ± SD
DNA concentration (µg/ml)	74.43± 13.18
DNA purity	1.94 ± 0.42

3.5.2 Results of amplification reactions

The amplification of KISS 1 gene showed an amplicon of 300 bp as illustrated in Figure (3-1).

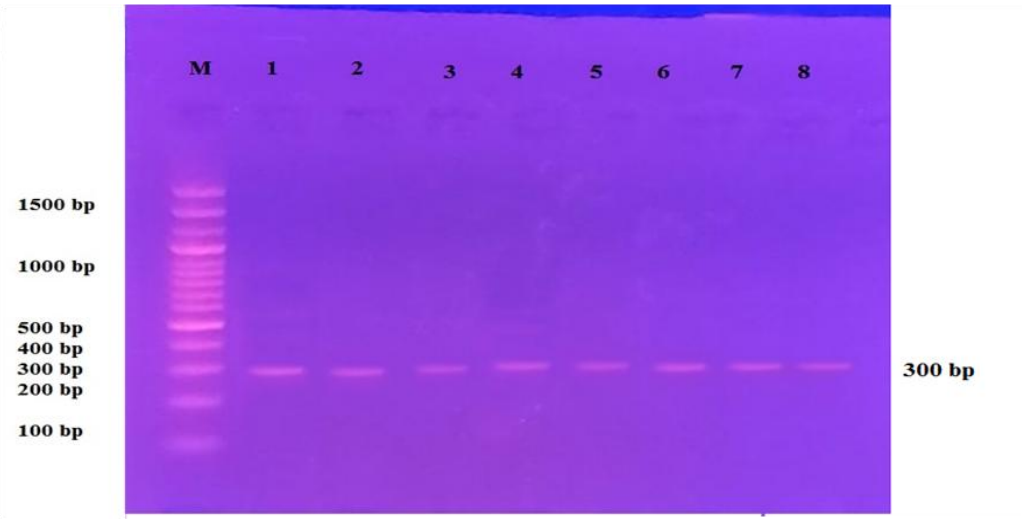


Figure (3-1): The electrophotography of SNP of the KISS1 gene from PCR product loaded in each well on 2% agarose gel electrophoresis, 70 V, 120 minute and 5 µL. Lanes 1- 8: PCR product (one band 300 bp), lane M: DNA Ladder (100- 1500 bp).

3.5.3 DNA Sequencing of KISS1 gene polymorphism

The molecular detection was carried out by using the technology of DNA sequencing, where the sequence of nitrogen bases was determined in *Kiss-1* polymorphism region, that amplified by PCR technique. The results were obtained from Macrogen Company/ Korea (Macrogen Inc. Geumchen, Seoul, South Korea) and analyzed using the SnapGene Viewer software, as illustrated in Fig 3-2 and 3-3.

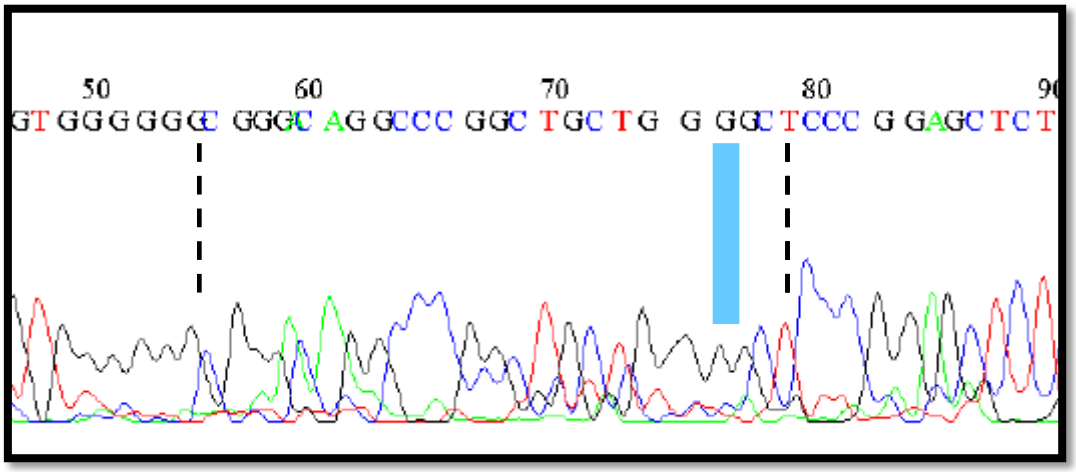


Figure (3-2): The result reading of KISS1 gene polymorphism of the sample of control group by sequencing chromatograms.

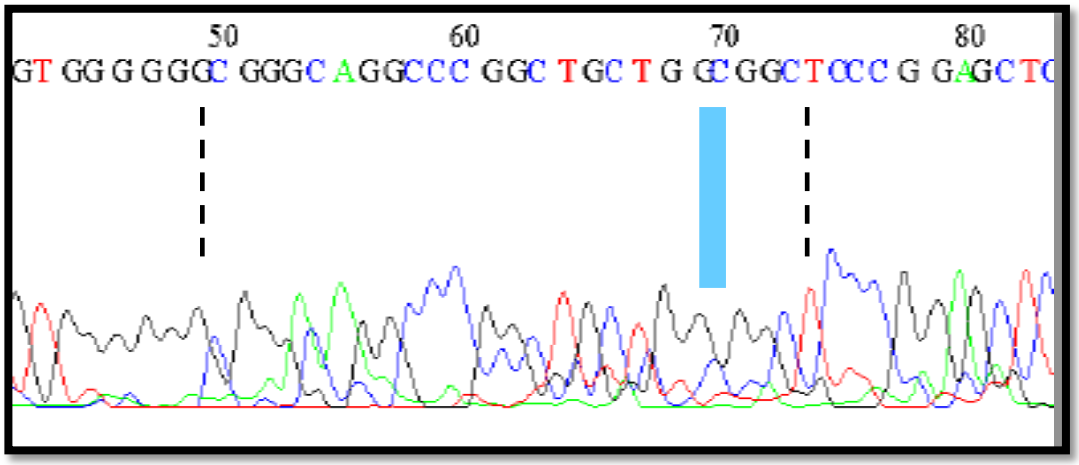
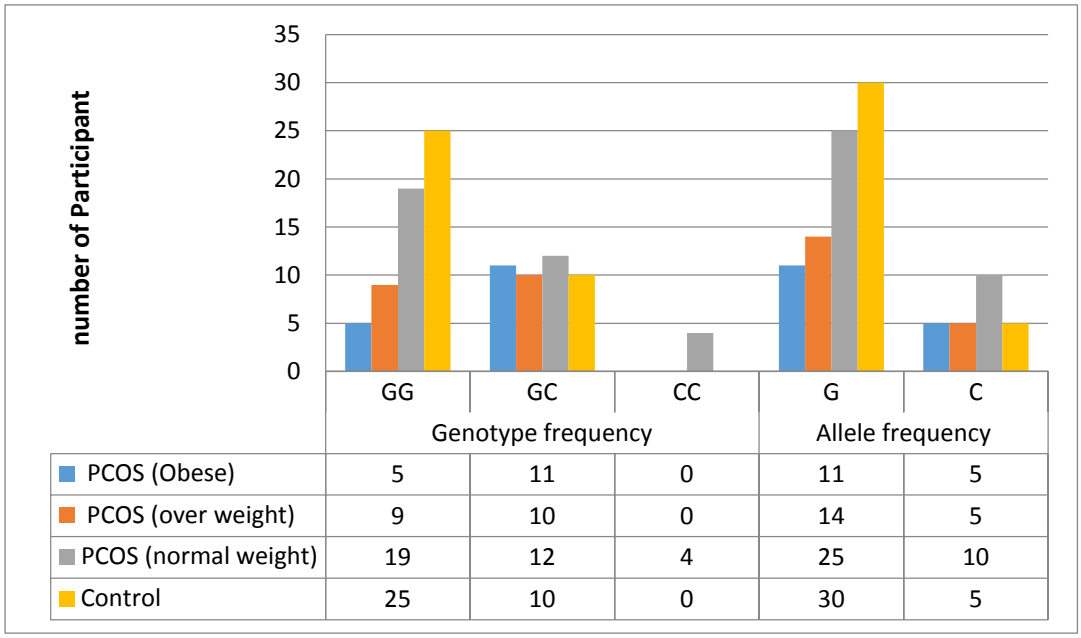


Figure (3-3): The result reading of KISS1 gene polymorphism of the sample of PCOS patient by sequencing chromatograms

3.5.4 Genotype and allelic frequency

The non-synonymous SNP rs4889 (C/G) was found more frequently in the obese women with PCOS than in the overweight and normal weight women with PCOS and control groups, and this SNP causes P81R substitution where a substitution of proline for arginine at the position 81. Figure (3-4) summarizes the allele and genotype rates in PCOS and control groups, as well as the distribution of rs4889 C/G in the KISS 1 gene



	GG	GC	CC	G	C
PCOS (Obese)	5	11	0	11	5
PCOS (over weight)	9	10	0	14	5
PCOS (normal weight)	19	12	4	25	10
Control	25	10	0	30	5

Figure (3-4): Genotype and allele frequency among study groups.

Allele C showed higher frequency in obese women with PCOS (31.2%) than overweight and normal weight women with PCOS (26.3%, 28.5%) respectively, and control group (14.2%).

Genotype counts and frequencies in obese woman with PCOS and control groups are shown in Table (3-11). The frequencies of the

identified polymorphisms of PCOS patients were significantly different in comparison with controls. The frequency of heterozygous allele (GC) was higher in PCOS than in controls, while the homozygous (CC) allele was not present in both groups. In this study, the allelic frequency of SNP rs4889 G/C was detected more frequently with non-significant association in obese women with PCOS than in control with odd ratio 2.72 and confidence interval (0.65-11.2) (P value = 0.25).

