Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Babylon
College of Medicine
Department of Biochemistry

Genetic Polymorphisms of *KISS1* with Biochemical Features of kisspeptin and Gonadotrophic Hormones in Polycystic Ovary Syndrome

A Thesis

Submitted to the Council of the College of Medicine University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Clinical Biochemistry

By

NOOR JAMAL TALIB

B.Sc. chemistry -University of Babylon(2009)

Supervised By

Professor
Dr. Suhayr Aesa Al-Qaysi
Clinical Biochemistry
PhD. Clinical Biochemistry

Professor
Dr. Suha Jasim Witwit
Obstetrics and Gynecology
F.I.B.O.G. D.O.G. M.B.Ch.B

2022 A.D. **1443 A.H.**

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل/ كلية الطب

فرع الكيمياء والكيمياء الحياتية

تعدد الأشكال الجينية لجين *KISS1* مع السمات البيوكيميائية لبروتين الكيسبيبتين وهرمونات الغدة النخامية في متلازمة تكيس المبايض

رسالة مقدمة إلى

مجلس كلية الطب- جامعة بابل كجزء من متطلبات نيل درجة الماجستير في علم الكيمياء الحياتية السريرية

من قبل الطالبة

نـور جمـال طـالـب

بكالوريوس علوم كيمياء / جامعة بابل سنة 2009

الاشراف

الاستاذ

د. سـهير عيسـى القيسـي

كيمياء حياتية سريرية

دكتوراه كيمياء حياتية سريرية

الاستاذ

د. سـها جاسـم وتـوت

نسائية وتوليد

بورد نسائية وتوليد

٢٠٢٢ ميلادي

١٤٤٣هجري

﴿ قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ

إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ ﴾

صدق الله العلي العظيم

(سورة البقرة اية 32)

Supervisor Certification

We certify that this thesis entitled ((Genetic Polymorphisms of *KISS1* with Biochemical Features of kisspeptin and Gonadotrophic Hormones in Polycystic Ovary Syndrome)) has been prepared under our supervision at the Department of Biochemistry, College of Medicine, University of Babylon, in partial requirements for the degree of master in Clinical Biochemistry.

Professor

Dr. Suhayr Aesa Al-Qaysi

Date: / /2022

Professor

Dr. Suha Jasim Witwit

Date: / /2022

In the view of the available recommendation, I forward this thesis for debate by the Examination Committee.

Head of the Department of Biochemistry

College of Medicine, University of Babylon

Professor Dr. Abdulsamie Hassan Altaee

Date: / /2022

Dedication

To

Who helped me to achieve my goals, the greatest Father…

who accompanied me in prayer, the secret of my success, my mother is my life

who supported me... my husband's father and mother…

who bore me so much with patience, my husband " Ali AL-Kufaishi"…

who helped me and supported me... my brothers and my sister…

Everyone who stood with me to complete this research my relatives and all my friends, especially dear Sanaa…

For precious gifts and beautiful flowers.. my son "Yousif" and daughter "Sana"…
I dedicate this thesis with love…

NOOR -2022

Acknowledgements

First of all, thank Allah for helping me in performing this work.

I would like to introduce my deepest thanks to my supervisors Professor **Dr. Suhayr Aesa Al-Qaysi** and Professor **Dr.Suha Jasim Witwit** for their supervision, guidance and advice throughout the work.

Great thanks to the Dean of the College of Medicine, University of Babylon.

I want to thank the staff of the Department of Biochemistry, College of Medicine, University of Babylon to

provide all facilities to complete the requirements of the present study.

I would like to thank the staff of the DNA Research Center at the University of Babylon for their supporting me.

Great thanks to Imam Sadiq General Hospital staff, especially to Dr. Haydar Hantush Al-Eataar for his kind assistance during the work and for facilitating the contact with PCOS patients where the sample was collected.

I would like to express my deepest thanks to all participants for their help in agreeing to give them blood samples and all information about their medical condition to participate in this study.

NOOR -2022

Summary

Polycystic ovarian syndrome is one of the most common endocrinopathies diseases categorized by hyperandrogenism that affects early reproductive age, the *KISS1* gene has play role in regulating the hypothalamic-pituitary-gonad axis, the abnormal regulation that developed in PCOS. The present study is designed to estimate the association between *KISS1* gene polymorphisms (rs3727354G>A and rs4889G>A) with the pathogenesis of PCOS, and also to evaluate the association of kisspeptin level with gonadotrophin hormone and its relationship with PCOS.

The blood samples were collected from 120 females (60 control were divided into 30 normal weight and 30 obese) and (60 patients were divided into 30 normal weight and 30 obese). Deoxyribonucleic acid was extracted from blood samples and analysis of *KISS1* gene variants (rs3727354 and rs4889) were measured by a high-resolution melt technique for real-time polymerase chain reaction and the level of

kisspeptin in serum was measured by enzyme-linked immunosorbent assay, while luteinizing hormone, follicle-stimulating hormone, dehydroepiandrosterone sulfate and free testosterone by chemiluminescence immunoassay.

The present results indicated that levels of luteinizing hormone, free testosterone, and dehydroepiandrosterone sulfate were elevated ($P<0.05$) significantly in patients group, while serum levels of follicle-stimulating hormone declined in the PCOS females group, otherwise kisspeptin level increase ($P>0.05$) insignificantly in patients (494.5±57.7) than control (388.1±7.2).

Furthermore, gene analysis revealed that rs372790354 and rs4889 were associated significantly ($P<0.01$) with PCOS in dominant, recessive, and co-dominant models, while the over dominant model was significant [$P=0.04$, OR=2.14, CI (2.7-167.9)] with PCOS at rs4889 only.

Herein rs37279054 AA was linked significantly ($P=0.008$) in normal weight groups (PCOS female compared to control), while for rs4889 genotypes GA and AA were linked significantly ($P<0.05$) with all subgroups in the present study except the GA genotype in obese females were changed insignificantly, and mostly related with waist/hip ratio, luteinizing hormone to follicle-stimulating hormone ratio, free testosterone and dehydroepiandrosterone sulfate in PCOS females when compared with control. In the design of patient's subgroup, GA and AA genotypes for the same SNP were changed significantly ($P<0.01$) when compared between normal weight and obese related to the levels of the waist to hip ratio while kisspeptin (mean ± SE in normal weight 1062 ± 187, in obese 372.2 ± 51.4) exhibited highly significant ($P=0.007$) association with AA genotype only.

Receiver operating characteristic analysis result for kisspeptin of AA genotype in normal weigh PCOS females for rs4889 was recorded

that area under the curve equal to 0.91 with specificity 93% and 67% sensitivity at a cut-off value was 484.2 ng/ml.

The current study concluded that dysregulation of kisspeptin lead to an increased level of LH that contributes to the pathogenesis of PCOS. Polymorphisms of *KISS1*gene (rs3727354, rs4889) play an important role in development of PCOS, especially at the level of mutant GA and AA genotypes that related to the elevated levels of waist to hip ratio, luteinizing hormone to follicle-stimulating hormone ratio, free testosterone and dehydroepiandrosterone sulfate.

Finally, kisspeptin considers a good indicator for normal weight PCOS females that have AA genotype of *KISS1* gene rs4889 and no strong effect on obese females.

Contents

List of Tables

List of Figures

List of Scheme

Abbreviations

Abbreviation	Details
Abs	Absorbance
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of Variables Test
AE-PCOS	Androgen Excess & PCOS Society
AR	Androgen Receptor
AVPV	Anteroventral Periventricular Nucleus
Ab	Antibody
Ag	Antigen
ARC	Arcuate Nucleus
BMI	Body Mass Index
CVD	Cardiovascular Disease
CLIA	Chemiluminescent immunoassay technology
X^2	Chi – Square
CAH	Congenital Adrenal Hyperplasia

R	Correlation Coefficient
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
DAG	Diacylglycerol
ER	Endoplasmic Reticulum
E2R	Estrogen Receptor
E1	Estrone
EDTA	Ethylenediaminetetraacetic Acid
FSH	Follicle-stimulating hormone
FFA	Free fatty acid
GPR54	G Protein-Coupled Receptor
GWAS	Genome wide association studies
Gly	Glycine
GnRH	Gonadotrophin releasing hormone
HWE	Hardy-Weinberg Equilibrium
HRM	High resolution melt
HRP	Horseradish Peroxidase
HPG	Hypothalamic pituitary gonad axis
HPO	Hypothalamic pituitary ovary axis
IP_3	Inositol 1,4,5-Trisphospate
IR	Insulin resistance
KNDy	Kisspeptin, Neurokinin B, And Dynorphin Neurons

Leu	Leucine
LH	luteinizing hormone
LH/FSH ratio	Luteinizing hormone /Follicle-stimulating hormone ratio
KISS1	Metastasis suppressor gene
Met	Methionine
NIH	National Institutes of Health
OR	Odds Ratio
OD	Optical Density
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PLC	Phospholipase C
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovarian syndrome
PCR	Polymerase Chain Reaction
POA	Preoptic Area
Pro	Proline
PKC	Protein Kinase C
RNA	Ribonucleic acid
ROT	Rotterdam Criteria
SE	Sandard Error
SHBG	Sex-hormone binding globulin
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package for Social Sciences
ELISA	The Enzyme-Linked Immunosorbent Assay

TSH	Thyroid Stimulating Hormone
T2DM	Type2 diabetic mellitus
U/S	Ultra Sound
UTR	Un translation region
WHR	Waist To Hip Ratio
WHO	World Health Organization
Xg	Multiple gravity
E2	17-beta estradiol
17-HSD	17-beta hydroxyl steroid dehydrogenases

الخلاصة :

متلازمة تكيس المبايض (PCOS) هي واحدة من أكثر أمراض الغدد الصماء شيوعًا المصنفة بفرط الأندروجين الذي يؤثر على سن الإنجاب المبكر ، يلعب جين *KISS1* دورًا في تنظيم محور الغدة النخامية – الغدة تحت المهاد ، وهو التنظيم غير الطبيعي الذي تطور في متلازمة تكيس المبايض. تم تصميم هذه الدراسة لتقدير العلاقة بين تعدد الأشكال الجيني *KISS1* (rs3727354G> A , rs4889G> A)مع التسبب في متلازمة تكيس المبايض ، وكذلك لتقييم ارتباط مستوى الكيسبيبتين بهرمون الغدد التناسلية وعلاقته بحدوث متلازمة تكيس المبايض.

تم جمع عينات الدم من 120 أنثى (60 عينة مقسمة إلى 30 وزن طبيعي و 30 سمنة) و (60 مريض مقسمة إلى 30 وزن طبيعي و 30 سمنة). تم استخلاص الحمض النووي من عينات الدم وتحليل المتغيرات الجينية (rs3727354, rs4889) *KISS1*, حيث تم قياسها بواسطة تقنية ذوبان عالية الدقة (HRM-PCR) وتم قياس مستوى كيسبيبتين في المصل بواسطة مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA) ، بينما الهرمون اللوتيني (LH) ، والهرمون المنبه للجريب (FSH) ، وكبريتات ديهيدرو إيبي أندروستيرون (DHEA-S) والتستوستيرون الحر عن طريق المقايسة المناعية الإشعاعية.(CLIA)

أشارت النتائج الحالية إلى أن مستويات الهرمون اللوتيني والتستوستيرون الحر وكبريتات ديهيدرو إيبي أندروستيرون ارتفعت بشكل ملحوظ في مجموعة المرضى ، بينما انخفضت مستويات الهرمون المنبه للجريب في المصل لمجموعة إناث متلازمة تكيس المبايض ، وانخفضت مستويات الكيسبيبتين (57.7±494.5) تزداد (P> 0.05) بشكل ضئيل في المرضى عن المجموعة الضابطة (388.1 ± 7.2).

علاوة على ذلك ، كشف تحليل الجينات أن rs372790354 و rs4889 ارتبطا معنويا (P <0.01)مع متلازمة تكيس المبايض في النماذج السائدة والمتنحية والسيطرة المشتركة ، في حين أن النموذج السائد كان معنويا((P = 0.04, OD = 2.14, CI (2.7- 167.9) مع متلازمة تكيس المبايض عند rs 4889 فقط ، هنا تم ربط AA rs37279054 بشكل كبير (P = 0.008) في مجموعات الوزن الطبيعي PCOS أنثى مقارنة بالمجموعة الضابطة ، بينما في rs4889 تم ربط الأنماط الجينية GA و AA بشكل كبير (P <0.05) مع جميع المجموعات الفرعية في هذه الدراسة ، تم تغيير النمط الجيني GA في الإناث البدينات بشكل طفيف ، فيما يتعلق بالمستويات المرتفعة لنسبة الخصر إلى الورك ، ونسبة الهرمون اللوتيني إلى نسبة

الهرمون المنبه للجريب ، وهرمون التستوستيرون الحر وكبريتات ديهيدرو إيبي أندروستيرون في إناث متلازمة تكيس المبايض.

في تصميم المجموعة الفرعية للمريض ، تم تغيير الأنماط الجينية GA و AA لنفس SNPبشكل ملحوظ (P <0.01) عند المقارنة بين الوزن الطبيعي والسمنة المرتبطة بمستويات نسبة الخصر إلى الورك بينما كيسبيبتين (mean ± SE في الوزن الطبيعي أظهر 1062 ± 187 ، في السمنة 372.2 ± 51.4) ارتباطًا ذا دلالة عالية (P = 0.007) بالنمط الجيني AA فقط. تم تسجيل نتيجة تحليل ROC لـ kisspeptin من النمط الجيني AA في الإناث ذات الوزن الطبيعي من متلازمة تكيس المبايض لـ rs4889 كانت المنطقة تحت المنحنى (AUR) تساوي 0.91 مع خصوصية 93٪ و 67٪ حساسية عند قيمة قطع كانت 484.2 نانوغرام / مل.

لخصت الدراسة الحالية إلى أن عدم انتظام مادة الكيسبيبتين يؤدي إلى زيادة مستوى الهرمون اللوتيني الذي يساهم في التسبب في متلازمة تكيس المبايض. تلعب الأشكال المتعددة لجين (*KISS1* rs3727354, rs4889) دورًا مهمًا في تطوير متلازمة تكيس المبايض ، خاصة على مستوى الأنماط الجينية GA و AA الطافرة المرتبطة بالمستويات المرتفعة لنسبة الخصر إلى الورك ، الهرمون اللوتيني إلى نسبة الهرمون المنبه للجريب والتستوستيرون الحر وكبريتات ديهيدرو إيبي أندروستيرون. أخيرًا ، يعتبر كيسبيبتين مؤشرًا جيدًا للإناث ذات الوزن الطبيعي من متلازمة تكيس المبايض التي لديها نمط وراثي AA من جين *KISS1* rs4889 وليس لها تأثير قوي على الإناث البدينات.

متلازمة تكيس المبايض (PCOS) هي واحدة من أكثر أمراض الغدد الصماء شيوعًا المصنفة بفرط الأندروجين الذي يؤثر على سن الإنجاب المبكر لدى السيدات حيث يلعب جين *KISS1* دورًا في تنظيم محور الغدة النخامية – الغدة تحت المهاد ، وهو التنظيم غير الطبيعي الذي تطور في متلازمة تكيس المبايض وأن عدم انتظام مادة الكيسبيبتين يؤدي إلى زيادة مستوى LH الذي يساهم في التسبب في متلازمة تكيس المبايض حيث تلعب الأشكال المتعددة لجين (*KISS1* rs3727354, rs4889) دورًا مهمًا في تطوير متلازمة تكيس المبايض ، خاصة على مستوى الأنماط الجينية GA و AA الطافرة المرتبطة بالمستويات المرتفعة لنسبة الخصر إلى الورك ، LH/FSH Ratio والتستوستيرون الحر و DHEA-S. أخيرًا ، يعتبر كيسبيبتين مؤشرًا جيدًا للإناث ذات الوزن الطبيعي من متلازمة تكيس المبايض التي لديها نمط وراثي AA من جين *KISS1* rs4889 وليس لها تأثير قوي على الإناث البدينات.

تم جمع عينات الدم من 120 أنثى (60 عينة مقسمة إلى 30 وزن طبيعي و 30 سمنة) و (60 مريض مقسمة إلى 30 وزن طبيعي و 30 سمنة). تم استخلاص الحمض النووي من عينات الدم وتحليل المتغيرات الجينية (rs3727354, rs4889) *KISS1*, حيث تم قياسها بواسطة تقنية ذوبان عالية الدقة (HRM-PCR) وتم قياس مستوى كيسبيبتين في المصل بواسطة (ELISA) ، بينما LH، FSH، DHEA-S والتستوستيرون الحر عن طريق CLIA

أشارت النتائج إلى أن مستويات LH والتستوستيرون الحر ,DHEA-S ارتفعت بشكل ملحوظ في مجموعة المرضى ، بينما انخفضت مستويات FSHفي المصل لمجموعة إناث متلازمة تكيس المبايض ، وانخفضت مستويات الكيسبيبتين (57.7±494.5) تزداد (P> 0.05) بشكل ضئيل في المرضى عن المجموعة الضابطة (388.1 ± 7.2).

علاوة على ذلك ، كشف تحليل الجينات أن rs372790354 و rs4889 ارتبطا معنويا (P <0.01)مع متلازمة تكيس المبايض في النماذج السائدة والمتنحية والسيطرة المشتركة ، تم ربط AA rs37279054 بشكل كبير في مجموعات الوزن الطبيعي PCOS أنثى مقارنة بالمجموعة الضابطة ، بينما في rs4889 تم ربط الأنماط الجينية GA و AA بشكل كبير (P <0.05)مع جميع المجموعات الفرعية في هذه الدراسة ، تم تغيير النمط الجيني GA في الإناث البدينات بشكل طفيف. تم تسجيل نتيجة تحليل ROC لـ kisspeptin من النمط الجيني AA في الإناث ذات الوزن الطبيعي من متلازمة تكيس المبايض لـ rs4889 كانت المنطقة تحت المنحنى (AUR)تساوي 0.91 مع خصوصية 93٪ و 67٪ حساسية عند قيمة قطع كانت 484.2 نانوغرام / مل.

COMMITTEE CERTIFICATION

We, the examination committee, certify that we have read the thesis entitled "**Genetic Polymorphisms of *KISS1* with Biochemical Features of kisspeptin and Gonadotrophic Hormones in Polycystic Ovary Syndrome"** and have examined the student Noor Jamal Talib in its contents and that in our opinion it is accepted as a thesis for degree of Master of Science in clinical biochemistry with excellent estimation.

<table>
<tr><td colspan="2">Signature:
Professor
Dr. Dhafer Abd Al Mahdi Faisal
Faculty of Pharmacy
University of Kufa
Date: / / 2022
(Chairman)</td></tr>
<tr><td>Signature:
Professor
Dr. Bushra Jaber AL-Rubaie
Department of Obstetrics and Gynecology
College of Medicine
University of Babylon
Date: / / 2022
(Member)</td><td>Signature:
Assistant Professor
Dr. Zinah Abbass Ali
Department of Biochemistry
College of Medicine
University of Babylon
Date: / / 2022
(Member)</td></tr>
<tr><td>Signature:
Professor
Dr. Suhayr Aesa Al-Qaysi
Department of Biochemistry
College of Medicine
University of Babylon
Date: / / 2022
(Supervisor)</td><td>Signature:
Professor.
Dr. Suha Jasim witwit
Department of Obstetrics and Gynecology
College of Medicine
University of Babylon
Date: / / 2022
(Supervisor)</td></tr>
<tr><td colspan="2">Approved by the council of the college of medicine</td></tr>
<tr><td colspan="2">Signature
Professor
Dr. Mohend AL Shalah
Department of Surgery
College of Medicine-University of Babylon
Date: / / 2020</td></tr>
</table>

1 Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common endocrinopathies diseases that affect early reproductive age in females, the first description of PCOS in 1935 by Stein and Leventhal. The diagnose is achieved by three different criteria that were used in this field and was developed by the National Institutes of Health (NIH) in 1990, Rotterdam Criteria (ROT) in 2003, Androgen Excess and PCOS Association (AE-PCOS) criteria in 2006 [1].

The prevalence of PCOS is depended on the method of diagnosis, Word Health Organizations (WHO) estimate the prevalence of PCOS worldwide is 2% to 26% [1, 2], with a high prevalence in obese patients at approximate 73%, the type of obesity is android, with a higher waist to hip ratio and fat in the front abdominal wall [3].

The main pathophysiological characteristic of PCOS is androgen excess with a prevalence of 60–80%[5,6], the biochemical hyperandrogenism includes an increase in total testosterone, free testosterone, dehydroepiandrosterone(DHEA), the dehydroepiandrosterone sulfate(DHEA-S) and androstenedione [6].

A dysfunctional interaction of behavioral, environmental, and genetic factors causes PCOS. Enlargement of ovaries, as well as secreting higher levels of androgens than normal theca cells are the most common clinical presentations of PCOS. Increased androgenic secretion results from increased enzyme activity in the steroid production pathway [7].

The hypothalamic-pituitary-gonadal (HPG) axis controls reproductive function, kisspeptin is a neuropeptide encoded by metastasis suppressor gene (*KISS1*) that release from kisspeptin neuron and effective stimulator of gonadotropin-releasing hormone(GnRH) neurons in the hypothalamus to

controlling the production and release of gonadotropins that regulate gonadal function, the abnormal regulation of kisspeptin leads to an abnormal hypothalamic- pituitary- ovary (HPO) axis that developed into PCOS [8].

Kisspeptin is also influenced by leptin and insulin leading to an increase in luteinizing hormone (LH) level and activating reproductive axis and stimulating GnRH release, therefore, obesity has been associated with abnormal HPO axis function that leads to the development of PCOS [9].

1.1 Hypothesis

1. The *KISS1* gene polymorphism has been implicated in the development of the PCOS.
2. One or more SNPs are expected to be associated with disease severity.

1.2 Polycystic Ovarian Syndrome

1.2.1 Definition

Stein and Leventhal, described seven females suffering from hirsutism, amenorrhea, and enlarged ovaries with multiple cysts [10,11], multiple ovarian cysts, which signify immature follicles, are a common finding on ultrasound examination, giving the disease its name. The follicles formed from primordial follicles, but due to ovarian dysfunction, they stopped developing at an early antral stage, on ultrasound (U/S), the follicles may be positioned along the ovarian perimeter and appear as a string of pearls as shown in figure (1-1) [12].

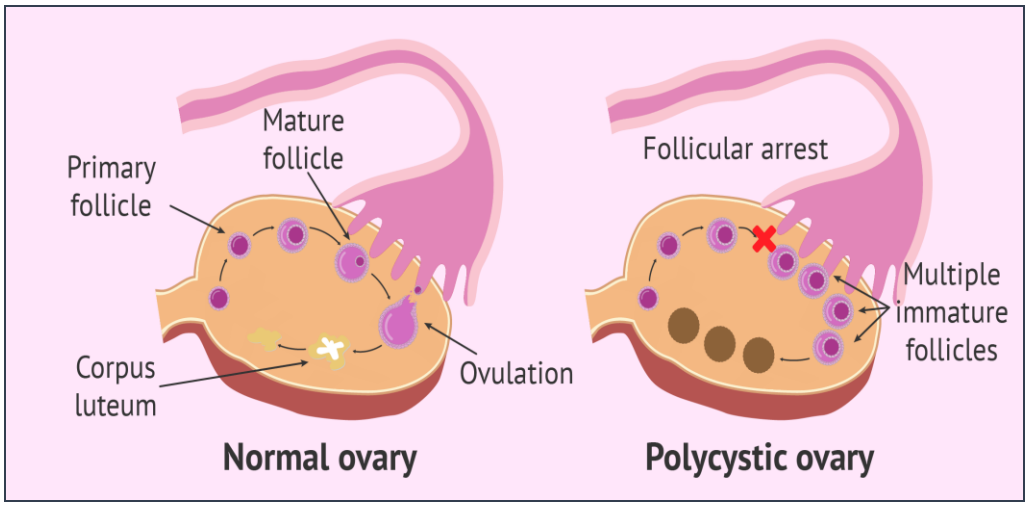


Figure 1-1: characters of Polycystic ovaries [13]

Polycystic ovarian syndrome is a heterogeneous genetic disorder characterized by hyperandrogenism, it's can cause impaired ovulation and infertility. PCOS negatively affects the endocrine, menstrual disturbance, heart, metabolic and vascular health due to the presence of insulin resistance and hyperinsulinemia are common and puts those affected at risk of diabetes, cardiovascular disease, and other long term of endometrial cancer and metabolic syndrome [14].

Obesity contributes to the pathogenesis of the characteristics of PCOS containing hyperandrogenemia(HA), insulin resistance (IR), and infertility which appears to play an important role in the manifestation of this syndrome [17 ,16].

1.2.2 Phenotypic Classification of Polycystic Ovarian Syndrome

The classification of PCOS based on the presence or absence of key features using phenotypic classification into four groups [17]:

- ‘‘Classic’’ PCOS (Phenotypes A): Hyperandrogenism; ovulatory dysfunction, polycystic ovarian morphology.
- ‘‘Classic’’ PCOS (Phenotypes B): Hyperandrogenism, ovulatory dysfunction.
- ‘Ovulatory PCOS’’ (Phenotype C): Hyperandrogenism, polycystic ovarian morphology.
- ‘Non hyperandrogenic PCOS’’ (Phenotype D) : Polycystic ovarian morphology, ovulatory dysfunction.

1.2.3 Diagnostic Criteria of Polycystic Ovarian Syndrome

The diagnosis method of PCOS is different by phenotype, all phenotypes are diagnosed according to ROT, the two phenotypes (A, B) are diagnosed by NIH, and the three phenotypes (A, B, C) are diagnosed by AE-PCOS [18,13]. The definition of PCOS patients as having at least two of the following characteristics according to the AE-PCOS association in 2006:

a. Ovarian dysfunction(OD) (oligo or amenorrhea, infertility) less than 6-9 menstruation per year [18]**.**

b. Polycystic ovarian morphology (PCOM) the presence of stromal hypertrophy (numerous follicles and enlarged ovaries) and high ovarian volume with ultrasound for patients with polycystic ovaries (≥12 small follicles in ovary), excluding medical conditions such as Cushing's syndrome, androgen-secreting tumors or congenital adrenal hyperplasia [2,19,20].

c. Clinical hyperandrogenism signs such as hirsutism, acne, and androgenetic alopecia (modified Ferriman-Gallwey score >8) [20], the modified Ferriman-Gallwey score is one of the methods known to measure and evaluate the severity of hirsutism in females, the modified Ferriman-Gallwey score assesses hair growth in nine different areas of the body sensitive to excess androgen: the chin, upper lip, lower and upper back, chest, around the abdomen (up and down), thigh and upper arm [11].

Where score 4 represents normal overall growth hair while score 8 represents the excess of the hair growth or higher to be called hirsutism, 0 score represents no terminal hair growth [21], the maximum degree is 36, as it is degree 8-16: hirsutism is mild and degree 17-24: moderate while severe ones are more than 24 by using a visual examination [21,22].

1.2.4 Epidemiology of Polycystic Ovarian Syndrome

The characteristics of PCOS are irregular menstruation, and hirsutism, as well as an elevated risk of a variety of disorders, including insulin resistance, dyslipidemia, and infertility. PCOS is a heritable disorder if there is a family history of the condition [23], a higher prevalence of PCOS has been linked to a measured of health problems. Obesity history that increases frequently precedes and follows the emergence of PCOS clinical characteristics [4,6].

The prevalence of PCOS depends on the population studied and the criteria used for diagnosis, it affects 6.1-7.1% of females, according to NIH definitions, and increase two to three times reaching approximately 14.6% and 19.9%, according to ROT, and from 11.7% and 15.3% according to the AE-PCOS criteria were applied in Iran and Turkey populations respectively [2]. The association between PCOS and obesity supported by epidemiological data, the prevalence of overweight or obese female with PCOS are between 38-88% [5].

1.2.5 Aetiology for Polycystic Ovarian Syndrome

Many functions implications in the aetiology of PCOS whether internal or external alteration like hyperandrogenism (HA), insulin resistance (IR), inflammation, oxidative stress, and obesity while the external factors including environmental factors, physical and emotional stress, unhealthy lifestyle, diet, and epigenetics factors [16,24].

a. **Internal Factors Effect:** The low response cell to insulin leads to a high level of insulin in blood (hyperinsulinemia) that androgen direct triggers production in ovarian theca cell and reduces production of hepatic sex hormone binding globulin (SHBG) and increases the level of free testosterone.

   Pro-inflammatory cytokines produce during follicular development and participate in inducting ovulation while continuous chronic inflammation can impair follicular growth such as interleukin1(IL-1) effect on the LH and FSH receptors inhibition of these receptors leads to inhibition of follicular maturation and ovulation [25]. Autoimmune disease, systemic lupus, epilepsy and endocrine disruptor chemicals (including certain drugs) has been shown to play a role in some females with PCOS [24,25].

b. External Factors Effect:

- Epigenetic mechanism refers to inheritable alterations in genome and gene expression without any change in DNA sequence, these changes involve adding or deleting chemical compounds to DNA or histone, for example, the hypomethylation process of the LH receptor in theca cell leads to higher gene expression and sensitivity to LH and increased LH activity response to follicle development and increase steroidogenesis process and finally increase androgen level that is responsible for increases risks of PCOS [24,28,29].
- Genetic factors also play a role in the pathogenesis of PCOS as single nucleotide polymorphism (SNP) in genes. Changes can causes a defect in the transcriptional activity of several mutations which associated with gene that leads to PCOS [31–34], and having different function and affecting varied biochemical pathways like:
  - Ovarian and adrenal steroidogenesis.
  - Pathway of *KISS1*, kisspeptin and hypothalamus-pituitary-ovary axis (*KISS1* and gonadotrophic releasing hormone gene GnRH/GnRHR).
  - Gonadotropin action and regulation (LH receptor and FSH receptor).
  - Gene of insulin action and secretion.
  - Inflammation are associated with PCOS[34].

1.2.6 Pathogenesis of Polycystic Ovarian Syndrome

The pathogenesis of PCOS is interactions between endocrine, metabolic, genetic, and environmental factors, abnormality of HPO axis or adrenal axis. Kisspeptin a hypothalamic peptide encoded by the *KISS1* gene, the role of kisspeptin is activation signals GnRH neurons directly through its action on the kisspeptin receptor to release GnRH into the portal circulation. Theca cell hyperactivity cause hypersecretion of LH, hypofunction of granulosa cell axis cause a low level of FSH, also the abnormal regulation of LH/FSH ratio from the gonadotrophs of the anterior pituitary gland [35–37] leads to hyperandrogenism that is present in more than 60% of females with PCOS [38].

The ratio of LH to FSH in healthy females normally between 1 to 2, this ratio is increased in females with PCOS, and it can reach as high as 2 or 3 in some cases because of the increased frequency of GnRH [37,38], leading to impaired follicular maturation, and reduced inhibition of the GnRH pulse generator by progesterone, production of steroid hormones are shared by the ovaries and adrenal glands response to LH and adrenocorticotrophic hormone (ACTH) respectively. The adrenal gland is the primary producers of DHEA and its sulfate ester (DHEA-S) (90 percentage of the total circulating amount).

Although roughly 40-70 percentage of females with PCOS have elevated DHEA-S levels. The specific process that causes the adrenal gland to produce androgens is yet unknown, DHEA also appeared to have a positive effect on the number of follicles and ovarian volume in female with PCOS [41], also obesity plays a role in hyperinsulinemia, insulin resistance(IR), and hyperandrogenism as it increases the frequency of GnRH pulses, the dominance of LH over FSH, ovarian androgen production, follicular maturation, and SHBG binding. All of these factors play a role in the progression of PCOS [6,31].

Although skeletal muscles, adipose tissue, and liver lose insulin sensitivity while the ovary and adrenal gland do not. Hyperinsulinemia increases GnRH neuron activity and insulin stimulates androgen synthesis in ovarian theca cells, and the combination of LH by insulin stimulates steroidogenic activity. Hyperinsulinemia promotes LH binding sites and the androgen-producing response to LH with lowering hepatic SHBG, resulting in higher blood levels of free testosterone [24,35–37]. In addition, environmental factors may initially cause IR or bind as agonists to estrogen or androgen receptors, eventually contributing to PCOS [27,37,46], 30% of females with PCOS will have normal menses, approximately 85%–90% of females with oligomenorrhea have PCOS while 30%–40% of females with amenorrhea will have PCOS that summarized in figure (1-2) [63,89].

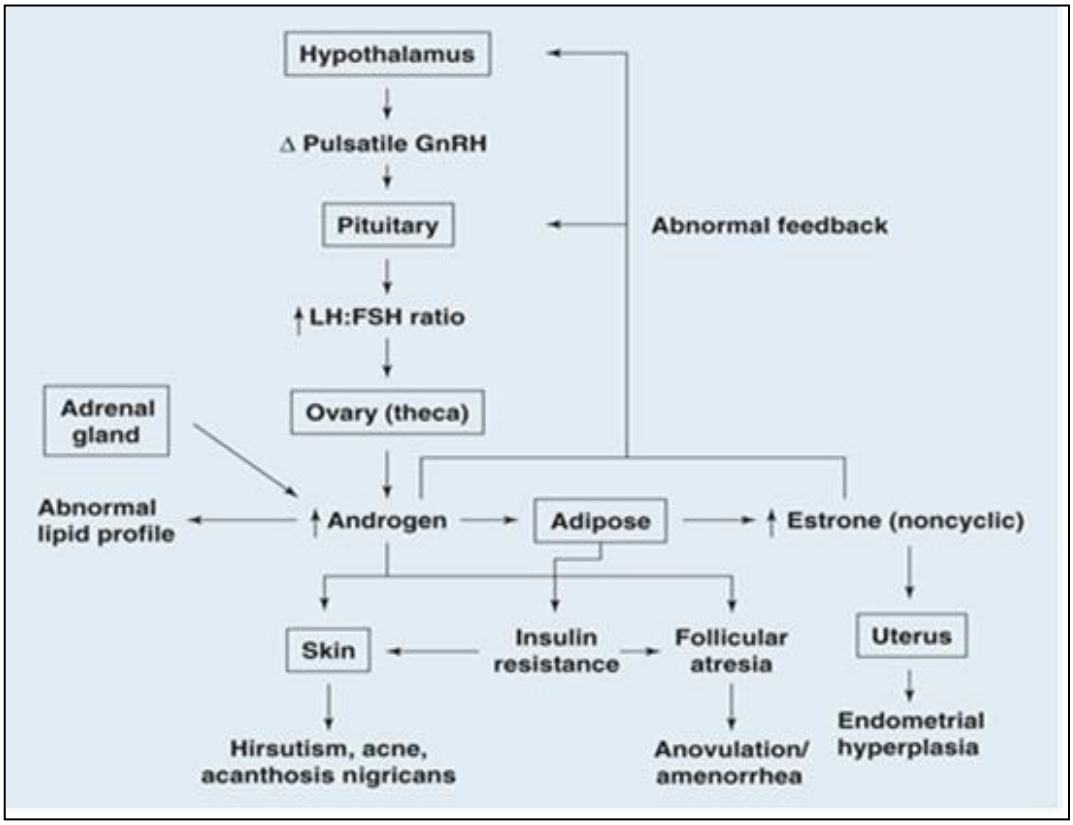


Figure (1-2) Pathogenesis of polycystic ovarian syndrome [47,48]
symbols: ↑ (increased), ↓ (decreased), GnRH (Gonadotropin-releasing hormone), LH (luteinizing hormone), FSH (follicle stimulate hormone).

1.2.7 Clinical Feature for Polycystic Ovarian Syndrome

The commonly clinical observed in PCOS are menstrual disturbances including oligomenorrhea, amenorrhea, and prolonged erratic menstrual bleeding caused by infrequent or absent ovulation [51], and another clinical feature of PCOS are hirsutism and acne as results of hyperandrogenism, approximately 15-30% of an adult females with PCOS present with acne [52], as well as the characteristic morphological appearance of polycystic ovary "cysts are a sign instead of the cause of the disease" due to excess androgen in PCOS stimulates multiple ovarian follicles in an equivalent time thus prevents the development of a single dominant follicle and leads to ovulation failure and relative resistance to gonadotropins [53].

1.2.8 Treatment for Polycystic Ovarian Syndrome

The United Food and Drug Administration(UFDA) information's there is no treatment for PCOS, oral contraceptives, antiandrogens therapy, anti-insulin drugs such as metformin, and follow ovulation with used inducers are commonly used, as well as PCOS is treated by an arrangement of lifestyle changes, including weight loss [24,36].

1.2.9 Complications for Polycystic Ovarian Syndrome

The polycystic ovarian syndrome can cause many abnormalities throughout the life of affected female including impaired fertility, and even if pregnancy is achieved, females with PCOS have a greater risk of pregnancy related complications such as gestational diabetes, preeclampsia, increased risk of miscarriage and premature delivery, and about 10 times more common in PCOS females compared to healthy controls [55].

In addition, patients with PCOS have three times increased risk for endometrial hyperplasia and carcinoma [9,55], as well as mood disorders (psychological disorders) including anxiety and depression binge eating, psychosexual dysfunction, and insufficient sleep and stress [57]. PCOS is one of the most common reasons of insulin resistance, obesity, metabolic syndrome, cardiovascular diseases, dyslipidemia, and type 2 diabetes mellitus (T2DM).

Metabolic syndrome is a collection of metabolic abnormalities that predispose people to metabolic diseases such as T2DM, cardiovascular disease, hypertension, and dyslipidemia.

Obesity, insulin resistance, and high blood pressure are all essential to the diagnosis of metabolic syndrome [58]. On the other hand, obesity, insulin resistance, and dyslipidemia are all common symptoms in PCOS patients. These diagnostic similarities between metabolic syndrome and PCOS also result in females with PCOS having higher risks of metabolic syndrome than females without PCOS.

The frequency of metabolic dysfunction in patients with PCOS is reported to be as high as 43% in these patients [25,49] as in the figure (1-3). PCOS is often also associated with excess body weight (increasing body mass index), females with PCOS have an increase of androgens, which can result in increased abdominal and subcutaneous fat and increase fatty acids [60].

Hypertension was present in 22% of females with PCOS compared to 2.1% of controls [11] that summarize in the figure (1-3), females with PCOS have an increased risk of dyslipidemia (higher levels of total cholesterol, low-density lipoprotein cholesterol, and triglyceride) than healthy females, so obesity, diabetes mellitus, hypertension, and dyslipidemia are well-recognized risk factors for cardiovascular disease [46,47] in females with PCOS.

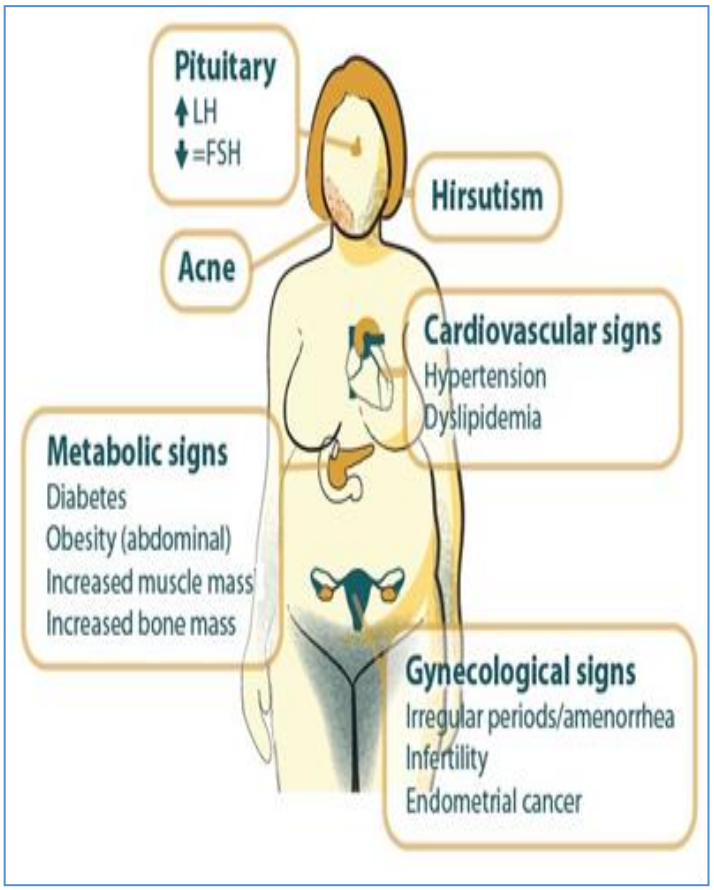


Figure 1-3 : Clinical features and complications of polycystic ovary syndrome [63,89]
↑ (increased), ↓ (decreased), LH (luteinizing hormone), FSH (follicle stimulate hormone).

Insulin resistance develops most commonly in females with PCOS which may lead to dyslipidemia and type 2 diabetes mellitus at all body weights [25,54].

The skeletal muscles, adipose tissue, and the liver of females with PCOS have lost their insulin sensitivity, they uptake free fatty acids as the energy source instead of glucose. The hyperglycemia leads to a pancreas rapid reaction and hyperinsulinemia [63].

1.3 Hormonal Changes in Polycystic Ovarian Syndrome

Polycystic ovarian syndrome may occur due to impaired neuronal pathways in the brain that control of HPO axis by the effect of kisspeptin neuron to produce kisspeptin that stimulating the GnRH neuron to produce the GnRH from the hypothalamus then it's effect on gonadotrophic cell in pituitary gland to produce LH and FSH [64] as shown in figure (1-4), continuously with figure (1-5) that explain the direct effect of LH and FSH to stimulate ovary to produce steroid hormones (estrogen, progesterone, and androgens), the steroid hormone has a regulation effect on the HPO axis in normal status through negative feedback on the hypothalamus and pituitary gland to decrease gonadotrophin secretion. In PCOS, increased GnRH pulsatility results the increased LH release, which in turn stimulates increased ovarian androgen secretion [65].

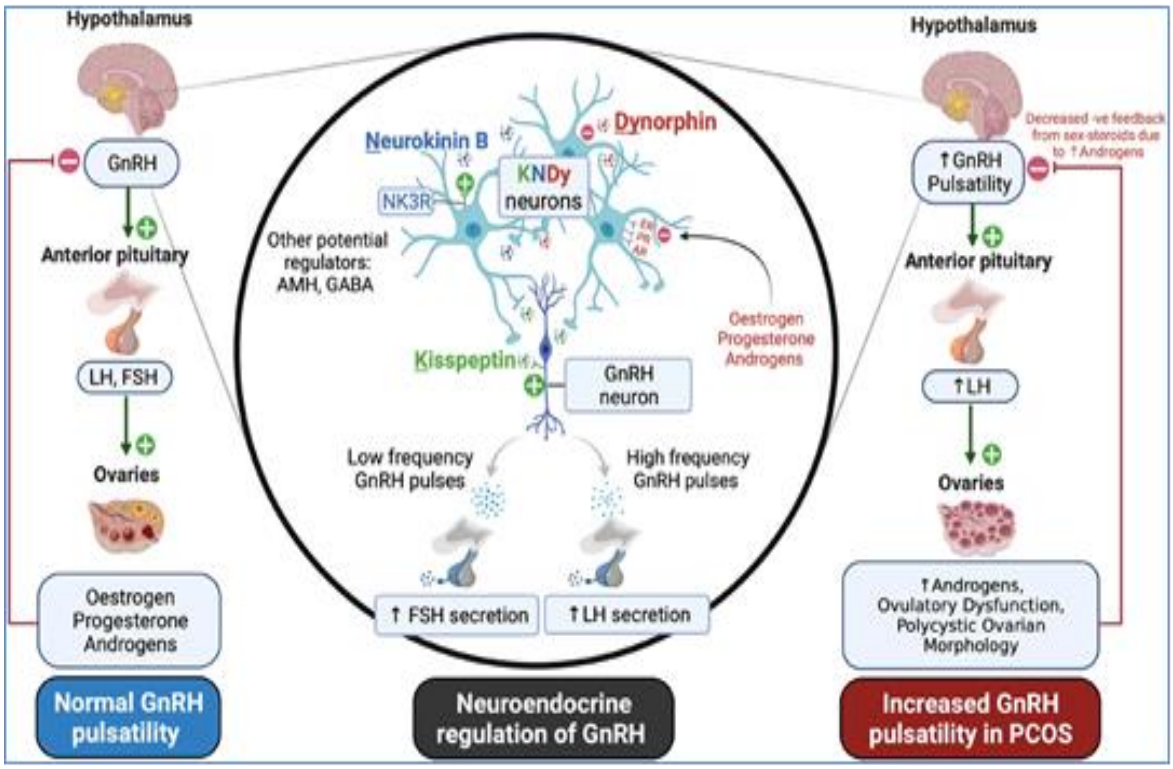


Figure 1-4: Regulation of neuroendocrine in hypothalamus-pituitary-ovary axis[48].

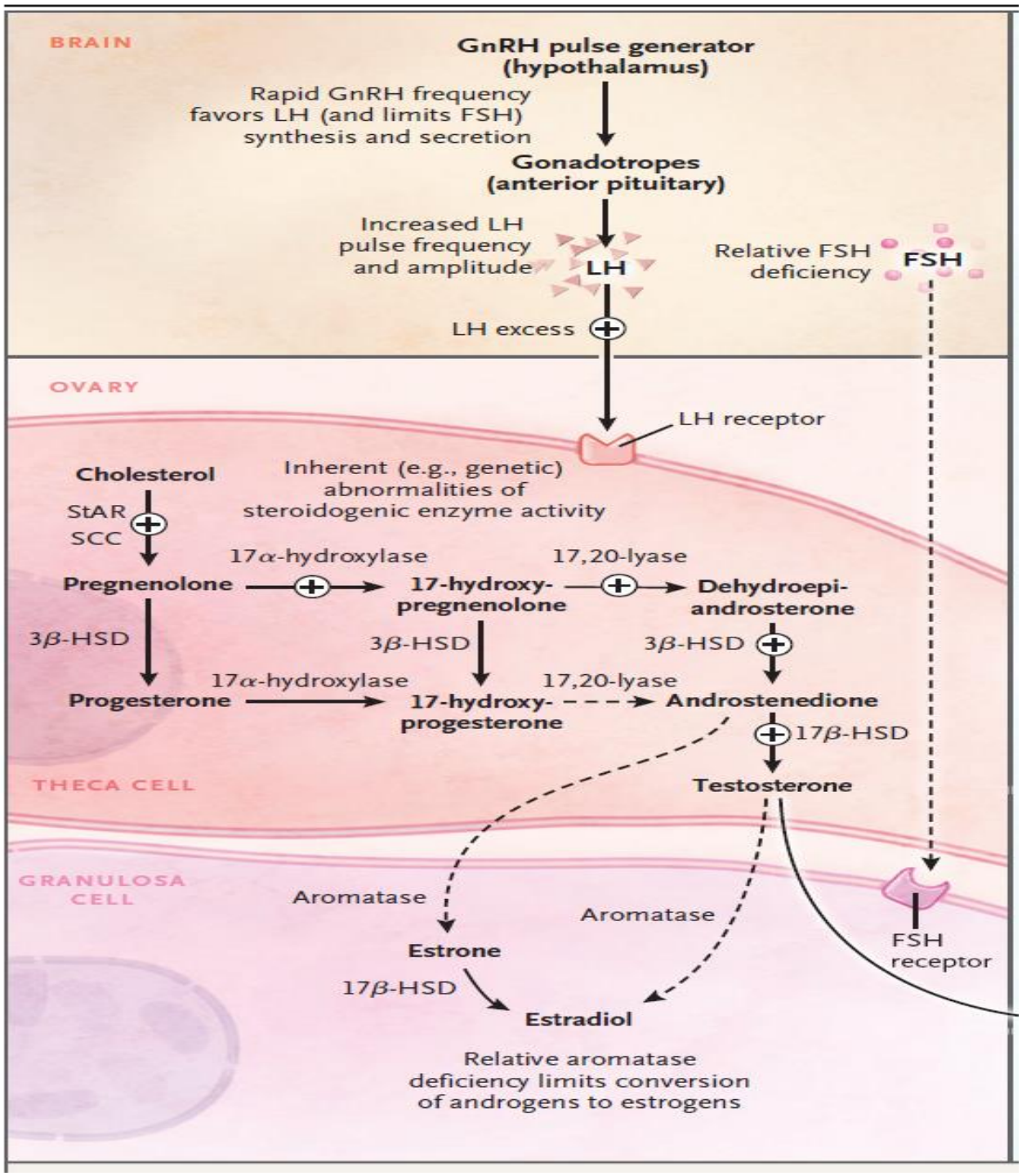


Figure 1-5: Hormonal changes in polycystic ovarian syndrome [66]

Symbol: 3β-HSD: 3β-hydroxysteroid dehydrogenase, 17β-HSD 17β-hydroxysteroid dehydrogenase, SCC cholesterol side-chain cleavage enzyme, and StAR steroidogenic acute regulatory protein.

1.3.1 Gonadotropin Secretion

a. Follicle Stimulating Hormone

Glycoprotein hormone the molecular weight of FSH is 25000 Kilo Dalton, dimers of α and β-chains linked by noncovalent bond. β-chain of FSH is 118 amino acids each chain has several disulfide bridges [67], it is synthesized and secreted by gonadotrophs β-cells in the anterior pituitary gland.

The essential for gonadal development is FSH, as well as the follicular growth, pubertal maturation, and reproductive activities, Which enhances the release of estrogen and prepares the follicular development to LH, in reproduction, FSH and LH act simultaneously [68], FSH affects the function of granulosa cell, allowing the aromatase enzyme to convert the resulting androgen to estrogen as in figure (1-6). It acts by binding to specific receptors found only in the gonads. The FSH receptor is the G protein-coupled receptor family [69].

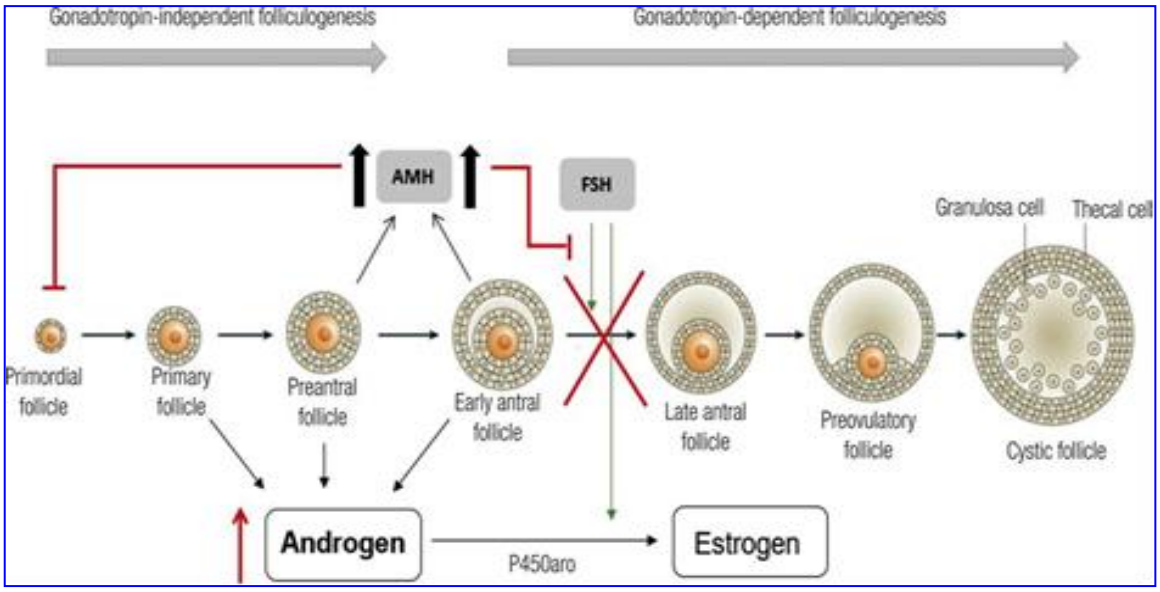


Figure 1-6: Follicular development [70]
↑ (increased), ↓ (decreased), FSH (follicle stimulate hormone) AMH (anti moliarian hormone), P450 (cytochrome P450, aromatase).

b. Luteinizing Hormone

Luteinizing Hormone (LH) is a diverse glycoprotein hormone, molecular weight of LH is 40000 Kilo Dalton, heterodimers of α and β-chains subunit linked by noncovalent bond. The β-chain LH is 112 amino acids each chain has several disulfide bridges [67]. It is synthesized and secreted by gonadotrophs β-cells in the anterior pituitary gland**.**

The role of LH is responsible for follicular and oocyte development, ovulation, and the formation of the corpus luteum, LH causes the ovarian theca cells to create androgens during folliculogenesis production from cholesterol, LH stimulates the corpus luteum to secrete estrogen and progesterone after ovulation [71], the activity of LH during ovulation that is LH required for final follicular growth and ovulation. The follicle will not develop to the phase of ovulation without this hormone, even if significant amounts of FSH are available, LH acts synergistically with FSH to cause rapid follicle swelling just before ovulation.

Ovulation and the development of the corpus luteum are triggered by an acute rise in LH ("LH surge"). LH surge is secreted by the pituitary during the day immediately prior to ovulation [72].

c. Luteinizing Hormone to Follicle Stimulating Hormone Ratio

Early follicular development required a high balance of LH and FSH, in normal females LH/FSH ratio in the early follicular phase is normally one, abnormal gonadotropin secretion has contributed to the pathogenesis of PCOS as LH levels are too high, up to 2:1 or 3:1[73].

The decrease in FSH level for a long time leads to a decrease in follicular maturation, the immature follicles may remain as a cysts

(polycystic morphology), subsequently, and don't occur the ovulation. Also, progesterone hormone in serum will be low and lose the negative feedback on kisspeptin and GnRH released [74–76] as in figure (1-7).

1.3.2 Androgens

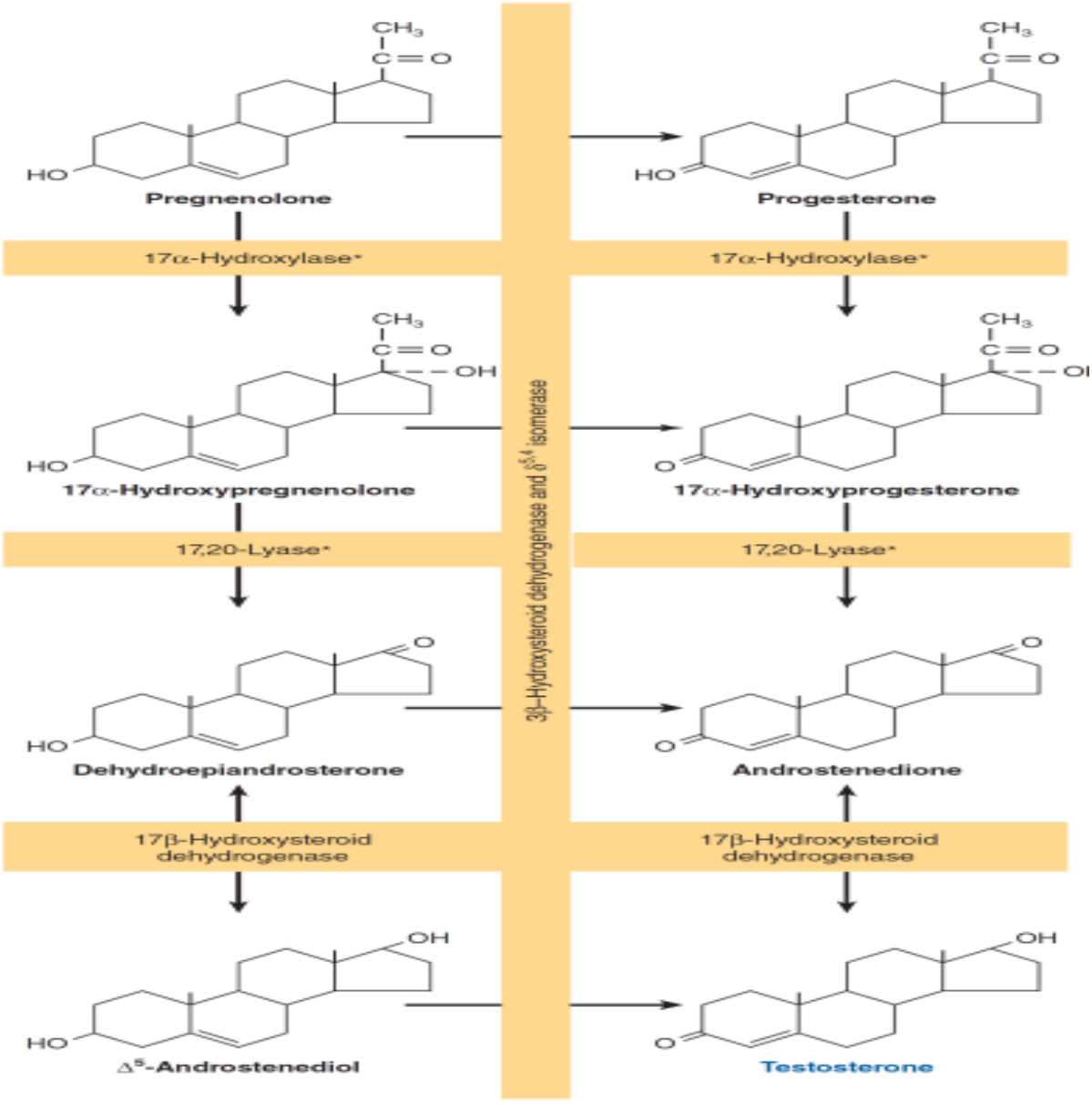
The adrenal cortex synthesizes the major androgens DHEA-S, DHEA, total testosterone, androstenedione, and dihydrotestosterone (DHT) in small amounts from the ovaries. The DHEAS, DHEA, and androstenedione are really hormone precursors, that are converted to total testosterone and DHT which are the active form[77].

Testosterone and other androgens that are important for maintaining ovarian function, bone metabolism, sexual function, and female reproduction. As well as the androgens lead to increase levels of receptors for the FSH hormone in the follicles, which is responsible for inducing follicular growth and development. So high androgen levels can lead to excessive growth with dysfunctional formation of antral follicles, as in PCOS. On the other hand, too low levels of androgen are associated with less ovarian response and ovarian insufficiency[78].

Approximately (90-99%) of DHEA is produced from the adrenal cortex while androstenedione is produced in both ovaries and adrenal gland, 25% of total testosterone is synthesized in ovaries while 25% in the adrenal gland and the remaining part is produced through peripheral conversion from androstenedione in the skin, adipose tissue, and liver.

As well as abdominal obesity is also associated with an increased testosterone production rate[79]. Synthesis of androgen from cholesterol which converted to pregnenolone then is converted to 17α-hydroxypregnenolone and follows the removal of the two-carbon side chain

through the action of 17,20-lyase. The lyase activity catalyzes 17α-hydroxylation that important in both the adrenals and the gonads and acts exclusively on 17α-hydroxy-containing molecules, to produce DHEA-S by the actions of 17α-hydroxysteroid dehydrogenase, convert the DHEA into the androstenedione, reduction of androstenedione at the C17 position results in the formation of testosterone, that summarized in the scheme (1-1) [77].



Scheme 1-1: Biosynthasis of testosterone and dehydroepiandrosterone in ovary [77]

The causes of hyperandrogenism in patients with PCOS as summarized in the figure below:

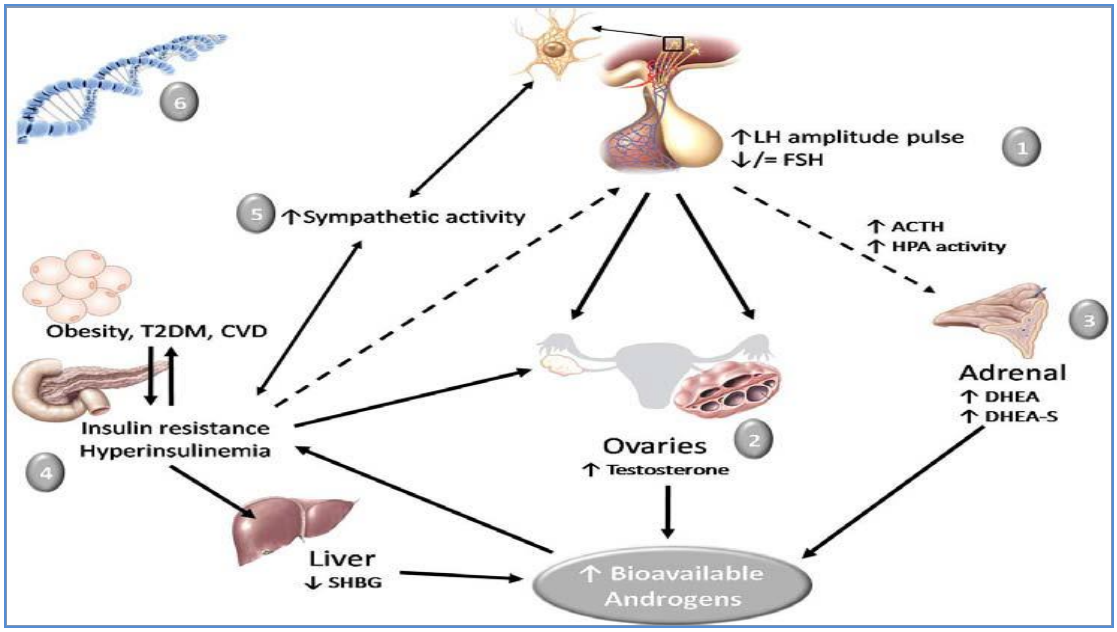


Figure 1-7: Hyperandrogenism in patients with polycystic ovarian [80]

Sympol: ACTH adreno corticotrophic hormone, CVD – cardiovascular disease, DHEA – dehydroepiandrosterone, DHEAS – dehydroepiandrosterone sulfate, FSH – follicle stimulating hormone, HPA – hypothalamic-pituitary-adrenal, LH – luteinizing hormone, SHBG – sex hormone binding globulin, T2DM – type 2 diabetes mellitus.

This figure show as:

(1) Neuroendocrine defects: impaired the normal negative feedback from sex-steroids on the hypothalamus and resulting high GnRH pulse frequencies high LH and limited production of FSH which promote androgen production.

(2) Ovarian cell, the ovary's theca cell response to high level of LH, a key factor in the hyperandrogenemia. The quantity of testosterone secreted varies approximately in proportion to the amount of LH available. Progesterone is the primary regulator of gonadotropin-releasing hormone (GnRH) pulse frequency by the negative feedback effects of progesterone [81], produced a large androgens level causing disrupt the

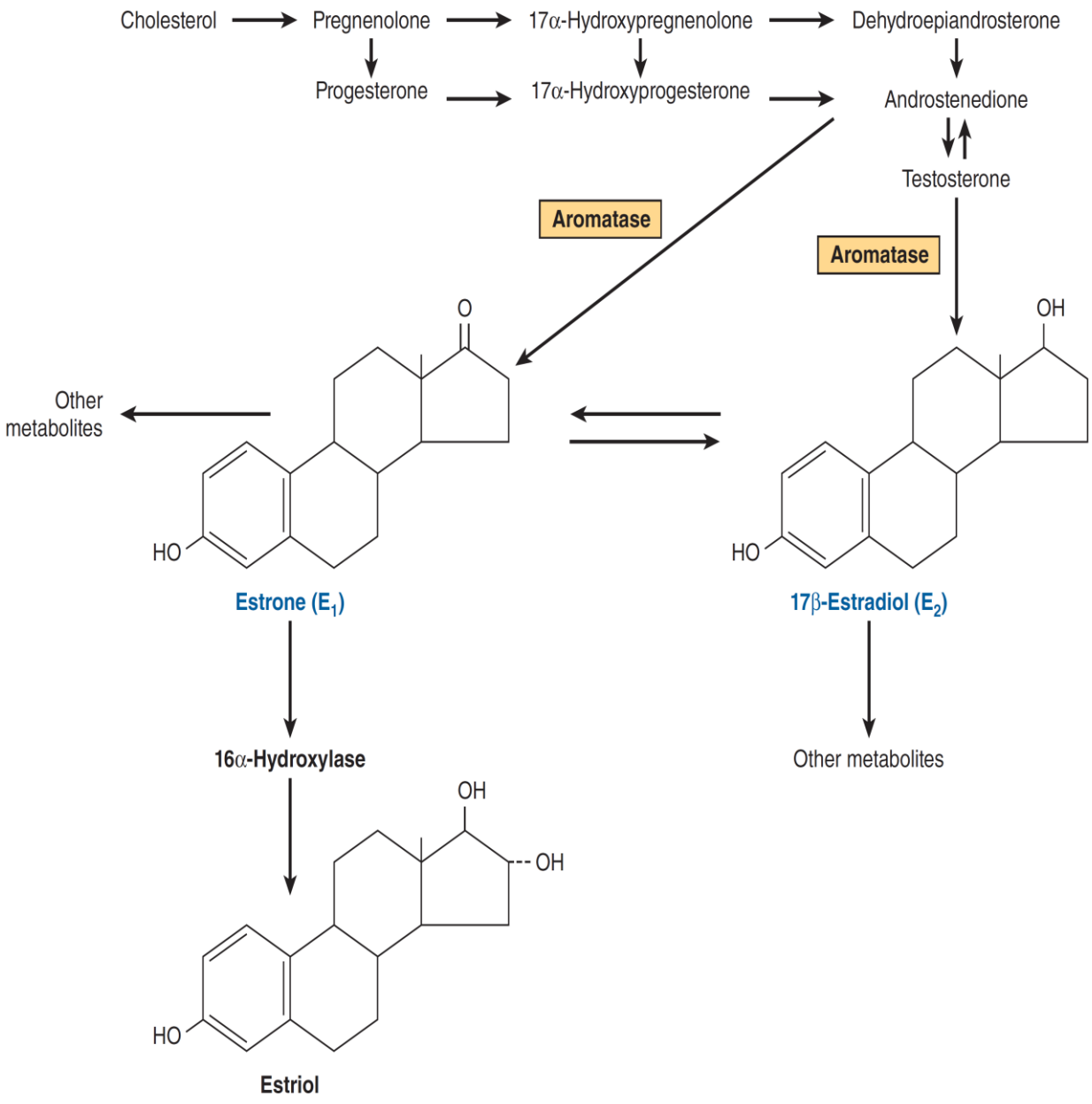
menstrual cycle [82]. Follicular atresia and leading to an ovulatory process by inhibit follicle growth [12,82]. 17β-hydroxysteroid dehydrogenases (17β-HSD) enzymes found in both theca and granulosa cell responsible for converting androstenedione to testosterone and converted testosterone to in theca cell and converting estrone to estradiol in granulosa cell and the aromatase enzyme found only in granulosa cell response to convert the androgen to estrogen therefor the defect of FSH in granulosa cell lead to inhibition the aromatase enzyme and accumulation of androgen as shown in scheme (1-2) [66].

(3) Impaired adrenal androgen production.

(4) Insulin resistance with hyperinsulinemia which effect directly or synergistically with LH stimulates androgen production from the ovarian theca cells and effect of by inhibits the hepatic synthesis of sex hormone-binding globulin (SHBG) may cause increases free testosterone concentration in the ovary [79,83].

(5) Increase sympathetic nerve activity.

(6) Genetic defects [80].



Scheme 1-2: Biosynthasis of estrogen from testosterone[77]

1.4 Genetic Basis of Polycystic Ovarian Syndrome

The genetic aetiology of PCOS has been associated with a common pathway both within and between families. There are two types of gene studies including Genome-Wide Association Studies (GWAS) and candidate gene studies. GWAS studies are used to determine if there is a link between genetic polymorphisms to loci associated with disease [87-89].

Candidate gene studies are used to specialized genes and identify SNP, more than 100 candidate genes were investigated, and it was discovered that several mutations or polymorphisms in genes involved in steroid hormone metabolism, obesity, energy regulation, insulin secretion, action pathways, gonadotropin and gonadal hormone action, insulin secretion and action pathways interact and initiate the development of PCOS and hypothalamic-pituitary-ovarian axis genes have associated with PCOS hyperandrogenism such as *KISS1* gene[88–90].

These investigations are useful approaches for detecting genetic variants, but they have significant limitations, such as sample size and diagnostic criteria, which cause variation in results[90, 91].

The pathogenesis of PCOS is a complex multigenic disorder, the genetic component of being inherited in an autosomal dominant, single gene mutation can give rise to the phenotype of the syndrome[92, 93].

1.4.1 Metastasis Suppressor Gene (*KISS1*)

Metastasis Suppressor Gene was discovered as a metastasis-suppressor gene in 1996, *KISS1* part was called for its role as a suppressor sequence (SS); the letters "KI" were added to "SS" to form "KISS" in homage of the

discovery place [94], the *KISS1*gene is located on the long arm of chromosome1 (1q32.1) as in figure (1-8). The human *KISS1* mRNA is transcribed from *KISS1* gene, it contains four exons, of which only third and fourth exons are finally translated into the 145 amino acid peptide called kisspeptin-145, and it is cleaved into four forms of active kisspeptin containing (10, 13, 14, and 54) amino acid as shown in the figure (1-9) [95].

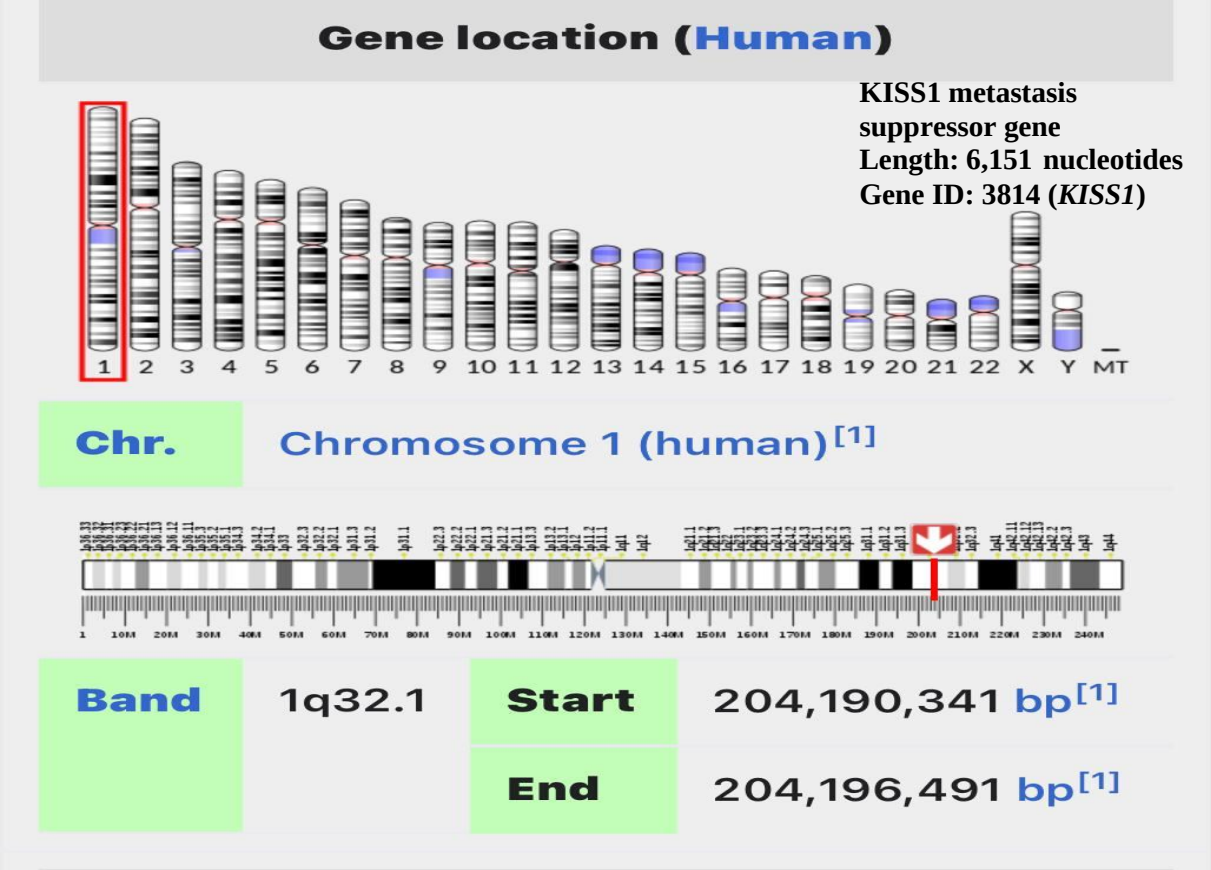


Figure 1-8 : *KISS1* gene information[96]

The *KISS1* gene is one of the candidate genes contributing to a regulatory role in the female reproductive system with an essential function in gonadotropin secretion of the HPG axis [97], some SNPs are found in the *KISS1*gene to disrupt the healthy functioning of the female reproductive system through disturbing the HPG axis and play an essential role in PCOS

etiopathogenesis such as missense effect (the base change of codon in first or second location lead to different amino acid depending upon its location in the specific protein might be acceptable, partially acceptable, or unacceptable to the function of that protein molecule), that increased risk of PCOS[98, 99].

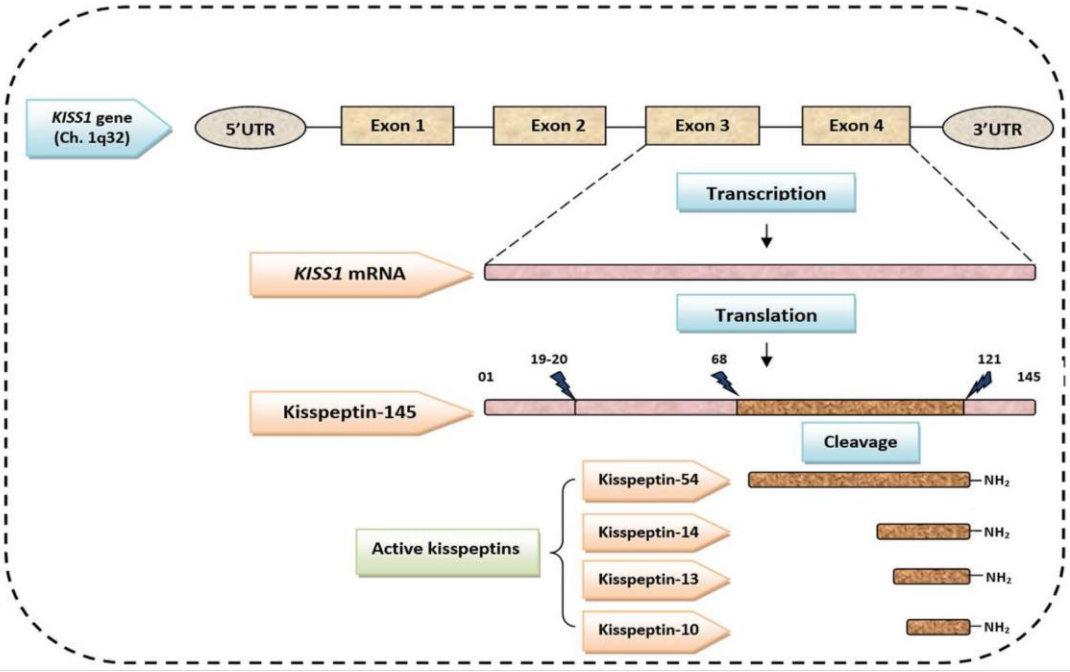


Figure 1-9: Human *KISS1* gene and kisspeptin [100]

1.5 Kisspeptin

Kisspeptin(Kp) is the neuropeptide defines as family of peptide hormones of varying amino acid lengths cleaved from the product of *KISS1* gene[101], consisting of 145 amino acids called kisspeptin and cleaved to different amino acid lengths (Kp-10, Kp-13, Kp-14, and Kp-54), encoded by the *KISS1* gene, the alternative name for kisspeptin is metastin that because it helps prevent metastasis in melanoma and breast cancer owing to its ability to inhibit cell invasion, altering cellular motility and adhesion [102]. Figure (1-10) show structure of kisspeptin that contain in location 81 is proline and in 1 position is methionine [103].

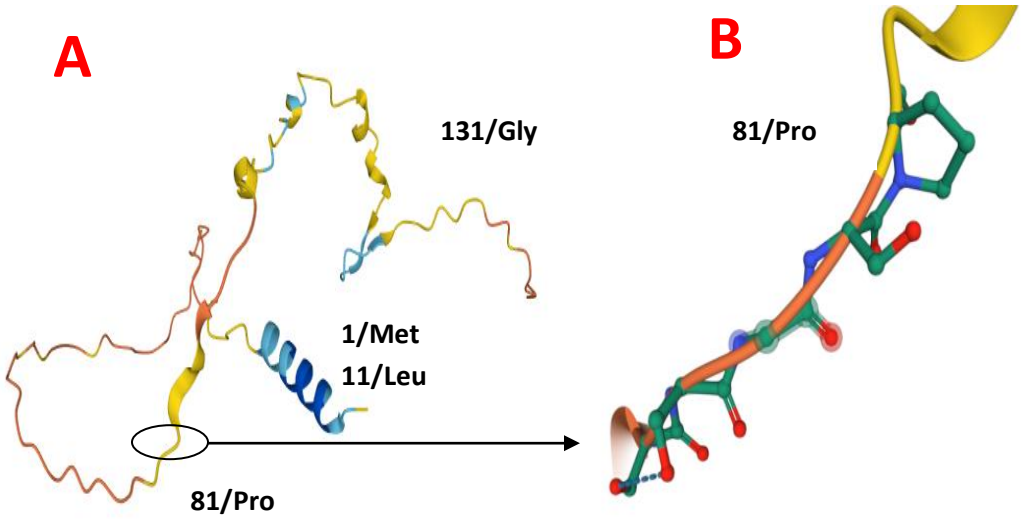


Figure 1-10 : (A) Structure of kisspeptin, (B) Proline at 81 position of kisspeptin[103]
Sympol: Pro :proline, Leu: leucine, Met: methionine, Gly: glycine.

1.5.1 Sources of Kisspeptin

Kisspeptin was first isolated from human placenta, hypothalamus and ovary. Hypothalamic kisspeptin neurons are mainly localized in two regions: the anterior region of the hypothalamus called the anteroventral periventricular nucleus (AVPV) in rodents, or in the preoptic area (POA)in human and the posterior region of the hypothalamus called the arcuate nucleus (ARC), the anterior kisspeptin neurons (POA) regulates ovulation by controlling surge-mode GnRH/LH secretion, and ARC kisspeptin regulates follicular growth by controlling pulse-mode GnRH/gonadotropin secretion [104,105].

1.5.2 Kisspeptin Signaling

Kisspeptin neuron secreted kisspeptin neuropeptide by activity of *KISS1*gene which has direct action on GnRH neuron to release GnRH into the

portal circulation by binding and activation G protein-coupled receptor known as GPR54 to regulate the function of the HPG-axis as summarized in figure (1-11) [105].