Table (3-11): Genotypes and allelic distribution in obese with PCOS and controls groups.

		Patients with PCOS (Obese)	**Control group**	**Odds Ratio**	**CI 95 %**	***p*-value**
Genotyping	**GG**	5	25	**Reference**	-	-
	GC	11	10	5.5	(1.51-19.91)	0.01
	CC	0	0	5	(0.08-282.9)	1
Allele frequency	**G (%)**	11 (68.7)	30 (85.7)	**Reference**	-	-
	C (%)	5 (31.2)	5 (14.2)	2.72	(0.65-11.2)	0.25

Genotype counts and frequencies in overweight weight with PCOS and control groups are shown in Table (3-12). The frequencies of the identified polymorphisms were non-significantly different between cases and controls. The frequency of heterozygous allele (GC) was 10 in cases

and controls, while the homozygous (CC) allele was not present in both groups. In this study, the allelic frequency of SNP rs4889 G/C was detected more frequently with non-significant association in overweight weight with PCOS than in control with odd ratio 2.14 and confidence interval (0.53-8.62) (P value = 0.46).

Table (3-12): Genotypes and allelic distribution in overweight with PCOS and controls groups.

		Patients with PCOS (Over weight)	Control group	Odds Ratio	CI 95 %	*p*-value
Genotyping	**GG**	9	25	**Reference**	-	-
	GC	10	10	2.77	(0.86-8.87)	0.13
	CC	0	0	2.77	(0.05-150.65)	1
Allele frequency	**G (%)**	14 (73.6)	30 (85.7)	**Reference**	-	-
	C (%)	5 (26.3)	5 (14.2)	2.14	(0.53-8.62)	0.46

Genotype counts and frequencies in PCOS normal weight and control groups are shown in Table (3-13). The frequencies of the identified polymorphisms were significantly different between cases and controls. The frequency of heterozygous allele (GC) was 12 in cases higher than in controls 10 while the homozygous (CC) allele was present only in PCOS

with normal weight. In this study, the allelic frequency of SNP rs4889 G/C was detected more frequently with non-significant association in PCOS normal weight than in control with odd ratio 2.4 and confidence interval (0.72-7.94), (P value = 0.24).

Table (3-13): Genotypes and allelic distribution in PCOS normal weight and controls groups.

		Patients with PCOS (normal weight)	Control	Odds Ratio	CI 95 %	p-value
Genotyping	GG	19	25	Reference	-	
	GC	12	10	1.57	(0.56-4.42)	0.43
	CC	4	0	10.52	(0.52-211.4)	0.15
Allele frequency	G (%)	25 (71.4)	30 (85.7)	Reference	-	
	C (%)	10 (28.5)	5 (14.2)	2.4	(0.72-7.94)	0.24

In KISS 1 gene polymorphisms rs4889, Table (3-14) showed the homozygous genotypes (CC) was present just in the normal weight women with PCOS, and not available in the obese and overweight women with PCOS, and it is also less than those of their wild type (GG) and heterozygous genotypes in both cases, on the other hand our result

found non-significant differences between normal weight women with PCOS and overweight among genotype and allelic frequency.

Table (3-14): Genotypes and allelic distribution in overweight women and normal weight with PCOS groups.

		Patients with PCOS (Normal weight)	**Patients with PCOS (Over weight)**	**Odds Ratio**	**CI 95 %**	***p*-value**
Genotyping	**GG**	19	9	**Reference**	-	-
	GC	12	10	0.56	(0.17-1.80)	0.38
	CC	4	0	3.78	(0.18-79.62)	0.52
Allele frequency	**G (%)**	25 (71.4)	14 (73.6)	**Reference**	-	-
	C (%)	10 (28.5)	5 (26.3)	1.12	(0.31-3.93)	1

Through our current study, in Tables (3-11), (3-12),(3-13) and (3-14) we did not notice significant differences between the allele frequencies of patients and the control group, this result was in agreement with Branavan, *et al*., 2019 [99] who found the frequencies of the identified polymorphisms were not significantly different between cases and controls.

The KISS1 gene plays a critical role in the regulation of the hypothalamic-pituitary-gonadal axis, so, numerous studies have looked into polymorphisms or haplotypes in the gene, particularly in the context of central precocious puberty [155] .

3.5.4.1. Relationships between clinical traits with genotype among PCOS obese women

Genotype frequency among KISS 1 gene polymorphisms were shown to be associated with a variety of clinical trait, as illustrated in Table (3-15), due to the absence of a type allele CC among obese woman with PCOS, we cannot associate the homozygote mutant allele with the clinical characteristics of the patients. The result showed the most cases of Hirsutism in the group having heterozygous allele GC, among 16 obese women have PCOS, 7 (43.75%) and 3 (18.75%) have GC and GG respectively, and 4 (25%) and 2 (12.5%) they were negative for GC and GG genotype, respectively.

Table (3-15): Relationships between clinical traits with genotype among PCOS obese women.

Clinical parameters	Genotyping frequency			X^2	P value
	GG	GC	CC		
No. of patient	5	11	0	-	-
Hirsutism					
Positive: (%)	3: (18.75%)	7: (43.75%)	0	0.01	0.99
Negative: (%)	2: (12.5%)	4: (25%)	0		
Acne					
Positive: (%)	1: (6.25%)	3: (18.75%)	0	0.09	0.95
Negative: (%)	4: (25%)	8: (50%)	0		
Hair Loss					
Positive: (%)	5: (31.25%)	7: (43.75%)	0	2.42	0.29
Negative: (%)	0	4: (25%)	0		
Menstrual cycle					
Regular: (%)	3: (18.75%)	3: (18.75%)	0	1.57	0.45
Irregular: (%)	2: (12.5%)	8: (50%)	0		

This result was in agreement with Eriksen, *et al*.,2012 [156] who found none of the SNPs were associated with hirsutism or increased metabolic parameters. Hirsutism is excessive body hair on parts of the body where hair is normally absent or minimal, it may refer to a "male" pattern of hair growth that may be a sign of a more serious medical condition, especially if it develops well after puberty [157].

The result shown in Table (3-15) that cases of acne in the group have heterozygous allele GC, among 16 obese women with PCOS, 3 (18.75%), and 1(6.25%) have GC and GG respectively with no significant

difference (P> 0.05), otherwise, 8 (50%) and 4 (25%) they are negatives for acne in the GC and GG genotype, respectively.

The second most prevalent symptom of hyperandrogenism is acne. Acne prevalence varies by ethnicity; Indo-Asian women have the highest reported incidence, while Pacific Islanders have the lowest. The prevalence of acne in Kashmiri PCOS women is 48% [158]. In Allahabad, the prevalence of acne was observed to be 17.7% [159] . Other studies have estimated the frequency of acne in PCOS patients to be between 9.8% and 34% [160] .

The inflammation of the pilosebaceous glands causes acne. Increased testosterone encourages the synthesis of the more potent dihydrotestosterone, which boosts sebum production in the sebaceous glands and promotes aberrant desquamation in the follicular epithelial cells. The bacterium *Propioni bacterium* acnes colonizes this buildup of sebum and epithelial cell debris, causing acne [161].

The result showed that most cases of hair loss in the group have heterozygous allele (GC), among 16 obese women with PCOS, 7 (43.75%) and 5 (31.25%) get hair loss in the women that have GC and GG allele respectively, while, 4 (24%) were negatives for GC genotype.

Another sign of PCOS women's hyper androgenic condition is androgenic alopecia, or hair loss. It appears to be widespread in people with PCOS, with rates ranging from 3.2 to 34.8 percent in different

groups [162]. Miniaturization is a condition in which mature terminal hair on the scalp shortens its anagen (growth) phase and gradually transforms into fewer, finer vellus hair. On the one hand, women with PCOS struggle with excessive facial hair development; while on the other hand, they struggle with thinning scalp hair. This is due to the fact that women with PCOS have high amounts of testosterone, which causes hair loss in the same way that it occurs in men. However, in PCOS women with androgenic alopecia, the hair follicle remains alive; increasing the likelihood that the hair lost will regrow with hair therapy [163].

We did not notice significant differences (P> 0.05) in the regularity of the menstrual cycle for obese women with PCOS. The menstrual cycle features are influenced by a variety of factors, including body size, smoking, alcohol consumption, and physical activity, as well as pathologic disorders such as PCOS. PCOS affects 85-90 percent of women with oligomenorrhea, which is characterized as a cycle length of more than 35 days [138].

3.5.4.2. Relationships between clinical trait with genotype among PCOS overweight women

The results revealed no significant differences (P>0.05) in the most cases of hirsutism in the group that have homozygous allele (GG), among 19 women overweight have PCOS, 6 (31.57%) and 6 (31.57%) get hirsutism for women who have GG and GC respectively, on the other hand, 3 (15.78%) and 4 (31.05%) were negatives for GG and GC genotype, respectively as shown in Table (3-16).

Table (3-16): Relationships between clinical traits with genotype among PCOS overweight women.

Clinical parameters	Genotyping frequency			X^2	P value
	GG	GC	CC		
No. of patient	9	10	0	-	-
Hirsutism					
Positive: (%)	6: (31.57%)	6: (31.57%)	0	0.09	0.95
Negative: (%)	3: (15.78%)	4: (31.05%)	0		
Acne					
Positive: (%)	8: (42.10%)	8: (42.10%)	0	0.28	0.86
Negative: (%)	1: (5.26%)	2: (10.52%)	0		
Hair Losse					
Positive: (%)	7: (36.84%)	10: (52.63%)	0	2.48	2.88
Negative: (%)	2: (10.52%)	0	0		
Menstrual cycle					
Regular: (%)	4: (31.05%)	2: (10.52%)	0	1.31	0.51
Irregular: (%)	5: (26.31%)	8: (42.10%)	0		

The results reveal no significant differences (P>0.05) in the most cases of acne in the group that have homozygous allele (GG), among 19 overweight women with PCOS, 8 (42.10%), 8 (42.10%) get acne in their faces for women that have GG and GC respectively, on the other hand 1 (5.26%) and 2 (10.52%) were negatives for GG and GC, respectively.