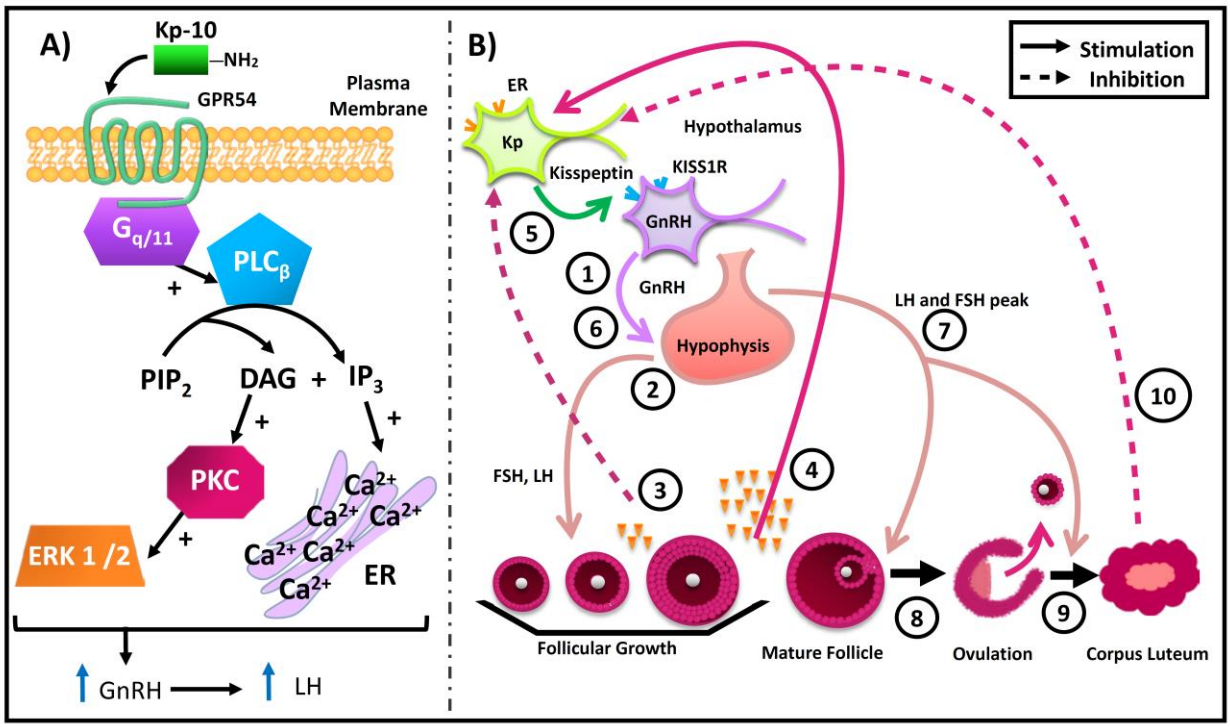


Figure 1-11 : (A) GPR54 and intracellular signaling, (B) The kisspeptin role in LH pulse[106].

The figure above show the kisspeptin/GPR54 cellular signaling and physiologic role in menstrual cycle [106]. **(A)** GPR54 and intracellular signaling: The GPR54 is coupled to G_q protein, when kisspeptin stimulates its receptor, the G_q activates phospholipase C (PLCβ) that hydrolyzes phosphatidylinositol 4,5-bisphosphate(PIP_2) producing second messengers: inositol 1,4,5-trisphospate(IP_3) and diacylglycerol(DAG). IP3 stimulates the endoplasmic reticulum (ER) to mobilizes calcium (Ca^{2+}). Increasing intracellular Ca^{2+} levels activate calcium-dependent signaling pathways in GnRH neurons. DAG activates calcium-dependent protein kinase C (PKC) that activates extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2).

(B) The kisspeptin role in LH pulse: ①GnRH stimulates the hypophysis, ②that releases FSH and LH, ③low level of estradiol inhibit LH by inhibit kisspeptin and stimulates the follicular growth then begin to produce estradiol, ④The high level of estradiol stimulates kisspeptin then produce LH ⑤In late follicular phase when main follicle achieves around 12mm estradiol level increase and stimulates the kisspeptin (Kp) neurons which have an estradiol receptor (ER) releasing the kisspeptin, ⑥Kp neurons stimulate the GnRH neurons, ⑦that induces the LH release, ⑧ovulation, ⑨and corpus luteum development, ⑩Corpus luteum produces progesterone, estradiol and inhibin A that inhibit the HPG axis via inhibit kisspeptin neuron, in PCOS cannot formation corpus luteum.

Kisspeptin has a stimulatory role in LH preovulatory surge, causing pulses stimulatory activity of LH and FSH with the release of androgen [107], therefore, it is important to measure levels of kisspeptin, LH, and FSH in serum of PCOS patients to understand the pathology of PCOS[30,93].

Estrogen increased histone acetylation level of AVPV/POA *KISS1* promoter region with an increase in *KISS1* expression, while estrogen has an opposite effect on the acetylation level and *KISS1* expression in the ARC [110,111], kisspeptin, neurokinin B, and dynorphin co-expressing neurons (KNDy neurons) produce kisspeptin to regulate GnRH release at the hypothalamus level. These neuropeptides work together to control the pulsatile release of kisspeptin from KNDy neurons in an auto/paracrine way, with dynorphin acting as an inhibitor and neurokinin B acting as a stimulator, GnRH secretion is controlled by kisspeptin. High frequency GnRH pulses promote LH release from pituitary gonadotrophs, whereas low frequency GnRH pulses promote FSH secretion from pituitary gonadotrophs. Oestrogen, progesterone, and

androgen receptors (E_2R, PR, and AR) on KNDy neurons provide feedback to the HPG axis, as shown in figure (1-12) [63,89].

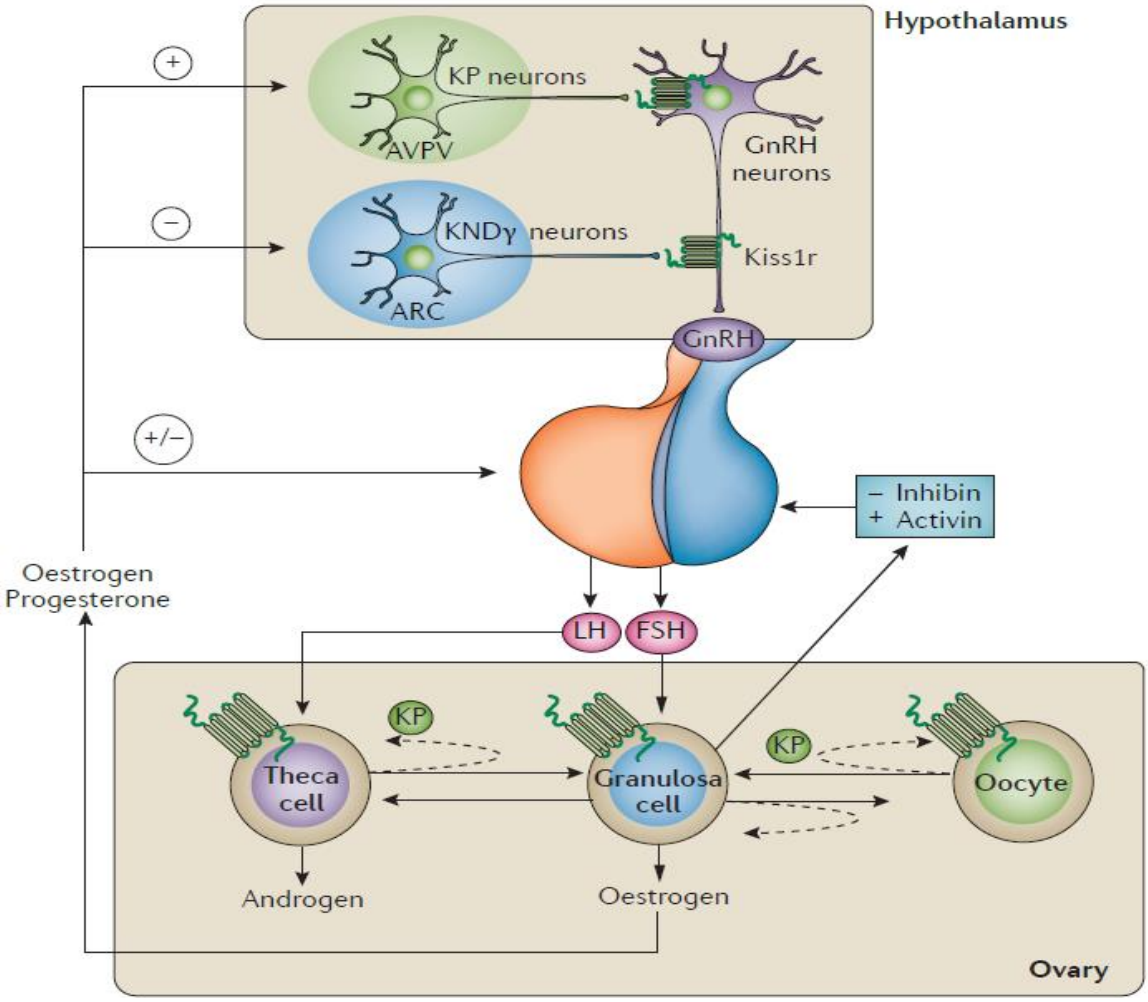


Figure 1-12: Role of kisspeptin and other hormones in reproductive system[111].
Symbol: ARC, arcuate nucleus of the hypothalamus; AVPV, the anterior ventral periventricular nucleus of the hypothalamus; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; KNDγ neurons, kisspeptin, neurokinin B and dynorphin containing neurons: KP, kisspeptin; LH, luteinizing hormone**.**

1.5.3 Biological Function

Metastin can stimulate the secretion of aldosterone and the release of insulin, it's directly activates GnRH neuron, and induces the production of LH and FSH which are required for females menstruation.

It's role in the onset of puberty, sexual patterns, desires, ovum development in females, sperm quality in males, feedback mechanisms, pregnancy, and lactation [112].

a. Role in Puberty

The onset of puberty is marked by an increase in gonadotropin secretion, which leads to sexual maturity and the ability to reproduce, the primary event that leads to the beginning of puberty is the activation of GnRH neurons by kisspeptin/GPR54 signalling, which leads to the activation of GnRH neurons, kisspeptin causing GnRH release which leads to the release of FSH and LH. Loss of *KISS1* gene function is reported to be associated with idiopathic hypogonadotropic hypogonadism in humans which is associated in delay of puberty and sexual maturation [112].

b. Role in Tumor Suppression

Kisspeptin plays a large role in tumor suppression, when it is active in cells the tumor stays constant and does not spread or grow [113]**.**

c. Role in Kidney Function

Kisspeptin and its receptor were found in various sites in the kidney, including in the collecting duct, vascular smooth muscle, and the renal tubule cell, kisspeptin directly increases the release of aldosterone, that is causes reabsorption of filtrate leads to increased blood pressure [67, 93]**.**

d. Role in Reproduction

Kisspeptin has a regulatory role in the female reproductive system, follicular maturation, oocyte formation, ovulation, ovarian steroidogenesis, and placenta. Kisspeptin is highly expressed during

pregnancy, in early-term placenta GPR54 was expressed at a higher rate than in placentas late-term, the expression of kisspeptin, however, remains unchanged in the placenta throughout pregnancy[114]. The elevated level of *KISS1* expression demonstrates positive feedback on kisspeptin association with PCOS[102]**.**

e. Role in Ovary

Kisspeptin and KISS1R are found on ovarian theca cells, granulosa cells, and oocytes, it's possible the local kisspeptin has both autocrine and paracrine functions. Kisspeptin has an indirect effect on ovarian activity by LH and FSH affect steroidogenesis and androgenizes via acting on theca and granulosa cells of the ovary as show in the figure (1-12) P. 29 [111].

f. Role in Polycystic Ovarian Syndrome

Known and unknown factors such as the mutation that causes the hyperactivity to the *KISS1* gene that is specifically expressed in kisspeptin neurons in each nucleus may be involved in making the difference between the anterior and posterior population of kisspeptin neurons to control GnRH/LH surge and pulse generation systems[47].

The increased GnRH pulse frequency can promotes LH synthesis over FSH synthesis, leading to a high LH/FSH ratio in females with PCOS.

First, LH promote the synthesis of androgen in ovarian theca cells which leads to hyperandrogenemia and arrested follicle development.

Second, increased LH pulse frequency impairs estrogen and FSH synthesis, thus inhibiting follicle growth and ovulation and impairing of negative feedback effect of estrogen on the hypothalamus, it’s still unclear whether the abnormal GnRH function is the primary dysfunction

of hypothalamus and pituitary or secondary to the complicated effect of reproductive and metabolic disorder[65]

1.5.4 Polymorphisms in *KISS1*Gene

Many studies illustrate polymorphisms of the *KISS1* gene in PCOS patients along with the gene *KISS1*. At least 294 SNPs have been described of which 42 correspond to mutations located in un translation regions (UTR), 30 in exonic and the rest in intronic regions, multiple candidate genes associated with PCOS should be screened to determine its exact genetic basis. The genes encoding gonadotropins and their receptors might influence the ovarian function and reproductive success, in this study determined two SNPs in the *KISS1* gene that were suggested by candidate gene studies that associated with PCOS, determined by HRM –PCR technique [115]:

1- rs4889 G>C , A polymorphism location in exon the types of mutation is missense.
2- rs372790354 G>A polymorphism location in 5-prime untransulation region on *KISS1*gene

1.6 Polycystic Ovarian Syndrome and Obesity with Kisspeptin

Obesity is strongly associated with PCOS, both obese and non-obese PCOS patients have an increase of androgens due to the present defect of the hypothalamus gland and abnormal GnRH pulses that lead to LH hypersecretion and the high response of theca cell in ovary to produce androgen that leads to induce abdominal adipose tissue accumulation, and induce insulin resistance in subcutaneous adipose tissue and inhibit insulin-stimulated glucose uptake by impairing phosphorylation of protein kinase C [116].

Hyperinsulinemia can directly increase androstenedione secretion in theca cell, which affects the fatty acid with lipid accumulation in the liver leading to reduced SHBG production, so, abdominal obesity and insulin resistance together lead to an increase in the level of androgen in blood[52], as shown in figure (1-13).

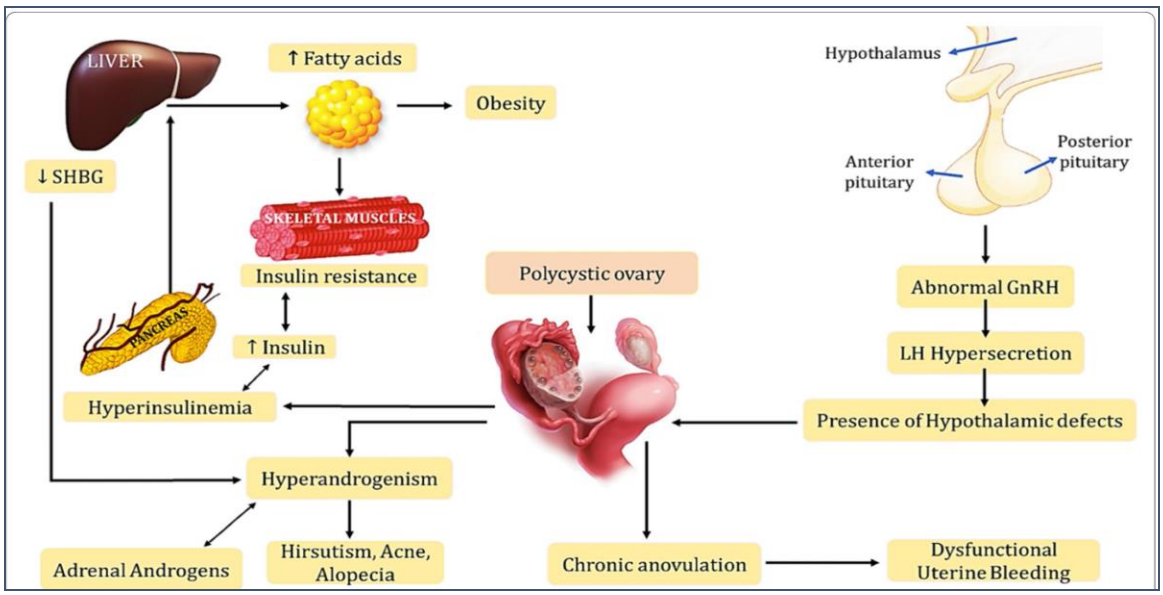


Figure 1-13 : Polycystic ovarian syndrome and obesity[55]

Symbol : LH: luteinizing hormone, GnRH: gonadotropin-releasing hormone, PCOS: polycystic ovarian syndrome, SHBG: sex hormone binding globulin**.**

The androgens play role in appetite regulation, adipose tissue secretes substances known as adipokines or adipocytokines, which have an endocrine function, leptin and adiponectin are produced by adipocytes [24,41], while the insulin secreted by the pancreas, are two long-term peripheral signals that transmit information about adipose tissue. Ghrelin is the major hormone, an appetite-regulating hormone, secreted by the stomach in response to fasting and regulates meal frequency by triggering the commencement of eating [119]. They can reach their receptors through blood brain barrier at the level of the arcuate nucleus, and their plasma levels are proportional to body fat [120] as in figure (1-14).

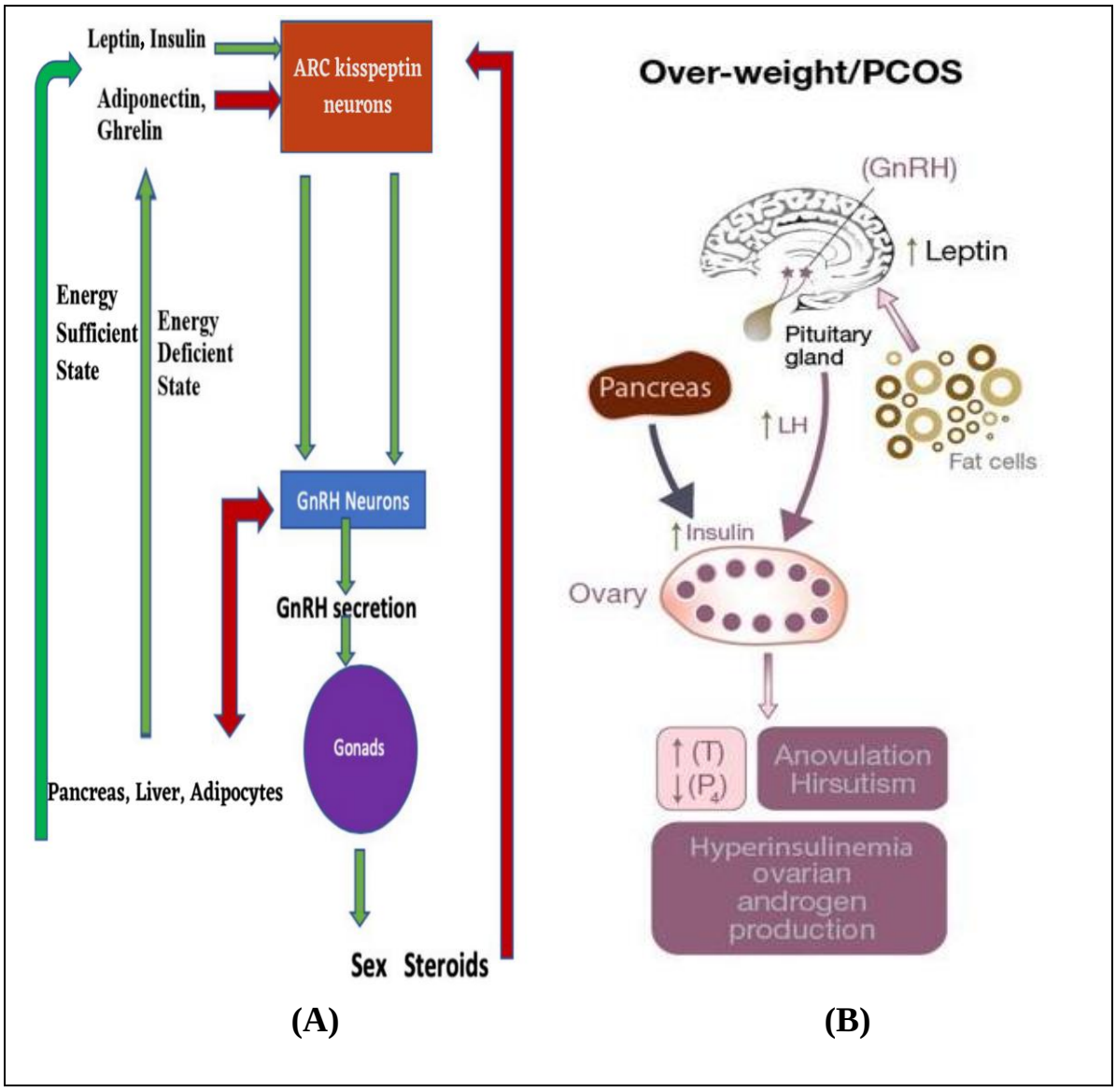


Figure 1-14 (A) Effect of leptin and adiponectin on kisspeptin to the regulation of the reproductive cycle of humans, (B) Effect of leptin on females with polycystic ovarian syndrome [102]

ARC: Arcuate nucleus, GnRH: Gonadotropin-releasing hormone. Estrogen, progesterone, and testosterone, which are sex hormones, have a negative feedback control on kisspeptin neurons. The excitatory inputs are represented by green arrows and inhibitory inputs by red arrows.

Adiponectin has an effect that causes a reduction in FFA uptake and gluconeogenesis. It is also plays a role in progesterone and estrogen production, ovulation, and decreased GnRH secretion and reduces LH secretion from the pituitary, triggers estradiol secretion in granulosa cells by increasing aromatase expression and is associated with androgen production in ovaries [116].

The effect of leptin on females with PCOS and on kisspeptin: A high concentration lead to

a. The granulosa cells store and produce leptin and a high level of leptin inhibits the expression of aromatase which affects the ability of a dominant follicle to produce sufficient amounts of estrogen causing accumulation of androgen, don't conversion of androgens to estrogen [70,71].

b. Increase leptin levels are related to the absence of folliculogenesis [118].

c. Kisspeptin neurons are also influenced by leptin and insulin (excitatory) leading to increase LH levels as in figures (1-14(A and B)) as well as ghrelin and adiponectin (inhibitory), kisspeptin binding to its receptor on gonadotropin-releasing hormone (GnRH) neurons, activates reproductive axis and stimulates GnRH release [70,71].

1.7 Aims of the study

To:

1. Evaluate the kisspeptin level and its association with gonadotrophin hormone, and its relationship with PCOS occurrence.
2. Estimate the association between *KISS1* gene polymorphisms (rs3727354G>A, rs4889 G>A) with the pathogenesis of PCOS.
3. Estimate the relationship of *KISS1* gene variants with kisspeptin level in PCOS development.
4. Introduce a new specific technique(high resolution melting (HRM)-PCR assay) in the estimation of SNPs detection with appropriate cost and speed time with highly accurate results.

2.1 Materials

2.1.1 Subjects and Samples

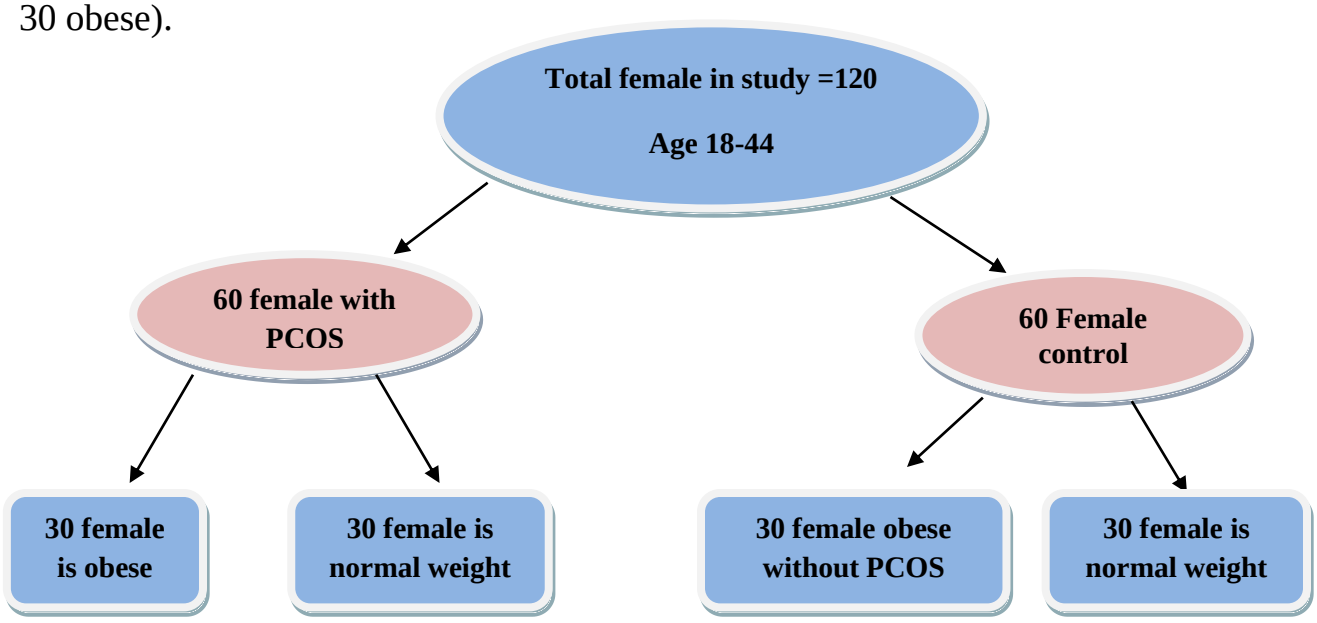
2.1.1.1 Study Design : Case control study.

2.1.1.2 Subjects Group

The study samples was collected from patients who attended the Imam Sadiq General Hospital (infertility unit), as well as in private clinics in the Babylon province. Samples were collected during the period from September 2021, to January 2022. As well as the practical aspect of the study was carried out in the laboratory of Biochemistry Department in College of the Medicine / University of Babylon.

The study involved independent 120 females, the age ranged between (18-44) years old, which were divided into two groups:

1. Control groups: Included 60 apparently healthy females (30 normal weight and 30 obese).
2. Patient groups: Included 60 females with PCOS(30 normal weight and 30 obese).



Scheme(2-1) Design of sample groups

The selection of the sample size was calculated by the following equation:

$$n = \frac{Z^2 p(1-p)}{d^2}$$

n: number, Z statistic: For the level of confidence of 95%, which is conventional, Z value is 1.96 , p: prevalence ,
d: margin of error(precision) = 5%[123].

2.1.1.3 Ethical Considerations

a. Approval by the Babylon Medical College (University of Babylon, Iraq) scientific committee and the Biochemistry Department of the same college.
b. Approval of the Scientific Committee of the Imam Sadiq General Hospital in Hilla city.
c. All volunteers in the current study were verbally accepted once the objectives and methodology of the investigation were explained to them.

2.1.1.4 Sample Collection

The selection of patients was depended on androgen excess criteria (AE-PCOS), which required the presence of hyperandrogenism and/or clinical features such as(hirsutism, acne, and pigmentation), also the vaginal ultrasound was used to assess the morphology of ovary, blood samples were collected from all females in periods between the 2nd and 5th day of the cycle.

At room temperature, venous blood samples were drawn from all subjects by using a disposable syringe (5 mL) in the sitting position, and slowly pushed into two tubes for genetic study two milliliters of the blood was drained into ethylenediaminetetraacetic acid (EDTA) tube and mixed gently, the blood in the EDTA tube was stored at -20 ˚C and used for DNA extraction.

The rest of 3ml of blood was drained into plain tube gel for serum preparation, which was used in hormonal test after separation centrifuged at 3000 Xg for approximately 5-10 minutes, the blood test tubes used were manufactured of high-purity polypropylene created without plasticizers or biocides to provide the best quality of the study, tubes have a safe-lock seal to prevent inadvertent tube opening and sample loss (Eppendorf, Germany), then the serum was divided into four parts and stored at -20˚C until analysis.

2.1.1.5 Selection Criteria

The inclusion and exclusion criteria for this study were as follows:

a. Inclusion Criteria

All females in this study included :

- Reproductive age (18-44).
- Positive family history of PCOS for patients only.
- Normal weight and obese.

b. Exclusion Criteria

The following conditions were ruled out of the study:

- Pregnant females.
- Overweight (BMI 25-30).
- Chronic diseases (metabolic and endocrine) such as diabetes, kidney disease, and liver disease that affect the SHBG level and free androgen[124].
- Patients who were taken any hormonal medicine for PCOS treatment prior to the collection of a blood sample for about three months like oral contraceptive and metformin
- Who were in the nursing period.

2.1.1.6 Study Requirements

a. Questionnaire

Age, family history, marital status, and medical history are all demographic factors, the survey was attached as an appendix.

b. Anthropometric Measurement

Including:

- **Body Mass Index:**

Weight (kg) and height (m), were measured practically BMI was calculated as weight (in kilograms) divided by the square of height (in meters)[125], weight and height are measured on the same scale for all members of the sample.

$$\mathbf{BMI(Kg/m^2)} = \frac{\mathbf{Weight\ (Kg)}}{\mathbf{(Hight)^2\ m^2}} \quad [125]$$

- **Waist-to-Hip Ratio**

Waist circumference was measured in this way (breathing while standing straight) the distance around the smallest area of the waist, just above the belly button, was measured using a tape measure, and then measurement was taken around the widest area of the hips - the widest part of the buttocks, this is the measurement of the circumference of the hips.WHR was calculated by

WHR = Waist circumference(cm)/ Hip circumference(cm)[125]

2.1.2 Chemicals

All of the chemicals and materials that were utilized, as well as the standard kits, were listed in Table (2-1).

Table 2-1: Chemical and materials utilized in the analysis of research

No.	Substance	Company and Country
Hormonal kit utilized in this study		
1	DHEA kit	Maglumi / China
2	Free Testosterone kit	Maglumi / China
3	FSH kit	Maglumi / China
4	Kisspeptin kit	Bioassay technology laboratory/ China
5	LH kit	Maglumi / China
Nucleic acid extraction kits utilized in this study		
6	DNA extraction kit (blood)	INtRON / Korea
7	Mini DNA extraction kit (blood)	Favorgene (china)
Chemical utilized in molecular part		
8	Absolute Ethanol (100%)	China
9	Agarose	Promega https://worldwide.promega.com
10	EvaGreen qPCR Master Mix.	Biotium Co.
11	Loading dye (bromophenol blue)	SCR (china)
12	Nuclease free water	Promega / USA

13	Red-Safe	INtRON / Korea
14	Tris-borate EDTA buffer (TBE buffer) 10X	Promega / USA
Primer utilized in this study		
15	Primers HRM –PCR(F,R, wild and mutant) for 4889	Alpha AND : Custom DNA synthesis
16	Primer HRM- PCR(F,R, wild and mutant) for 372790354	Alpha AND : Custom DNA synthesis
Enzyme utilized in this study		
17	RNAase	INtRON / Korea
18	Proteinase K (10 mg/mL)	INtRON / Korea

2.1.3. Instruments and Materials

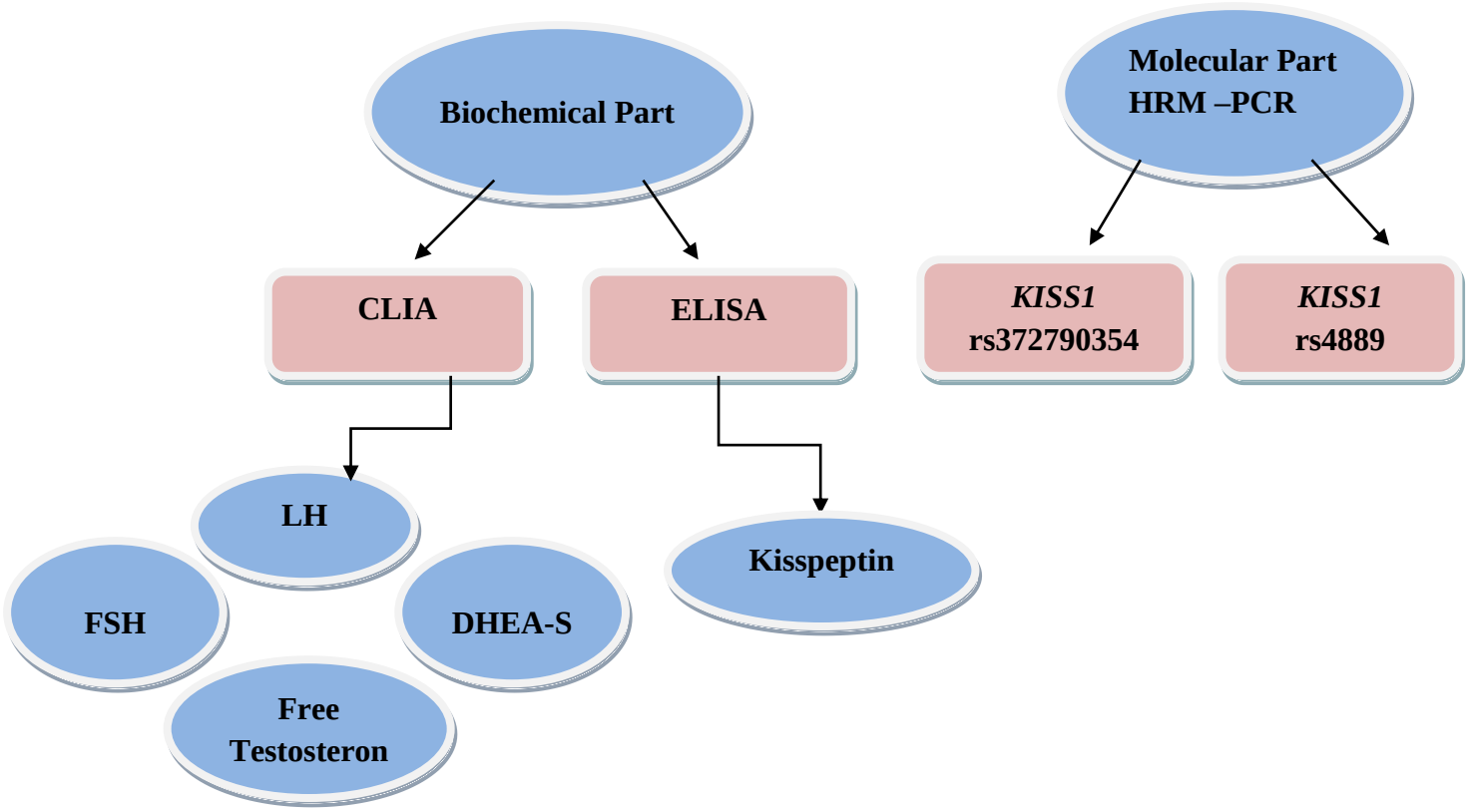
Table 2-2: The instruments and tools utilized in the analysis of research

No.	Instruments and Tools	Company and country
1	Autoclave	Haramaya / Japan
2	Centrifuge	Hettish/india
3	Centrifuge / vortex for PCR plates	Biosan/ industrial
4	Chemiluminescence Immunoassay (CLIA) analyzers	Maglumi 800/ China
5	Cooling Centrifuge	PrismR / USA
6	Deep Freeze	GFL / Germany
7	Disposable syringes (5 mL)	Medical jet / Syria
8	Distillation device	GFL / Germany
9	EDTA tube (5ml)	AFCO , Jordan
10	ELISA reader and washer	Biotech /USA
11	Eppendorf tube (1.5ml,0.2ml)	Eppendorf tube (1.5ml,0.2ml)
12	Gel tube (2.5mL)	AFCOVAC / Jordan
13	Heater	China
14	Hood/UV	labtech / Korea
15	Horizontal gel electrophoresis	Autto/ Japan

16	Hot plate	Grant / England
17	Incubator	Fisher Scient./Germany
18	Nanodrop	Analytikjena/Germany
19	Nanodrop 2000 spectrophotometer	Thermo scientific / USA
20	Oven	Binder/ China
21	Photo documentation	E-Graph/ Japan
22	Pipette different size	Slamed / Germany
23	Real time PCR Thermo cycler Rotor-Gene Q	Qiagene / USA
24	Sensitive Balance	Sartorius / Germany
25	UV Trans-illumination	E-Graph/ Japan
26	Vortex (Electronic)	Kunkel /Germany
27	Water bath	GFL / Germany
The online website of the bioinformatics program that was used to design primers.		
28	HRM-Real Time -PCR primer design	Primer3web NCBI Primer blast

2.2 Methods

Methods of present study summarized in this scheme :-



Scheme(2-2) Study methods

2.2.1 Biochemical Part

2.2.1.1 Determination of Serum Luteal Hormone Concentration

a. Principle of The Test

Chemiluminescent immunoassay technology(CLIA) has been used an anti-LH monoclonal antibody was conjugated to N-(4-aminobutyl)-*N*-ethyl-isoluminol (ABEI) label as show in the figure (2-1) and (2-2)[126].

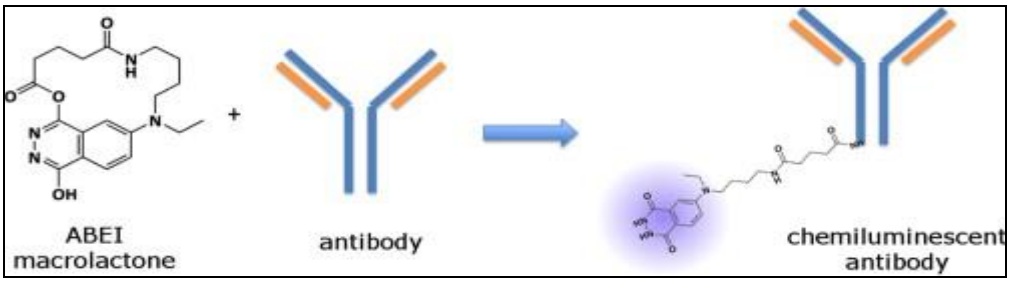


Figure 2-1: Contact between (ABEI) aminobutyl-n-ethyl-isoluminol and antibody[126]

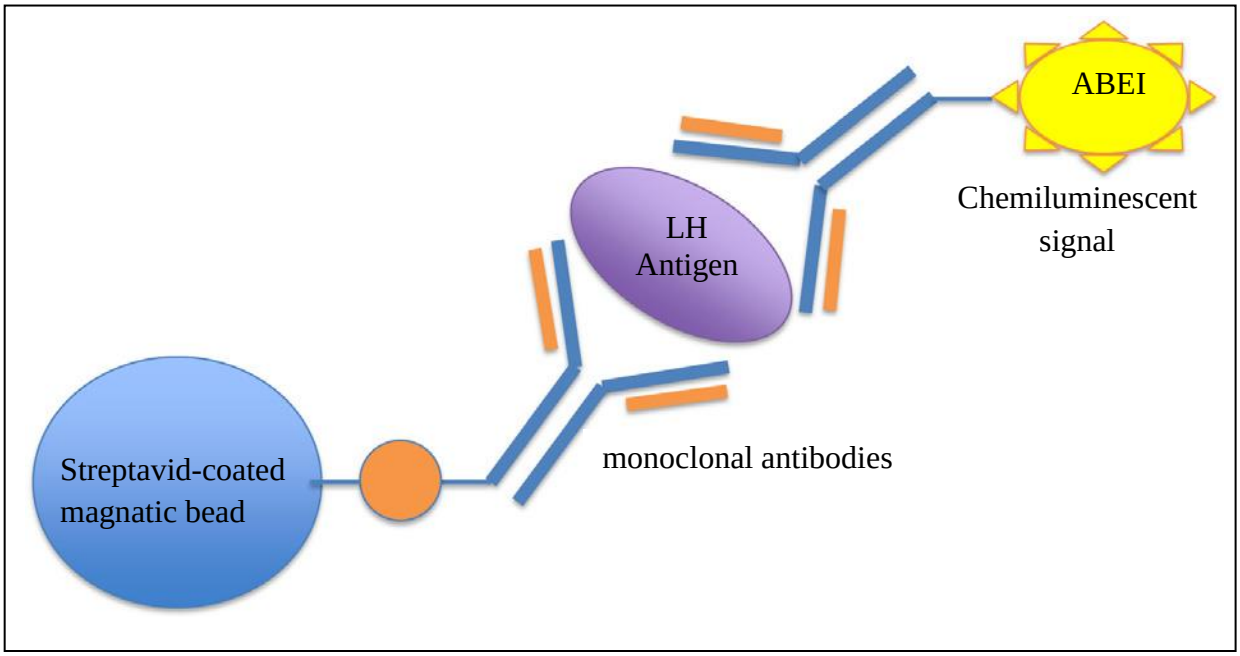


Figure 2-2: Sandwich of chemiluminescent assay [126]

Symbol: ABEI: aminobutyl-n-ethyl-isoluminol and antibody

Another monoclonal antibody was used to coat magnetic microbeads. Samples, ABEI labels, and magnetic microbeads, were mixed thoroughly and incubated at 37℃, forming a sandwich, after the sediment in a magnetic field, sucked the supernatant and then cycle wash it for 3 times. Subsequently, the starter reagents were added and a flash chemiluminescent reaction was initiated, the light signal measured by a photomultiplier as relative light units (RLU) within 3 seconds and is proportional to the concentration of LH present in samples[127].

b. Reagents Integral Preparation

Microbeads were mixed prior to loading the reagent assembly onto the system for the first time to resuspend the microbeads that had settled during shipment.

The micro wheel in the microbeads chamber was rotated back and forth until the color of the suspension changed to brown, waited for 30 minutes for the integrator to sit in the reagent compartment, the magnetic microbeads were automatically agitated and completely resuspend during this period.

To preparation the wash buffer was added one bottle of concentrated washing to 10 liters of distal water.

c. Calibration and Traceability

The instrument was calibrated by pressing the calibration button, as well as standard solution was added instead of the sample by replacing the sample with a standard solution.

The control for the maglumi system has been added to ensure that it was within the expected range, and if the room temperature changes more than 5 °C, the control must be added to ensure an accurate result via Maglumi's fully automatic chemiluminescence immunoassay (CLIA) analyzer.

d. Procedure

1. The device reagents had been downloaded after scanning the device space automatically in the device test, the group had been suspended on the bead in the automatic confusion solution that occurs after loading for 30 minutes.
2. A volume of 40µL sample and control tube had been entered into the device and then the starting was determined.

3. The sample had been mixed with maglumi system (80µL ABEI label, 20µL nanomagnetic microbeads) and then incubated for 15 minutes at 37°C.
4. Cuvettes had been transferred to the washing station for a cycle of washing 3 times to remove the unbond content by 400 µL washing buffer.
5. Cuvettes had been moved to the measurement in a dark room, here, 2 substrates had been added (starter 1+2) and then the light signal was measured by a photomultiplier within 3 seconds
6. After finished measured, cuvette has been paid to the waste bag and the end of the test.

e. Calculation Result

The starter detector has been added to start the chemiluminescent interaction, and the production of a light signal is measured by a photomultiplier, which suits the LH concentration in the sample, the analyzer automatically calculates LH concentration in each sample through the calibration curve created by 2 points in the calibration curve, which was automated in advice, the results are expressed in mIU / mL, the detection limit was between1.5 and 225.5 mIU/mL.

2.2.1.2 Determination of Serum Follicle-Stimulating Hormone Concentration

a. Principle

Sandwich immunoluminometric assay was used an anti-FSH monoclonal antibody to N-(4-aminobutyl)-*N*-ethyl-isoluminol (ABEI) label, and has been used another monoclonal antibody to coat magnetic microbeads.

Sample, calibrator or control, with ABEI label and magnetic microbeads were mixed thoroughly and incubated at 37℃, forming a sandwich, after sediment in a magnetic field, suck the supernatant and then cycle wash it for 3 times. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated, the light signal is measured by a photomultiplier as relative light units (RLU) within 3 seconds and proportional to the concentration of FSH present in samples[128].

b. Calibration and Traceability

The instrument was calibrated by pressing the titration button and the sample was replaced with a standard solution instead of the sample, calibration was repeated if control were outside the expected range, or if there was a change in room temperature of more than 5 °C.

c. Procedure

1- The reagents had been loaded into the device after scanning the code using the automatic area scanning of the device in order to take the test information, the group containing the bead was suspended in the solution via the automatic mixing that occurs after loading for 30 minutes.
2- A volume of 40µL sample and control were placed in a collection tube, entered into the device and then the starting was determined.
3- Sample needle and reagent sampler were taken into a cuvette, serum was combined with 80µL of ABEI label and 20 µL of nanomagnetic microbeads from the Maglumi system kit.
4- Mixture had been incubated at 37 °C for 15 minutes.
5- The cuvettes had been transferred to the washing station and washed cycle washed three times with 400 µL wash buffer to remove non-agglutinating contents.

6- The cuvettes had been moved to a three-second measuring room, which was a dark room, starters 1 and 2 had been added, they reacted with the ABEI chemiluminescence label by oxidative reaction of NaOH and H_2O_2 to indicate the chemical product.

7- The cuvettes had been pushed into the waste bag after being measured, and the test was completed.

d. Calculation Result

The initiator reagent was added to start a luminous chemical reaction, which resulted in a photomultiplier as relative luminous units(RLU) which are proportional to the concentration of FSH present in the sample, and the analyzer automatically calculated the FSH concentration in each sample by means of the generated calibration curve by performing a two-point master calibration curve. The results were expressed in mIU/mL and the detection limit was between (0.02-78.92) mIU/mL.

2.2.1.3 Determination of Serum Dehydroepiandrosterone-Sulfate Concentration

a. Principle

Competitive immunoluminometric assay; label fluorescein isothiocyanate (FITC), combines with amine groups to produce protein conjugates (an anti-DHEA-S antigen labeled FITC), and ABEI coated with a purified DHEA-S monoclonal antibody while the magnetic microbeads that coated with anti-FITC polyclonal antibody.

Herein, Sample, calibrators or control, FITC label, ABEI label, and nanomagnetic microbeads are completely combined and incubated at 37°C to form complexes Ab-Ag, then sediment in a magnetic field, decant the supernatant, and cycle wash.

The beginning reagents are then added, and the flash chemiluminescent process is started, within three seconds, a photomultiplier measures the light signal as RLU which is proportional to the amount of DHEA-S in the samples, the half-life of DHEA-S is 7-10hours, whereas the half-life of DHEA is only 15-30 minutes[129].

b. Preparation of The Reagent Integral

The solutions were prepared in the same way as mentioned in the previous analyzes of LH and FSH.

c. Procedure

1- The reagents had been loaded into the device after scanning the code with the automatic area scanning of the device until, the device took the test information, the group containing the bead was suspended in the solution by automatic mixing that occurs after loading for 30 minutes.
2- A volume of 10µL of sample and control had been placed into the collection tube, entered into the device and then the starting was determined.
3- The samples had been withdrawn by needle pipettes and 50 µL ABEI Label, 50 µL FITC Label, 20 µL nanomagnetic into the cuvette; antigen of serum is competitive with FITC-labeled DHEA-S antigen for fusion with ferromagnetic nanomicrobeads 20 µL of the maglumi system array and then combined with 50 µL of purified ABEI-labeled DHEA-S monoclonal antibody.
4- The mixture had been incubated at 37°C for 15min.
5- The cuvettes were transferred to the washing station and the cycle was washed three times with 400 µL wash buffer to remove unconjugated contents.

6- The cuvettes had been moved to a three-second measuring room, which was a dark room, starters 1 and 2 had been added, they reacted with the ABEI chemiluminescence label by oxidative reaction of NaOH and H_2O_2 to indicate the chemical product.
7- The cuvettes were pushed into the waste bag after being measured and tested.

d. Calculation Result

Concentration was calculated automatically by the device, the concentration of DHEA-S in each sample was calculated using a calibration curve generated by a two-point titration curve procedure. which results had been measured in a photomultiplier as RLUs proportional to the concentration of DHEA-S present in the sample.

2.2.1.4 Determination of Serum Free Testosterone Concentration

a. Principle

A purified testosterone antigen had been used to label FITC, and an anti-testosterone monoclonal antibody to label ABEI in this competitive immunoassay. The samples, calibrator, or control with ABEI label, FITC label, and magnetic microbeads coated with anti FITC polyclonal antibody were properly incorporated and incubated at 37 °C to generate antibody and antigen complexes, the supernatant after deposition in a magnetic field had been poured, and then washed. Primary reagents are then introduced, and the chemiluminescence process begins, within 3 seconds, a photomultiplier was measured by measuring the light signal as RLU, which was proportional to the amount of testosterone displayed in the sample[130].

b. Procedure

The reagents had been loaded into the device.

1. Samples and control (40µL) were placed into the collection tube and then pressed to start.
2. The samples were withdrawn with the samples needle and 40 µL ABEI Label, 40 µL FITC Label, 20 µL nanomagnetic were added in the cuvette. Antigen of serum is competitive with FITC-labeled purified testosterone antigen for fusion with ferromagnetic nanomicrobeads.
3. Mixture was incubated for 15 min at 37 °C.
4. The cuvettes were transferred to the washing station and the cycle was washed three times with 400 µL wash buffer to remove unconjugated contents.
5. The cuvettes had been moved to a three-second measuring room, which was a dark room, and starters 1 and 2 were applied, they reacted with the ABEI chemiluminescence label by oxidative reaction of NaOH and H_2O_2 to indicate the chemical product.
6. The cuvettes were pushed into the waste bag after being measured and test finished.

c. Calculation Result

A calibration curve analyzer was used and developed by a two-point master calibration curve process to calculate the concentration of free testosterone in each sample, results are given in (pg/ml).

2.2.1.5 Determination of Serum Kisspeptin Concentration

a. Principle

An enzyme-linked immunosorbent assay (ELISA) kit was included in the human KISS-1 antibody was used to pre-coat the plate. *KISS1*, which was present in the sample, was introduced into the wells and bound to the antibodies in which they were coated.

The biotin-conjugated human KISS1 antibody was then added to the sample, where it binds to *KISS1*. Next, Streptavidin-HRP, which binds to Biotinylated KISS1, was added. During the washing phase, any unbound Streptavidin-HRP were eluted.

The gradient develops in proportion to the amount of human *KISS1* in the substrate solution. After added an acidic stop solution, the reaction was stopped and the absorbance was measured at 450 nm as show in figure (2-3)[131].

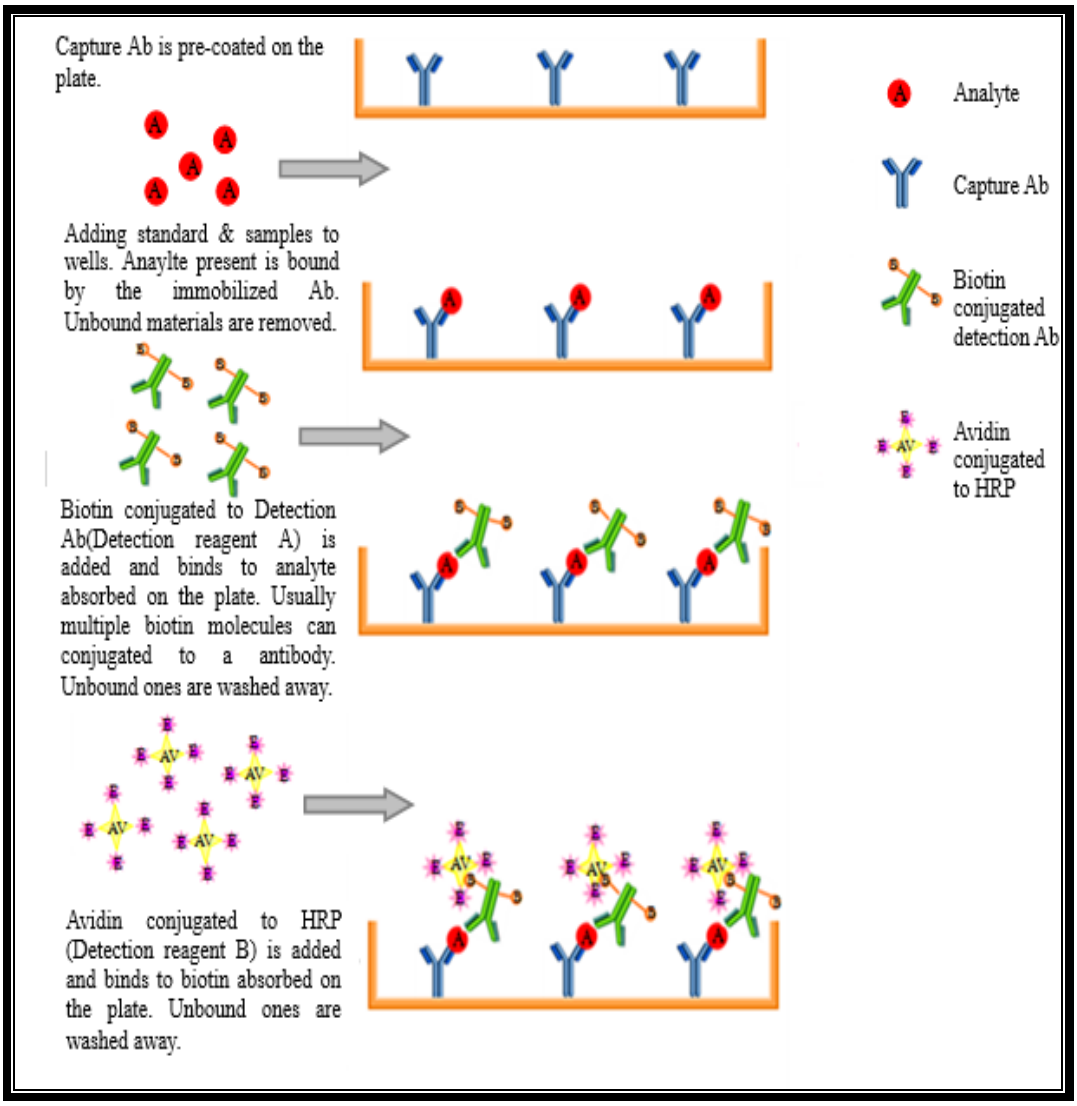


Figure 2-3: Sandwich of enzyme-linked immunosorbent assay[131]

b. Reagents Integral Preparation

1. Before was used, all reagents were brought at room temperature.
2. Wash buffer was diluted by added 20mL of wash buffer concentrate 25X into 480mL deionized or distilled water to yield 500 mL of 1X wash buffer, mixed gently until the crystals have completely dissolved.
3. The standard was prepared by incorporating 120 µ L of the standard (1600 ng/L)with 120 µL of standard diluent.

4. A standard stock solution of 800 ng/L had been prepared. Before dilution, the standard was allowed to sit for 15 minutes with moderate stirring, duplicated standard points were made by diluting the standard stock solution (800 ng/L) 1:1 with standard diluent to produce solutions of 400 ng/L, 200 ng/L, 100 ng/L, and 50 ng/L, on straight, the zero standard is the standard diluent (0 ng/L) as in Figure (2-4).
5. The proposed standard solutions were diluted by adding 120 µL of the standard diluent to a 120 µL standard solution to the prepared solution at a concentration of 400 ng/L and taking 120 µL of the first resulting solution to 120 µL of the standard diluent to prepared 200 ng/L in the same way the solutions were prepared at a concentration of 100 and 50 ng/L

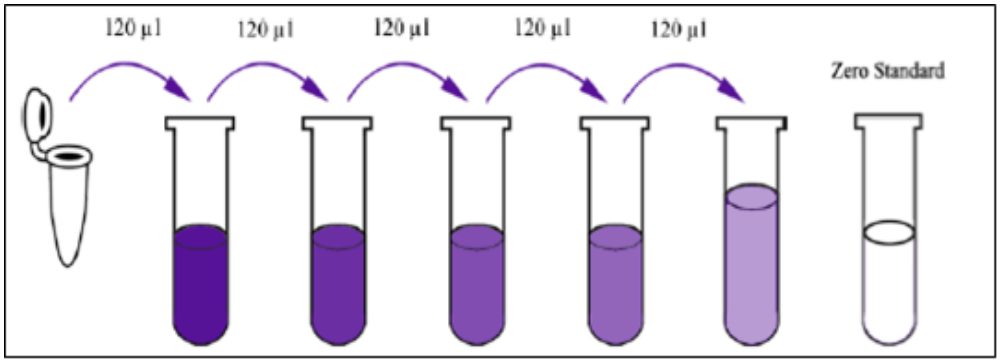


Figure 2-4: Preparation of standard concentration[131]

c. Procedure

1- All reagents, buffers, and samples had been prepared before being used and at room temperature.
2- A standard solution of 50 µL was added to the standard well without adding the antibody to the standard well.
3- A sample of 40 µL was added to sampling wells and then 10µL of anti-KISS-1 antibody had been added to the samples wells, then 50

μL of streptavidin-HRP was added to sampling and standard wells, mix well and cover the panel with a sealant, incubate 60min at 37°C.

4- The sealant was removed, and the plate was cleaned 5 times with washing solution, for each wash, wells were covered in at least 0.35 mL of wash solution was added for 30 s to 1 min. The wells were then aspirated and washed 5 times with wash solution before filled with an automated washer, paper wipes were placed out on the plate.

5- A volume of 50μL substrate solution A was added to each well, then 50μL of substrate solution B was added to each well, the covered plate was incubated with fresh plug for 10 min, at 37°C in the dark.

6- Stop solution of 50μL was added to each well, the blue color immediately turned yellow.

7- The optical density (OD-value) of each well was determined immediately by used a micro plate reader set to 450 nm within 10min after the addition of the stop solution.

d. Calculation Result

A standard curve was plotted by plotting the OD of each standard on the Y-axis versus the focus on the X-axis and plotting the best fit curve through the point on the graph as figure (2-5).

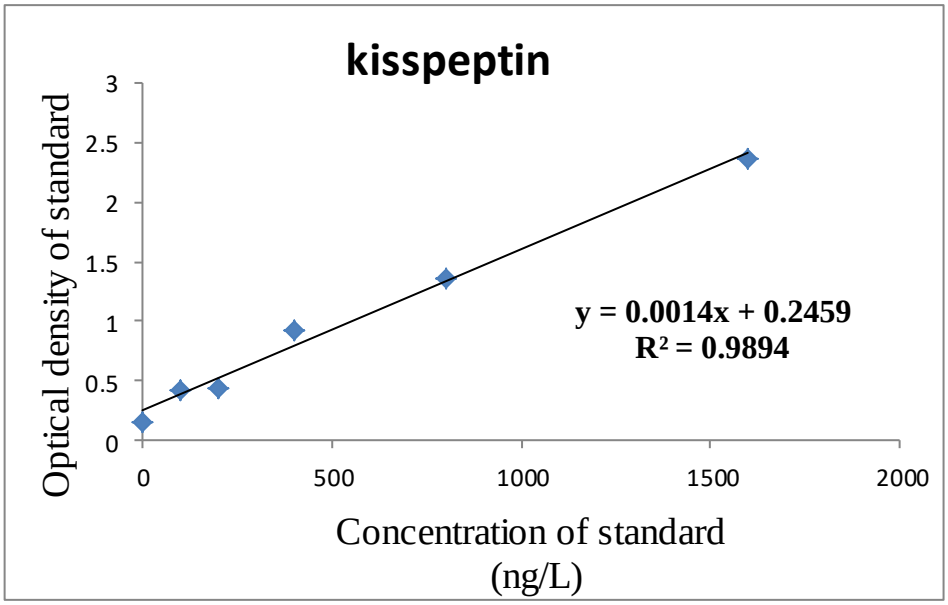


Figure 2-5: The standard curve for kisspeptin concentration

2.2.2 Molecular Part

2.2.2.1 Deoxyribonucleic Acid Extraction

a. Principle of Extraction

Genomic DNA was extracted from patients' peripheral blood in the laboratory of the Department of Biochemistry at College of Medicine, University of Babylon, the DNA was kept at (-20)°C.

Total DNA can be extracted from a variety of sources quickly and easily using a frozen DNA extraction kit (Whole Blood), peripheral blood monocytes can be used for DNA collecting, the Chaotropic salt causes cell lysis and protein degradation, allowing DNA fragments to adhere to the spindle fiberglass matrix. Elution buffer, low salt was used to elute purified DNA after DNA contaminants were removed with wash buffer solution containing ethanol as figure (2-6)[132].

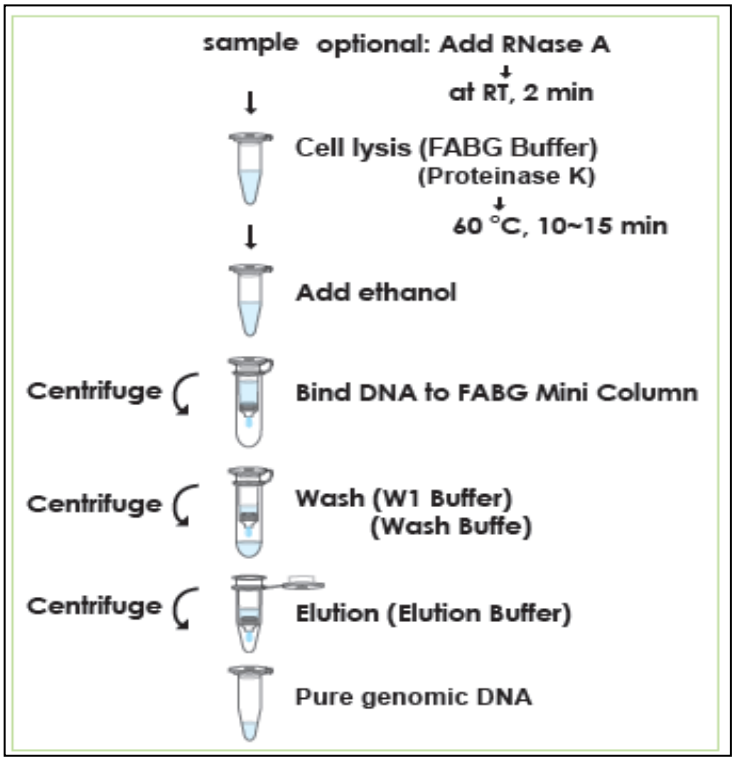


Figure 2-6: Principle of deoxyribonucleic acid extraction[132].

b. Preparation of Solutions

As recommended on the kit's leaflet, with a few changes DNA was extracted from venous blood by means of the preferred genomic DNA purification kit and another G-spin, total DNA Extraction buffer was prepared, briefly as follows:

1- Proteinase K was dissolved in 1.1 mL of deionized water.
2- Before the first used, a volume of 100 mL of absolute ethanol was placed in the washing solution.
3- The elution buffer (EL) solution was heated in a 70 °C water bath, before starting the extraction process.

Note: Fresh blood and frozen blood were used.

Table 2-3 : Components of deoxyribonucleic acid extraction kit

No.	Item	Quantity
1	Collection tube	200 pcs
2	Elution Buffer	20 mL
3	Elution Tube	100 pcs
4	FABG Buffer	30 mL
5	FABG Mini Column	100 pcs
6	Proteinase K, lyophilized	25mg , 2 vial
7	Protocol sheet	1 pcs
8	RNase A	10mg/mL ,3 vial
9	Wash Buffer (concentrate)	20 mL
10	Washing Buffer W1(concentrate)	44 mL

c. Procedure

Step 1:

- **For Frozen Blood**

1- A volume of 200 µL from frozen blood in 1.5mL microcentrifuge tube was added.

2- The blood tube had been mixed thoroughly by pulse- vortex with 40µL of proteinase K.

3- The mixture was incubated at 60 ºC for 15 minutes to lysis the leucocytes, the tube was inverted 2-3 times during the incubation period.

4- A volume of 5 µL RNase (10mg/mL) had been added to the mixture and it was shaken strongly then followed the DNA binding step.

- **For Fresh Blood (RBC lysis)**

1- A volume of 300 µL from fresh blood was transferred to eppendorf tube, then 900 µL of RBC lysis buffer was added and mix it, then incubated the mixture at 25ºC for 10 min, during incubation, inverted the tube every 3 minutes.

2- Centrifuged at 3000 Xg for 5 min and the supernatant was removed then added 100 µL of RBC lysis buffer and the cell was resuspend.

3- A volume was required of elution buffer prepared to pre-heated calculated the required volume (100µL/sample) to 70ºC (for Step 4 DNA Elution).

Step 2: Cell Lysis for Fresh and Frozen Blood

1- A volume of 200 µL FABG buffer was added to the sample mixture and it shaken by vortex then incubated the mixture at 25ºC for 10 min, during incubated, inverted the tube every 3 minutes.

2- A volume of 5 µL RNase (10mg/mL) had been added to the mixture and it was shaken strongly.

Step 3: DNA Binding for Fresh and Frozen Blood

1- A volume of 200 µL absolute ethanol had been added and was mixed by Shaking for 10 min, then it centrifuged at 18000 Xg for 5 minutes.

2- A column of FABG was placed in a 2mL collection tube.

3- Supernatant including any precipitate was transferred to FABG column, and centrifuged at 18000 Xg for 1 minute.

Step4: Wash

1- Collection tube containing the flow-through was discarded and 400 µL of W1 buffer was added, then centrifuged at 18000 Xg for 1 minute.
2- Collection tube containing the flow-through was discarded and 600 µl of wash buffer was added, then centrifuged at 18000 Xg for 1 minute.
3- The FABG column placed in a new 2 mL collection tube, re-centrifuged after discarded flow rate for 3 minutes at the same speed to dry matrix column.

Step 5: DNA Elution

1- Eluted DNA in new eppendorf tube 100 µL of pre-heated elution buffer was added to column and left 10 minutes at 37ºC to absorb it.
2- The tube was centrifuged at 8.000 Xg for one minute to elute the purified DNA.
3- Additional DNA samples were obtained by placing the FABG column in a new eppendorf and added 50 µl of pre-heated elution buffer solution and left 10 minutes at 37ºC to absorb it.
4- Repeated centrifuged at 8.000 Xg for one minute to elute the purified DNA.

2.2.2.2 Determination of DNA Concentration and Purity

The absorbance was measured by a nanodrop device, figure (2-7) shows how absorbance measurements are used to determine the concentration and purity of DNA after each DNA extraction. The concentration is proportional to the absorbance, proteins have a maximum absorbance capacity of 280 nm and DNA has a maximum absorbance capacity of 260 nm where the A260/A280 ratio was used to determine DNA purity. If it is approximately to 1.8, the DNA is considered pure.

Similar to the absorbability at 230 nm, the absorbability at 230 nm was the result of other pollutants, and thus A260/A230 was also evaluated, the DNA concentration assessed by the nanodrop was usually in the range of 1.8–2.2 for unstained DNA.

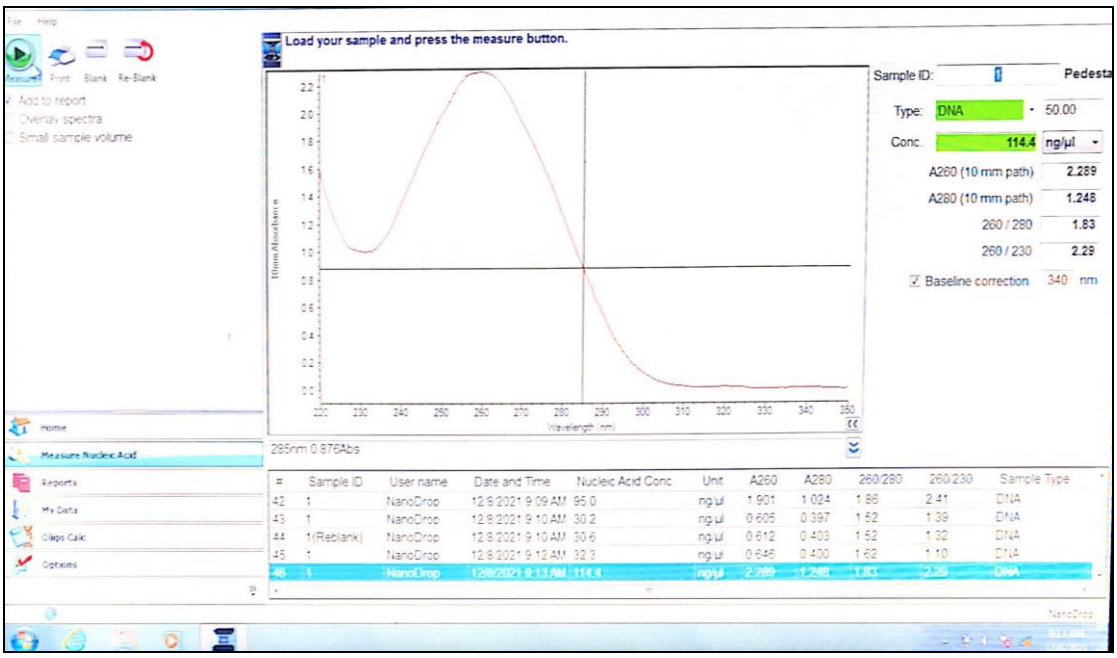


Figure 2-7: Concentration and purity of deoxyribonucleic acid

2.2.2.3 Detection of DNA Presence

The detected of DNA by used agarose gel electrophoresis technique.

a. Agarose Gel Electrophoresis

The standard method for separating, identify, and purify the DNA fragments is electrophoresis on agarose gel, bands containing less than 1-10ng of DNA can be directly determined by staining with low concentration of safe red fluorescent dye.

Electrophoresis is the process through which charged molecules in solution, primarily protein and nucleic acids, travel in response to an electric field, their rate of migration or mobility in the electric field, was high, and it

was dependent on the field's strength, net charge, size, and shape of molecules as well as the ionic strength, viscosity, and temperature of the medium in which the molecules were moving. The following process was used to move of DNA in the gel-based on its molecular weight, conformation, and concentration of the agarose, the voltage applied, and strength of the electrophoresis buffer[133].

1- To make 100 mL of a 2% agarose solution, 2 g of agarose was weighted and putted into conical flask, then 100 mL of TBE 1X was added.
2- The TBE buffer (10X) PH 8.3 of the stock solution (100mL of stock solution 10X dissolved in 900mL DW to prepared 1X of TBE).
3- The agarose was completely dissolved and the solution becomes clear, the solution was allowed to cool to about 45-50°C and 4 µL of red stain was added.
4- The gel was cast in the gel tray and allowed to harden at room temperature for about 20 minutes, with a thickness of less than 0.5 cm because a thick gel could reduce sensitivity.
5- The comb placed in the gel tray about 1 inch from one end of the tray and position the comb vertically, so that the teeth are about 1–2mm above the surface of the tray.
6- After carefully removing the comb, the tray was placed in the electrophoresis chamber and electrophoresis solution (1X-TBE) was applied to the wells until just covered (the same buffer used for agarose preparation).
7- At this step the DNA product (4µL) was mixed with (3µL) loading dye (bromophenol blue and glycerol) had been loaded on each well with extreme cautions to avoid damages of the wells and cross contamination of neighboring wells.

8- Later supplement to the power supply , run at 50 V, for 10 min and then at 100 V for 30 min.
9- Observed the DNA bands which will be visible under short wave UV light.

b. Photo Documentation

The agarose gel was positioned a top the UV trans illuminator device, the agarose gel was visualized in a UV supplied with the gel documentation device. the gel was exposed to UV light, and images were taken using the gel documentation of the computing device's digital camera, a tracking dye (bromophenol blue) was added to DNA in loading step of the electrophoresis method because DNA was colorless.

2.2.3 Markers Selection

Candidate studies had been useful in finding polymorphisms linked to a higher risk of developing PCOS[28], association of PCOS with *KISS1* that located in chromosome 1, also, the two SNP (rs4889 G>A,C in exon 3 of *KISS1* and rs372790354 G>A in 5UTR of *KISS1*) were selected because they were more polymorphic, in addition to it related with the target protein (kisspeptin), used the website below to take information about SNPs, as shown in figure (2-8).

- https://www.ncbi.nlm.nih.gov/variation/view
- https://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000170498;r=1:204190341-204196491;t=ENST00000367194.

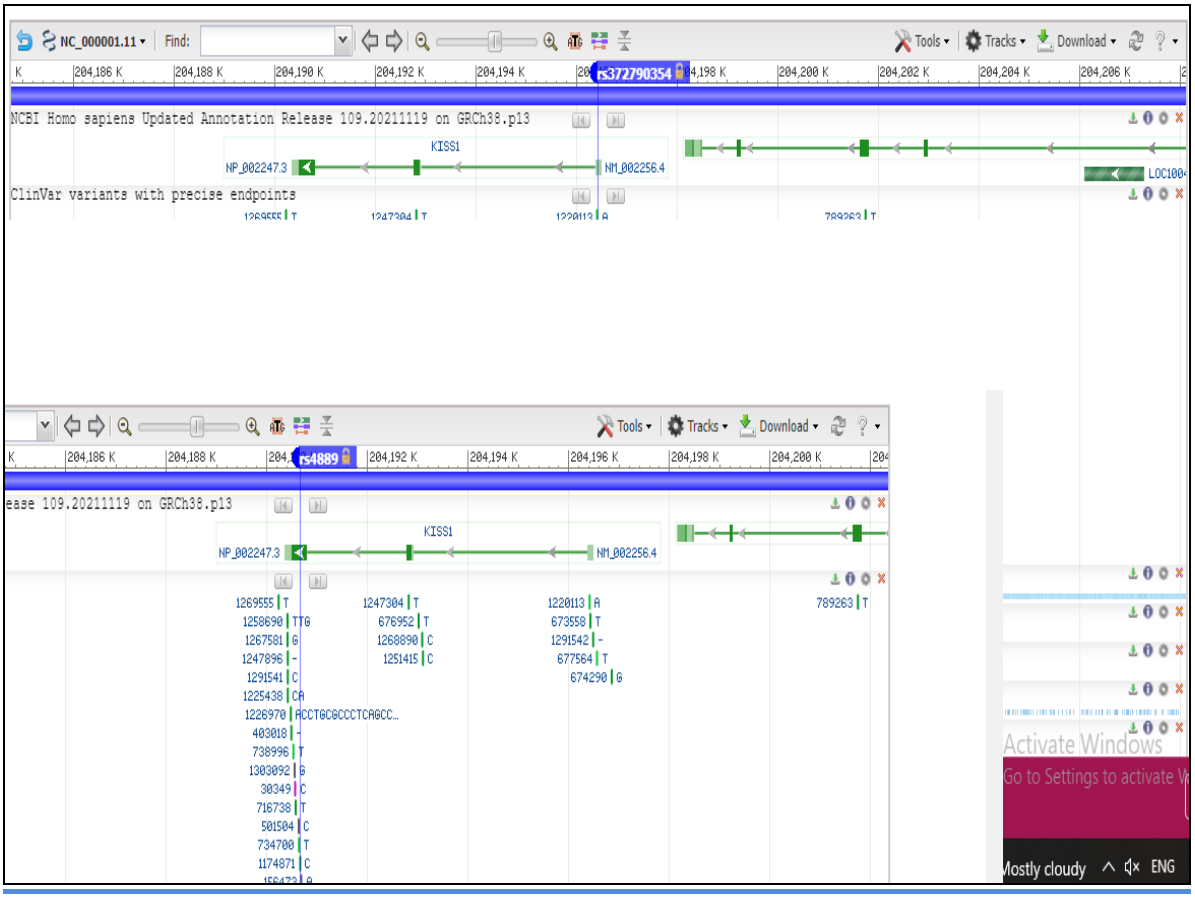


Figure 2-8: Location of single nucleotide polymorphism in *KISS1* Gene[96]

2.2.4 High Resolution Melt Assay for Genotyping Method

- **Principle**

analysis of HRM was performed on double-stranded DNA samples, typically using the real-time polymerase chain reaction prior to HRM analysis to amplify the DNA region in which their mutation, essentially the real-time PCR process turns a tiny amount of region DNA into a large amount, this region that was amplified known as the amplicon, after the PCR process, the HRM analysis begins. The process is simply precise warming of the amplicon DNA from around 55°C up to around 95°C as in figure (2-9). At some point

during this process the melting temperature of the amplicon was reached and the two strands of DNA "melt" apart[137,138].

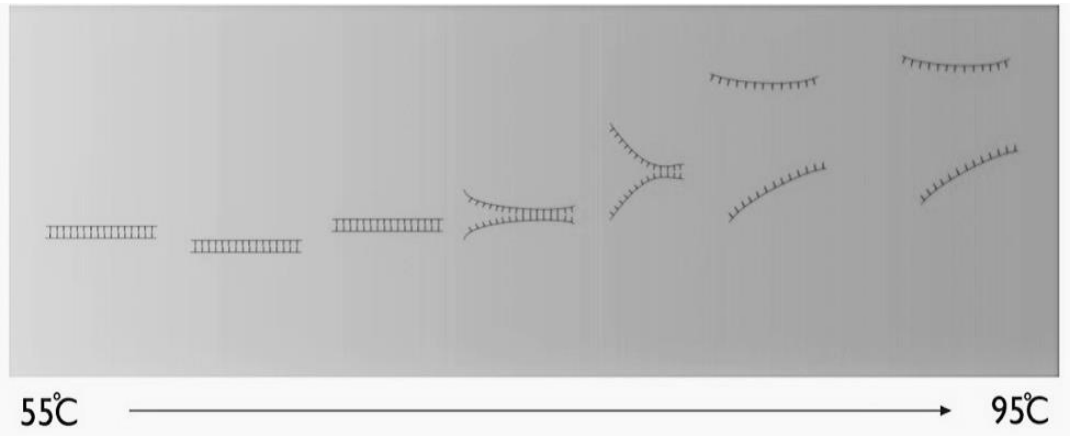


Figure 2-9: Temperature gradient for high resolution melt analysis[138]

The secret of HRM to monitor this process happening in real-time, was achieved by using a fluorescent dye, the dyes that used for HRM known as intercalating dyes that bind specifically to double-stranded DNA and when they are bound they fluoresce brightly.

So at the beginning of the HRM analysis there is a high level of fluorescence in the sample because of the billions of copies of the amplicon. But as the sample is heated up and the two strands of the DNA melt apart there is no longer any double stranded DNA present and thus fluorescence was reduced, the HRM PCR has a camera that watches this process by measuring the fluorescence, the machine then simply plots this data as a graph known as a melt curve showing the level of fluorescence vs. the temperature. HRM real time PCR was simple, fast, cost-effective and efficient genotyping technique, there was no need for costly probe synthesis and labelling, also time-saving (completed in about two hours) and had a low risk for DNA contamination, one current limitation of HRM is the possibility had designed amplicon that as short.

The three genotypes are known as "Wild–type", "Heterozygote" or "homozygote" respectively to compression with unknown DNA samples. Each gives a melt curve that is slightly different with a high-quality HRM assay, it is possible to distinguish between all three of these models as show in figure (2-10).

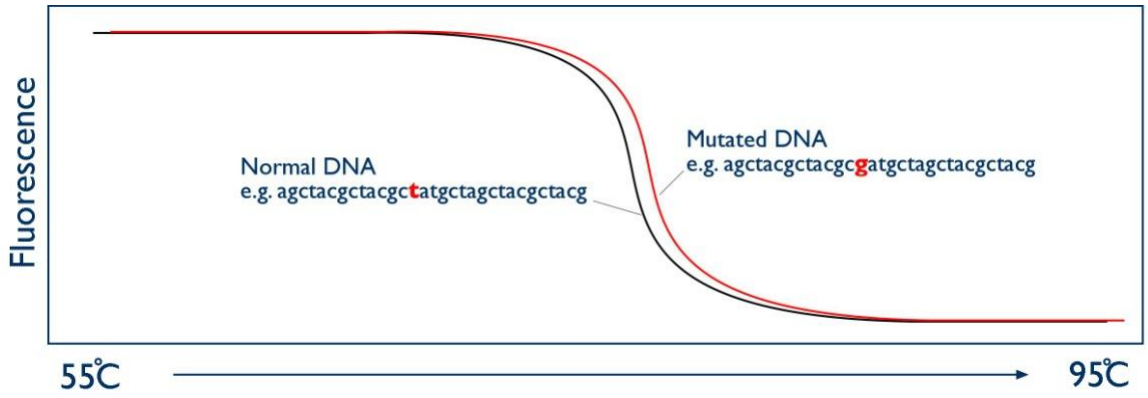


Figure 2-10: Genotyping curve by high resolution melt

- **Real Time- PCR- HRM primer and controls DNA was design as a following**

1- The sequence of the *KISS1* gene was taken from NCBI site according to the add gene site, and then the required SNPs were identified and through (flank) selection was taken the sequence.