Our study did not find significant differences (P> 0.05) in the hair loss and menstrual cycle in the overweight women with PCOS, the results of the study showed that the most common allele and genotype frequency was GC genotype in the two clinical trait, where it was 10: (52.63%) and

7: (36.84%) get hair loss in woman that have GC and CC respectively, while, 2 (10.52%) are negative for GG genotype.

3.5.4.3. Relationships between clinical traits with genotype among PCOS normal weight women

The results revealed no significant differences (P>0.05) in the most cases of hirsutism in the group that have homozygous allele (GG), among 35 women normal weight have PCOS, 16 (45.71%), 8 (22.85%) and 2 (5.71%) get hirsutism for women who have GG and GC and CC respectively, on the other hand, 3 (8.57%) , 4 (11.42%) and 2 (5.71%) were negatives for GG , GC and CC genotype, respectively as shown in Table (3-17).

Table (3-17): Relationships between clinical trait with genotype and allele frequency among PCOS normal weight women.

Clinical parameters	Genotyping frequency			X^2	P value
	GG	GC	CC		
No. of patient	19	12	4	-	-
Hirsutism Positive: (%) Negative: (%)	 16: (45.71%) 3: (8.57%)	 8: (22.85%) 4: (11.42%)	 2: (5.71%) 2: (5.71%)	2.57	0.27
Acne Positive: (%) Negative: (%)	 9: (25.71%) 10: (2857%)	 5: (14.28%) 7: (20%)	 2: (5.71%) 2: (5.71%)	0.13	0.93
Hair Loss Positive: (%) Negative: (%)	 15: (42.85%) 4: (11.42%)	 12: (34.28%) 0	 4: (11.42%) 0	3.80	0.14
Menstrual cycle Regular: (%) Irregular: (%)	 2: (5.71%) 17: (48.57%)	 10: (28.57%) 2: (5.71%)	 1: (2.85%) 3: (8.57%)	16.98	< 0.01

The results reveal no significant differences (P>0.05) in the most cases of acne in the group that have homozygous allele (GG), among 35 normal weight women with PCOS, 9 (25.71%), 5 (14.28%) and 2 (5.71%) get acne in their faces for women that have GG, GC, and CC respectively, on the other hand 10 (28.57%), 7 (20%) and 2 (5.71%) were negatives for GG, GC, and CC, respectively.

Our study did not find significant differences (P> 0.05) in the hair loss and menstrual cycle in the normal weight women with PCOS, the results of the study showed that the most common allele and genotype frequency was GG genotype in the two clinical trait, where it was recorded 15: (42.85%) and 4: (11.42%), respectively, followed by GC allele it was recorded as 12 (34.28%) finally, among CC allele it was found 4: (11.42%).

3.5.4.4. The statistical differences between biochemical parameters, and genotype of obese women with PCOS.

We note through our current study that there were no significant differences (P>0.05) between the homogenous allele and the heterozygous allele when measuring the levels of hormones LH and FSH in the blood of women with PCOS, Table (3-18).

Ovulation is aided by the hormones LH and FSH. The pituitary gland in the brain produces both LH and FSH. The hormones levels typically range from 5- 20 mlU/ml at the start of the cycle during the early part of their cycle and most women have roughly equal quantities of LH and FSH. However, there is an LH surge 24 hours before ovulation, when the amount of LH rises to around 25-40 mlU/ml; LH levels return to normal after the ovary releases the egg [164]. In the pathogenesis of polycystic ovarian disease, abnormality of the hypothalamic-pituitary-ovarian or adrenal axis has been suggested. The gonadotrophin-releasing hormone (GnRH) secretion pattern is disrupted, resulting in a relative increase in LH to FSH release [165] . Ovarian estrogen is to blame for an aberrant feedback process that resulted in an increase in LH production [166]. The ratio between LH and FSH in healthy women is normally

between 1 and 2. This ratio is inverted in women with polycystic ovarian disorder, and it can reach as high as 2 or 3 in some cases [167], there is an increase in the proportion of the FSH/LH ratio hormone with significant differences (P<0.05) in women who carry the homozygous allele compared to women who carry the heterozygous allele, Table (3-18).

Table (3-18): The differences in biochemical parameters according to GG and GC genotype in the obese women with PCOS.

Biochemical parameters	Genotyping frequency		P value
	GG	GC	
LH (mIU/ml) Mean ± SD	8.22 ±2.91	10.29 ±2.15	0.13
FSH (mIU/ml) Mean ± SD	5.68 ±0.91	5.78 ±1.32	0.89
LH/FSH ratio Mean ± SD	1.25 ±0.16	1.79 ±0.19	< 0.01
Free Testosterone (ng/ml) Mean ± SD	0.87 ±0.48	0.59 ±0.36	0.22
Prolactin (ng/ml) Mean ± SD	20.96 ±5.24	23 ±4.55	0.43
Kisspeptin1 (ng/L) Mean ± SD	450.206 ±119.478	371.827 ±142.972	0.30

Serum testosterone level was higher but non-significant in the GG genotype compared with the GC genotype (0.87 vs. 0.59 ng/ml; P=0.22). Women who have PCOS frequently have elevated levels of both total testosterone and free testosterone in their bodies. Furthermore, even a

modest rise in testosterone levels in a woman's body can interfere with her ability to have a normal menstrual cycle and ovulation.

High prolactin levels were found in 16 obese women with PCOS. Of 16 patients; high prolactin level (23) was in the GG allele rather than GC allele which had (20.96) prolactin levels. Prolactin levels in women with PCOS are typically normal, with levels less than 25 ng/ml in the majority of cases. However, it is necessary to check for high prolactin levels in order to rule out other disorders, such as a pituitary tumor, that could be causing the symptoms of PCOS.

The KISS1 gene encodes a hypothalamic peptide initially identified from the human placenta. Kisspeptin has been implicated in the regulation of the HPG axis since its discovery. Some investigations found that administering kisspeptin lead to increased LH levels while decreasing or eliminating FSH levels in PCOS [168].

3.5.4.5 The statistical differences between biochemical parameters and genotype in overweight women with PCOS

The two genotypes had comparable serum FSH level, prolactin level, free testosterone, and Kisspeptin level. However, patients with overweight women with PCOS showed no significance in levels of all hormones, except the LH was higher in GC genotype compared with GG genotype (13.4 ±5.05 and 7.22 ±3.23 ng/ml, $P<0.01$) and LH/FSH ratio was (2.27 ±0.59 and 1.58 ±0.62 ng/ml, $P=0.01$) in the GC and GG genotype respectively. The comparison between the two groups in terms of biochemical analysis results is shown in Table (3-19).

Table (3-19): Differences between biochemical parameters and genotype of the overweight women with PCOS.

Biochemical parameters	Genotyping frequency		P value
	GG	GC	
LH (mIU/ml) Mean ± SD	7.22 ±3.23	13.41 ±5.05	< 0.01
FSH (mIU/ml) Mean ± SD	4.63 ±1.06	5.94 ±1.90	0.08
LH/FSH ratio Mean ± SD	1.58 ±0.62	2.27 ±0.59	0.01
Free Testosterone (ng/ml) Mean ± SD	0.80 ±0.53	0.79 ±0.56	0.94
Prolactin (ng/ml) Mean ± SD	18.86 ±8.73	20.15 ±9.51	0.71
Kisspeptin1 (ng/L) Mean ± SD	418.589 ±168.88	419.688 ±139.438	0.98

3.5.4.6 The statistical differences between biochemical parameters and genotype in normal weight women with PCOS.

All the three genotypes had comparable serum LH level, FSH level, LH/FSH ratio, prolactin level, free testosterone, and Kisspeptin level. However, patients with normal weight women with PCOS showed no significance in levels of all hormones, Table (3-20). Serum testosterone level was higher in the GC genotype compared with homozygous wild and mutation allele (0.76 ± 0.53, 0.93± 0.73 and 0.33 ± 0.27 ng/ml, P=0.23). The LH/FSH ratio was (1.17 ± 0.67, 1.71 ± 0.92, 1.67 ±0.59) in the GG, GC and CC genotype, the percentage of women with LH/FSH

ratio showed no statistical difference between the three groups. The comparison between the two groups in terms of biochemical analysis results is shown in Table (3-20)

Table (3-20): Differences between biochemical parameters and genotype of the normal weight women with PCOS.

Biochemical parameters	Genotyping frequency			P value
	GG	GC	CC	
LH (mIU/ml) Mean ± SD	6.13 ± 4.39	7.67 ± 4.14	8.55 ± 4.85	0.47
FSH (mIU/ml) Mean ± SD	5.17 ± 1.06	4.47 ± 0.79	4.85 ± 0.98	0.16
LH/FSH ratio Mean ± SD	1.17 ± 0.67	1.71 ± 0.92	1.67 ± 0.59	0.14
Free Testosterone (ng/ml) Mean ± SD	0.76 ± 0.53	0.93 ± 0.73	0.33 ± 0.27	0.23
Prolactin (ng/ml) Mean ± SD	23.79 ± 14.75	23.07 ± 11.78	17.75 ± 5.90	0.7
Kisspeptin1 (ng/L) Mean ± SD	387.783 ±63.839	337.829 ± 112.481	389.719 ± 82.925	0.3

3.5.4.7. The difference between biochemical parameters and genotype in control women

The control group showed non-significant differences in levels of all hormone parameters, as shown in Table (3-21). A slight but not significant increase was observed for each of serum LH, LH/FSH ratio, testosterone level and Kisspeptin1 levels in GC genotype, Table (3-21).