2- Transferred the sequence to the primer site 3, then determined the length of the required primer around the SNPs.

3- The control DNA was designed two fragment of DNA, firstly the sequence of DNA fragment was contained wild G SNPs and secondly sequence of DNA fragment was contained mutant A SNPs. Also the sequence of DNA complementary the selected primers, and then obtained a sequence of the resulting primer.

4- After going through all the steps, the prefix for the SNPs on *KISS1* gene (rs4889 sequencing and rs372790354 sequencing) was using NCBI to avoid false-positive amplification to determine the primer (forward and reverse) location and SNPs on the gene as in Table (2-4).

NOTE:

- The HRM PCR required fragment of mutant and wild DNA for each SNP.
- For each SNP was designed a primer pair amplify around 80-100 bp. to be suitable for HRM analysis as show in figure (2-11)[134].

Table 2-4: Primers used in present study

Primers sequences of SNPs	Name of primer	Seq. of primer	Length	Product size	Tm	GC %
HRM rs4889 G>A	Forward	CTGGGGTGCGGGGATCTG	18	91	62	72
HRM rs4889 G>A	Reverse	GACCTCGCTGTCCCCGCC	18	91	65	77
Wild rs4889 Allele G	CTGGGGTGCGGGGATCTGGCGGCTGTGGGGGGCGGACAGG CCCGGCTGCTGGGGGCTCCCGGAGCTCTCGGGGGGCGGGG ACAGCGAGGTC					
Mutant rs4889 Allele A	CTGGGGTGCGGGGATCTGGCGGCTGTGGGGGGCGGACAGG CCCGGCTGCTGGAGGCTCCCGGAGCTCTCGGGGGGCGGGG ACAGCGAGGTC					
HRM rs372790354 G>A	Forward	CTCAGTCCTGGCCTGGG	17	78	58	70
HRM rs372790354 G>A	Reverse	CCCTCTGGACATTCACCCA	19	78	58	57
Wild rs372790354 Allele G	CTCAGTCCTGGCCTGGGCAGGAGTCTGGCGGAGCCTCTGAG GTGACGAGACCACCTGGCTGGGTGAATGTCCAGAGGG					
Mutant rs372790354 Allele A	CTCAGTCCTGGCCTGGGCAGGAGTCTGGCGGAGCCTCTGAG GTGACAAGACCACCTGGCTGGGTGAATGTCCAGAGGG					

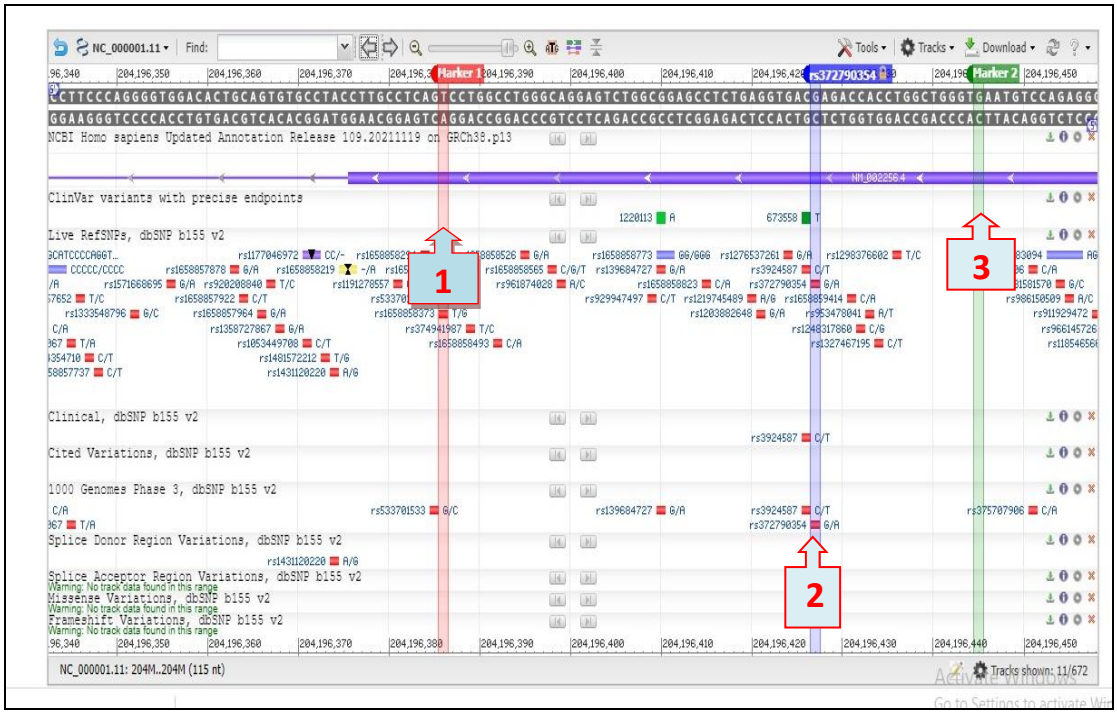


Figure 2-11: Designs primers pair for single nucleotide polymorphisms.
(1) Forward primer,(2)Selected SNP,(3)Revers primer

- **Prepare The Primers**

Primers were commonly shipped in a lyophilized state, the units of a lyophilized primer were given as a mass, in picomoles.

To create a stock of primers in sterile free nuclease water to obtain a master stock that would be used again to obtain a working stock, the solution was divided into a number of single-use tubes to reduce thawing and refreezing of the primers then both primers were stored at -20°C.

The following steps were followed for reconstituting and diluting the primers

1- The tubes were spin before the covers opened.
2- The concentration of 100 Picomoles /µL was obtained by adding the necessary amount of sterile and free nuclease water according to the instructions of the manufacturer (Master Stock).
3- A volume of 10µL of the main stock was transferred to a 0.2 mL Eppendorf tube containing 90µL of sterile, free nuclease water (working stock) for each forward and reverse primers.
4- The main stock was kept at -20°C.
5- The working stock was kept at -20°C.
6- Before being used in the PCR, the working stock was melted on ice and shaken, and then stored at -20°C.

- **Running the Polymerase Chain Reaction**

The real time-PCR HRM reaction was done by mixing PCR components with DNA solution and used the several different thermal attempts to PCR as in Tables (2-5) and (2-6) respectively.

Table 2-5 : Mix reaction of polymerase chain reaction for genotyping of *KISS1* rs372790354 G>A and rs4889 G>A

Component	Volume /Total 20µL			
PCR super mix	10 µL			
Forward Primer	1 µL			
Reverse Primer	1 µL			
free nuclease H_2O	5 µL			
Sample DNA	3 µL			
Wild DNA Known		3 µL		1.5 µL
Mutant DNA Known			3 µL	1.5 µL
	Sample DNA	Wild DNA	Mutant DNA	Hetero DNA

Table 2-6 : Optimization of polymerase chain reaction for genotyping of *KISS1* gene rs372790354 G>A and rs4889 G>A

No. of Program	Tm	Time	No. of cycle	Product
Pre heating	94	30sec	1	Failure
Denaturation	94	5 sec	35	
Annealing	--		-	
(rs372790354)	54,55,57,58	10sec	-	
(rs4889)	58,59,61			
Extension	72	20sec		
HRM	55-95		1	
Pre heating	94	30sec	1	Succses
Denaturation	94	5 sec	35	
Annealing	--		-	
(rs372790354)	56	10sec	-	
(rs4889)	60			
Extension	72	20sec		
HRM	55-95		1	

2.2.5 Statistical Analysis

Data of the study participants were transferred into computerized database, revised for errors or inconsistency and then managed, processed and analyzed using the statistical package for social sciences (IBM-SPSS) version 23. All continuous (scale) variables were expressed as the mean ± standard error (mean ± SE). Scale variables like age, BMI. Following the statistical normal distribution, parametric tests were applied. To compare the studied parameters between studied groups, student ***t*** test for two groups was used, while for more than two groups analysis of variables test (ANOVA) was applied[136].

Correlation coefficient (r) was used to find the relationship between two continuous variables. A *p*-value of ≤ 0.05 was considered as a significant.

Test for Hardy-Weinberg equilibrium of allelic or genotypic association in cases versus control were evaluated by Chi – square (x^2) test, this analysis was performed for all genotypes in this study using Hardy-Weinberg equilibrium online calculator.

To assess the predictability of PCOS, logistic analysis of both SNPs was applied, this yielded odds ratio (OR). Also the 95% confidence interval was calculated which is good estimator for the significance of the OR; when the value of "one" included within interval, this is an indicator that the OR is not significant, web sites were used for statistical analysis [137], as shown in below.

1- odds ratio was measured by an online software program (https://www.medcalc.org/calc/odds_ratio.php)

2- Hardy Weinberg equilibrium was measured by (https://scienceprimer.com/hardy-weinberg-equilibrium-calculator)

3 Results and Discussion

3.1 Demographic Characterization of Studied Groups

Demographic data of the studied groups summarize in Table (3-1), the results were expressed as means ± standard error (SE) between patient and control, (27.78 ±0.9 and 29.15 ±0.89) for age and (28.07 ±0.81 and 28.2 ±0.75) for BMI with P-value 0.28 and 0.9 respectively, also the mean±SE of the waist to hip ratio for patient and control was (0.95 ±0.01 and 0.83 ±0.006 with P-value <0.001).

Table 3-1 Demographic data of patients and control groups

Variable	groups	No.	Means ±SE	P- value
Age (years)	Patients	60	27.78 ±0.9	0.28
	Control	60	29.15 ±0.89	
BMI Kg/m2	Patients	60	28.07 ±0.81	0.90
	Control	60	28.2 ±0.75	
Waist/ Hip ratio	Patients	60	0.95 ±0.01	**<0.001**
	Control	60	0.83 ±.006	

BMI (body mass index) ,SE(Standard Error), P-value ≤0.05 was significant.

The demographic characteristics of all subdivided groups. also, measured and summarized in Table (3-2), for descriptive age mean±SE of normal weight PCOS patients was (28.72 ± 1.31), normal-weight controls (28.4 ± 1.23), and the mean ±SE for obese PCOS(26.85 ± 1.23), obese without PCOS (29.9 ± 1.30) with (P>0.05). between all subdivided groups more than 0.05 were no significant difference.

On the other hand, mean ± SE of BMI for normal-weight PCOS patients was (22.4 ± 0.30), normal-weight controls was (23.2 ±0.31), and the mean ±SE for obese PCOS (33.66 ± 0.69), obese without PCOS (33.14 ±0.55) with (P>0.05).

According to the WHR mean ± SE for normal-weight PCOS patients (0.9±0.01) and normal-weight controls (0.82±0.01) with (P<0.001), while the mean ± SE for obese PCOS (1.0±0.01) and obese without PCOS (0.85±0.007) with (P<0.001), that indicated highly significant differences in all subgroups, PCOS patients had a higher WHR than controls.

Table 3-2 Demographic data within studied groups

Variable	group	Study groups	No.	Means ±SE	P- value
Age (years)	N.Wt. PCOS	Ob. PCOS	30	26.8 ±1.23	0.72
		N.Wt. Control	30	28.4±1.23	0.99
		Ob. Without PCOS	30	29.9±1.30	0.91
	Ob. PCOS	N.Wt. PCOS	30	28.72 ±1.31	0.72
		N.Wt. Control	30	28.4±1.23	0.82
		Ob. Without PCOS	30	29.9±1.30	0.33
BMI (kg/m2)	N.Wt. PCOS	Ob. PCOS	30	33.66±0.69	**<0.001**
		N.Wt. Control	30	23.2±0.31	0.74
		Ob. Without PCOS	30	33.14±0.55	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	22.4±0.30	**<0.001**
		N.Wt. Control	30	23.2±0.31	**<0.001**
		Ob. Without PCOS	30	33.14±0.55	0.90
Waist/ Hip ratio	N.Wt. PCOS	Ob. PCOS	30	1.0±0.01	**<0.001**
		N.Wt. Control	30	0.82±0.01	**<0.001**
		Ob. Without PCOS	30	0.85±0.007	**0.019**
	Ob. PCOS	N.Wt. PCOS	30	0.9±0.01	**<0.001**
		N.Wt. Control	30	0.82±0.01	**<0.001**
		Ob. Without PCOS	30	0.85±0.007	**<0.001**

N.Wt. (normal weight) Ob.(obese), BMI (body mass index), PCOS (polycystic ovary syndrome), SE(Standard error), P value ≤0.05 was significant.

3.1.1 Age

All females in this study were in reproductive age, matching of age was used in the present study between patient and control groups to avoid variations in parameter results that may have originated as a result of the large variance in age [36].

3.1.2 Body Mass Index

The classification of body mass index According to the WHO as show in Table (3-A), BMI is considered an anthropometric index for assessing obesity [138].

Table 3-A : Classification of body mass index [138]

Definition	BMI category (kg/m2)
Underweight	less than 18.5
normal weight	(18.5-24.9)
overweight	(25-29.9)
Obesity	greater than or equal to 30

Barber *et al*. (2021)[5] and Ghatnatti *et al*. (2022)[3] confirmed that weight gain and obesity occur in approximately (73-76)% of females with PCOS[139]. Even though Azziz and Ricardo (2008)[140] suggested that the development of obesity in PCOS patients is caused due to surrounding environment, unhealthy lifestyle and insulin resistance. To investigate the obesity effects on biochemical parameters in PCOS patients were selected obese subjects to determine these effects.

3.1.3 Waist to Hip Ratio

Abdominal obesity is defined as a WHR of more than 0.85 for females, the waist to hip ratio is used as a measurement of obesity [141], risk level of WHR is summarized in Table (3-B) [125] :

Table 3-B : Risk level of waist to hip ratio [125]

Definition	WHR
Low	0.80 or less
Moderate	0.81-0.85
High	0.86 or higher

As indicated in Table (3-1), there was a highly significant difference between patients and control groups, also the highly significant differences ($P<0.01$) present in all subgroups as in Table(3-2), PCOS patients had a higher WHR than control that confirming the contribution of abdominal fat as an etiological mechanism of PCOS[142].

Moreover, the WHR was increased in normal weight PCOS patients compared to obese controls could help in indicating that PCOS has an effect on abdominal fat, this fact was supported in studies recorded most PCOS patients exhibit some form of abdominal obesity [144,145].

3.2 Biochemical Results

The serum LH level was significantly lower in the control group than PCOS group, while the serum FSH level was conversely different, it was higher in the control group than PCOS group with a significant increase in the LH/FSH ratio in PCOS group compared to control group, there was a significant increase in kisspeptin, testosterone and DHEA levels in PCOS group compared to controls. Table (3-3) shows mean differences in biochemical results including (LH, FSH, LH/FSH Ratio, Free testosterone, DHEA-S and Kisspeptin).

Table 3-3: Biochemical variables in studied groups

Dependent Variable	Group	Study group	No.	Mean ±SE	95% Confidence Interval		P- value
					Lower	Upper	
LH (mIU/ml)	N.Wt. PCOS	Ob. PCOS	30	8.84±1.24	6.21	11.3	0.65
		N.Wt. Control	30	5.35±0.42	4.4	6.2	**0.002**
		Ob. Without PCOS	30	5.71±0.34	5.01	6.42	**0.004**
	Ob. PCOS	N.Wt. PCOS	30	9.41±1.15	7.05	11.74	0.65
		N.Wt. Control	30	5.35±0.42	4.4	6.2	**0.007**
		Ob. Without PCOS	30	5.71±0.34	5.01	6.42	**0.015**
FSH (mIU/ml)	N.Wt. PCOS	Ob. PCOS	30	4.49±0.43	3.6	5.37	0.65
		N.Wt. Control	30	7.42±0.54	6.3	8.53	**<0.001**
		Ob. Without PCOS	30	7.43±0.36	6.6	8.1	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	4.23±0.30	3.6	4.8	0.65
		N.Wt. Control	30	7.42±0.54	6.3	8.53	**<0.001**
		Ob. Without PCOS	30	7.43±0.36	6.6	8.1	**<0.001**
LH/FSH Ratio	N.Wt. PCOS	Ob. PCOS	30	1.9±0.11	1.66	2.1	**0.01**
		N.Wt. Control	30	0.73±0.30	0.66	0.79	**<0.001**
		Ob. Without PCOS	30	0.76±0.24	0.71	0.81	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	2.46±0.28	1.8	3.0	**0.01**
		N.Wt. Control	30	0.73±0.30	0.66	0.79	**<0.001**
		Ob. Without PCOS	30	0.76±0.24	0.71	0.81	**<0.001**
kisspeptin ng/L	N.Wt. PCOS	Ob. PCOS	23	382.9±20.5	340.3	425.5	**0.005**
		N.Wt. Control	22	374.4±10.7	352.1	396.7	**0.004**
		Ob. Without PCOS	23	401.2±9.3	381.9	420.5	**0.010**
	Ob. PCOS	N.Wt. PCOS	22	611.2±112	378.9	844.3	**0.005**
		N.Wt. Control	22	374.4±10.7	352.1	396.7	0.91
		Ob. Without PCOS	30	401.2±9.3	381.9	420.5	0.81
F.Testosterone (pg/ml)	N.Wt. PCOS	Ob. PCOS	30	2.7±0.27	2.23	3.3	**<0.002**
		N.Wt. Control	30	0.86±0.03	0.7	0.96	**<0.001**
		Ob. Without PCOS	30	0.89±0.06	0.7	1.0	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	2.05±0.14	1.72	2.3	**0.002**
		N.Wt. Control	30	0.86±0.03	0.7	0.96	**<0.001**
		Ob. Without PCOS	30	0.89±0.06	0.7	1.0	**<0.001**
DHEA-S (µg/dl)	N.Wt. PCOS	Ob. PCOS	30	320±31.6	255.23	384.9	0.91
		N.Wt. Control	30	173.5±11.1	150.70	196.3	**<0.001**
		Ob. Without PCOS	30	174.7±11.6	150.9	198.5	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	323.6±31.8	258.3	388. 8	0.91
		N.Wt. Control	30	173.5±11.1	150.70	196.3	**<0.001**
		Ob. Without PCOS	30	174.7±11.6	150.9	198.5	**<0.001**

LH(Luteinizing Hormone), FSH(Follicle-Stimulating Hormone), DHEA (Dehydroepiandrosterone Sulfate) , PCOS (polycystic ovarian syndrome), N.Wt. (normal weight) Ob.(obese), BMI (body mass index) ,SE(standard error), P value $\leq$0.05 was significant.

3.2.1 Concentration of Gonadotropic Hormones Levels of Studied Groups

a. Luteinizing Hormone

According to the analysis of one way ANOVA between the groups as shown in Table (3-3) a significant differences (P= 0.002) between the mean of LH level for the normal weight (patients and control groups), (9.41 ± 1.15), (5.35 ± 0.42) respectively and significant differences (P=0.004) between normal weight patient with an obese without PCOS (5.71 ± 1.26).

Also significant differences (P= 0.015) between the mean ±SE for obese PCOS(8.84 ± 1.24) and obese without PCOS, while no significant differences (P>0.05) between normal-weight PCOS patients and obese PCOS.

Derived from Table 3-3: Luteinizing hormone level

Dependent Variable	Group	Study group	No.	Mean ±SE	95% Confidence Interval		P- value
					Lower	Upper	
LH (mIU/ml)	N.Wt. PCOS	Ob. PCOS	30	8.84±1.24	6.21	11.3	0.65
		N.Wt. Control	30	5.35±0.42	4.4	6.2	**0.002**
		Ob. Without PCOS	30	5.71±0.34	5.01	6.42	**0.004**
	Ob. PCOS	N.Wt. PCOS	30	9.41±1.15	7.05	11.74	0.65
		N.Wt. Control		5.35±0.42	4.4	6.2	**0.007**
		Ob. Without PCOS		5.71±0.34	5.01	6.42	**0.015**

Current study was observed no significant difference between normal-weight and obese patient, that there was no effect of obesity on the level of LH, and an increase of LH secretion in PCOS patients.

The findings of this study was supported by Blank *et al.*(2006), and Saadia (2020) they also found that there is a highly significant difference of LH between patient and control and also found no statistically difference between the obese and non-obese groups [9,10].

On other hand, the results were not concordant with the study of Lal *et al.* (2017) they found a significant differences between obese and non-obese females regarding LH, FSH, and LH/FSH ratio [147].

The increase of LH level in PCOS patients may be due to decreased sensitivity of the GnRH pulse generator to feedback inhibition by ovarian steroids that lead to rapid GnRH pulse frequency and distribution in gonadotropin secretion that prefers the secretion of LH over FSH [148].

b. Follicle-Stimulating Hormone

As in Table (3-3) a significant difference (P<0.001) between means of FSH levels (4.49 ± 0.43), (7.43± 0.36) of obese groups (with and without PCOS) respectively. While a significant difference (P<0.001) between means (7.42 ± 0.54), (4.23±0.30) of the normal weight groups (control and patient) respectively and no significant difference (P>0.05) between the means of patient groups (obese and normal weight), but finding a significant difference between the mean of normal weight PCOS with an obese females without PCOS group.

Derived from Table 3-3: Follicle stimulate hormone level

Dependent Variable	Group	Study group	No.	Mean ±SE	95% Confidence Interval		P- value
					Lower	Upper	
FSH (mIU/ml)	N.Wt. PCOS	Ob. PCOS	30	4.49±0.43	3.6	5.37	0.65
		N.Wt. Control	30	7.42±0.54	6.3	8.53	**<0.001**
		Ob. Without PCOS	30	7.43±0.36	6.6	8.1	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	4.23±0.30	3.6	4.8	0.65
		N.Wt. Control	30	7.42±0.54	6.3	8.53	**<0.001**
		Ob. Without PCOS	30	7.43±0.36	6.6	8.1	**<0.001**

The result confirms that decreased level of serum FSH concentration in PCOS patients more than in control group.

Indeed result of FSH in the current study was in agreement with the results of previous studies by Yasmen *et al.* (2019), and Suresh *et al.* (2015) [4,147] which showed reduced FSH levels in females with PCOS.

Ibrahim and Abdelsalam (2015) [150] reported in their study that FSH level was insignificantly increased in PCOS females. Cascella *et al.* (2008) [151], reported that the level of FSH did not alter significantly. along with another study that disagreement Haneen *et al*. (2020) study which measured FSH showed a non-significant difference in serum FSH level was observed in the patient's group when compared with control [152].

The interpretation of the results in present study is a defect in pulsating GnRH secretion which leads to the decreased FSH concentration along with increased LH resulting in elevated androgen with decreased estrogen [153].

c. Luteinizing Hormone to Follicle-Stimulating Hormone Ratio

The Table (3-3) shows a significant difference (P<0.001) between means of all groups, for obese groups (with and without PCOS) were (1.9 ± 0.11), (0.76 ± 0.24) respectively, and means of the normal weight groups (control and patient) were (0.73 ± 0.30), (2.46 ± 0.28) respectively.

Derived from Table 3-3: Luteinizing hormone to follicle-stimulating hormone ratio level

Dependent Variable	Group	Study group	No.	Mean ±SE	95% Confidence Interval		P- value
					Lower	Upper	
LH/FSH Ratio	N.Wt. PCOS	Ob. PCOS	30	1.9±0.11	1.66	2.1	**0.01**
		N.Wt. Control	30	0.73±0.30	0.66	0.79	**<0.001**
		Ob. Without PCOS	30	0.76±0.24	0.71	0.81	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	2.46±0.28	1.8	3.0	**0.01**
		N.Wt. Control	30	0.73±0.30	0.66	0.79	**<0.001**
		Ob. Without PCOS	30	0.76±0.24	0.71	0.81	**<0.001**

Regarding LH/FSH ratio, the results recorded a highly significant elevation in the patient groups when compared with the control, the results that

appear in Table (3-3), these findings agreed with Lewandowski *et al.*(2011) study which explains the role of ratio in PCOS etiology and found that PCOS females have an increased speed of hypothalamic GnRH pulses, leading to an increase in the LH/FSH ratio [154].

As known, LH/FSH >2.5 is considered a PCOS disease, but the present results showed that females with PCOS may have LH/FSH levels <2.5. but this ratio was higher than control females as in previous studies[155].

The absence of a normal feedback mechanism on GnRH by ovarian estrogen and progesterone lead to an increase of LH release in approximately 60% of the patients with PCOS which agree with the results obtained in this study where about 2/3rd of the females with PCOS have high LH/FSH ratio[156].

In addition, the current study failed to show any significant correlation coefficients between BMI and the serum hormone levels of PCOS females including LH, FSH, and LH/FSH ratio in PCOS patients subject, on other hand, positive correlations between LH with LH/FSH Ratio observed in normal weight and obese patient as shown in figures (3-1,3-2) with ($P<0.001$, $r = 0.73$), ($P=0.002$, $r = 0.74$) respectively.

Otherwise, the correlation coefficients between FSH and LH/FSH in normal-weight patient is negative as appeared in figure (3-2) with ($P=0.03$, $r = - 0.39$) while the correlation coefficients in an obese patient are positively between LH and FSH ($P<0.001$, $r = 0.47$) as in figure (3-3) and no significant correlation was observed between FSH and LH/FSH.

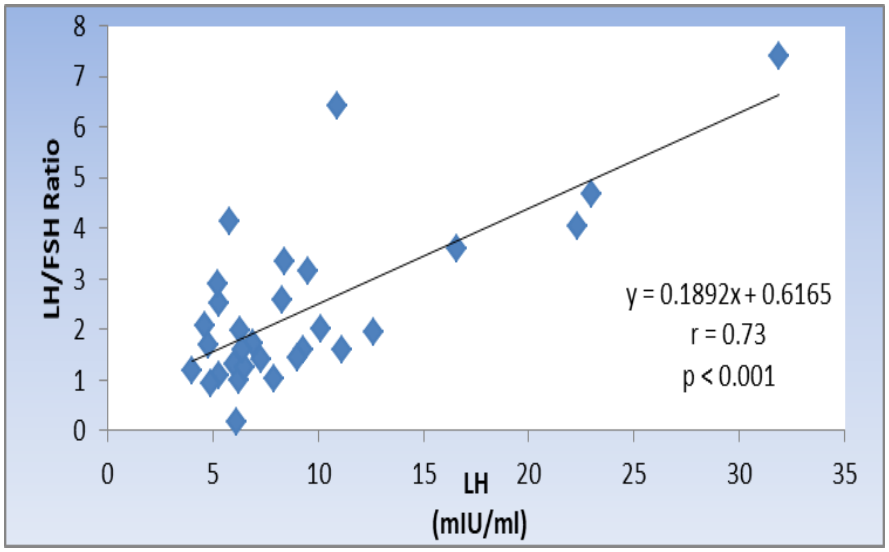


Figure 3-1: Correlation between luteinizing hormone to follicle-stimulating hormone ratio and luteinizing hormone for PCOS normal weight patients

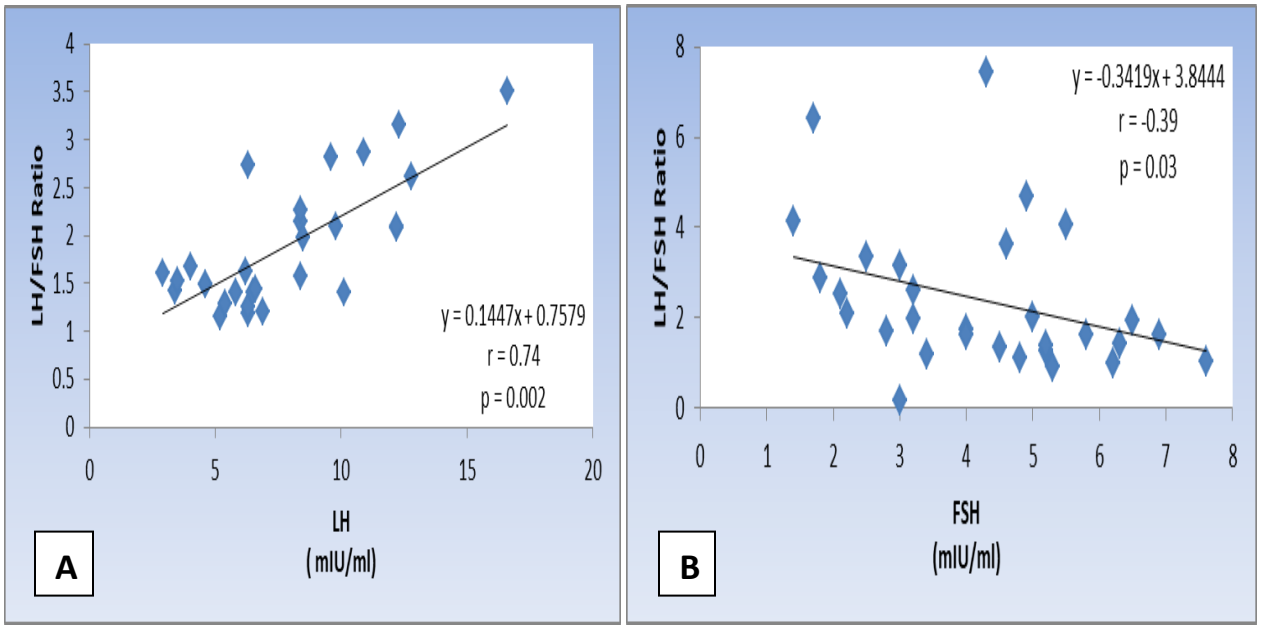


Figure 3-2 : Correlation between luteinizing hormone to follicle-stimulating hormone ratio and A) luteinizing hormone, B) follicle stimulating hormone for PCOS obese patients

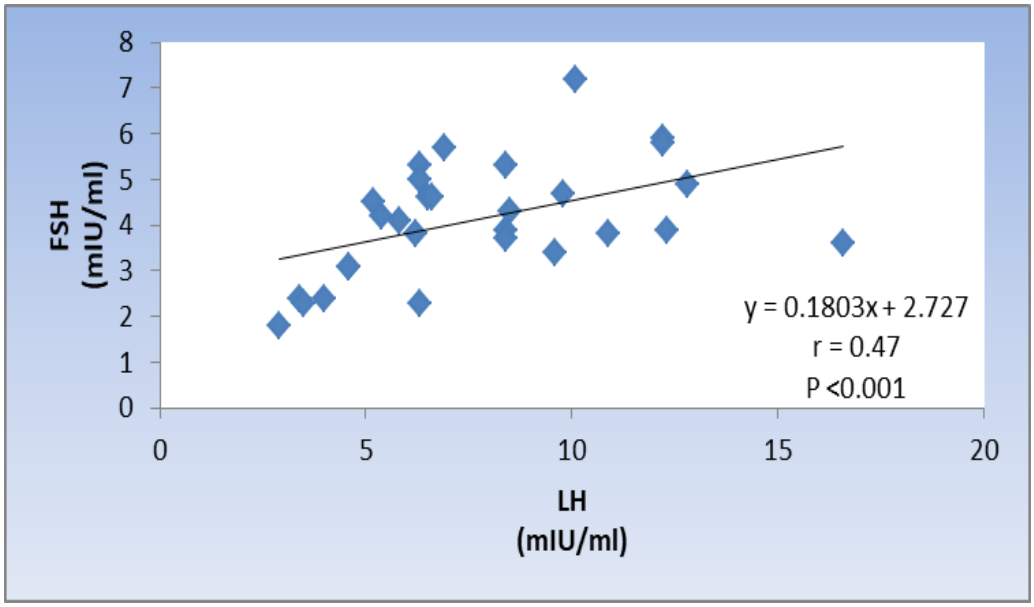


Figure 3-3 : Correlation between luteinizing hormone and follicle stimulating hormone for PCOS obese patients

A study by Alnakash *et al.* (2007) [157] has found no significant statistical correlation between BMI and LH/FSH ratio. Kiddy *et al*. (1990) [158] found a negative correlation between BMI and FSH levels in obese females.

The present study recorded a direct correlation between LH/FSH ratio and LH at a highly significant level, which agrees with a study that showed a correlation between the ratio and LH level in normal-weight patients more than in obese patients [152].

Hyperinsulinemia and IR that occur in an obese patients can inhibit follicular development and ovulation as a result of hyperandrogenic intraovarian and by altering gonadotropin [159].

3.2.2 Androgen

a. Free Testosterone

The significant difference in free testosterone (P< 0.05) among patients (normal weight and obese), mean ± SE were (2.05 ± 0.14),(2.7 ±0.27) respectively and also within control groups mean±SE for (normal weight and obese) were (0.86 ± 0.03), (0.89 ± 0.06) respectively with highly significant difference (P< 0.001), as in Table (3-3).

Derived from Table 3-3: Free testosterone level

Dependent Variable	Group	Study group	No.	Mean ±SE	95% Confidence Interval		P- value
					Lower	Upper	
F.Testosterone (pg/ml)	N.Wt. PCOS	Ob. PCOS	30	2.7±0.27	2.23	3.3	**0.002**
		N.Wt. Control	30	0.86±0.03	0.7	0.96	**<0.001**
		Ob. Without PCOS	30	0.89±0.06	0.7	1.0	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	2.05±0.14	1.72	2.3	**0.002**
		N.Wt. Control	30	0.86±0.03	0.7	0.96	**<0.001**
		Ob. Without PCOS	30	0.89±0.06	0.7	1.0	**<0.001**

The highly significant difference (P<0.01) between patients and control means that PCOS causes this increment in free testosterone, as well as the elevated level of free testosterone concentration in obese patients with PCOS more than in normal-weight PCOS patients group, this agreement with previous studies by Eilerman *et al.*(2009) Sadeghi *et al.*(2022) [23,24], that show that obese females had higher total and free testosterone compared to normal weight females.

This may be due to the effect of obesity on the level of androgen, obesity induced hyperinsulinemia stimulating ovarian and adrenal androgen production lead to increasing the sensitivity of the pituitary to the effect of gonadotrophin-releasing hormone, and enhancing the ovarian response to gonadotrophin[44],

the obese females has high LH lead to androgens production in ovarian theca cells[43], hyperinsulinemia reduces hepatic SHBG leads to a higher concentration of free testosterone levels in blood circulation[159,160]**.** Renato *et al.* (1993) [163] showed that obesity may have an important role in the appearance of features of hyperandrogenism, not only in PCOS females but also in females with normal ovaries**.**

The main endocrine disturbance is an extreme androgen secretion or activity, with frequent clinical signs of hyperandrogenism such as hirsutism, acne, and alopecia can appear as a result of hypersecretion of androgen by the ovary in PCOS females [164].

b. Dehydroepiandrosterone -Sulfate

There is no significant differences (P=0.91) of DHEA-S concentration among patients groups, while the highly significant results (P <0.001) presented between PCOS patients and control subgroups with mean±SE that summarized in Table below.

Derived from Table 3-3: Dehydroepiandrosterone sulfate level

Dependent Variable	Group	Study group	No.	Mean ±SE	95% Confidence Interval		P- value
					Lower	Upper	
DHEA-S (µg/dl)	N.Wt. PCOS	Ob. PCOS	30	320±31.6	255.23	384.9	0.91
		N.Wt. Control	30	173.5±11.1	150.70	196.3	**<0.001**
		Ob. Without PCOS	30	174.7±11.6	150.9	198.5	**<0.001**
	Ob. PCOS	N.Wt. PCOS	30	323.6±31.8	258.3	388. 8	0.91
		N.Wt. Control	30	173.5±11.1	150.70	196.3	**<0.001**
		Ob. Without PCOS	30	174.7±11.6	150.9	198.5	**<0.001**

Actually, ovarian theca cells increase androgen production in response to chronically elevated LH and insulin levels, hyperinsulinemia promotes androgen biosynthesis via the insulin receptor on theca cells lead to increase of levels of circulating androgen as well as by suppressing hepatic production of

SHBG and albumin by effect of hyperinsulinemia that reduces the hepatic biosynthesis of protein that lead to decrease SHBG and albumin, also 80% of testosterone is bound with a high affinity to SHBG, 19% is bound to albumin, and only 1% circulates as a free fraction [165].

In contrast, DHEA-S is bound to albumin with low affinity and available for peripheral conversion, it's have little to no capacity to bind to the androgen receptor and require conversion to testosterone to exert androgenic effects. Androstenedione is the most important precursor of testosterone, while DHEA accounts for only 5% and 13% of circulating testosterone in reproductive age women, therefor, the level of DHEA-S remaining higher in serum [162].

On the other hand, stimulates visceral adipose tissue that generates free fatty acids (FFA's) which contribute to insulin resistance and finally lead to an increase in DHEA-S from the ovary [29], these factors may refers to increase of DHEA-S of PCOS females in current study. This result similar to previous study [165].

Also, the figure (3-5 A and B) were shows DHEA-S was positively correlated with FSH and levels in obese patients only. The obesity, insulin resistance, and dyslipidemia are PCOS-related morbidities and were found to be correlated with the LH/FSH ratio, as well as positive correlation observed between DHEA-S and free testosterone hormone in obese patient groups as show in figure (3-6) furthermore, the figure (3-4) shows directly positive correlation between LH, FSH with free testosterone in normal weight and in obese patients, high level of free testosterone (2.7 µg/dl) in obese compared with normal weight patients (2.0 µg/dl) that lead to accumulation of DHEA-S in the obese patients, that is an intermediate product, produced by the ovaries and adrenal glands [33-35].

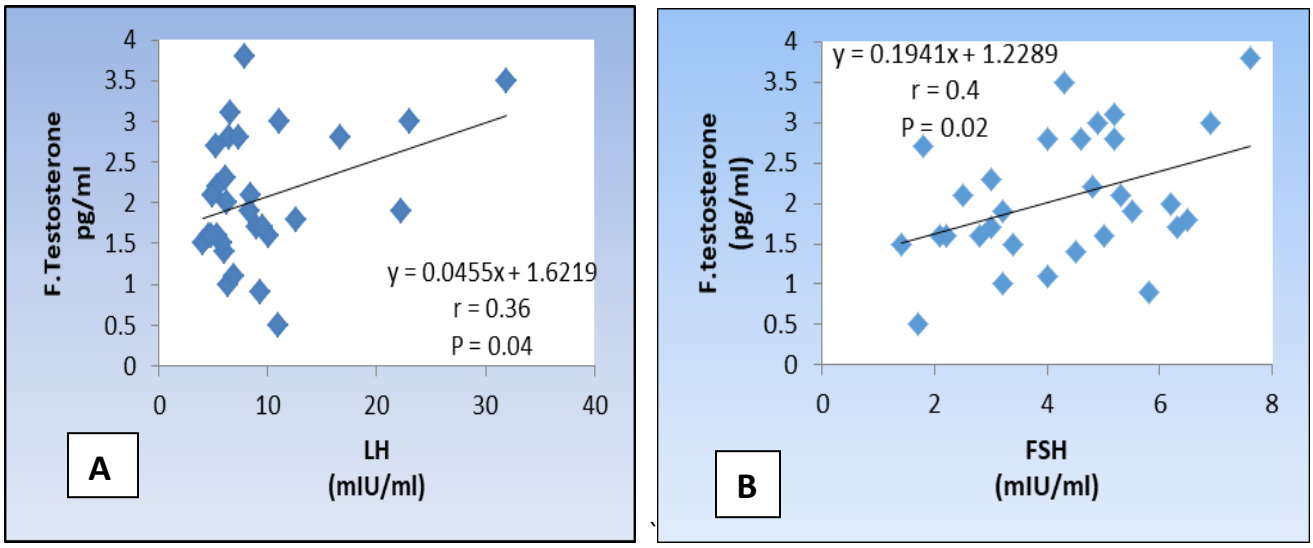


Figure 3-4 : Correlation between free testosterone and A) luteinizing hormone, B) follicle stimulating hormone, for PCOS normal weight patients

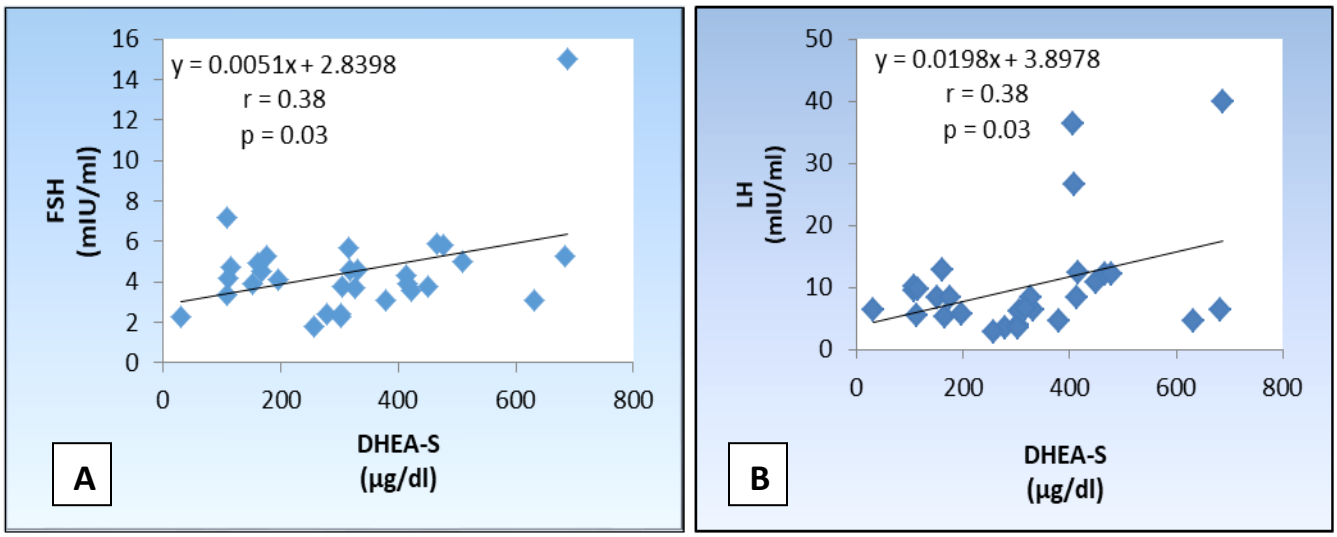


Figure 3-5 : Correlation between dehydroepiandrosterone –sulfate and, A)luteinizing hormone, B)follicle stimulating hormone for PCOS obese patients

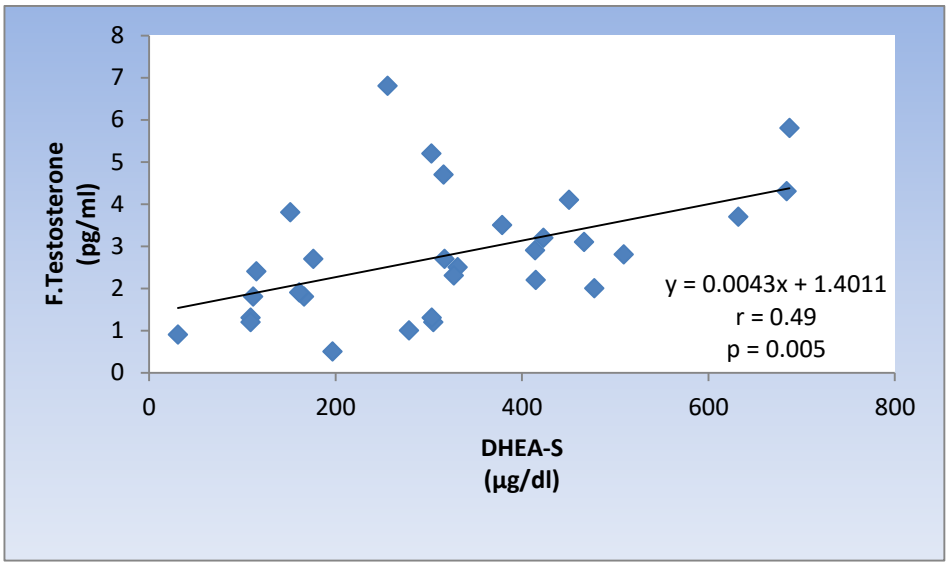


Figure 3-6 : Correlation between dehydroepiandrosterone -sulfate and free testosterone hormone for PCOS obese patients

3.2.3 Kisspeptin

Kisspeptin levels were also found to be insignificantly (P=0.07) in females with PCOS (494.5±57.7) versus of control (388.1±7.2). While based on BMI the highly significant difference (P<0.05) of kisspeptin among all subdivided groups except of obese PCOS patients with normal weight and obese controls, the result was recorded higher mean of normal weight patients (611.2±112) than other studied groups.

The Table (3-3) appears the means of kisspeptin level of the normal weight control group (374.4 ± 10.7), an obese females without PCOS group (401.2 ±9.3) and obese PCOS (382.9 ± 20.54).

Derived from Table 3-3: Kisspeptin level

Dependent Variable	Group	Study group	No.	Mean ±SE	95% Confidence Interval		P-value
					Lower	Upper	
kisspeptin ng/L	Patient		45	494.5±57.7	9.2	222.0	0.07
	control		45	388.1±7.2			
	N.Wt. PCOS	Ob. PCOS	23	382.9±20.54	340.3	425.5	**0.005**
		N.Wt. Control	22	374.4±10.7	352.1	396.7	**0.004**
		Ob. Without PCOS	23	401.2±9.3	381.9	420.5	**0.010**
	Ob. PCOS	N.Wt. PCOS	22	611.2±112	378.9	844.3	**0.005**
		N.Wt. Control	22	374.4±10.7	352.1	396.7	0.91
		Ob. Without PCOS	23	401.2±9.3	381.9	420.5	0.81

The current study was indicated that kisspeptin level in patients higher insignificantly than control, from four studies assessed kisspeptin in patients with PCOS, three of them [23–25] reported higher kisspeptin levels in female with PCOS, similar to the data of the present study, while one of them [169] reported lower levels in females with PCOS than controls.

Supporting the present study's findings by Yerlikaya *et al*.(2013) [170] and Nearmeen *et.al*.(2019) [171] founded that kisspeptin level among PCOS group, significantly lower in underweight, overweight and obese compared to normal weight group. The result may be because the stimulatory effect of obesity on kisspeptin neurons are also influenced by leptin and insulin resistance (excitatory), as well as ghrelin and adiponectin that effected as inhibitory on kisspeptin neuron [102].

In normal-weight patients, kisspeptin level was higher than obese group that lead to higher LH level in normal weight (9.41mIU/mL), more than obese (8.84 mIU/mL), although the way of direct pituitary effects of *KISS1* neurons, which produce kisspeptin (encoded by the *KISS1* gene) [28,41].

The main endocrine changes observed that hyperandrogenism from the theca cell, inhibit the activity of aromatase in the granulosa cells that leading to decrease the estrogen in granulosa cells and a decrease the progesterone from the corpus luteum [29], which leads to decrease in the effect of negative feedback of estrogen and progesterone on the kisspeptin and GnRH that lead to higher kisspeptin, which has an important role in GnRH secretion, increased speed of hypothalamic GnRH pulses, and up-regulated expression of GnRH mRNA, which explains the elevated LH/FSH ratio [1], kisspeptin levels are likely to be related to LH levels, and therefore to the pathophysiology of PCOS [17,34].

Therefore, kisspeptin levels showed a positive significant correlation with LH for PCOS normal weight patients only ($P < 0.01$, $r = 0.49$) as show in figure (3-7), also positive significant correlation with LH/FSH ratio in both groups obese and normal weight ($P < 0.01$, $r = 0.58$ and 0.72 respectively) as in figure (3-8).

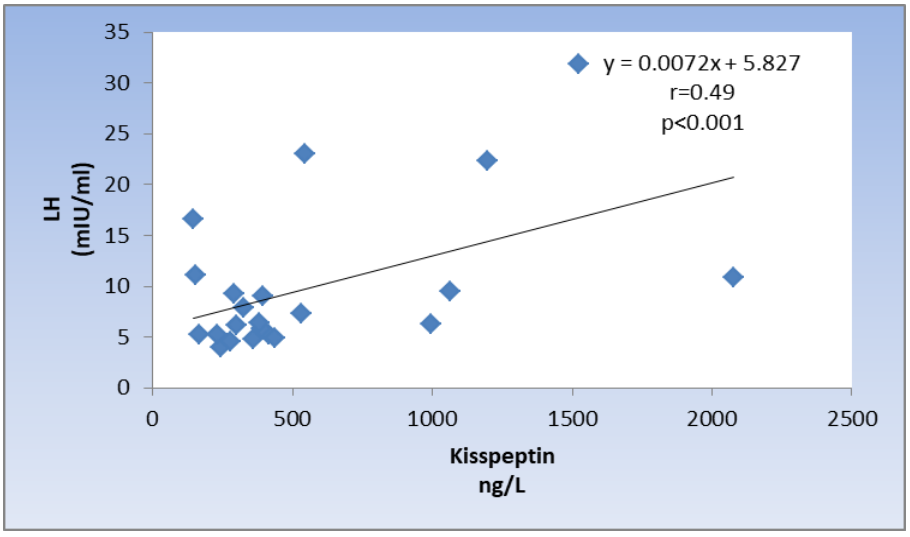


Figure 3-7 : Correlation between kisspeptin and luteinizing hormone for PCOS normal weight patients

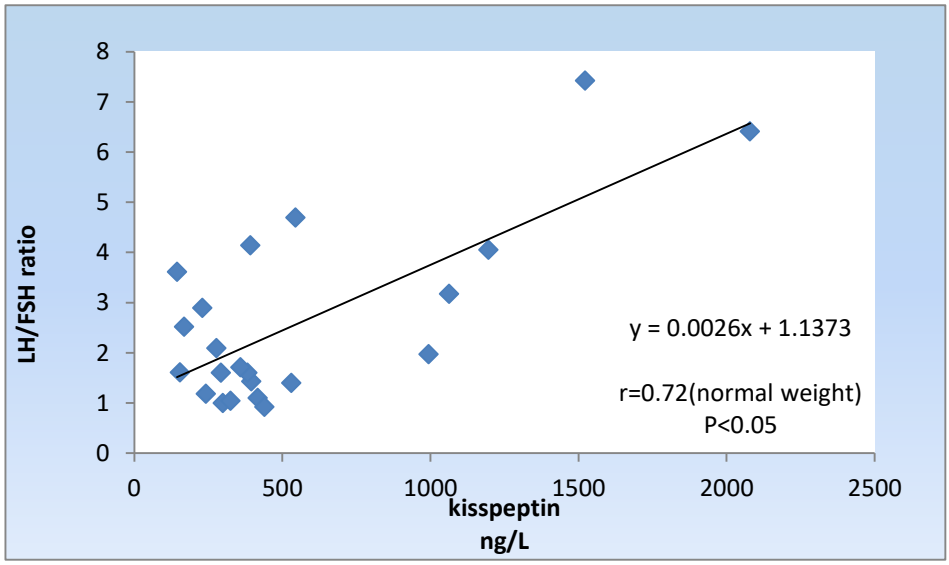


Figure 3-8 : Correlation between kisspeptin and luteinizing hormone to follicle-stimulating hormone ratio for PCOS normal weight patients

Razaw *et al*.(2020) [173] his study showed also a positive correlation between kisspeptin and free testosterone, while the present study that no significant correlation ($P > 0.05$, $r < 0.3$) of kisspeptin with free testosterone levels and DHEA-S among the studied groups, may be not found directly effected of kisspeptin on androgens level but the condition of hyperandrogenism is induced by the direct effect of high stimulation of LH on gonads, on other hand, the elevated of LH relation to FSH that leads to increase level of free testosterone and DHEA-S in normal weight and obese respectively [174].

3.3 Genetic Analysis

3.3.1 Detection of Genomic Deoxyribonucleic acid

Figures (3-9),(3-10), and (3-11) shows DNA extraction of some studied samples that show high accuracy of the band, purity, and concentration from fresh blood compared to frozen blood. Agarose gel electrophoresis staining with low concentrations of a safe red fluorescent dye.

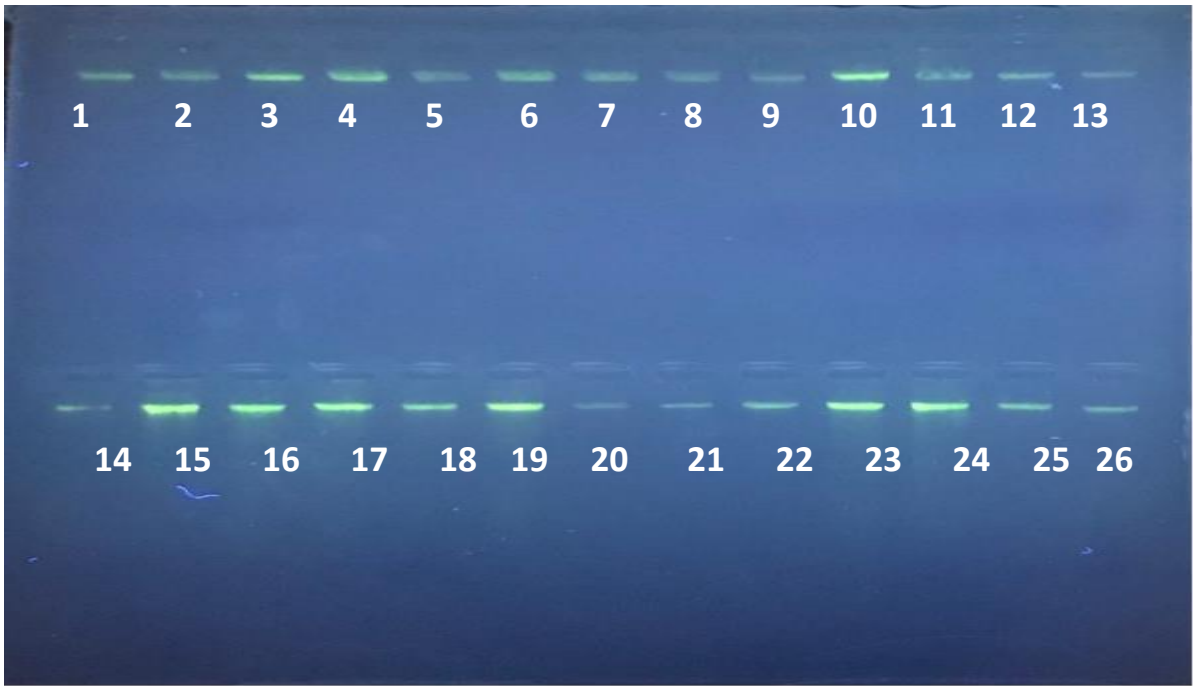


Figure 3-9: Detection the presence of genomic

(1-13)DNA extraction from frozen blood (15-19,23and 24) DNA extraction from fresh blood, (14,20-22,25,26) DNA extraction from the same sample that fresh blood after diluent

3.3.2 Measurement of Concentration and Purity of Deoxyribonucleic Acid

A ratio of absorbance at 260/280 nm and 260/230 nm were used to measure the DNA purity that expressed as (mean ± SD) 1.78 ± 0.15 and 2.1± 0.23 respectively, the mean of DNA concentration was 96.9 ± 6.53. Data was demonstrated in Table(3-4).

Table 3- 4: Concentration and purity of deoxyribonucleic acid

DNA concentration and purity	No.	Mean ± SD
DNA concentration(ng/µl)	120	96.9± 6.53
DNA purity(260/280)	120	1.78±0.15
DNA purity(260/230)	120	2.1± 0.23

The acceptable range of absorbance at 260/ 280 ratio that ~1.8 is optimal. A ratio of < 1.7 suggests protein or acidic phenol contamination, while a value > 2.0 suggests remnants of the basic solution used to lyse the cells, figure (3-10A) show the present result of DNA purity at 260/280nm [175].

In contrast, the 260/230 ratio is used to indicate the presence of unwanted organic compounds such as Trizol, phenol, Guanidine HCL, and guanidine thiocyanate. Generally, acceptable 260/230 ratios are in the range of 2.0 – 2.2, figure (3-10B) show the present result of DNA purity at 260/230nm.

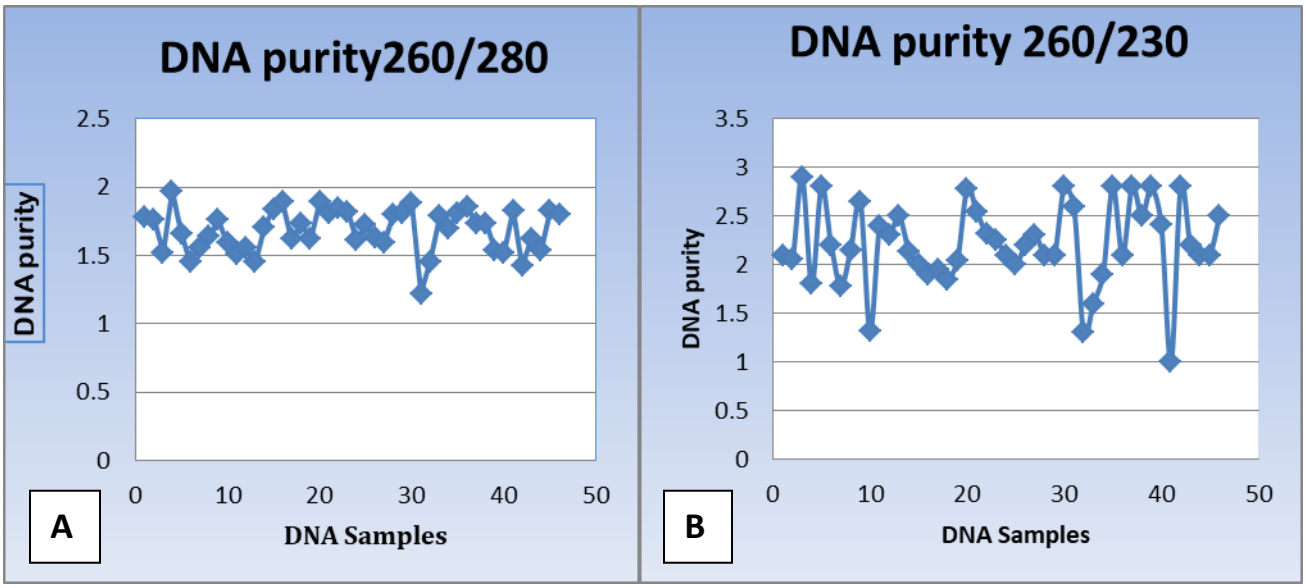


Figure 3-10: Purity of deoxyribonucleic acid A) at 260/280nm, B) at 260/230nm

Figure below shows the result of DNA concentration for extraction is higher in fresh blood when compared with frozen blood.

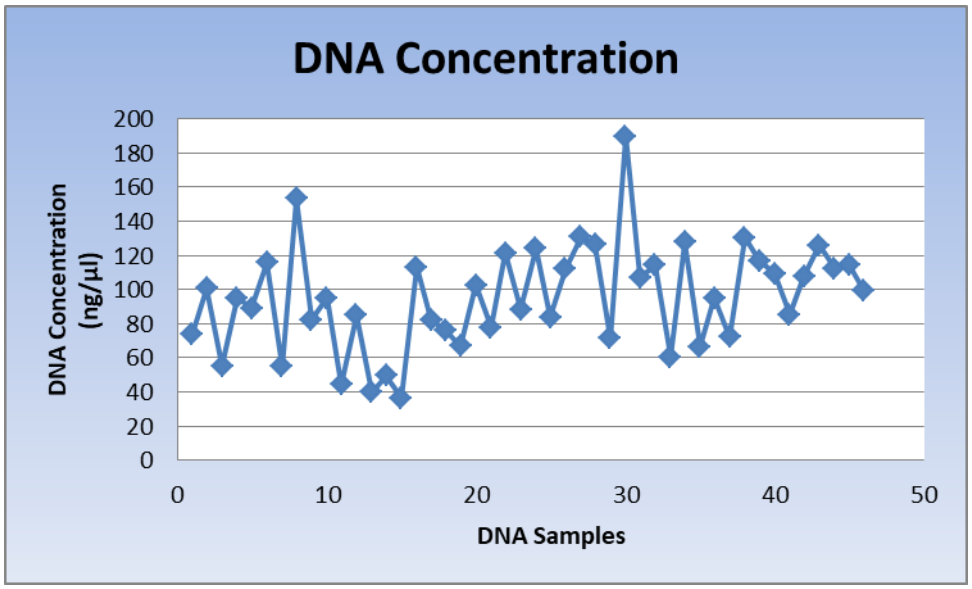


Figure 3-11: Concentration of deoxyribonucleic acid
DNA(1-23)DNA extraction from frozen blood, (24-46) DNA extraction from fresh blood

The DNA extraction from fresh blood used the RBC lysis buffer to broking RBC cell and precipitate WBC cell, then added detergent solution to denaturation the proteins and WBC wall that used it to DNA extraction without proteinase K that used in frozen blood, because found RBC and crystal formation in frozen blood that may be caused denaturation high number of WBC and proteins, used proteinase K that have high activity to detect even low amount of WBC and proteins, also it act on all condition(even at high temperature and different range of pH), for this cause the DNA concentration from fresh blood higher than frozen blood [177,178].

3.3.3 Gene Polymorphism of *KISS1* in Studied Groups

The gene polymorphism of *KISS1* was studied in patients with PCOS and apparently healthy control groups. The genotyping was detected by HRM-real time PCR, there are two SNPs rs4889 G>A and rs372790354 G>A within

the *KISS1* gene included in the present study, from data base in NCBI allele A for rs4889 G>A considered as new SNPs studied in PCOS patients, while the other Allele of (rs4889G>C) studied previously in other population[88,175] Table (3-5) shows the information of SNPs in this study.

Table 3-5: Gene polymorphism of *KISS1* in studied groups

Gene	Position in Chromosome	Allele	Location	dbSNP ID	Amino Acid Change
KISS1	1:204196427 (GRCh38)	G > A	5-prime UTR variant	rs372790354	----
KISS1	1:204190659 (GRCh38)	G > A	Exon 3	rs4889	Pro-Leu

Pro : prolin, Leu: leucine, dbSNP ID : single nucleotide polymorphism data base ID, GRCh38:Genomic Reference Consortium Human Build 38 in 2013.

3.3.3.1 Analysis of rs372790354 G>A and rs4889 G>A Polymorphism

a. Analysis Results of Amplification Reaction and Genotype of rs372790354 G>A

The illustrated fluorescent signal of amplification curve for rs372790354 (G>A) shown in figure (3-12).

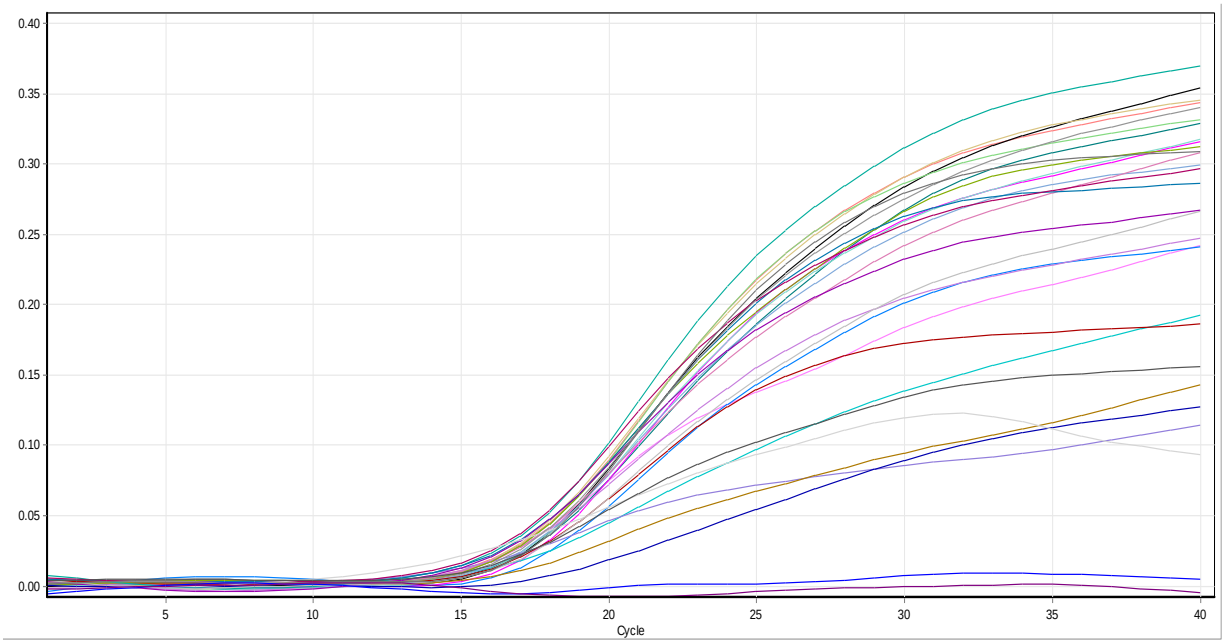


Figure 3-12: Amplification curve of rs372790354 G>A

Genotyping analysis by HRM was performed from 55˚C to 95˚C with a temperature increase of 0.1˚C/s. Each sample was run in duplicate and each genotyping was carried out with a set of sequentially confirmed control samples of known DNA genotypes (wild type, heterozygous and homozygous alleles). HRM assay melt curve results were normalized to identify the genotype as figure (3-13) the top, middle and bottom lines represent GG,GA and AA for known DNA genotypes respectively, while the colors lines, each sample has a specific color as shows in the appendix(5).

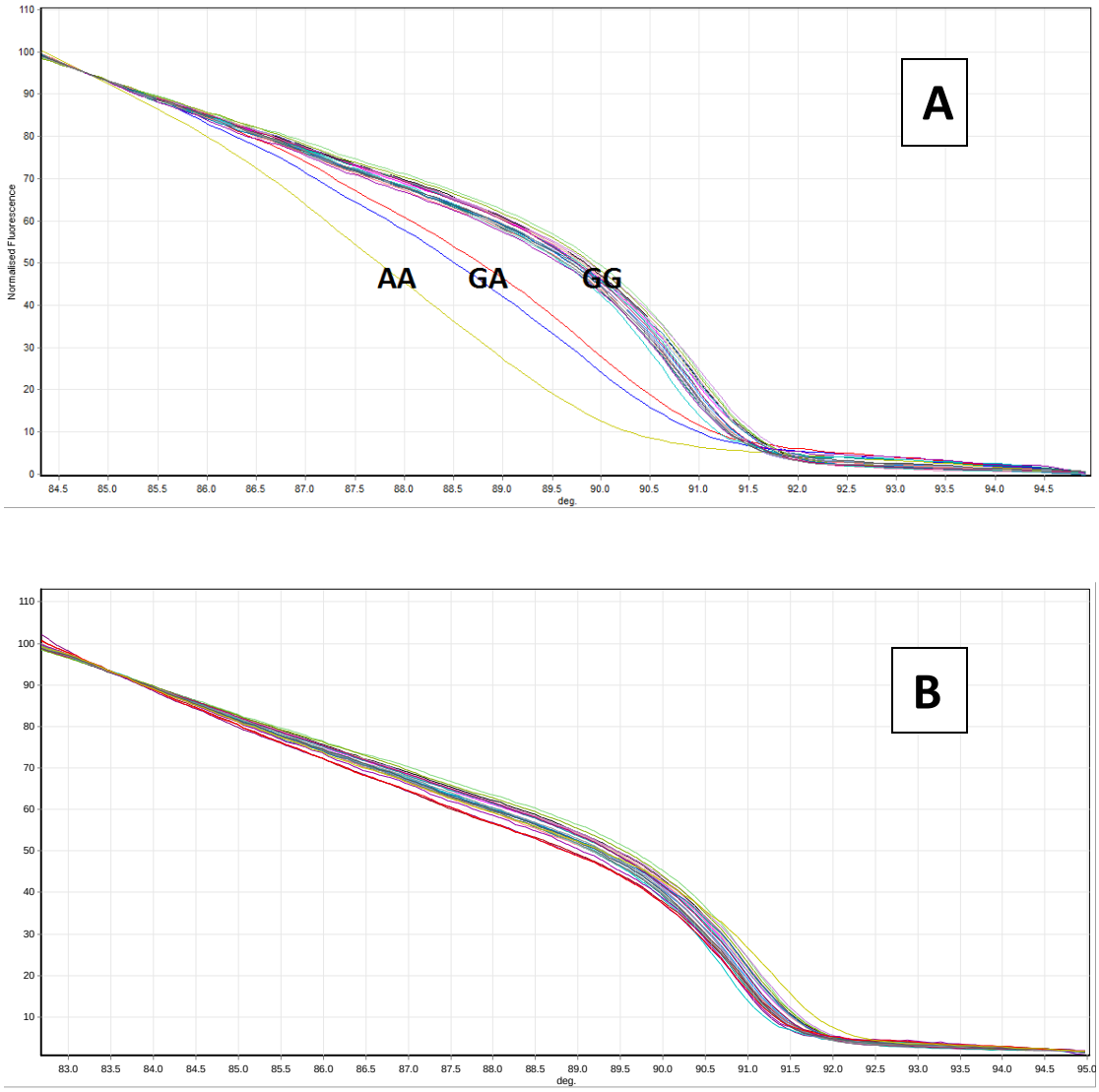


Figure 3-13: Genotype curve for control DNA was known genotype and samples of rs372790354 G>A ,

A) Comparison between (GG wild, GA hetero, AA mutant) was known DNA and unknown DNA of samples. B) curve for 1 run of PCR (36 samples)

b. Analysis Results of Amplification Reaction and Genotype of rs4889 G>A

The amplification curve for rs4889 (G>A) shown in figure (3-14).

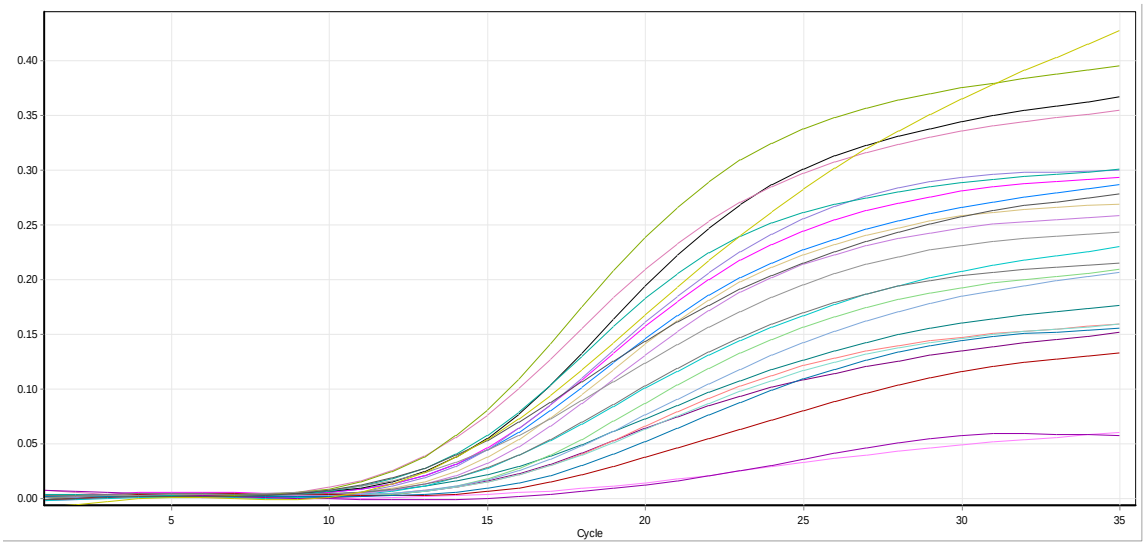
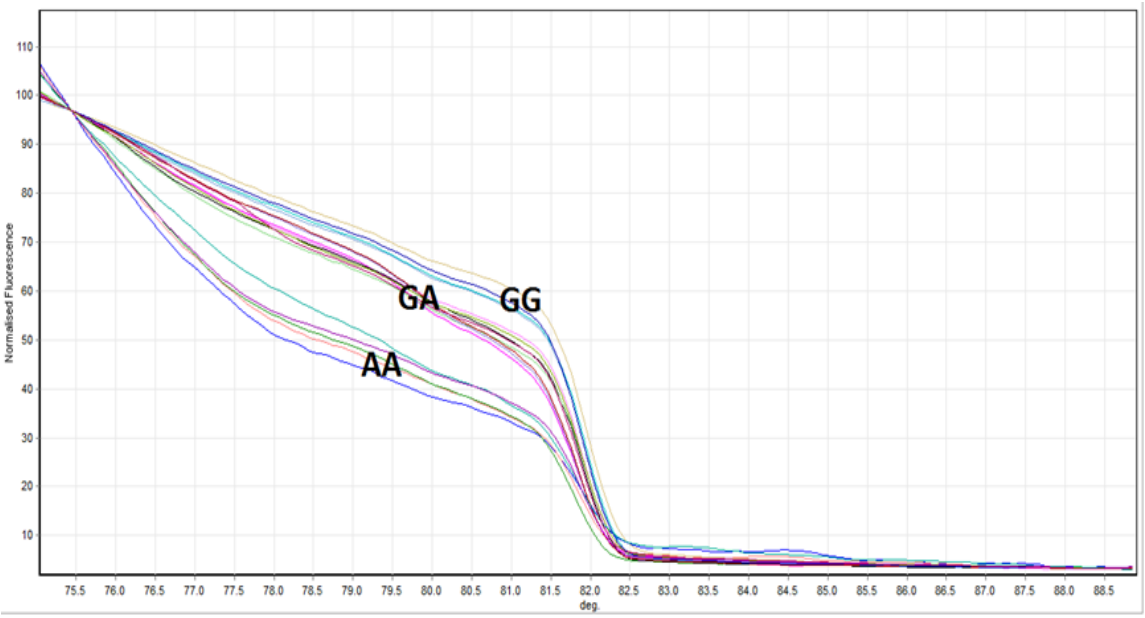


Figure 3-14: Amplification curve of rs4889 G>A

HRM assay melt curve results were normalized to identify the genotype in figure (3-15) for control was known genotype and samples of rs4889G>A.



Figure(3-15) Genotype curve for control DNA was known genotype and samples of rs4889 G>A

Comparison between (GG wild, GA hetero, AA mutant) was known DNA and unknown DNA of samples.

3.3.3.2 Alleles Frequency for rs372790354 G>A and rs4889G>A

Allele frequency of *KISS1* gene variants rs372790354 (G>A) and rs4889(G>A) in PCOS patients were represented (55 and 48) % of G allele, (45 and 52)% of A allele respectively, while in control groups were represented (78 and 82)% of G allele, (22 and 18) % of A allele respectively.

Table (3-6) shows alleles frequency in a case-control study and their association with PCOS, which was highly significant (P<0.001) between the distribution of allele frequency (rs372790354 and rs4889) with the odd ratio for G allele = 0.34 and 0.2, the confidence interval at 95% level of (0.2-0.5), (0.1-0.3) respectively for two SNPs, while the odd ratio for A allele = 2.96 and 4.9, the confidence interval at 95% level of (1.68-5.19),(2.7-8.8) respectively.

Table 3-6: Alleles frequency and allelic association of rs372790354 G>A and rs4889G>A by hardy-weinberg equilibrium law of *KISS1* gene polymorphism between (patient and control) groups.

KISS1 SNPs	Alleles	Frequencies (%)		Odd Ratio (95% CI)	P value
		Control (n=60)	Patients (n=60)		
rs372790354	G	78% (n=94)	55% (n=66)	0.34 (0.2 to 0.5)	0.0002
	A	22% (n=26)	45% (n=54)	2.96 (1.68 to 5.19)	
rs4889	G	82% (n=98)	48% (n=57)	0.2 (0.1 to 0.3)	0.0001
	A	18% (n=22)	52% (n=63)	4.9 (2.7 to 8.8)	

OR: odd ratio, G- major allele, A-minor allele, CI: confidence interval, SNP(single nucleotide polymorphism), P value ≤0.05 was significant.

The effects of *KISSI* gene polymorphisms on PCOS have been investigated by the current study. The results showed that A allele of two SNPs was high risk in PCOS, in contrast, the G allele was protective with higher frequency in control.

Maha *et al*. (2020)[100], was showed that significant association between rs372790354 polymorphism with PCOS the G allele was protective, and the frequency of allele A was significantly higher in PCOS patients compared to controls.

3.3.3.3 The *KISS1* Gene Variants rs372790354G>A

a. Hardy–Weinberg Equilibrium and Inheritance Model for rs372790354G>A

Genotype frequencies of rs372790354 of *KISS1* gene polymorphism were accordance with Hardy Weinberg Equilibrium (P>0.05) in PCOS patient and control groups shown in Table (3-7).

Table 3-7 :Hardy-weinberg equilibrium law of *KISS1* gene polymorphism rs372790354 observed and expected genotype frequency for control and patients

rs372790354	GG	GA	AA	P -Value
Control	58% (n=35)	40.4% (n=24)	1.6% (n=1)	0.388
Patients	30% (n=18)	50% (n=30)	20% (n=12)	0.93

G- major allele, A-minor allele, P value ≤0.05 was significant

b. Genotyping

The analyses were conducted to assess the effects SNP rs372790354 variants [GG (wild type), GA (heterozygous type), and AA (mutated type)] on PCOS development, the results were appeared in Table (3-8) that indicated a higher risk model was recorded in genotypes AA recessive model which was [P<0.05, OR (95% CI) = 14.7(1.85-117.5)], followed by AA co-dominant model which was [P<0.05; OR (95% CI) = 23.3(2.8-193.9)], while lower risk for disease in GA co-dominant model which was [P<0.05; OR (95% CI) = 2.4(1.1-5.3)], also GA-AA dominant model which was [P<0.05; OR (95% CI) = 3.26(1.5-6.9)]. Finally, no significant association found in GA over dominant model (P>0.05).

Table 3-8 : Association of rs372790354 genotypes with polycystic ovarian syndrome under different models of inheritance

Models	Genotypes	Control (No.60)	Patients (No.60)	Odd Ratio (95% CI)	P. Value
Co.dominant	**GG**	58% (n=35)	30% (n=18)	**References(OR=1)**	
	GA	40% (n=24)	50% (n=30)	2.4 (1.1 to 5.3)	**0.026**
	AA	1.6% (n=1)	20% (n=12)	23.3 (2.8 to 193.9)	**0.0036**
Dominant	**GG**	58% (n=35)	30% (n=18)	**References (OR=1)**	**0.0021**
	GA-AA	41% (n=25)	70% (n=42)	3.26 (1.5 to 6.9)	
Recessive	**GG-GA**	98% (n=59)	80% (n=48)	**References (OR=1)**	**0.011**
	AA	1.6% (n=1)	20% (n=12)	14.7 (1.85 to 117.5)	
Over dominant	**AA-GG**	60% (n=36)	50% (n=30)	**References (OR=1)**	0.27
	GA	40% (n=24)	50% (n=30)	1.50 (0.73 to 3.1)	

OR: odd ratio, G- major allele, A-minor allele, CI: confidence interval, P value ≤0.05 was significant

Also the comparison was conducted in obese and normal-weight PCOS and to determine the obesity effect, Table (3-9) was shows the highly significant difference (P<0.001) of AA genotype in normal-weight patients compared to control, while no significant different between other groups.

Table 3-9: Genotype frequency of rs372790354 G>A of *KISS1* gene in patient and control subgroups (normal weight and obese).

KISS1 rs3727354	Groups	Control (%) n	Patients	P. Value	
GG	Normal weight	(26%) 16	(11%)7	**References**	
	Obese	(32%)19	(19%)11		
GA	Normal weight	(21%)13	(24%)14	0.12**	0.6*
	Obese	(19%)11	(26%)16	0.09***	
AA	Normal weight	(1%)1	(15%)9	**0.008****	0.051*
	Obese	0	(5%)3	0.11***	
Total	All subject	60	60		

*** between obese (patients and control), **between normal weight (patients and control) ,* between normal weight and obese in patients only, P value ≤0.05 was significant

The current study results are similar to the study of Maha *et al.* (2020) [100] which is case control study that included the SNP rs372790354, the genotypes AA and GA exhibited a significant association with the risk of PCOS. The frequency of the GA genotype was significantly higher in control females, the mutant AA genotype was found only in PCOS patients.