Table (3-21): The difference between clinical parameters and genotype in the control group.

Biochemical parameters	Genotyping frequency		P value
	GG	GC Mean ± SD	
LH (mIU/ml) Mean ± SD	4.94 ± 1.18	5.42 ± 1.34	0.30
FSH (mIU/ml) Mean ± SD	6.92 ± 1.16	6.91 ±1.21	0.97
LH/FSH ratio Mean ± SD	0.71 ± 0.12	0.78 ± 0.14	0.15
Free Testosterone (ng/ml) Mean ± SD	0.34 ± 0.22	0.48 ± 0.26	0.1
Prolactin (ng/ml) Mean ± SD	17.87 ± 3.74	16.89 ± 2.75	0.45
Kisspeptin1 (ng/L) Mean ± SD	322.36 ± 53.28	326.62 ± 52.24	0.83

3.5.4.8. Association of biochemical parameters with genotype frequency among four groups.

The results of the current study showed that when measuring hormones levels with the GG allele, there were significant differences between FSH, free testosterone levels and LH/FSH ratio, it was more in obese, overweight and normal weight women with PCOS patients then control groups, except FSH levels, found the concentration of FSH hormone (6.92 ± 1.16) in control groups was higher than patients with PCOS.

Table (3-22): The biochemical markers relationship of patients with alleles

Biochemical parameters	Genotyping frequency				P value	Genotyping frequency				P value
	Patient with PCOS (Obese) GG	Patient with PCOS (over weight) GG	Patient with PCOS (normal weight) GG	Control GG		Patient with PCOS (Obese) GC	Patient with PCOS (over weight) GC	Patient with PCOS (normal weight) GC	Control GC	
LH Mean ± SD	8.22 ±2.91	7.22 ±3.23	6.13 ± 4.39	4.94 ± 1.18	0.07	10.29 ±2.15	13.41 ±5.05	7.67 ± 4.14	5.42 ± 1.34	P < 0.01
FSH Mean ± SD	5.68 ±0.91	4.63 ±1.06	5.17 ± 1.06	6.92 ± 1.16	P < 0.01	5.78 ±1.32	5.94 ±1.90	4.47 ± 0.79	6.91 ±1.21	P < 0.01
LH/FSH ratio Mean ± SD	1.25 ±0.16	1.58 ±0.62	1.17 ± 0.67	0.71 ± 0.12	P < 0.01	1.79 ±0.19	2.27 ±0.59	1.71 ± 0.92	0.78 ± 0.14	P < 0.01
Free Testosterone Mean ± SD	0.87 ±0.48	0.80 ±0.53	0.76 ± 0.53	0.34 ± 0.22	P < 0.01	0.59 ±0.36	0.79 ±0.56	0.82 ± 0.79	0.48 ± 0.26	0.43
Prolactin Mean ± SD	20.96 ±5.24	18.86 ±8.73	23.79 ± 14.75	17.87 ± 3.74	0.22	23 ±4.55	20.15 ±9.51	23.07 ± 11.78	16.89 ± 2.75	0.27
Kisspeptin1 Mean ± SD	450.206 ±119.478	418.589 ±168.88	387.783 ±63.839	322.36 ± 53.28	P < 0.01	371.827 ±142.972	419.688 ±139.438	337.829 ±112.481	326.62 ± 52.24	0.29

From this Table, observed that the allele frequency GG is statistically significant between the four groups (PCOS groups and control) in FSH, LH/FSH ratio, free testosterone and Kisspeptin1. While, the GC allele showed significant difference with LH, FSH, LH/FSH ratio between the four groups (PCOS groups and control).

In patients with PCOS, FSH levels are within the reference range or low. The LH/FSH ratio is usually greater than 2 in comparison to control group (0.71), FSH and LH are often both in the range of about 4-8 in young fertile women. In women with polycystic ovaries the LH / FSH ratio is often higher – for example 2:1, or even 3:1 [169] .

On the other hand the results of the current study showed that when measuring hormone levels with the GC allele, there were significant differences between LH, FSH and LH/FSH ratio levels, it was more in obese, overweight and normal weight women patients with PCOS from that of control group, except FSH levels, we found the concentration of FSH hormone (6.91 ±1.21) in control group more than that of PCOS patient where it was (5.78 ±1.32 , 5.94 ±1.90 and 4.47 ±0.79) for PCOS obese, overweight and normal weight, respectively.

No significant differences (P> 0.05) were observed for both assays when compared with the homozygous wild and heterozygous alleles.

Conclusions

&

Recommendations

Conclusions

From the results of this study, the following can be concluded:

1. No correlation was observed between KISS1 level and the measured hormones in patients have PCOS.

2. The age of the patients has no effect on KISS1 level.

3. The non-synonymous SNP rs4889 (C/G) was found more frequently in the obese women with PCOS than in the overweight and normal weight women with PCOS and control groups.

4. The frequencies of the identified polymorphisms were significantly different between cases and controls, and the frequency of heterozygous allele (GC) was higher in cases 21 than in controls 10 while the homozygous (CC) allele was not present in both groups. The allelic frequency of SNP rs4889 G/C was detected more frequently with non-significant association in obese women with PCOS than in control.

5. The homozygous genotypes (CC) were found just in the PCOS with normal weight, and not found in obese and overweight women with PCOS, and it is also less than those of their wild type (GG) and heterozygous genotypes in both cases.

6. The result showed the most cases of Hirsutism, Acne and hair loss found in obese women with PCOS have heterozygous allele (GC).

7. The genotyping frequency of KISS1 gene showed significant association with LH, FSH, and LH/ FSH ratio in PCOS. There is an increase in the proportion of the LH/FSH ratio hormone with significant differences ($P< 0.05$) in women who carry the homozygous allele compared to women who carry the heterozygous allele.

8. Serum testosterone level was higher in the GC genotype compared with homozygous wild and mutation allele

Recommendations

1. It is recommended to evaluate serum kisspeptin levels in different menopausal stages (pre, post and transitional) & correlate it with the clinical and biochemical parameters.
2. Neurokinin and Dynorphin are neuropeptides that work with kisspeptin in the hypothalamus for regulation GnRH secretion. They can be detected in the circulation. Evaluation of them with kisspeptin in different phases of the menstrual cycle may give further findings.
3. Evaluation of kisspeptin level in other types of subfertility is recommended.
4. Make further comparison of serum kisspeptin level between treated and untreated sub-fertile women.
5. Study the correlation between KISS1 level and insulin level in PCOS patients with diabetes.

References

References

1. Xue, Y., et al., *Identification of microRNAs and genes associated with hyperandrogenism in the follicular fluid of women with polycystic ovary syndrome.* Journal of cellular biochemistry, 2018. **119**(5): p. 3913-3921.
2. El Hayek, S., et al., *Poly cystic ovarian syndrome: an updated overview.* Frontiers in physiology, 2016. **7**: p. 124.
3. De Leo, V., et al., *Genetic, hormonal and metabolic aspects of PCOS: an update.* Reproductive Biology and Endocrinology, 2016. **14**(1): p. 1-17.
4. Johansson, J. and E. Stener-Victorin, *Polycystic ovary syndrome: effect and mechanisms of acupuncture for ovulation induction.* Evidence-Based Complementary and Alternative Medicine, 2013. **2013**.
5. Khan, M.J., A. Ullah, and S. Basit, *Genetic basis of polycystic ovary syndrome (PCOS): current perspectives.* The application of clinical genetics, 2019. **12**: p. 249.
6. Umayal, B., et al., *Polycystc ovary syndrome: Genetc Contributons from the hypothalamic-pituitary-gonadal Axis.* Int Arch Endocrinol Clin Res, 2018. **4**(013): p. 1-8.
7. Valkenburg, O., et al., *Genetic polymorphisms of GnRH and gonadotrophic hormone receptors affect the phenotype of polycystic ovary syndrome.* Human reproduction, 2009. **24**(8): p. 2014-2022.
8. Prapas, N., et al., *Genetics of polycystic ovary syndrome.* Hippokratia, 2009. **13**(4): p. 216.
9. Zadeh-Vakili, A., *The Role of Kisspeptin in Female Reproduction.* International Journal of Endocrinology and Metabolism, 2017. **15**(3): p. e44337-e44337.
10. Albalawi, F.S., et al., *rs4889 polymorphism in KISS1 gene, its effect on polycystic ovary syndrome development and anthropometric and hormonal parameters in Saudi women.* Journal of biomedical science, 2018. **25**(1): p. 1-6.
11. Pinilla, L., et al., *Kisspeptins and reproduction: physiological roles and regulatory mechanisms.* Physiological reviews, 2012. **92**(3): p. 1235-1316.
12. Daghestani, M.H., et al., *Influence of KISS1 gene polymorphisms on the risk of polycystic ovary syndrome and its associated variables, in Saudi women.* BMC endocrine disorders, 2020. **20**(1): p. 1-10.