Since the SNP 372790354 G>A is located in the 5'-UTR, the genotype of AA was higher frequency in normal-weight patients, therefore the activity of *KISS1* gene was increased in patients compared with the controls, the disturbance of *KISS1* promoter activity and the variation in this region may lead to irregular of HPG axis expression that leads may be a risk of PCOS[178].

c. Influence of *KISS1* Gene Polymorphisms (rs372790354G>A) on PCOS Associated Endocrine and Obesity Linked Variables

The present study describes the probable influence of *KISS1* gene SNP rs372790354 G>A in PCOS pathogenesis. Actually, the influence of these SNP on the (kisspeptin, LH, FSH, LH- FSH ratio, DHEA, and free testosterone) and obesity-linked parameters (BMI and waist-hip ratio) were studied, in females

with and without PCOS. Mutant genotype(AA) has significant association with PCOS and found higher frequency in normal-weight patients, the results were presented in Tables (3-10), exhibited no significant influence on the values of endocrine as well as obesity liked parameters between different genotype in the PCOS patients compared to the control group.

Table 3-10 :The influence of rs372790354 G>A polymorphism of *KISS1* gene on the mean differences of parameters between genotypes AA, normal weight (patient and control) groups

Parameters	Group	No.	AA Mean ±SE	Difference with Other Genotype (P-value)
WHR	PCOS Patients	9	0.89±0.02	
	control	1	0.83	0.38
LH (mIU/ml)	PCOS Patients	9	9.2±2.05	
	control	1	6.1	0.63
FSH (mIU/ml)	PCOS Patients	9	3.8±0.65	
	control	1	7.2	0.14
LH/FSH Ratio	PCOS Patients	9	2.49±0.34	
	control	1	0.84±	0.16
kisspeptin ng/L	PCOS Patients	9	477±129	
	control	1	290.1	0.66
F.Testosterone (pg/ml)	PCOS Patients	9	2.27±0.26	
	control	1	0.9±	0.13
DHEA (µg/dl)	PCOS Patients	9	384.5±57.2	
	Control	1	142	0.21

PCOS (polycystic ovary syndrome) , SE(Standard error), LH(Luteinizing Hormone), FSH(Follicle-Stimulating Hormone), DHEA-S (dehydroepiandrosterone sulfate), WHR(waist to hip ratio), P value ≤0.05 was significant.

The result in the current study showed no significant of influence SNP rs372790354 G>A on the value of parameters, which disagree with study of Maha *et.al*. (2020), which highlights a significant difference in kisspeptin level, between homozygous wild type (GG) and mutant (AA) genotypes of this

SNP[100]. This study disagrees with present results may be due to the small sample size and inclusion criteria chosen.

Moreover, when both study groups were compared for the level of kisspeptin in genotype AA, no significant difference was obtained between PCOS patients and controls. With highly elevation of kisspeptin level in PCOS(477±129) ng/L compared to control (290), rs3727354 is located in the 5'UTR (untranslated region) of the *KISS1* gene. The 5'UTR has been recognized as playing an important role in the regulation of gene expression through regulating the process of RNA transcription, mRNA stability and its localization, and translational efficiency[178]. Moreover, 5'UTR efforts great contribution in the initiation step of translation. The multi-step process of gene expression is primarily depended on this initiation translation step with this in view, the role of 5'UTR in gene expression, this step may indirectly promote alteration in the functional activity of the KISS1 gene product kisspeptin.

3.3.3.4 The *KISS1* Gene polymorphism rs4889G>A

a. The Hardy–Weinberg Equilibrium and Inheritance Models for rs4889G>A

Genotype frequencies of rs4889 of *KISS1* gene polymorphism were accordance with Hardy Weinberg Equilibrium (P>0.05) in PCOS patient and control groups shown in Table (3-11)

Table 3-11: Hardy-weinberg equilibrium law of *KISS1* gene polymorphism rs4889 observed and expected genotype frequency for control and patients

rs4889	GG	GA	AA	P value
Control	65%(n=39)	33.4%(n=20)	1.6%(n=1)	0.678
Patients	22% (n=13)	52% (n=31)	26% (n=16)	0.96

G- major allele, A-minor allele, P value ≤0.05 was significant.

.

b. Genotyping

The analyses were conducted to assess the association of *KISS1* gene SNP rs4889 [GG (Wild type), GA (heterozygous type), and AA(mutated type)] with the pathogenesis of PCOS according to the results were shown in Table (3-12) higher association risk was observed in AA of co-dominant model which was [P<0.05; OR(95% CI) = 48(5.8-398.1)], followed by recessive model with AA genotype which was [P<0.05; OR(95% CI) = 21.5(2.7-167.9)], also other risk association recorded in GA-AA of dominant model [P<0.05; OR (95% CI) = 6.7(2.98-15.11)] and GA of co-dominant model [P<0.05; OR (95% CI) = 4.65(2.0-10.8)], finally, GA of over dominant model have lower risk compare other models was significant [P<0.05; OR (95% CI)= 2.14(1.02-4.47)].

Table 3-12 : Association of rs4889 genotypes with polycystic ovarian syndrome under different models of inheritance

Models	Genotypes	Control (No.60)	Patients (No.60)	Odd Ratio (95% CI)	P. Value
Co. dominant	**GG**	65% (n=39)	22%(n=13)	**References(OR=1)**	
	GA	33% (n=20)	51%(n=31)	4.65 (2.0 to 10.8)	**0.0004**
	AA	1.6% (n=1)	27% (n=16	48 (5.8 to 398.1)	**0.0003**
Dominant	**GG**	65% (n=39)	22%(n=13)	**References(OR=1)**	**0.0001**
	GA-AA	35% (n=21)	78% (n=47	6.7 2.98 to 15.11	
Recessive	**GG-GA**	%98 (n=59)	73% (n=44	**References(OR=1)**	**0.0035**
	AA	1.6% (n=1)	27% (n=16	21.5 (2.7 to 167.94)	
Over dominant	**AA-GG**	67% (n=40)	49% (n=29	**References(OR=1)**	**0.04**
	GA	33% (n=20)	51%(n=31)	2.14 1.02 to 4.47	

OR: odd ratio, G- major allele, A-minor allele, CI: confidence interval P value ≤0.05 was significant.

The genotype frequencies were calculated and compared between PCOS patients and controls, also *KISS1* genotype studied in obese and normal weight to show the effect of obesity, Table (3-13) was show the influence of rs4889 variant in studied subgroups, significant frequency (P<0.05) for both AA and GA in patient compared to control also among patients (normal-weight when compared with obese), the frequency of AA and GA genotype was greater among normal weight PCOS when compare to obese PCOS patients while the GG allele was seen in a greater proportion of controls. While no significant difference (P>0.05) in GA genotype among obese groups (patient and control).

Table 3-13 : Genotype frequency of rs4889 G>A of *KISS1* gene in patients and control subgroups (normal weight and obese).

***KISS1* rs4889**	**Groups**	**Control**	**Patients**	**P. Value**	
GG	Normal weight	(35%) 21	(5%)3	**References**	
	Obese	(30%)18	(17%)10		
GA	Normal weight	(14%)8	(30%)18	**0.0002****	**0.042***
	Obese	(20%)12	(21%)13	0.23***	
AA	Normal weight	(1%)1	(15%)9	**0.0007****	**0.07***
	Obese	0	(12%)7	**0.03*****	
Total	All subject	60	60		

*** between obese (patients and control), **between normal weight (patients and control), * between normal weight and obese in patients only, P value ≤0.05 was significant

Polymorphism of rs4889 was founded in exon3 the coding region introduced a substitution of proline at the 81 position. This is a substitution that was observed in kisspeptin-54, but not in the other three forms of kisspeptin (kisspeptin-14, -13,-10)[34], that lead to a change in the DNA sequence could alter the structure, function, and binding capacity of kisspeptin to its receptor GPR54.

This study observed of allele A instead allele C that rs4889 G >A had a significant association with PCOS and genotypes AA and GA exhibited significant associated with the risk of PCOS which differs from studies concordant with Fadwa *et al*. (2018) [34], Umayal *et al*. (2019) [36], Farsimadan *et.al*. (2021) [124], their results the frequency of the GC and CC genotype was significantly higher in PCOS patients, rs4889G>C had a significant association with PCOS.

c. Influence of *KISS1* Gene Polymorphisms(rs4889G>A) on PCOS Associated Endocrine and Obesity Linked Variables

The current study describes the probable influence of identified SNP (rs4889 G>A) in the *KISS1* gene in PCOS pathogenesis. Mainly, the influence of these SNP on the endocrine (kisspeptin, LH, FSH, LH- FSH ratio, DHEA, and free testosterone) and obesity-linked parameters (BMI and waist-hip ratio) were studied, in females with and without PCOS, the effect of rs4889 AA and GA genotype among patients and control as show the results in Table (3-14) exhibited highly significant influence on the values of WHR, LH/FSH ratio, DHEA, and free testosterone as well as between normal weight and obese patients as the Table (3-15) that explain the influence mutant genotype(AA) has significant association with PCOS and found higher frequency in normal-weight patients exhibited significant influence on the values kisspeptin and WHR levels expect GA genotype significant influence on WHR only.

Table 3-14: The influence of rs4889 G>A polymorphism of *KISS1* gene on the mean differences of characteristics and parameters between genotypes normal weight (patient and control) groups

Parameters	Groups	Genotype of rs4889		(P-Value)
		GA Mean ±SE	AA Mean ±SE	
No. of GA/AA	PCOS Patients	18	9	
	control	8	1	
WHR	PCOS Patients	0.89 ±0.01	0.90±0.02	GA<**0.01**
	Control	0.81±0.01	0.77	AA=0.09
LH (mIU/ml)	PCOS Patients	8.79 ±1.11	11.58±3.1	GA=0.19
	control	6.26±1.3	3.2	AA=0.4
FSH (mIU/ml)	PCOS Patients	6.06±1.6	3.8±0.43	GA=0.35
	control	8.59±1.48	4.8	AA=0.52
LH/FSH ratio	PCOS Patients	2.11±0.28	3.18±0.77	GA<**0.01**
	control	0.81±0.04	0.66	AA=0.33
kisspeptin ng/L	PCOS Patients	300.4±38.3	1062±187	GA=0.2
	control	363.7±20	220.1	AA=0.19
F.Testosterone (pg/ml)	PCOS Patients	2.16±0.17	1.98±0.31	GA<**0.01**
	control	0.81±0.04	1.0	AA=0.34
DHEA (µg/dl)	PCOS Patients	312.9±35.3	402±67.4	GA<**0.01**
	Control	175±22.6	224	AA=0.42

WHR (waist to hip ratio) , LH(Luteinizing Hormone), FSH(Follicle-Stimulating Hormone), DHEA-S (dehydroepiandrosterone sulfate), PCOS (polycystic ovary syndrome), SE(Standard error), P value ≤0.05 was significant.

Table 3-15: The influence of rs4889 G>A polymorphism of *KISS1* gene on the mean differences of characteristics and parameters between genotypes patients (normal weight and obese) groups

Parameters	Groups	Genotype of rs4889		(P-value)
		GA Mean ±SE	AA Mean ±SE	
No. of GA/AA	Normal weight	18	9	
	Obese	13	7	
WHR	Normal weight	0.89 ±0.01	0.90±0.02	GA<**0.01**
	Obese	1.0±0.02	1.02±0.03	AA=**0.009**
LH (mIU/ml)	Normal weight	8.79 ±1.11	11.58±3.1	GA=0.54
	Obese	10.3±2.62	8.98±1.82	AA=0.51
FSH (mIU/ml)	Normal weight	6.06±1.6	3.8±0.43	GA=0.66
	Obese	5.13±0.93	4.22±0.51	AA=0.62
LH/FSH Ratio	Normal weight	2.11±0.28	3.18±0.77	GA=0.24
	Obese	1.94±0.55	2.04±0.35	AA=0.33
kisspeptin ng/L	Normal weight	300.4±38.3	1062±187	GA=0.09
	Obese	363.7±20	372.2±51.4	AA=**0.007**
F.Testosterone (pg/ml)	Normal weight	2.16±0.17	1.98±0.31	GA=0.32
	Obese	2.53±0.37	3.34±0.68	AA=0.07
DHEA (µg/dl)	Normal weight	312.9±35.3	402±67.4	GA=0.86
	Obese	323.3±24.2	330.9±46.6	AA=0.42

WHR (waist to hip ratio) , LH(Luteinizing Hormone), FSH(Follicle-Stimulating Hormone), DHEA-S (dehydroepiandrosterone sulfate), PCOS (polycystic ovary syndrome), SE(Standard error), P value ≤0.05 was significant

The comparison analysis was performed between the studied groups of wild type homozygous and mutant allele carriers (mutant homozygous and heterozygous). When compared between the patients and control within AA genotype the result showed no influence while GA genotype exhibit a

significant influence (P<0.05) on the obesity-linked parameters (WHR) also the effect on the endocrine that lead to increase the value of LH/FSH ratio, FT, and DHEA-S, while kisspeptin, FSH and LH showed no significant difference compared to GA genotype control.

As well as this study showed the higher significant (P<0.01) of WHR in (obese compared to normal weight) patients with both genotype GA and AA. In other study the allele C instead of allele A for this SNP, these concordant with the results of mazin *et. al.* study (2021) [179], the importance of WHR as prognostic marker, conventional anthropometric markers for the description of obesity.

They are proved to have direct association with PCOS as increased adiposity under high-nutrient conditions increases leptin secretion from adipocytes, which in turn activates the kisspeptin-HPG axis and leads to activation of the reproductive system.

However, in a low nutritional state, the amount of fat decreases and the amount of leptin decreases, resulting in decreased activity of the kisspeptin-HPG axis, which leads to a decrease in the reproductive system, their effect creating disturbances in metabolic as well as endocrine system[45,51], confirms the contribution of abdominal fat as an etiological mechanism in PCOS by higher WHR in PCOS compared to obese control, it was found that obese females had significant hyperandrogenemia compared to normal weight, findings indicating that obesity plays an exciting role in the early progress of androgen excess [4].

In addition, finding higher level of kisspeptin in normal weight compare to obese (P= 0.007) patients with genotype AA. Previous study, reported by Albalawi *et al.*(2019)[34], found no significant difference in the kisspeptin

level between PCOS females and control. Other study by Umayal *et.al*.(2019) [36] found no association of the *KISS1* gene rs4889 G>C and serum kisspeptin levels in PCOS.

No significant elevation of the kisspeptin level in PCOS patients compare to control, may be due to the sample size was small. The effect of rs4889 GA and AA polymorphism of the *KISS1* gene may have a direct effect on the functional activity of kisspeptin in term of its altered behavior and binding capacity of kisspeptin to its receptor GPR54. Consequently, the disturbed kisspeptin-GPR54 pathway and dysregulation in GnRH secretion, which leads to the hypersecretion of LH that lead to hyperandrogenism I.e. increase testosterone level and induced by the direct action of high stimulation of LH on gonads. Appears that rs4889 influences the mechanism by which kisspeptin activates secretion of LH but not FSH [181].

3.3.3.5 Haplotype Analysis

Haplotype analysis results were presented in Table (3-16). The haplotypes exhibited polymorphism in the PCOS and control groups. The most frequently identified haplotype in both groups were G allele for each SNPs (rs372790354, and rs4889). The results in the two groups (patients and control) were significantly different ($P<0.05$) and high risk to probable influence of PCOS in the females that have any haplotypes of (AA and GA) of (rs4889 with rs372790354).

Finally, the females who have both genotypes of AA and GA from the two SNPs in this study, have a higher risk of developing PCOS. Also the present study, finding that females who have a type of mutation AA in both SNPs have a high severity of PCOS.

Table 3-16: Genotype combination of SNPs rs4889 and rs372790354 in *KISS1* gene between (patient and control) groups

rs4889 + rs372790354	Control (No.60)	Patients (No.60)	Odd Ratio (95% CI)	P. Value
Normal/Normal GG+GG	24	5	References(OR=1)	
Normal/Hetero GG+GA	14	6	1.05 0.5 to 7.9	0.29
Normal/Mutant GG+AA	1	2	9.6 0.7 to 127.5	0.086
Hetero/Normal GA+GG	11	8	3.49 0.92 to 13.1	0.064
Hetero/Hetero GA+GA	9	18	9.6 2.74 to 33.5	**0.0004**
Hetero/Mutant GA+AA	0	5	49.0 2.3 to 1023.57	**0.0121**
Mutant/Normal AA+GG	0	5	49.0 2.3 to 1023.57	**0.0121**
Mutant/Hetero AA+GA	1	6	28.8 2.8 to 294.8	**0.0046**
Mutant/Mutant AA+AA	0	5	49.0 2.3 to 1023.57	**0.0121**

OR: odd ratio, G- major allele, A-minor allele, CI: confidence interval P value ≤0.05 was significant.

3.3.4 Receiver Operating Characteristic Analysis of The Kisspeptin in rs4889- AA Genotype

The role of kisspeptin (a major product of *KISS1* gene) has been well identified in puberty, ovulation, brain sex differentiation, and fertility, with an essential regulatory function in the normal release of hypothalamic GnRH and consequently in LH secretion, The pathophysiological mechanism of PCOS is also reflected in the inappropriate GnRH/LH secretion[106].

Kisspeptin was showed good ability to predict PCOS that cause from mutant in *KISS1* gene with kisspeptin genotype AA vs. GA and GG in normal weight patient with an AUC (area under the curve) of 0.91(P-value <0.001), since sensitivity is (67%) and specificity of (93%) at a cut off value 484.2 ng/L,

this suggests it may be a more useful tool for conformation of the diagnosis of PCOS, the study of Manal *et al*. (2020) that agree with this finding [37] as shown in figure (3-17), while kisspeptin between other groups were no significant.

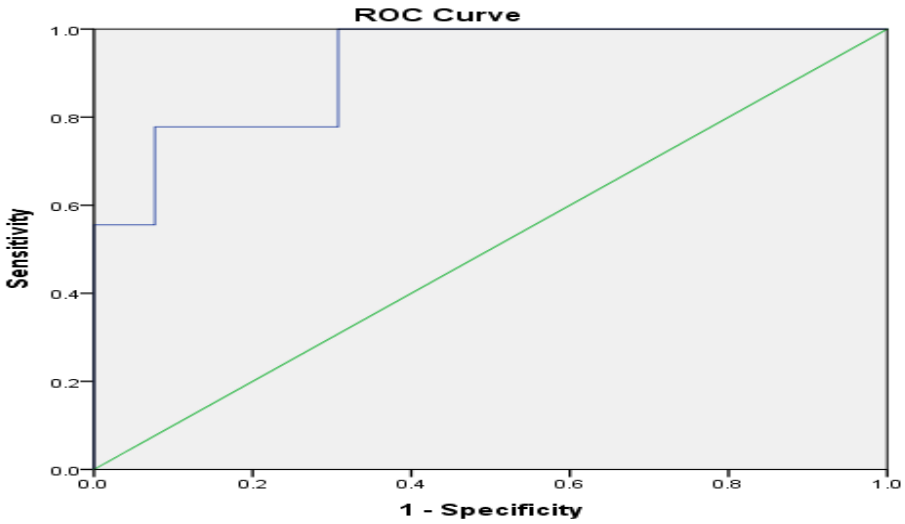


Figure 3-16: Receiver operating characteristic (ROC) curve of serum level of kisspeptin for PCOS patients (AA Against GA and GG) group with patient rs4889 genotype, AUC: area under the curve

Conclusions

1. Dysregulation of kisspeptin lead to increase level of LH that contribute to pathogenesis of PCOS.

2. Polymorphisms of *KISS1*gene (rs3727354G>A, rs4889 G>A) play an important role in development of PCOS.

3. At the level of genotypes mutant GA and AA of rs4889 play an important role on the elevated levels of LH/FSH ratio, free testosterone and DHEA that consider as a major factors for developing PCOS.

4. Kisspeptin is a good indicator for normal weight with PCOS females that have AA genotype of *KISS1* gene rs4889 and no strong effect on obese females.

5. Real time HRM PCR is a simple, fast, cost-effective and efficient genotyping technique, with low risk for DNA contamination

Recommendations

1. Evaluate the role of other candidate genes (such as genes involved in insulin action and secretion , genes involved in pathway of *KISS1*gene- G-protein coupled receptor (GPR54), and fat-mass gene) in predisposing individuals for PCOS.
2. Using more than molecular techniques for detection of other *KISS1* genetic polymorphisms.

References

[1] H. Chaudhary, J. Patel, N. K. Jain, and R. Joshi, “The role of polymorphism in various potential genes on polycystic ovary syndrome susceptibility and pathogenesis,” Journal of Ovarian Research, vol. 14, no. 1. 2021.

[2] R. Deswal, V. Narwal, A. Dang, and C. S. Pundir, “The Prevalence of Polycystic Ovary Syndrome: A Brief Systematic Review,” Journal of Human Reproductive Sciences, vol. 13, no. 4. pp. 261–271, 2020.

[3] V. Ghatnatti, S. Patil, and H. Kour, “Assessment of clinical, biochemical, and hormonal profile of lean versus overweight polycystic ovarian syndrome patients: A cross-sectional study,” APIK J. Intern. Med., vol. 10, no. 1, p. 13, 2022.

[4] Y. L. Alsaadi and B. J. Mohamad, “Prevalence of hyperandrogenism in Iraqi women with polycystic ovary syndrome,” Iraqi Journal of Science, vol. 60, no. 12. pp. 2600–2608, 2019.

[5] Thomas M. Barber and S. Franks, “Obesity and polycystic ovary syndrome,” Clinical Endocrinology, vol. 95, no. 4. pp. 531–541, 2021.

[6] A. E. Jones, “Diagnosis and treatment of polycystic ovarian syndrome.,” Nursing times, vol. 101, no. 3. pp. 40–43, 2005.

[7] G. Conway et al., “The polycystic ovary syndrome: a position statement from the European Society of Endocrinology Gerard,” no. 8 (72). 2015.

[8] J. M. Castellano and M. Tena-Sempere, “Metabolic regulation of kisspeptin — the link between energy balance and reproduction Víctor,”

Advances in Experimental Medicine and Biology, vol. 784. pp. 363–383, 2013.

[9] A. Raffone et al., “Clinical Predictive Factors of Response to Treatment in Patients Undergoing Conservative Management of Atypical Endometrial Hyperplasia and Early Endometrial Cancer,” J. Adolesc. Young Adult Oncol., vol. 10, no. 2, pp. 193–201, 2021.

[10] T. Mehreen, H. Ranjani, R. Kamalesh, U. Ram, R. Anjana, and V. Mohan, “Prevalence of polycystic ovarian syndrome among adolescents and young women in India,” J. Diabetol., vol. 12, no. 3, p. 319, 2021.

[11] S. M. Sirmans and K. A. Pate, “Epidemiology, diagnosis, and management of polycystic ovary syndrome,” Clinical Epidemiology, vol. 6, no. 1. pp. 1–13, 2013.

[12] S. Haider, N. Manan, A. Khan, and M. A. Qureshi, “Prevalence of elevated luteinizing hormone [LH]/follicle stimulating hormone [FSH]ratio in polycystic ovary syndrome [PCOS]women among local population.,” JDUHS-Journal of the Dow University of Health Sciences, vol. 5. pp. 17–20, 2019.

[13] “https://www.invitra.com/en/polycystic-ovary-syndrome/normal-ovary-vs-polycystic-ovary/.” .

[14] D. Lizneva, L. Suturina, W. Walker, S. Brakta, L. Gavrilova-Jordan, and R. Azziz, “Criteria, prevalence, and phenotypes of polycystic ovary syndrome,” Fertility and Sterility, vol. 106, no. 1. pp. 6–15, 2016.

[15] M. O. Goodarzi, D. A. Dumesic, G. Chazenbalk, and R. Azziz,

"Polycystic ovary syndrome: Etiology, pathogenesis and diagnosis," Nature Reviews Endocrinology, vol. 7, no. 4. pp. 219–231, 2011.

[16] S. S. Lim, R. J. Norman, M. J. Davies, and L. J. Moran, "The effect of obesity on polycystic ovary syndrome: A systematic review and meta-analysis," Obesity Reviews, vol. 14, no. 2. pp. 95–109, 2013.

[17] W. A. March, V. M. Moore2, K. J. Willson1, D. I. W. Phillips3, R. J. Norman1, and and M. J. Davies, "The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria Wendy." ORIGINAL ARTICLE Reproductive epidemiology The, pp. 544–551, 2010.

[18] F. A. K. Khazaal, A. H. Liebi, and I. J. Mahmoud, "Prevalence and Presenting Features of Polycystic Ovarian Syndrome in Iraqi Obese Females." iraqi journal of infertility researches, baghdad, pp. 14–18, 2014.

[19] S. Kiconco et al., "Pcos phenotype in unselected populations study (P-pup): Protocol for a systematic review and defining pcos diagnostic features with pooled individual participant data," Diagnostics, vol. 11, no. 11, 2021.

[20] M. K. C. C. Ilagan, E. Paz-Pacheco, D. Z. Totesora, L. R. Clemente-Chua, and J. R. K. Jalique, "The modified Ferriman-Gallwey score and hirsutism among Filipino women," Endocrinology and Metabolism, vol. 34, no. 4. pp. 374–381, 2019.

[21] R. N. Hussein1, K. I. Al Hamdi2, and A. A. Mansour, "Association between biochemical hyperandrogenism parameters and modified

Ferriman-Gallwey score in patients with hirsutism in Basrah (Southern Iraq).” pp. 603–607, 2021.

[22] M. H. Hunter, P. J. Carek, and S. C. Guest, “Evaluation and Treatment of Women with Hirsutism - American Family Physician.” pp. 2565–2572, 2003.

[23] B. Eilerman, M. Salehi, and Y. Tomer, “The genetics of polycystic ovary syndrome,” Diagnosis and Management of Polycystic Ovary Syndrome. pp. 83–91, 2009.

[24] H. M. Sadeghi et al., “Polycystic Ovary Syndrome: A Comprehensive Review of Pathogenesis, Management, and Drug Repurposing.” International Journal of Molecular Sciences Review, 2022.

[25] Y. Liu et al., “The release of peripheral immune inflammatory cytokines promote an inflammatory cascade in PCOS patients via altering the follicular microenvironment,” Frontiers in Immunology.vol.12, p. 1834, 2021.

[26] A. Z. Rutkowska and E. Diamanti-Kandarakis, “Polycystic ovary syndrome and environmental toxins,” Fertility and Sterility, vol. 106, no. 4. pp. 948–958, 2016.

[27] Y. Kawakami, S. Fujii, G. Ishikawa, A. Sekiguchi, A. Nakai, and M. Takase, “Valproate-induced polycystic ovary syndrome in a girl with epilepsy: A case study,” J. Nippon Med. Sch., vol. 85, no. 5, pp. 287–290, 2018.

[28] A. Sacerdote, “Rare and Underappreciated Causes of Polycystic Ovarian

Syndrome," in Polycystic Ovary Syndrome [Working Title], 2022.

[29] N. Ajmal, S. Z. Khan, and R. Shaikh, "Polycystic ovary syndrome (PCOS) and genetic predisposition: A review article," European Journal of Obstetrics and Gynecology and Reproductive Biology: X, vol. 3. 2019.

[30] S. Joseph, R. S. Barai, R. Bhujbalrao, and S. Idicula-Thomas, "PCOSKB: A knowledgebase on genes, diseases, ontology terms and biochemical pathways associated with polycystic ovary syndrome," Nucleic Acids Res., vol. 44, no. D1, pp. D1032–D1035, 2016.

[31] T. Strachan and A. P. Read, "Instability of the human genome: mutation and DNA repair," Hum. Mol. Genet., vol. 2, pp. 209–217, 1999.

[32] N. Xita, I. Georgiou, and A. Tsatsoulis, "The genetic basis of polycystic ovary syndrome," European Journal of Endocrinology, vol. 147, no. 6. pp. 717–725, 2002.

[33] R. A. Condorelli, A. E. Calogero, M. Di Mauro, and S. La Vignera, "PCOS and diabetes mellitus: from insulin resistance to altered beta pancreatic function, a link in evolution," Gynecological Endocrinology, vol. 33, no. 9. pp. 665–667, 2017.

[34] F. S. Albalawi1, M. H. Daghestani1*, M. H. Daghestani2, and A. s. Eldali3 Abdelmoneim and Warsy4, "rs4889 polymorphism in KISS1 gene, its effect on polycystic ovary syndrome development and anthropometric and hormonal parameters in Saudi women.pdf." 2018.

[35] R. Tang, X. Ding, and J. Zhu, "Kisspeptin and polycystic ovary syndrome," Frontiers in Endocrinology, vol. 10, no. MAY. 2019.

[36] U. Branavan, K. Muneeswaran, W. S. S. Wijesundera, A. Senanayake, N. V. Chandrasekharan, and C. N. Wijeyaratne, "Association of Kiss1 and GPR54 Gene Polymorphisms with Polycystic Ovary Syndrome among Sri Lankan Women," BioMed Research International, vol. 2019. 2019.

[37] M. M. Abdalqader and S. S. Hussein, "Metastatin as a Marker for Hyperandrogenemia in Iraqi Women with Polycystic Ovary Syndrome," Obstet. Gynecol. Int., vol. 2020, 2020.

[38] A. J. morales, G. A. laughlin, T. BijTZOW$, H. maheshwari, G. baumann, and S. S. C. YENS, "Insulin, Somatotropic, and Luteinizing Hormone in Lean and Obese Women with Polycystic Ovary Syndrome: Common and Distinct Features* Axes A." 1996.

[39] S. Bednarska and A. Siejka, "The pathogenesis and treatment of polycystic ovary syndrome: What's new?," Adv. Clin. Exp. Med. Off. organ Wroclaw Med. Univ., vol. 26, no. 2, pp. 359–367, 2017.

[40] M. Ganie, V. Vasudevan, I. Wani, M. Baba, T. Arif, and A. Rashid, "Epidemiology, pathogenesis, genetics & management of polycystic ovary syndrome in India," Indian Journal of Medical Research, vol. 150, no. 4. pp. 333–344, 2019.

[41] D. A. Dumesic, S. E. Oberfield, E. Stener-Victorin, J. C. Marshall, J. S. Laven, and R. S. Legro, "Scientific statement on the diagnostic criteria, epidemiology, pathophysiology, and molecular genetics of polycystic ovary syndrome," Endocrine Reviews, vol. 36, no. 5. pp. 487–525, 2015.

[42] C. Zhang, J. Hu, W. Wang, Y. Sun, and K. Sun, "HMGB1-induced aberrant autophagy contributes to insulin resistance in granulosa cells in

PCOS," FASEB J., vol. 34, no. 7, pp. 9563–9574, 2020.

[43] Y. M. Jeanes and S. Reeves, "Metabolic consequences of obesity and insulin resistance in polycystic ovary syndrome: Diagnostic and methodological challenges," Nutrition Research Reviews, vol. 30, no. 1. pp. 97–105, 2017.

[44] R. RL and E. DA, "The Pathogenesis of Polycystic Ovary Syndrome (PCOS): The Hypothesis of PCOS as Functional Ovarian Hyperandrogenism Revisited.," Endocrine Reviews, vol. 37. p. 467, 2016.

[45] S. A. Polyzos, J. Kountouras, G. Deretzi, C. Zavos, and C. S. Mantzoros, "The Emerging Role of Endocrine Disruptors in Pathogenesis of Insulin Resistance: A Concept Implicating Nonalcoholic Fatty Liver Disease," Current Molecular Medicine, vol. 12, no. 1. pp. 68–82, 2011.

[46] P. Shukla and S. Mukherjee, "Mitochondrial dysfunction: An emerging link in the pathophysiology of polycystic ovary syndrome," Mitochondrion, vol. 52, pp. 24–39, 2020.

[47] F. Matsuda et al., "Role of kisspeptin neurons as a GnRH surge generator: Comparative aspects in rodents and non-rodent mammals," J. Obstet. Gynaecol. Res., vol. 45, no. 12, pp. 2318–2329, 2019.

[48] A. Garg, B. Patel, A. Abbara, and W. S. Dhillo, "Treatments targeting neuroendocrine dysfunction in polycystic ovary syndrome (PCOS)." 2022.

[49] T. T. L. Yau, N. Y. H. Ng, L. P. Cheung, and R. C. W. Ma, "Polycystic ovary syndrome: A common reproductive syndrome with long-term

metabolic consequences," Hong Kong Medical Journal, vol. 23, no. 6. pp. 622–634, 2017.

[50] Muhjah Falah, "A Comparable Study of Infertile Females with and without Polycystic Ovary Syndrome Undergo Intracytoplasmic Sperm Injection and Evaluation of Oocyte Quality Outcome," no. September, 2017.

[51] H. G. Huddleston and A. Dokras, "Diagnosis and Treatment of Polycystic Ovary Syndrome," JAMA, vol. 327, no. 3, pp. 274–275, 2022.

[52] K. Unluhizarci, G. Kaltsas, and F. Kelestimur, "Non polycystic ovary syndrome-related endocrine disorders associated with hirsutism," European Journal of Clinical Investigation, vol. 42, no. 1. pp. 86–94, 2012.

[53] J. van Keizerswaard, A. L. P. Dietz de Loos, Y. V. Louwers, and J. S. E. Laven, "Changes in individual polycystic ovary syndrome phenotypical characteristics over time: a long-term follow-up study," Fertil. Steril., 2022.

[54] H. F. Escobar-Morreale, "Polycystic ovary syndrome: Definition, aetiology, diagnosis and treatment," Nature Reviews Endocrinology, vol. 14, no. 5. pp. 270–284, 2018.

[55] S. H. Yedulapuram, M. Gunda, N. R. Moola, and R. K. Kadarla, "an Overview on Polycystic Ovarian Syndrome," Asian Journal of Pharmaceutical Research and Development, vol. 7, no. 4. pp. 72–80, 2019.

[56] Z. Haoula, M. Salman, and W. Atiomo, “Evaluating the association between endometrial cancer and polycystic ovary syndrome,” Hum. Reprod., vol. 27, no. 5, pp. 1327–1331, 2012.

[57] A. L. Damone, A. E. Joham, D. Loxton, A. Earnest, H. J. Teede, and L. J. Moran, “Depression, anxiety and perceived stress in women with and without PCOS: A community-based study,” Psychological Medicine, vol. 49, no. 9. pp. 1510–1520, 2019.

[58] Y.-C. Hsieh1, P.-K. Yang, and M.-J. Chen, “Metabolic Syndrome in Polycystic Ovary Syndrome.” fertility & reproduction, pp. 125–135, 2022.

[59] T. Apridonidze, P. A. Essah, M. J. Iuorno, and J. E. Nestler, “Prevalence and characteristics of the metabolic syndrome in women with polycystic ovary syndrome,” Journal of Clinical Endocrinology and Metabolism, vol. 90, no. 4. pp. 1929–1935, 2005.

[60] J. Beumer et al., “Enteroendocrine cells switch hormone expression along the crypt-to-villus BMP signalling gradient.,” Nat. Cell Biol., vol. 20, no. 8, pp. 909–916, Aug. 2018.

[61] M. A. Sanchez-Garrido and M. Tena-Sempere, “Metabolic dysfunction in polycystic ovary syndrome: Pathogenic role of androgen excess and potential therapeutic strategies,” Molecular Metabolism, vol. 35. 2020.

[62] M. W. Elting, T. J. M. Korsen, P. D. Bezemer, and J. Schoemaker, “Prevalence of diabetes mellitus, hypertension and cardiac complaints in a follow-up study of a Dutch PCOS population,” Human Reproduction, vol. 16, no. 3. pp. 556–560, 2001.

[63] A. S. Momo et al., “Adiponectin levels and its relation with insulin secretion and insulin sensitivity in a group of sub-Saharan African women with polycystic ovary syndrome,” BMC Res. Notes, vol. 15, no. 1, pp. 1–6, 2022.

[64] A. M. Moore and R. E. Campbell, “The neuroendocrine genesis of polycystic ovary syndrome: a role for arcuate nucleus GABA neurons,” J. Steroid Biochem. Mol. Biol., vol. 160, pp. 106–117, 2016.

[65] B. Liao, J. Qiao, and Y. Pang, “Central Regulation of PCOS: Abnormal Neuronal-Reproductive-Metabolic Circuits in PCOS Pathophysiology,” Frontiers in Endocrinology, vol. 12. 2021.

[66] C. R. McCartney, M.D., J. C. Marshall, M.B., Ch.B, and M.D., “Polycystic Ovary Syndrome,” vol. 44, p.54-64, 2016.

[67] MN Chatterjea and Rana Shinde, Medical Biochemistry, 8 th ed., vol. 81. India, 2012.

[68] P. C. of the A. S. for R. Medicine, “Testing and interpreting measures of ovarian reserve: a committee opinion,” Fertil. Steril., vol. 98, no. 6, pp. 1407–1415, 2012.

[69] V. B. Mahesh, “Hirsutism, virilism, polycystic ovarian disease, and the steroid-gonadotropin-feedback system: a career retrospective,” Am. J. Physiol. Metab., vol. 302, no. 1, pp. E4–E18, 2012.

[70] R. P. Crespo, T. A. S. S. Bachega, B. B. Mendonça, and L. G. Gomes, “An update of genetic basis of PCOS pathogenesis,” Archives of Endocrinology and Metabolism, vol. 62, no. 3. pp. 352–361, 2018.

[71] P. Sonksen and J. Sonksen, “Insulin: understanding its action in health and disease,” Br. J. Anaesth., vol. 85, no. 1, pp. 69–79, 2000.

[72] W. E. Roudebush, W. J. Kivens, and J. M. Mattke, “Biomarkers of ovarian reserve,” Biomark. Insights, vol. 3, p. BMI-S537, 2008.

[73] N. Prapas, A. Karkanaki, I. Prapas, I. Kalogiannidis, I. Katsikis, and D. Panidis, “Genetics of polycystic ovary syndrome,” Hippokratia, vol. 13, no. 4, p. 216, 2009.

[74] E. Carmina, F. Z. Stanczyk, and R. A. Lobo, “Evaluation of hormonal status,” in Yen and Jaffe’s Reproductive Endocrinology, Elsevier, 2019, pp. 887–915.

[75] R. J. Chang and D. A. Dumesic, “Polycystic ovary syndrome and hyperandrogenic states,” in Yen and Jaffe’s reproductive endocrinology, Elsevier, 2019, pp. 520–555.

[76] S. F. Witchel and A. K. Topaloglu, “Puberty: gonadarche and adrenarche,” Yen Jaffe’s Reprod. Endocrinol., pp. 394–446, 2019.

[77] E. S. Han and A. goleman, daniel; boyatzis, Richard; Mckee, Illustrated Biochemistry, vol. 53, no. 9. 2019.

[78] E. Nieschlag and S. Nieschlag, “The history of discovery, synthesis and development of testosterone for clinical use,” European Journal of Endocrinology, vol. 180, no. 6. pp. R201–R212, 2019.

[79] H. G. Burger, “Androgen production in women,” Fertil. Steril., vol. 77, pp. 3–5, 2002.

[80] L. M. Holm, “Polycystic ovary syndrome Studies of metabolic and ovarian disturbances and effects of physical exercise and electro-acupuncture.” 2010.

[81] G. L. Hortin and B. A. Goldberger, Tietz Textbook of Clinical Chemistry and Molecular Diagnosis. 2015.

[82] V. L. Nelson et al., “The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome,” J. Clin. Endocrinol. Metab., vol. 86, no. 12, pp. 5925–5933, 2001.