13. Rutkowska, A.Z. and E. Diamanti-Kandarakis, *Polycystic ovary syndrome and environmental toxins.* Fertility and sterility, 2016. **106**(4): p. 948-958.
14. DeRijk, R., et al., *Exercise and circadian rhythm-induced variations in plasma cortisol differentially regulate interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα) production in humans: high sensitivity of TNFα and resistance of IL-6.* The Journal of Clinical Endocrinology & Metabolism, 1997. **82**(7): p. 2182-2191.
15. Pavicic Baldani, D., et al., *Clinical and biochemical characteristics of polycystic ovary syndrome in Croatian population.* Collegium antropologicum, 2012. **36**(4): p. 1413-1418.
16. Li, L., et al., *Clinical and metabolic features of polycystic ovary syndrome among Chinese adolescents.* Journal of pediatric and adolescent gynecology, 2012. **25**(6): p. 390-395.
17. Ates, S., et al., *Different phenotypes of polycystic ovary syndrome in Turkish women: clinical and endocrine characteristics.* Gynecological Endocrinology, 2013. **29**(10): p. 931-935.
18. Escobar-Morreale, H.F., *Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment.* Nature Reviews Endocrinology, 2018. **14**(5): p. 270.
19. Ibrahim, R.O., *Evaluation of Physiological Role of Kisspeptin and Other Physiological Parameteres among polycystic Ovarian Syndrome Patients in Kirkuk City (A Case-Control Study)*. 2020, University of Sulaimani.
20. Calcaterra, V., et al., *Polycystic Ovary Syndrome in Insulin-Resistant Adolescents with Obesity: The Role of Nutrition Therapy and Food Supplements as a Strategy to Protect Fertility.* Nutrients, 2021. **13**(6): p. 1848.
21. Balen, A.H., *Polycystic ovary syndrome (PCOS).* The Obstetrician & Gynaecologist, 2017. **19**(2): p. 119-129.
22. Wolf, W.M., et al., *Geographical prevalence of polycystic ovary syndrome as determined by region and race/ethnicity.* International journal of environmental research and public health, 2018. **15**(11): p. 2589.
23. Tabassum, R., F. Imtiaz, and S. Sharafat, *Prevalence and clinical profile of insulin resistance in young women of poly cystic ovary syndrome: A study from Pakistan.* Pakistan journal of medical sciences, 2013. **29**(2): p. 593.
24. Hussein, B. and S. Alalaf, *Prevalence and characteristics of polycystic ovarian syndrome in a sample of infertile Kurdish women attending IVF infertility center in maternity teaching*

hospital of Erbil City. Open Journal of Obstetrics and Gynecology, 2013. **2013**.

25. Mohammad, M.B. and A.M. Seghinsara, *Polycystic ovary syndrome (PCOS), diagnostic criteria, and AMH.* Asian Pacific journal of cancer prevention: APJCP, 2017. **18**(1): p. 17.
26. Tay, C.T., et al., *Updated adolescent diagnostic criteria for polycystic ovary syndrome: impact on prevalence and longitudinal body mass index trajectories from birth to adulthood.* BMC medicine, 2020. **18**(1): p. 1-11.
27. Carmina, E., et al., *AMH measurement versus ovarian ultrasound in the diagnosis of polycystic ovary syndrome in different phenotypes.* Endocrine Practice, 2016. **22**(3): p. 287-293.
28. Khazaal, F.A.K., A.H. Liebi, and I. Mahmoud, *Prevalence and presenting features of polycystic ovarian syndrome in Iraqi obese females.* Iraqi Journal of Embryo and Infertility Researches, 2014. **4**(2): p. 14-18.
29. Charifson, M.A. and B.C. Trumble, *Evolutionary origins of polycystic ovary syndrome: an environmental mismatch disorder.* Evolution, medicine, and public health, 2019. **2019**(1): p. 50-63.
30. Ali, R.J., *Genetic Polymorphism in MTHFR and PCO genes associated with the incidence of Polycystic Ovary Syndrome in A sample of Iraqi Women.* Master degree Msc, Al-Nahrain University, 2016.
31. Silva, M.S., et al., *Activation of arcuate nucleus GABA neurons promotes luteinizing hormone secretion and reproductive dysfunction: Implications for polycystic ovary syndrome.* EBioMedicine, 2019. **44**: p. 582-596.
32. Legro, R.S., V.D. Castracane, and R.P. Kauffman, *Detecting insulin resistance in polycystic ovary syndrome: purposes and pitfalls.* Obstetrical & gynecological survey, 2004. **59**(2): p. 141-154.
33. Yilmaz, B., et al., *Metabolic syndrome, hypertension, and hyperlipidemia in mothers, fathers, sisters, and brothers of women with polycystic ovary syndrome: a systematic review and meta-analysis.* Fertility and sterility, 2018. **109**(2): p. 356-364. e32.
34. Baskind, N.E. and A.H. Balen, *Hypothalamic–pituitary, ovarian and adrenal contributions to polycystic ovary syndrome.* Best Practice & Research Clinical Obstetrics & Gynaecology, 2016. **37**: p. 80-97.
35. Ciccone, N.A., et al., *Frequency-dependent regulation of follicle-stimulating hormone β by pulsatile gonadotropin-releasing hormone is mediated by functional antagonism of bZIP*

transcription factors. Molecular and cellular biology, 2010. **30**(4): p. 1028-1040.

36. Hall, J.E., *Neuroendocrine control of the menstrual cycle,* in *Yen and Jaffe's reproductive endocrinology*. 2019, Elsevier. p. 149-166. e5.
37. Carmina, E., F.Z. Stanczyk, and R.A. Lobo, *Evaluation of hormonal status*, in *Yen and Jaffe's Reproductive Endocrinology*. 2019, Elsevier. p. 887-915. e4.
38. Chang, R.J. and D.A. Dumesic, *Polycystic ovary syndrome and hyperandrogenic states*, in *Yen and Jaffe's reproductive endocrinology*. 2019, Elsevier. p. 520-555. e13.
39. Shaaban, Z., et al., *Pathophysiological mechanisms of gonadotropins–and steroid hormones–related genes in etiology of polycystic ovary syndrome.* Iranian journal of basic medical sciences, 2019. **22**(1): p. 3.
40. Qu, X. and R. Donnelly, *Sex hormone-binding globulin (SHBG) as an early biomarker and therapeutic target in polycystic ovary syndrome.* International journal of molecular sciences, 2020. **21**(21): p. 8191.
41. Surindar Singh, G.K., *Liquid chromatography tandem mass spectrometry measurements of steroids in biological samples for clinical research studies: methodology and application.* 2015.
42. Matalliotakis, I., et al., *Polycystic ovary syndrome: etiology and pathogenesis.* Archives of gynecology and obstetrics, 2006. **274**(4): p. 187-197.
43. McAllister, J.M., et al., *Overexpression of a DENND1A isoform produces a polycystic ovary syndrome theca phenotype.* Proceedings of the National Academy of Sciences, 2014. **111**(15): p. E1519-E1527.
44. Senthurselvi, R., *A cross sectional study on the status of inflammatory markers in polycystic ovary syndrome (pcos) patients in Indian population*. 2015, PSG Institue of Medical Sciences and Research, Coimbatore.
45. Wawrzkiewicz-Jałowiecka, A., et al., *In Search of New Therapeutics—Molecular Aspects of the PCOS Pathophysiology: Genetics, Hormones, Metabolism and Beyond.* International Journal of Molecular Sciences, 2020. **21**(19): p. 7054.
46. O'Reilly, M.W., et al., *AKR1C3-mediated adipose androgen generation drives lipotoxicity in women with polycystic ovary syndrome.* The Journal of Clinical Endocrinology & Metabolism, 2017. **102**(9): p. 3327-3339.
47. Kraynak, M., et al., *Extraovarian gonadotropin negative feedback revealed by aromatase inhibition in female marmoset monkeys.*

American Journal of Physiology-Endocrinology and Metabolism, 2017. **313**(5): p. E507-E514.
48. Luque-Ramírez, M. and H. F Escobar-Morreale, *Adrenal hyperandrogenism and polycystic ovary syndrome.* Current pharmaceutical design, 2016. **22**(36): p. 5588-5602.
49. Polak, K., et al., *New markers of insulin resistance in polycystic ovary syndrome.* Journal of endocrinological investigation, 2017. **40**(1): p. 1-8.
50. Sanchez-Garrido, M.A. and M. Tena-Sempere, *Metabolic dysfunction in polycystic ovary syndrome: Pathogenic role of androgen excess and potential therapeutic strategies.* Molecular metabolism, 2020. **35**: p. 100937.
51. Scaramuzzi, R.J., et al., *The effects of intravenous, glucose versus saline on ovarian follicles and their levels of some mediators of insulin signalling.* Reproductive Biology and Endocrinology, 2015. **13**(1): p. 1-14.
52. Coutinho, E.A. and A.S. Kauffman, *The role of the brain in the pathogenesis and physiology of polycystic ovary syndrome (PCOS).* Medical Sciences, 2019. **7**(8): p. 84.
53. Diamanti-Kandarakis, E., H. Kandarakis, and R.S. Legro, *The role of genes and environment in the etiology of PCOS.* Endocrine, 2006. **30**(1): p. 19-26.
54. Merkin, S.S., et al., *Environmental determinants of polycystic ovary syndrome.* Fertility and sterility, 2016. **106**(1): p. 16-24.
55. Gajbhiye, R., J.N. Fung, and G.W. Montgomery, *Complex genetics of female fertility.* NPJ genomic medicine, 2018. **3**(1): p. 1-10.
56. Mykhalchenko, K., et al., *Genetics of polycystic ovary syndrome.* Expert review of molecular diagnostics, 2017. **17**(7): p. 723-733.
57. Dadachanji, R., N. Shaikh, and S. Mukherjee, *Genetic variants associated with hyperandrogenemia in PCOS pathophysiology*. Genetics research international, 2018. **2018**.
58. Stener-Victorin, E., *Epigenetic and transgenerational transmission of polycystic ovary syndrome.* Current Opinion in Endocrine and Metabolic Research, 2020. **12**: p. 72-77.
59. Day, F., et al., *Large-scale genome-wide meta-analysis of polycystic ovary syndrome suggests shared genetic architecture for different diagnosis criteria.* PLoS genetics, 2018. **14**(12): p. e1007813.
60. Casadei, L., et al., *The diagnosis of PCOS in young infertile women according to different diagnostic criteria: the role of serum anti-Müllerian hormone.* Archives of gynecology and obstetrics, 2018. **298**(1): p. 207-215.

61. Zore, T., et al., *Minimal difference in phenotype between adolescents and young adults with polycystic ovary syndrome.* Fertility and sterility, 2019. **111**(2): p. 389-396.
62. Barbieri, R.L., *What is optimal hormonal treatment for women with polycystic ovary syndrome.* OBG Manag, 2020. **32**: p. 10-12.
63. Kazemi, M., et al., *MON-213 evaluation of type 2 diabetes and cardiovascular disease risk factors in reproductive-age women with polycystic ovary syndrome (PCOS) using the 2014 Androgen Excess and PCOS Society Criteria for the Polycystic Ovarian Morphology.* Journal of the Endocrine Society, 2019. **3**(Supplement_1): p. MON-213.
64. Nahar, K., *Polycystic Ovary Syndrome in Teenage and Young Women.* Journal of Bangladesh College of Physicians and Surgeons, 2019. **37**(2): p. 78-82.
65. Sirmans, S.M. and K.A. Pate, *Epidemiology, diagnosis, and management of polycystic ovary syndrome.* Clinical epidemiology, 2014. **6**: p. 1.
66. Li, H. and R.-C. Chian, *Follicular development and oocyte growth*, in *Development of In Vitro Maturation for Human Oocytes*. 2017, Springer. p. 37-57.
67. Mihailidis, J., et al., *Endocrine evaluation of hirsutism.* International journal of women's dermatology, 2017. **3**(1): p. S6-S10.
68. Woods, N.F. and N.J. Kenney, *Menstrual-Cycle-Related Disorders: Polycystic ovary syndrome, dysmenorrhea and menstrual migraine*, in *Routledge International Handbook of Women's Sexual and Reproductive Health*. 2019, Routledge. p. 101-126.
69. Sun, Y.-F., et al., *High BMI and insulin resistance are risk factors for spontaneous abortion in patients with polycystic ovary syndrome undergoing assisted reproductive treatment: a systematic review and meta-analysis.* Frontiers in endocrinology, 2020. **11**: p. 946.
70. Patel, S., *Polycystic ovary syndrome (PCOS), an inflammatory, systemic, lifestyle endocrinopathy.* The Journal of steroid biochemistry and molecular biology, 2018. **182**: p. 27-36.
71. Zeng, X., et al., *Polycystic ovarian syndrome: correlation between hyperandrogenism, insulin resistance and obesity.* Clinica Chimica Acta, 2020. **502**: p. 214-221.
72. Laven, J.S., et al., *New approach to polycystic ovary syndrome and other forms of anovulatory infertility.* Obstetrical & gynecological survey, 2002. **57**(11): p. 755-767.

73. Keevil, B.G. and J. Adaway, *Assessment of free testosterone concentration.* The Journal of steroid biochemistry and molecular biology, 2019. **190**: p. 207-211.
74. Sinha, P., et al., *Laparoscopic ovarian drilling reduces testosterone and luteinizing hormone/follicle-stimulating hormone ratio and improves clinical outcome in women with polycystic ovary syndrome.* Journal of human reproductive sciences, 2019. **12**(3): p. 224.
75. Walters, K.A., et al., *New perspectives on the pathogenesis of PCOS: neuroendocrine origins.* Trends in Endocrinology & Metabolism, 2018. **29**(12): p. 841-852.
76. Sheehan, M.T., *Polycystic ovarian syndrome: diagnosis and management.* Clinical Medicine & Research, 2004. **2**(1): p. 13-27.
77. Orio Jr, F., et al., *Is plasminogen activator inhibitor-1 a cardiovascular risk factor in young women with polycystic ovary syndrome?* Reproductive biomedicine online, 2004. **9**(5): p. 505-510.
78. Kanda, S., *Evolution of the regulatory mechanisms for the hypothalamic-pituitary-gonadal axis in vertebrates–hypothesis from a comparative view.* General and comparative endocrinology, 2019. **284**: p. 113075.
79. JE, H., *Guyton and Hall textbook of medical physiology.* Philadelphia, PA: Saunders Elsevier, 2011. **107**: p. 1146.
80. ESHRE, T.R. and A.-S.P.C.W. Group, *Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome.* Fertility and sterility, 2004. **81**(1): p. 19-25.
81. Rothenberg, S.S., et al., *Polycystic ovary syndrome in adolescents.* Best Practice & Research Clinical Obstetrics & Gynaecology, 2018. **48**: p. 103-114.
82. Hamza, D.H. and S.A. Hassan, *Polycystic ovary syndrome and some hormonal and physiological changes: A review.* EurAsian Journal of BioSciences, 2020. **14**(2): p. 5149-5156.
83. Findlay, J., et al., *Follicle selection in mammalian ovaries*, in *The Ovary*. 2019, Elsevier. p. 3-21.
84. Akhondi, M.M., et al., *Practical difficulties in estimating the prevalence of primary infertility in Iran.* International journal of fertility & sterility, 2019. **13**(2): p. 113.
85. DeUgarte, C.M., A.A. Bartolucci, and R. Azziz, *Prevalence of insulin resistance in the polycystic ovary syndrome using the homeostasis model assessment.* Fertility and sterility, 2005. **83**(5): p. 1454-1460.

86. Heijnen, E., et al., *A meta-analysis of outcomes of conventional IVF in women with polycystic ovary syndrome.* Human reproduction update, 2006. **12**(1): p. 13-21.
87. Moran, L.J., et al., *Predictors of lifestyle intervention attrition or weight loss success in women with polycystic ovary syndrome who are overweight or obese.* Nutrients, 2019. **11**(3): p. 492.
88. Tanco, V.M., et al., *Distribution and regulation of gonadotropin-releasing hormone, kisspeptin, RF-amide related peptide-3, and dynorphin in the bovine hypothalamus.* PeerJ, 2016. **4**: p. e1833.
89. Clarkson, J., *d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge.* J Neurosci, 2008. **28**(35): p. 86917.
90. De Roux, N., et al., *Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54.* Proceedings of the National Academy of Sciences, 2003. **100**(19): p. 10972-10976.
91. Seminara, S.B., et al., *The GPR54 gene as a regulator of puberty.* New England Journal of Medicine, 2003. **349**(17): p. 1614-1627.
92. Votsi, E., et al., *Kisspeptins: a multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system.* Hippokratia, 2008. **12**(4): p. 205.
93. Castellano, J., et al., *Expression of KiSS-1 in rat ovary: putative local regulator of ovulation?* Endocrinology, 2006. **147**(10): p. 4852-4862.
94. Pasquier, J., et al., *Comparative evolutionary histories of kisspeptins and kisspeptin receptors in vertebrates reveal both parallel and divergent features.* Frontiers in endocrinology, 2012. **3**: p. 173.
95. Witchel, S.F. and M. Tena-Sempere, *The Kiss1 system and polycystic ovary syndrome: lessons from physiology and putative pathophysiologic implications.* Fertility and sterility, 2013. **100**(1): p. 12-22.
96. Chaudhari, N., M. Dawalbhakta, and L. Nampoothiri, *GnRH dysregulation in polycystic ovarian syndrome (PCOS) is a manifestation of an altered neurotransmitter profile.* Reproductive Biology and Endocrinology, 2018. **16**(1): p. 1-13.
97. d'Anglemont de Tassigny, X. and W.H. Colledge, *The role of kisspeptin signaling in reproduction.* Physiology, 2010. **25**(4): p. 207-217.

98. Nejad, S.Z., F.R. Tehrani, and A. Zadeh-Vakili, *The role of kisspeptin in female reproduction.* International journal of endocrinology and metabolism, 2017. **15**(3).
99. Branavan, U., et al., *Association of Kiss1 and GPR54 gene polymorphisms with polycystic ovary syndrome among Sri Lankan women.* BioMed Research International, 2019. **2019**.
100. Mut, A., et al., *Serum kisspeptin levels correlated with anti-mullerian hormone levels in women with and without polycystic ovarian syndrome.* Gynecological Endocrinology, 2021. **37**(5): p. 462-466.
101. Matsuda, F., et al., *Role of kisspeptin neurons as a GnRH surge generator: Comparative aspects in rodents and non-rodent mammals.* Journal of Obstetrics and Gynaecology Research, 2019. **45**(12): p. 2318-2329.
102. Panidis, D., et al., *Plasma metastin levels are negatively correlated with insulin resistance and free androgens in women with polycystic ovary syndrome.* Fertility and sterility, 2006. **85**(6): p. 1778-1783.
103. Gorkem, U., et al., *Is there a role for kisspeptin in pathogenesis of polycystic ovary syndrome?* Gynecological Endocrinology, 2018. **34**(2): p. 157-160.
104. Emekci Ozay, O., et al., *Role of kisspeptin in polycystic ovary syndrome (PCOS).* Gynecological Endocrinology, 2016. **32**(9): p. 718-722.
105. Trevisan, C.M., et al., *Kisspeptin/GPR54 system: what do we know about its role in human reproduction?* Cellular Physiology and Biochemistry, 2018. **49**(4): p. 1259-1276.
106. Harter, C.J., G.S. Kavanagh, and J.T. Smith, *The role of kisspeptin neurons in reproduction and metabolism.* Journal of Endocrinology, 2018. **238**(3): p. R173-R183.
107. Latif, R. and N. Rafique, *Serum kisspeptin levels across different phases of the menstrual cycle and their correlation with serum oestradiol.* Neth J Med, 2015. **73**(4): p. 175-8.
108. Zhai, J., et al., *Kisspeptin: a new marker for human pre-ovulation.* Gynecological Endocrinology, 2017. **33**(7): p. 560-563.
109. Bacopoulou, F., et al., *Serum kisspeptin concentrations are negatively correlated with body mass index in adolescents with anorexia nervosa and amenorrhea.* Hormones, 2017. **16**(1): p. 33-41.
110. Kanasaki, H., et al., *Circulating kisspeptin and pituitary adenylate cyclase-activating polypeptide (PACAP) do not correlate with gonadotropin serum levels.* Gynecological Endocrinology, 2013. **29**(6): p. 583-587.

111. Ohtaki, T., et al., *Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor*. Nature, 2001. **411**(6837): p. 613-617.
112. Chen, X., et al., *Increased plasma metastin levels in adolescent women with polycystic ovary syndrome.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2010. **149**(1): p. 72-76.
113. Nyagolova, P.V., et al., *Kisspeptin and galanin-like peptide (galp) levels in women with polycystic ovary syndrome.* Int J Pharmaceut Med Res, 2016. **4**: p. 6-12.
114. Jeon, Y.E., et al., *Kisspeptin, leptin, and retinol-binding protein 4 in women with polycystic ovary syndrome.* Gynecologic and obstetric investigation, 2013. **75**(4): p. 268-274.
115. Yerlikaya, E., et al. *Relation of plasma kisspeptin levels with obesity and insulin resistance in polycystic ovary syndrome*. in *Endocrine Abstracts*. 2013. Bioscientifica.
116. Gupta, S., R.H. Rai, and R.H. Rai, *To determine the correlation of BMI, height and weight with peak expiratory flow rate in young adults.* Int J Sci Res Rev, 2019. **8**(08): p. 163-169.
117. Mazina, O., et al., *Determination of biological activity of gonadotropins hCG and FSH by Förster resonance energy transfer based biosensors.* Scientific reports, 2017. **7**: p. 42219.
118. Lawrenz, B., et al., *Step-Down of FSH-Dosage During Ovarian Stimulation–Basic Lessons to Be Learnt From a Randomized Controlled Trial.* Frontiers in Endocrinology, 2021. **12**: p. 346.
119. Liu, J., et al. *A rapid quantitative determination method of luteinizing hormone with gold immunochromatographic strip*. in *2017 39th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC)*. 2017. IEEE.
120. Zorovic, M., K. Kolmančič, and M. Živin, *Effects of L-dopa on expression of prolactin and synaptotagmin IV in 17-beta-estradiol-induced prolactinomas of ovariectomized hemiparkinsonian rats.* Bosnian Journal of Basic Medical Sciences, 2020.
121. Whyte, M., et al., *Importance of cannulated prolactin test in the definition of hyperprolactinaemia*. Pituitary, 2015. **18**(3): p. 319-325.
122. Patira, N.K., N. Salgiya, and D. Agrawal, *Correlation of thyroid function test with severity of liver dysfunction in cirrhosis of liver.* Journal of the Association of Physicians of India, 2019. **67**: p. 51-54.

123. Lau, K.K.E., et al., *Laboratory testing protocols for heparin-induced thrombocytopenia (HIT) testing*, in *Hemostasis and Thrombosis*. 2017, Springer. p. 227-243.
124. Tataurov, A.V., Y. You, and R. Owczarzy, *Predicting ultraviolet spectrum of single stranded and double stranded deoxyribonucleic acids.* Biophysical chemistry, 2008. **133**(1-3): p. 66-70.
125. Ehtisham, M., et al., *Polymerase chain reaction (PCR): back to basics.* Indian Journal of Contemporary Dentistry, 2016. **4**(2): p. 30-35.
126. Weissensteiner, T., et al., *PCR technology: current innovations*. 2003: CRC press.
127. Ye, J., et al., *Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction.* BMC bioinformatics, 2012. **13**(1): p. 1-11.
128. Denhart, M. and V. Doraiswamy, *Master Your PCR Domain!* Promega Notes, 2001. **78**: p. 9-12.
129. Green, M.R. and J. Sambrook, *Analysis of DNA by agarose gel electrophoresis.* Cold Spring Harbor Protocols, 2019. **2019**(1): p. pdb. top100388.
130. Westermeier, R., *Electrophoresis in practice: a guide to methods and applications of DNA and protein separations*. 2016: John Wiley & Sons.
131. Mohanty, A.K., M.L. Yadav, and S. Choudhary, *Gel electrophoresis of proteins and nucleic acids*, in *Protocols in semen biology (comparing assays)*. 2017, Springer. p. 233-246.
132. Nasiri, N., et al., *Abdominal obesity can induce both systemic and follicular fluid oxidative stress independent from polycystic ovary syndrome.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2015. **184**: p. 112-116.
133. Kshetrimayum, C., et al., *Polycystic ovarian syndrome: Environmental/occupational, lifestyle factors; an overview.* Journal of the Turkish German Gynecological Association, 2019. **20**(4): p. 255.
134. De Coster, S. and N. Van Larebeke, *Endocrine-disrupting chemicals: associated disorders and mechanisms of action.* Journal of environmental and public health, 2012. **2012**.
135. Kabir, E.R., M.S. Rahman, and I. Rahman, *A review on endocrine disruptors and their possible impacts on human health.* Environmental toxicology and pharmacology, 2015. **40**(1): p. 241-258.
136. Bazarganipour, F., et al., *Sexual functioning among married Iranian women with polycystic ovary syndrome.* International journal of fertility & sterility, 2014. **8**(3): p. 273.

137. Kriedt, K.J., A. Alchami, and M.C. Davies, *PCOS: diagnosis and management of related infertility.* Obstetrics, Gynaecology & Reproductive Medicine, 2019. **29**(1): p. 1-5.
138. Hart, R., M. Hickey, and S. Franks, *Definitions, prevalence and symptoms of polycystic ovaries and polycystic ovary syndrome.* Best Practice & Research Clinical Obstetrics & Gynaecology, 2004. **18**(5): p. 671-683.
139. Azziz, R., et al., *Criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an androgen excess society guideline.* The Journal of Clinical Endocrinology & Metabolism, 2006. **91**(11): p. 4237-4245.
140. Mara Spritzer, P., C. Rocha Barone, and F. Bazanella de Oliveira, *Hirsutism in polycystic ovary syndrome: pathophysiology and management.* Current pharmaceutical design, 2016. **22**(36): p. 5603-5613.
141. Sardana, K., et al., *A study comparing the clinical and hormonal profile of late onset and persistent acne in adult females.* International journal of dermatology, 2020. **59**(4): p. 428-433.
142. Shum, K.W., D.R. Cullen, and A.G. Messenger, *Hair loss in women with hyperandrogenism: four cases responding to finasteride.* Journal of the American Academy of Dermatology, 2002. **47**(5): p. 733-739.
143. Varothai, S. and W.F. Bergfeld, *Androgenetic alopecia: an evidence-based treatment update.* American journal of clinical dermatology, 2014. **15**(3): p. 217-230.
144. Ibrahim, R.O., S.H. Omer, and C.N. Fattah, *The correlation between hormonal disturbance in PCOS women and serum level of kisspeptin.* International journal of endocrinology, 2020. **2020**.
145. Abbott, D.H., et al., *Endocrine antecedents of polycystic ovary syndrome in fetal and infant prenatally androgenized female rhesus monkeys.* Biology of reproduction, 2008. **79**(1): p. 154-163.
146. Osuka, S., et al., *Kisspeptin in the hypothalamus of 2 rat models of polycystic ovary syndrome.* Endocrinology, 2017. **158**(2): p. 367-377.
147. Yarmolinskaya, M.I., et al., *Kisspeptin and polycystic ovary syndrome-is there any connection?* Journal of obstetrics and women's diseases, 2017. **66**(6): p. 73-80.
148. Cao, Y., et al., *Reproductive functions of Kisspeptin/KISS1R Systems in the Periphery.* Reproductive Biology and Endocrinology, 2019. **17**(1): p. 1-9.
149. Hu, K.-L., et al., *Kisspeptin/kisspeptin receptor system in the ovary.* Frontiers in endocrinology, 2018. **8**: p. 365.

150. Araújo, B.S., et al., *Kisspeptin Influence on Polycystic Ovary Syndrome—a Mini Review.* Reproductive Sciences, 2020. **27**(2): p. 455-460.
151. Owens, L., et al., *The direct and indirect effects of kisspeptin-54 on granulosa lutein cell function.* Human Reproduction, 2018. **33**(2): p. 292-302.
152. Kołodziejski, P., et al., *Serum levels of spexin and kisspeptin negatively correlate with obesity and insulin resistance in women.* Physiological research, 2018. **67**(1): p. 45-56.
153. Shenta, A., K. Saud, and A. Al-Shawi, *Assessment the Correlations of Hormones, Lipid Profiles, Oxidative Stress, and Zinc Concentration in Iraqi Women with Polycystic Ovary Syndrome.* Reports of Biochemistry & Molecular Biology, 2020. **9**(3): p. 270.
154. Li, Y., et al., *Effect of luteinizing hormone vs follicular stimulating hormone ratio on anti-Müllerian hormone secretion and folliculogenesis in patients with polycystic ovarian syndrome.* Zhonghua fu chan ke za zhi, 2010. **45**(8): p. 567-570.
155. Luan, X., et al., *Association study of the polymorphisms in the KISS1 gene with central precocious puberty in Chinese girls.* European Journal of Endocrinology, 2007. **157**(1): p. 113-118.
156. Eriksen, M.B., et al., *Association of polycystic ovary syndrome susceptibility single nucleotide polymorphism rs2479106 and PCOS in Caucasian patients with PCOS or hirsutism as referral diagnosis.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2012. **163**(1): p. 39-42.
157. Ghosh, K., *Hirsutism: Evaluation and treatment: A reader's dilemma.* Indian journal of dermatology, 2011. **56**(2): p. 246.
158. Keen, M.A., I.H. Shah, and G. Sheikh, *Cutaneous manifestations of polycystic ovary syndrome: A cross-sectional clinical study.* Indian dermatology online journal, 2017. **8**(2): p. 104.
159. Grover, S., R.K. Ranyal, and M.K. Bedi, *A cross section of skin diseases in rural Allahabad.* Indian journal of dermatology, 2008. **53**(4): p. 179.
160. Jones, G., et al., *The polycystic ovary syndrome health-related quality of life questionnaire (PCOSQ): a validation.* Human reproduction, 2004. **19**(2): p. 371-377.
161. Kircik, L.H., *Advances in the Understanding of the Pathogenesis of Inflammatory Acne.* Journal of drugs in dermatology: JDD, 2016. **15**(1 Suppl 1): p. s7-10.
162. Özdemir, S., et al., *Specific dermatologic features of the polycystic ovary syndrome and its association with biochemical markers of the metabolic syndrome and hyperandrogenism.* Acta obstetricia et gynecologica Scandinavica, 2010. **89**(2): p. 199-204.

163. Lee, A.T. and L.T. Zane, *Dermatologic manifestations of polycystic ovary syndrome.* American journal of clinical dermatology, 2007. **8**(4): p. 201-219.
164. Macklon, N.S., et al., *The science behind 25 years of ovarian stimulation for in vitro fertilization.* Endocrine reviews, 2006. **27**(2): p. 170-207.
165. Saadia, Z., *Follicle Stimulating Hormone (LH: FSH) Ratio in Polycystic Ovary Syndrome (PCOS)-Obese vs. Non-Obese Women.* Medical Archives, 2020. **74**(4): p. 289.
166. Sperling, M.A., *Sperling Pediatric Endocrinology E-Book*. 2020: Elsevier Health Sciences.
167. Scott, J.R., *Danforth's obstetrics and gynecology*. Vol. 597. 2003: Lippincott williams & wilkins Philadelphia.
168. De Bond, J.-A.P., et al., *Kisspeptin signaling is required for the luteinizing hormone response in anestrous ewes following the introduction of males.* PloS one, 2013. **8**(2): p. e57972.
169. Khmil, M., S. Khmil, and M. Marushchak, *Hormone Imbalance in Women with Infertility Caused by Polycystic Ovary Syndrome: Is There a Connection with Body Mass Index?* Open Access Macedonian Journal of Medical Sciences, 2020. **8**(B): p. 731-737.

Appendixes

PCOS Disease Questionnaire

Patients' information: **Code Number:** ☐

Name:	Age:	Residency:
Weight: Height:	Waist:	BMI:
Occupation: ☐ house wife	☐ employed	☐ student
Education: ☐ illiterate ☐ primary	☐ secondary	☐ university ☐ institute
Economic state: ☐ high	☐ low	☐ moderate
Social status: ☐ Married ☐ Unmarried	Number of children:	
Breastfeeding: ☐ Yes	☐ No	
Menstrual cycle: ☐ Regular	☐ Irregular	
(M.C) - duration :		
Infertility: ☐ primary	☐ secondary	
How many years since you:		
Female cause: ☐ ovarian ☐ PCOS	☐ other	
Hirsutism:		
Acne:		
Hair loss:		

Other Disease:

Diabetes	☐ ev
Hypertension	☐ ev
Cardiovascular disease	☐ ev

Medication:

Required tests:

LH		Testosterone	
FSH		Prolactin	

الخلاصة

المقدمة : يعد متلازمة تكيس المبايض (PCOS) من الامراض متعدد العوامل التي تصيب ملايين النساء حول العالم. يشارك كيسبيبتين في التحكم في التكاثر البشري لسد الفجوة بين مستويات الستيرويد الجنسي وآليات التغذية الراجعة التي تتحكم في إفراز هرمون الغدد التناسلية (GnRH) ؛ ومع ذلك ، فإن الدراسات التي تدرس هذا الببتيد والعقم محدودة. لم يتضح بعد التأثير الذي قد تلعبه المتغيرات الجينية في جين KISS1 في تطوير متلازمة تكيس المبايض.

كيسبيبتين هو ببتيد عصبي يحفز إنتاج هرمون إفراز الغدد التناسلية (GnRH). وهو ضروري لارتفاع الهرمون الملوتن (LH) ، والهرمون المنبه للجريب (FSH) ، والإباضة. هذا الببتيد العصبي ضروري لوظيفة الإنجاب الطبيعية وبدء سن البلوغ. يتم تغيير إفراز كل من GnRH و LH عند النساء المصابات بمتلازمة تكيس المبايض (PCOS). هدفت هذه الدراسة إلى دراسة تعدد الأشكال الجيني لجين KISS1 وتأثيره على النساء المصابات بمتلازمة تكيس المبايض.

طريقة العمل : تضمن العمل الحالي دراسة حالة لمجموعة من 105 عينة (16 مريضًا يعانون من السمنة مع متلازمة تكيس المبايض ، و 19 مريضًا يعانون من زيادة الوزن مع متلازمة تكيس المبايض ، و 35 مريضًا بوزن طبيعي مع متلازمة تكيس المبايض ، و 35 من النساء اللائي يبدو أنهن يتمتعن بصحة جيدة كمجموعة ضابطة) تم اختيارهم من قسم العقم / أمراض النساء ومستشفى التوليد التعليمي في كربلاء ومن مرضى العيادات الخارجية. تم جمع عينات الدم من المرضى والمجموعة الضابطة وقسمت كل عينة إلى جزئين لأستخدامها للتحليل الوراثي والمصل. تم إجراء تعدد الأشكال الوراثي لجين KISS1 ونظام مقايسة الممتز المناعي للوصلة الإنزيمية (ELISA) باستخدام طريقة الساندويتش لقياس تركيزات الكيسيبتين البشري 1 في الدم وكذلك تحليل الهرمونات.

النتائج : أشارت نتائج الدراسة إلى أن العقم الأولي وعدم انتظام الدورة الشهرية والشعرانية وحب الشباب وتساقط الشعر ارتبطت بمجموعتي متلازمة تكيس المبايض البدينة وغير البدينة.

كانت مستويات كيسبيبتين ، LH، FSH ، هرمون التستوستيرون الحر والبرولاكتين أعلى بشكل ملحوظ في كل من مجموعات متلازمة تكيس المبايض البدينة وغير البدينة مقارنة بمجموعة التحكم. ارتبط مستوى كيسبيبتين بشكل إيجابي مع العمر المبكر (18-23 سنة) في مجموعة متلازمة تكيس المبايض ذات الوزن الزائد. تم العثور على (SNP rs4889 (C / G غير المرادف بشكل متكرر في النساء البدينات المصابات بمتلازمة تكيس المبايض مقارنة بالنساء ذوات الوزن الزائد والوزن الطبيعي المصابات بمتلازمة تكيس المبايض والمجموعة الضابطة. أظهر Allele C تواترًا أعلى في النساء البدينات المصابات بمتلازمة تكيس المبايض (31.2٪) من النساء ذوات الوزن الزائد والوزن الطبيعي المصابات بمتلازمة تكيس المبايض (26.3٪) و (28.5٪) على التوالي والمجموعة الضابطة (14.2٪). كان تواتر أليل متغاير الزيجوت (GC) أعلى مرتين في حالات متلازمة تكيس المبايض مقارنة بالضوابط. تم العثور على الأنماط الجينية متماثلة اللواقح (CC) فقط في الوزن الطبيعي لمتلازمة تكيس المبايض.

الأستنتاج: وجدت هذه الدراسة أن (SNP rs4889 (C / G غير المرادف أكثر تواترًا في النساء البدينات المصابات بمتلازمة تكيس المبايض مقارنة بالنساء ذوات الوزن الزائد والوزن الطبيعي المصابات بمتلازمة تكيس المبايض والمجموعة الضابطة ، ويسبب هذا SNP استبدال P81R. وقد لوحظ أن تردد الأليل GG ذو دلالة إحصائية بين المجموعات الأربع (مجموعات PCOS والمجموعة الضابطة) في FSH و LH / FSH وهرمون التستوستيرون الحر و Kisspeptin1. بينما أظهر أليل GC فرقًا معنويًا مع نسبة LH و FSH و LH / FSH بين المجموعات الأربع (مجموعات PCOS والضابطة). ظهر اختلاف معنوي في مستويات كيسبيبتين 1 في الدم بين مجموعات متلازمة تكيس المبايض مقارنة مع المجموعة الضابطة. لم يتم العثور على ارتباطات ذات دلالة إحصائية في جميع المعلمات البيوكيميائية.

جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة كربلاء / كلية الطب

تأثير تعدد الأشكال الجيني KISS1 على تطور متلازمة تكيس المبايض لدى النساء العراقيات

رسالة مقدمة الى
مجلس كليةالطب جامعة / كربلاء كجزء من متطلبات نيل درجة الماجستيرفي الكيمياءالسريرية

من قبل

هبه مؤيد خضير

بكالوريوس كيمياء / جامعة كربلاء (2015)

و بأشراف

الاستاذ

د.شذى عبد الودود الشمري

كلية العلوم / جامعة بغداد

الاستاذ المساعد

د.موسى محسن العلاق

كلية الطب / جامعة كربلاء

2021 م

1443 هـ