[83] R. Azziz, E. Carmina, D. Dewailly, E. Diamanti-Kandarakis, H. F. Escobar-Morreale, and W. Futterweit, “Androgen Excess Society. Position statement: Criteria for defining pcos as a predominantly hyperandrogenic syndrome: An Androgen Excess Society guideline,” J Clin Endocrinol Metab, vol. 91, pp. 4237–4245, 2006.

[84] J. Schmidt, Polycystic ovary syndrome ovarian pathophysiology and consequences after the menopause. Geson Hylte Tryck, Göteborg, Sweden 2011, 2011.

[85] F. Day et al., “Large-scale genome-wide meta-analysis of polycystic ovary syndrome suggests shared genetic architecture for different diagnosis criteria,” PLoS Genet., vol. 14, no. 12, p. e1007813, 2018.

[86] H. Nautiyal et al., “Ovarian Syndrome: A Complex Disease with a Genetics Approach.” journal biomedicines, 2022.

[87] J. S. E. Laven, “Follicle stimulating hormone receptor (FSHR)

polymorphisms and polycystic ovary syndrome (PCOS)," Frontiers in Endocrinology, vol. 10, no. FEB. 2019.

[88] U. Branavan, S. Wijesundera, V. Chandrasekharan, and C. Wijeyaratne, "Potential Genetic Polymorphisms Predicting Polycystic Ovary Syndrome (PCOS) in Sri Lankan Women: Comparison with Different Ethnicity," Adv. Technol., vol. 1, no. 1, 2021.

[89] M. G. Hayes et al., "Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations," Nat. Commun., vol. 6, 2015.

[90] H. Lee et al., "Genome-wide association study identified new susceptibility loci for polycystic ovary syndrome," Hum. Reprod., vol. 30, no. 3, pp. 723–731, 2015.

[91] B. Yilmaz, P. Vellanki, B. Ata, and B. O. Yildiz, "Metabolic syndrome, hypertension, and hyperlipidemia in mothers, fathers, sisters, and brothers of women with polycystic ovary syndrome: a systematic review and meta-analysis," Fertil. Steril., vol. 109, no. 2, p. 356–364.e32, 2018.

[92] T. J. Housh, J. T. Cramer, J. P. Weir, T. W. Beck, and G. O. Johnson, "Laboratory Manual for Exercise Physiology, Exercise Testing, and Physical Fitness," Laboratory Manual for Exercise Physiology, Exercise Testing, and Physical Fitness. 2017.

[93] N. Draper et al., "Mutations in the genes encoding 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency," Nat. Genet., vol. 34, no. 4, pp. 434–439, 2003.

[94] N. Bhalakiya, N. Haque, and P. Patel, “Kisspeptin: A Novel Regulator in Reproductive Physiology,” Int. J. Livest. Res., p. 1, 2019.

[95] A. E. Oakley, D. K. Clifton, and R. A. Steiner, “Kisspeptin signaling in the brain,” Endocrine Reviews, vol. 30, no. 6. pp. 713–743, 2009.

[96] NCBI, “kiss1 gene location.” [Online]. Available: https://www.ncbi.nlm.nih.gov/gene/3814. [Accessed: 29-Mar-2022].

[97] S. Z. Nejad, F. R. Tehrani, and A. Zadeh-Vakili, “The role of Kisspeptin in female reproduction,” International Journal of Endocrinology and Metabolism, vol. 15, no. 3. 2017.

[98] C. B. Herber1 et al., “Estrogen signaling in arcuate Kiss1 neurons suppresses a sex-dependent female circuit promoting dense strong bones.” article nature communications, 2019.

[99] U. Branavan, C. NV, W. WSS, and W. Chandrika N, “Polycystic Ovary Syndrome: Genetic Contributions from the Hypothalamic-Pituitary-Gonadal Axis,” Int. Arch. Endocrinol. Clin. Res., vol. 4, no. 1, 2018.

[100] M. H. Daghestani et al., “Influence of KISS1 gene polymorphisms on the risk of polycystic ovary syndrome and its associated variables, in Saudi women,” British Journal of Biomedical Science, vol. 77, no. 4. pp. 185–190, 2020.

[101] A. N. Comninos and W. S. Dhillo, “Emerging Roles of Kisspeptin in Sexual and Emotional Brain Processing,” Neuroendocrinology, vol. 106, no. 2, pp. 195–202, 2018.

[102] J. Padda et al., “Role of Kisspeptin on Hypothalamic-Pituitary-Gonadal

Pathology and Its Effect on Reproduction," Cureus. 2021.

[103] "kisspeptin structure." [Online]. Available: https://www.uniprot.org/uniprot/Q15726#showFeaturesTable. [Accessed: 29-Mar-2022].

[104] K. Katulski, A. Podfigurna, A. Czyzyk, B. Meczekalski, and A. D. Genazzani, "Kisspeptin and LH pulsatile temporal coupling in PCOS patients," Endocrine, vol. 61, no. 1. pp. 149–157, 2018.

[105] X. D. A. De Tassigny and W. H. Colledge, "The role of Kisspeptin signaling in reproduction," Physiology, vol. 25, no. 4. pp. 207–217, 2010.

[106] C. M. Trevisan et al., "Kisspeptin/GPR54 System: What Do We Know about Its Role in Human Reproduction?," Cellular Physiology and Biochemistry, vol. 49, no. 4. pp. 1259–1276, 2018.

[107] L. Pinilla, E. Aguilar, C. Dieguez, R. P. Millar, and M. Tena-Sempere, "Kisspeptins and reproduction: Physiological roles and regulatory mechanisms," Physiological Reviews, vol. 92, no. 3. pp. 1235–1316, 2012.

[108] J. Johansson and E. Stener-Victorin, "Polycystic ovary syndrome: Effect and mechanisms of acupuncture for ovulation induction," Evidence-based Complementary and Alternative Medicine, vol. 2013. 2013.

[109] J. T. Smith, Q. Li, A. Pereira, and I. J. Clarke, "Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge," Endocrinology, vol. 150, no. 12, pp. 5530–5538, 2009.

[110] K. M. Estrada, C. M. Clay, S. Pompolo, J. T. Smith, and I. J. Clarke, "Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback," J. Neuroendocrinol., vol. 18, no. 10, pp. 806–809, 2006.

[111] F. Wahab, B. Atika, M. Shahab, and R. Behr, "Kisspeptin signalling in the physiology and pathophysiology of the urogenital system," Nature Reviews Urology, vol. 13, no. 1. pp. 21–32, 2016.

[112] Y.-J. Rhie, "Kisspeptin/G protein-coupled receptor-54 system as an essential gatekeeper of pubertal development," Annals of Pediatric Endocrinology & Metabolism, vol. 18, no. 2. p. 55, 2013.

[113] E. Votsi, D. Roussos, I. Katsikis, A. Karkanaki, M. Kita, and D. Panidis, "Kisspeptins: A multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system," Hippokratia, vol. 12, no. 4. pp. 205–210, 2008.

[114] M. Bhattacharya and A. V. Babwah, "Kisspeptin: Beyond the brain," Endocrinology (United States), vol. 156, no. 4. pp. 1218–1227, 2015.

[115] E. G. uadalup. C. Quevedo et al., "Polymorphisms rs12998 and rs5780218 in KiSS1 suppressor metastasis gene in Mexican patients with breast cancer," Dis. Markers, vol. 2015, p. 365845, 2015.

[116] X. Zeng, Y. jie Xie, Y. ting Liu, S. lian Long, and Z. cheng Mo, "Polycystic ovarian syndrome: Correlation between hyperandrogenism, insulin resistance and obesity," Clinica Chimica Acta, vol. 502. pp. 214–221, 2020.

[117] J. ling Zhu, Z. Chen, W. jie Feng, S. lian Long, and Z. C. Mo, "Sex hormone-binding globulin and polycystic ovary syndrome," Clinica Chimica Acta, vol. 499. pp. 142–148, 2019.

[118] A. P. Delitala, G. Capobianco, G. Delitala, P. L. Cherchi, and S. Dessole, "Polycystic ovary syndrome, adipose tissue and metabolic syndrome," Archives of Gynecology and Obstetrics, vol. 296, no. 3. pp. 405–419, 2017.

[119] M. Nunez-Salces, H. Li, C. Feinle-Bisset, R. L. Young, and A. J. Page, "The regulation of gastric ghrelin secretion.," Acta Physiol. (Oxf)., vol. 231, no. 3, p. e13588, Mar. 2021.

[120] M. Farhadipour and I. Depoortere, "The function of gastrointestinal hormones in obesity—implications for the regulation of energy intake," Nutrients, vol. 13, no. 6. 2021.

[121] B. Jaclyn Carr, B. Brianna Giannotte, and R. Monica Moore, MSN, "httpswww.obgproject.com20190612pcos-part-1-sensitive-care-of-the-pcos-patient." .

[122] S. Ghaderpour, R. Ghiasi, H. Heydari, and R. Keyhanmanesh, "The relation between obesity, kisspeptin, leptin, and male fertility," Horm. Mol. Biol. Clin. Investig., 2021.

[123] L. Naing, T. Winn, and B. N. Rusli, "Practical issues in calculating the sample size for prevalence studies," Arch. Orofac. Sci., vol. 1, pp. 9–14, 2006.

[124] M. Farsimadan, F. Moammadzadeh Ghosi, S. Takamoli, and H. Vaziri,

"Association analysis of KISS1 polymorphisms and haplotypes with polycystic ovary syndrome," Br. J. Biomed. Sci., 2021.

[125] R. Bhatia1, A. Bhatia, and K. Ganatara, "Correlation of body mass index and waist/hip ratio with severity of coronary artery disease." International Journal of Research in Medical Sciences, p. vol 10, ISSUE 2, PB 388-392, 2022.

[126] L. Cinquanta, D. E. Fontana, and N. Bizzaro, "Chemiluminescent immunoassay technology: what does it change in autoantibody detection?," Autoimmun. Highlights, vol. 8, no. 1, 2017.

[127] "snibe-maglumi-lh-clia.pdf." .

[128] "snibe-maglumi-fsh-clia.pdf." .

[129] L. Shenzhen New Industries Biomedical Engineering Co., "MAGLUMI™ DHEA-S (CLIA)." 2019.

[130] L. Shenzhen New Industries Biomedical Engineering Co., "MAGLUMI free Testosterone (CLIA)." .

[131] "Human-Kisspeptin1KISS-1ELISA-Kit-1600-1.pdf." .

[132] "favogene extraction DNA Kit." .

[133] A. J. Al-Aaraji, S. Aesa Al-Qaysi, and A. Salihbaay, "Haplotype in ABCC4 gene by PCR-SSCP technique in Iraqi Asthmatic patients," in Journal of Physics: Conference Series, 2019, vol. 1294, no. 6.

[134] U. Branavan, K. Muneeswaran, S. Wijesundera, S. Jayakody, V. Chandrasekharan, and C. Wijeyaratne, "Identification of selected genetic

polymorphisms in polycystic ovary syndrome in Sri Lankan women using low cost genotyping techniques," PLoS One, vol. 13, no. 12, 2018.

[135] "HRM." .

[136] I. Kalemis, "Anova Regression Correlation analysis. A portfolio of work in Statistical Techniques with SPSS." 2022.

[137] A. E. Yilmaz and S. Aktas Altunay, "Post-hoc comparison tests for odds ratios," Electron. J. Appl. Stat. Anal., vol. 15, no. 1, pp. 75–94, 2022.

[138] H. Wang et al., "Comparison of anthropometric indices for predicting the risk of metabolic syndrome and its components in Chinese adults: a prospective, longitudinal study," BMJ Open, vol. 7, no. 9, p. e016062, 2017.

[139] T. M. Barber and S. Franks, "Obesity and polycystic ovary syndrome," Clinical Endocrinology, vol. 95, no. 4. pp. 531–541, 2021.

[140] R. Azziz, "Polycystic ovary syndrome is a family affair," Journal of Clinical Endocrinology and Metabolism, vol. 93, no. 5. pp. 1579–1581, 2008.

[141] N. Naderpoor, S. Shorakae, B. de Courten, M. L. Misso, L. J. Moran, and H. J. Teede, "Metformin and lifestyle modification in polycystic ovary syndrome: Systematic review and meta-analysis," Human Reproduction Update, vol. 22, no. 3. pp. 408–409, 2016.

[142] R. Horejsi et al., "Android subcutaneous adipose tissue topography in lean and obese women suffering from PCOS: comparison with type 2 diabetic women," Am. J. Phys. Anthropol. Off. Publ. Am. Assoc. Phys.

Anthropol., vol. 124, no. 3, pp. 275–281, 2004.

[143] L. Ibáñez et al., “Normalizing ovulation rate by preferential reduction of hepato-visceral fat in adolescent girls with polycystic ovary syndrome,” J. Adolesc. Heal., vol. 61, no. 4, pp. 446–453, 2017.

[144] S. S. Lim, M. J. Davies, R. J. Norman, and L. J. Moran, “Overweight, obesity and central obesity in women with polycystic ovary syndrome: a systematic review and meta-analysis,” Hum. Reprod. Update, vol. 18, no. 6, pp. 618–637, 2012.

[145] S. K. Blank, C. R. McCartney, and J. C. Marshall, “The origins and sequelae of abnormal neuroendocrine function in polycystic ovary syndrome,” Hum. Reprod. Update, vol. 12, no. 4, pp. 351–361, 2006.

[146] Z. Saadia, “Follicle Stimulating Hormone (LH: FSH) Ratio in Polycystic Ovary Syndrome (PCOS) - Obese vs. Non- Obese Women,” Medical archives (Sarajevo, Bosnia and Herzegovina), vol. 74, no. 4. pp. 289–293, 2020.

[147] L. Lal, A. Bharti, and A. Perween, “To study the status of LH: FSH ratio in obese and non-obese patients of polycystic ovarian syndrome,” IOSR J Dent Med Sci, vol. 16, no. 01, pp. 20–23, 2017.

[148] G. N. Allahbadia and R. Merchant, “Polycystic ovary syndrome and impact on health,” Middle East Fertil. Soc. J., vol. 16, no. 1, pp. 19–37, 2011.

[149] S. Suresh and T. Vijayakumar, “Correlations of insulin resistance and serum testosterone levels with LH: FSH ratio and oxidative stress in

women with functional ovarian hyperandrogenism," Indian J. Clin. Biochem., vol. 30, no. 3, pp. 345–350, 2015.

[150] W. Ibrahim and K. E. A. Abdelsalam, "Levels of FSH, LH, SHBG, Total Testosterone, and LH/FSH ratio in Sudanese patients with polycystic ovary syndrome in relation to body mass index," Int. J. Curr. Res., vol. 7, no. 1, pp. 11919–11922, 2015.

[151] T. Cascella et al., "Visceral fat is associated with cardiovascular risk in women with polycystic ovary syndrome," Hum. Reprod., vol. 23, no. 1, pp. 153–159, 2008.

[152] HANEEN SUBHEE SHAHEED, W. A.-K. ABBASS, and S. Y. JASIM, "Correlation between Serum Bisphenol-A Level, Luteinizing Hormone (LH), Follicular Stimulating Hormone (FSH) In a Sample of Iraqi Women with PCOS," Int. J. Pharm. Res., vol. 13, no. 01, 2020.

[153] K. R. Nitin and R. Nataraj, "Hormones of HPG-axis and their Active Role during Chronic Stress and PCOS Induction : A Review," vol. 11, no. 1. pp. 1–8, 2022.

[154] K. C. Lewandowski, A. Cajdler-Łuba, I. Salata, M. Bieńkiewicz, and A. Lewiński, "The utility of the gonadotrophin releasing hormone (GnRH) test in the diagnosis of polycystic ovary syndrome (PCOS)," Endokrynol. Pol., vol. 62, no. 2, pp. 120–128, 2011.

[155] Z. Guleken, H. Bulut, P. Y. Bahat, S. Yılmaz, and D. Sarıbal, "Elevated Serum Level of DHEAS as a Hormone and IL-6 as a Proinflammatory Cytokine May Better Indicate Metabolic Syndrome in PCOS Women," J. Med. Physiol. Biophys., 2021.

[156] R. A.]Richard S., Lippincott Williams & Wilkins, Philadelphia, 8th editio. 2003.

[157] A. H. Alnakash and N. K. Al-Tae e, “Polycystic ovarian syndrome: the correlation between the LH/FSH ratio and disease manifestations,” Middle East Fertil. Soc. J., vol. 12, no. 1, p. 35, 2007.

[158] D. S. Kiddy et al., “Differences in clinical and endocrine features between obese and non-obese subjects with polycystic ovary syndrome: an analysis of 263 consecutive cases,” Clin. Endocrinol. (Oxf)., vol. 32, no. 2, pp. 213–220, 1990.

[159] A. Refaie, G. A. K. Ibrahim, and S. Al Oash, “Characteristics of polycystic ovary syndrome with and without insulin resistance and the role of insulin sensitizing drug (metformin) in its management,” 2005.

[160] A. I. Hamadamen, Z. B. Hasan, J. A. H. Albazaz, and R. T. Lak, “Differences in clinical and endocrine features between obese and non obese women in polycystic ovarian syndrome.” Journal of Kurdistan Board of Medical Specialties, Erbil-Iraq, pp. 54–61, 2017.

[161] L. Ibáñez et al., “An International Consortium Update: Pathophysiology, Diagnosis, and Treatment of Polycystic Ovarian Syndrome in Adolescence,” Hormone Research in Paediatrics, vol. 88, no. 6. pp. 371–395, 2017.

[162] D. Lizneva, L. Gavrilova-Jordan, W. Walker, and R. Azziz, “Androgen excess: Investigations and management,” Best Practice and Research: Clinical Obstetrics and Gynaecology, vol. 37. pp. 98–118, 2016.

[163] R. Pasquali et al., “Insulin and androgen relationships with abdominal body fat distribution in women with and without hyperandrogenism,” Horm. Res. Paediatr., vol. 39, no. 5–6, pp. 179–187, 1993.

[164] F. Tosi et al., “Insulin enhances ACTH-stimulated androgen and glucocorticoid metabolism in hyperandrogenic women,” Eur. J. Endocrinol., vol. 164, no. 2, p. 197, 2011.

[165] S. A. Yilmaz et al., “Metastin levels in relation with hormonal and metabolic profile in patients with polycystic ovary syndrome,” Eur. J. Obstet. Gynecol. Reprod. Biol., vol. 180, no. 1, pp. 56–60, 2014.

[166] A. T. Nanba, J. Rege, J. Ren, R. J. Auchus, W. E. Rainey, and A. F. Turcu, “11-Oxygenated C19 Steroids Do Not Decline with Age in Women,” Journal of Clinical Endocrinology and Metabolism, vol. 104, no. 7. pp. 2615–2622, 2019.

[167] X. Chen, Y. Mo, L. Li, Y. Chen, Y. Li, and D. Yang, “Increased plasma metastin levels in adolescent women with polycystic ovary syndrome,” Eur. J. Obstet. Gynecol. Reprod. Biol., vol. 149, no. 1, pp. 72–76, 2010.

[168] Y. E. Jeon et al., “Kisspeptin, leptin, and retinol-binding protein 4 in women with polycystic ovary syndrome,” Gynecol. Obstet. Invest., vol. 75, no. 4, pp. 268–274, 2013.

[169] D. Panidis et al., “Plasma metastin levels are negatively correlated with insulin resistance and free androgens in women with polycystic ovary syndrome,” Fertil. Steril., vol. 85, no. 6, pp. 1778–1783, 2006.

[170] E. Yerlikaya et al., “Plasma kisspeptin levels in polycystic ovary

syndrome," in Endocrine Abstracts, 2013, vol. 32.

[171] N. M. Rashad, R. M. Al-sayed, M. S. Yousef, and Y. S. Saraya, "Kisspeptin and body weight homeostasis in relation to phenotypic features of polycystic ovary syndrome; metabolic regulation of reproduction," Diabetes Metab. Syndr. Clin. Res. Rev., vol. 13, no. 3, pp. 2086–2092, 2019.

[172] J. M. Castellano et al., "Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition," Endocrinology, vol. 146, no. 9, pp. 3917–3925, 2005.

[173] R. O. Ibrahim, S. H. Omer, and C. N. Fattah, "The Correlation between Hormonal Disturbance in PCOS Women and Serum Level of Kisspeptin," vol. 2020, 2020.

[174] H. S. Mutasher and E. A. K. Jabbar, "Assessment of kisspeptin (Metastin) hormone and genetic aspect of polycystic ovary syndrome women in thi qar province, Iraq," Int. J. Pharm. Res., vol. 12, pp. 1423–1428, 2020.

[175] M. Cohen et al., "A fully automated high-throughput plasmid purification workstation for the generation of mammalian cell expression-quality DNA," SLAS Technol., vol. 3, no. 1, pp. 20–23, 2022.

[176] P. Guha, A. Das, S. Dutta, and T. K. Chaudhuri, "A rapid and efficient DNA extraction protocol from fresh and frozen human blood samples," J. Clin. Lab. Anal., vol. 32, no. 1, p. e22181, 2018.

[177] H. Khosravinia1, H. N. N. Murthy, D. T. Parasad, and N. Pirany, "Optimizing factors influencing DNA extraction from fresh whole avian

blood." African Journal of Biotechnology, p. vol 6 (4) pp.481-486, 2007.

[178] Q. Fan et al., "Functional polymorphism in the 5′-UTR of CR2 is associated with susceptibility to nasopharyngeal carcinoma," Oncology Reports, vol. 30, no. 1. pp. 11–16, 2013.

[179] M. H. Daghestani et al., "Adverse Effects of Selected Markers on the Metabolic and Endocrine Profiles of Obese Women With and Without PCOS," Front. Endocrinol. (Lausanne)., vol. 12, 2021.

[180] H. Ozawa, "Kisspeptin neurons as an integration center of reproductive regulation: Observation of reproductive function based on a new concept of reproductive regulatory nervous system," Reproductive Medicine and Biology. 2021.

[181] K. Daghestani, M. H.Daghestani Mazin H. Daghistani, Mamoon.Ambreen, "Relevance of KISS1 gene polymorphisms in susceptibility to Polycystic Ovary Syndrome and its associated Endocrine and Metabolic disturbances." Taylor & Francis & British Journal of Biomedical Science Journal:, 2020.

1. Research committee decision

جمهورية العـــراق

وزارة الصحة والبيئة
دائرة صحة محافظة بابـــل
المديـــر العـــام
مركـــز التدريب والتنمية البشرية
لجنة البحوث

Ministry Of Health
Babylon Health Directorate
Email:-
Babel_Healthmoh@yahoo.com
Tel:282628 or 282621

استمارة رقم :- ٢٠٢١/٠٣

رقم القرار :- ٧٥
تاريخ القرار :- ٢٠٢١/٢/٥

قرار لجنة البحوث

تحية طيبة ...

درست لجنة البحوث في دائرة صحة بابل مشروع البحث ذي الـرقم (٢٠٢١/٠٦٠/بابل) المعنون (التاثيرات الكيميائية الحيوية واجزيئية للكيسبيتين فيما يتعلق بالهرمونات المناعية للغدد التناسلية في الاناث المصابات بمتلازمة تكيس المبايض) والمقدم من الباحثة (نور جمال طالب) إلى وحدة أدارة البحوث والمعرفي مركز التدريب والتنمية البشرية في دائرة صحة بابل بتاريخ ٢٠٢١/١٢/١ وقررت :

قبول مشروع البحث أعلاه كونه مستوفيا للمعايير المعتمدة في وزارة الصحة والخاصة بتنفيذ البحوث ولا مانع من تنفيذه في مؤسسات الدائرة .

مـــــع الاحتـــرام

وزارة الصحة
دائرة صحة بابل
مركز التدريب والتنمية البشرية

الدكتور / محمد عبد الله عجرش
رئيس لجنة البحوث
٢٠٢١ / / ٢

نسخة منه إلى :
- مكتب المدير العام / مركز التدريب والتنمية البشرية / وحدة إدارة البحوث ... مع الأوليات.

سونران

دائرة صحة محافظة بابل / مركز التدريب والتنمية البشرية // ايميل المركز babiltrainning@gmail.com

2. Questionnaire

اسم المراجعة : العمر :

التاريخ و اليوم : رقم العينة :

السكن الحالي : نوع العينة : سمينة , ضعيفة

رقم الهاتف : تاريخ بدأ التكيس :

1	الحالة الاجتماعية	متزوجة // غير متزوجة
2	عدد الاطفال	لا يوجد // عدد
3	عدد مرات الاجهاض	عدد ( ) // لا يوجد
4	الدورة الشهرية	منتظمة // متقطعة كل ( ) اشهر // انقطاع تام للدورة
5	سحب الدم من المريضة	اثناء الدورة // بعد الدورة
6	تاريخ اخر دورة	
7	وهل يوجد عمليات للتنحيف	قص معدة او نظام تنحيف (كيتو او غيرها)
8	هل يوجد تاريخ عائلي بوجود التكيس	
9	هل يتم اخذ ادوية هرمونيه او موانع الحمل او اللولب الهرموني	
10	هل يوجد تاريخ عائلي للسمنة	
11	هل المريضة مصابة بالسكري	
12	الطول	
13	الوزن	
14	محيط الخصر	
15	محيط الورك	
	شعر خشن( )	حبوب ( ) تصبغات بالجلد( )
16	هل توجد اضطرابات الغدة الدرقية	
17	التحليل السابقة	Testosterone LH FSH PROLACTIN DHEA TSH

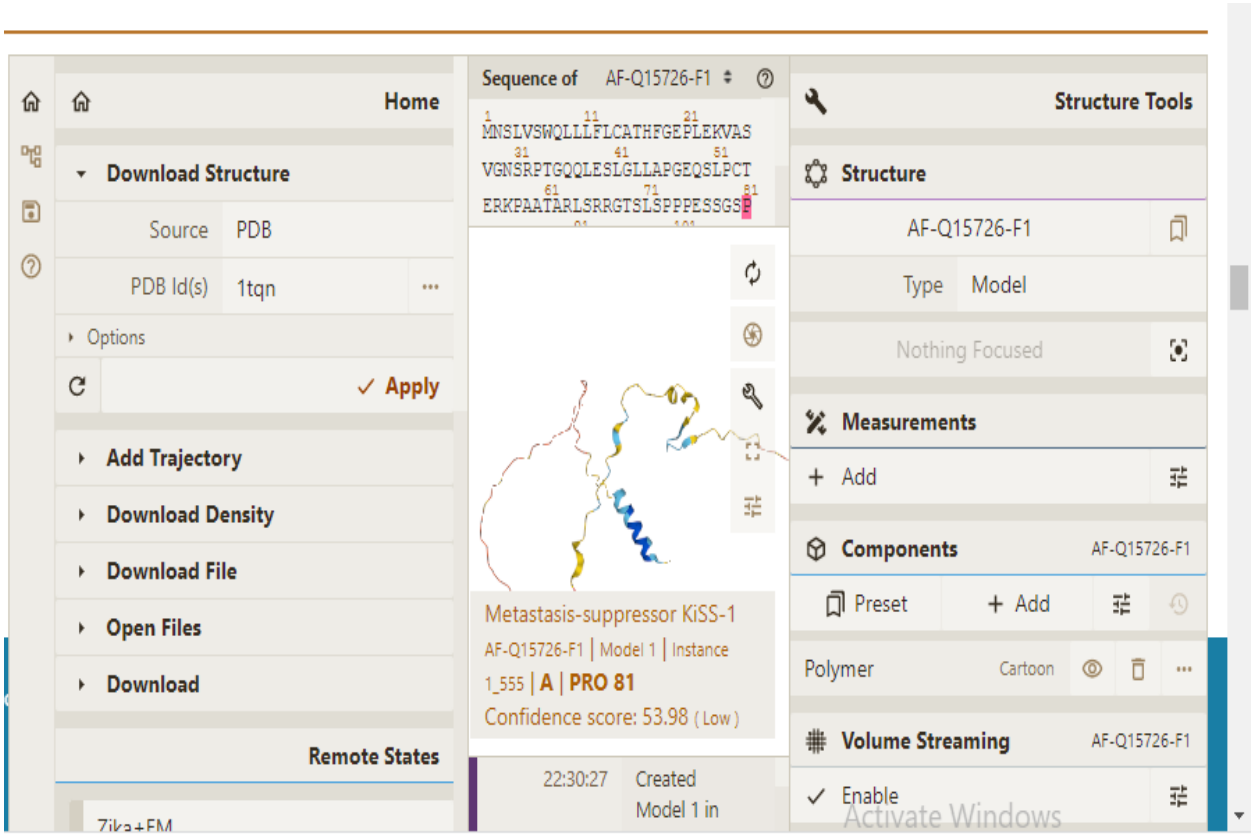
3. Web site medcalc

Odd Ratio calculated by on line calculate is:

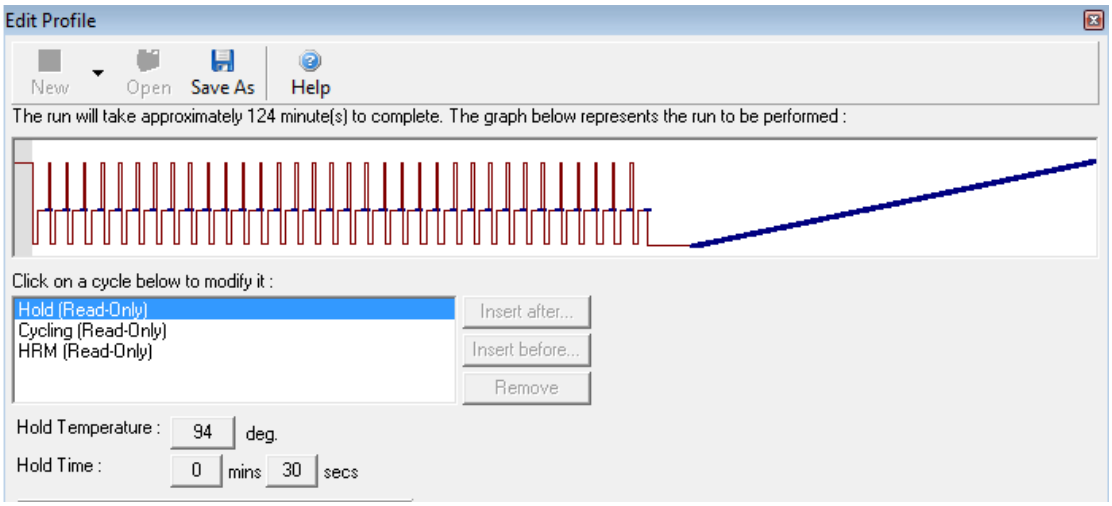
https://www.medcalc.org/calc/odds_ratio.php

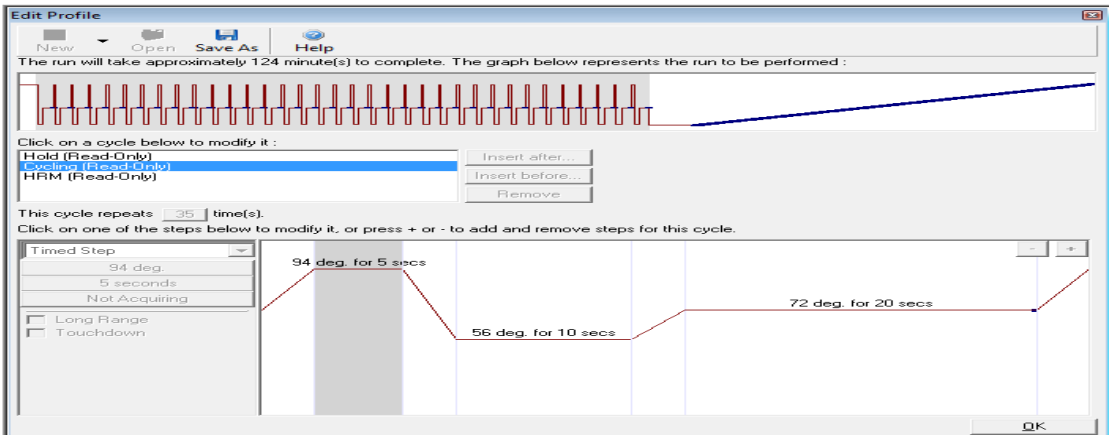
4. Web site uniprot

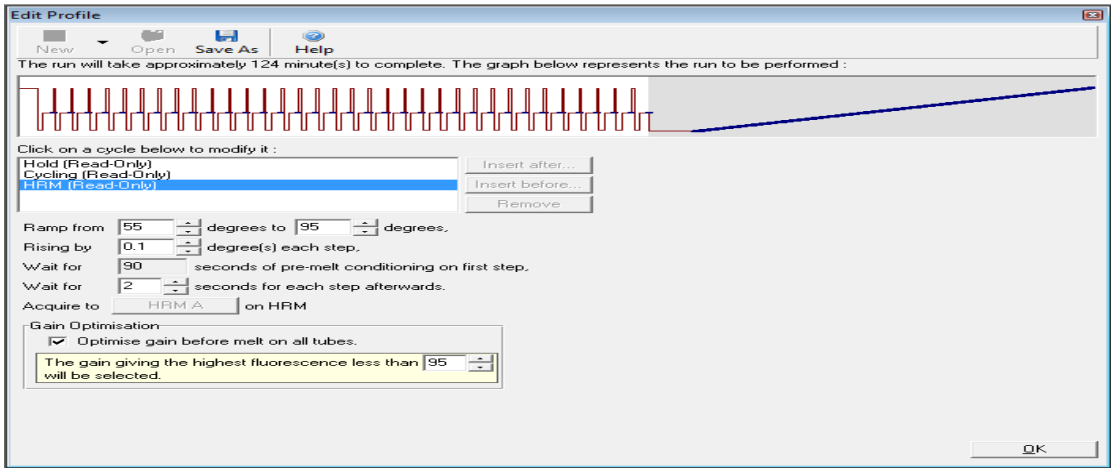
Kisspeptin structure



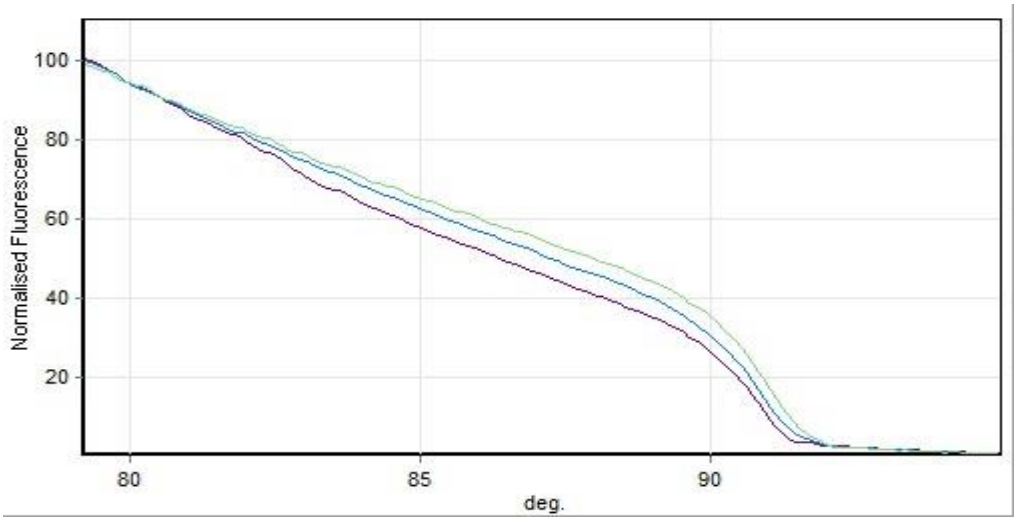
5. Thermo profile for SNP rs37279054 HRM







6. Genotyping step for SNP rs37279054 HRM



	Wild	GG
	Hetero	GA
	Mutant	AA

www.qiagen.com

HRM Report

Experiment Information

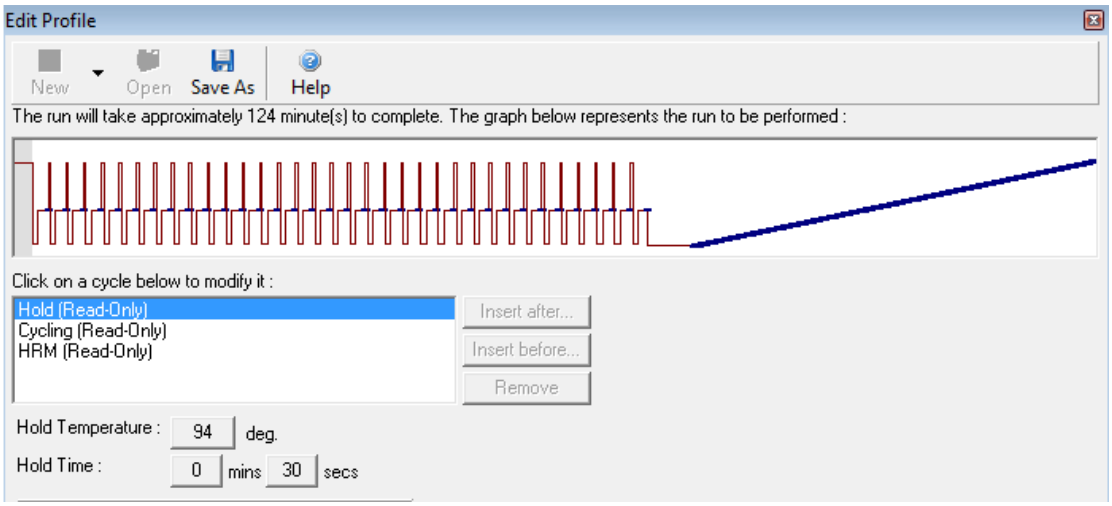
Run Name	Noor Hala SNP rs54-1
Run Start	31/01/2022 11:49:58 ص
Run Finish	31/01/2022 02:00:59 م
Operator	Noor hala
Notes	
Run On Software Version	Rotor-Gene 2.1.0.9
Run Signature	The Run Signature is valid.
Gain Green	5.33
Gain HRM	1.

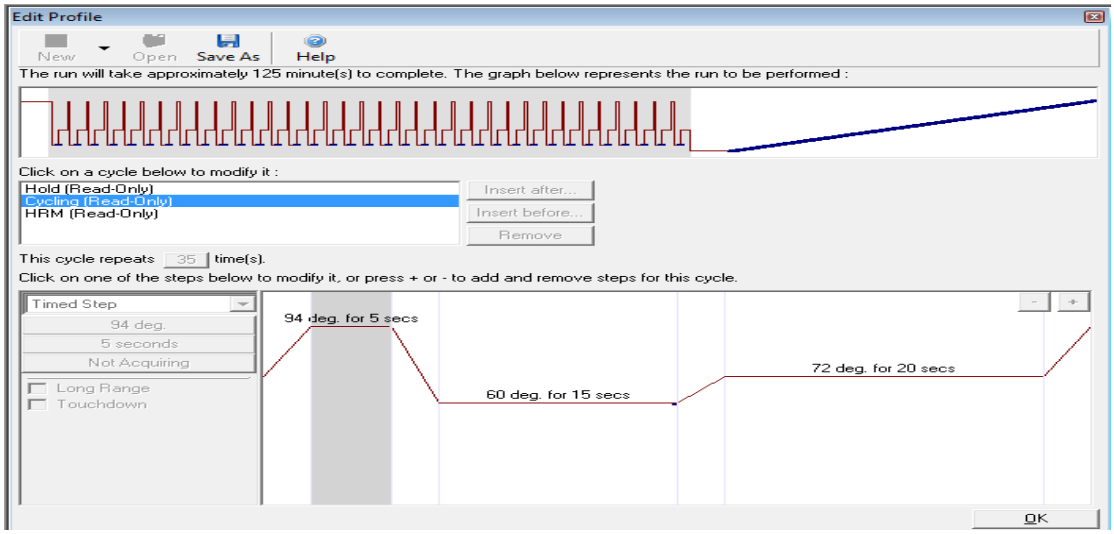
HRM Analysis Information

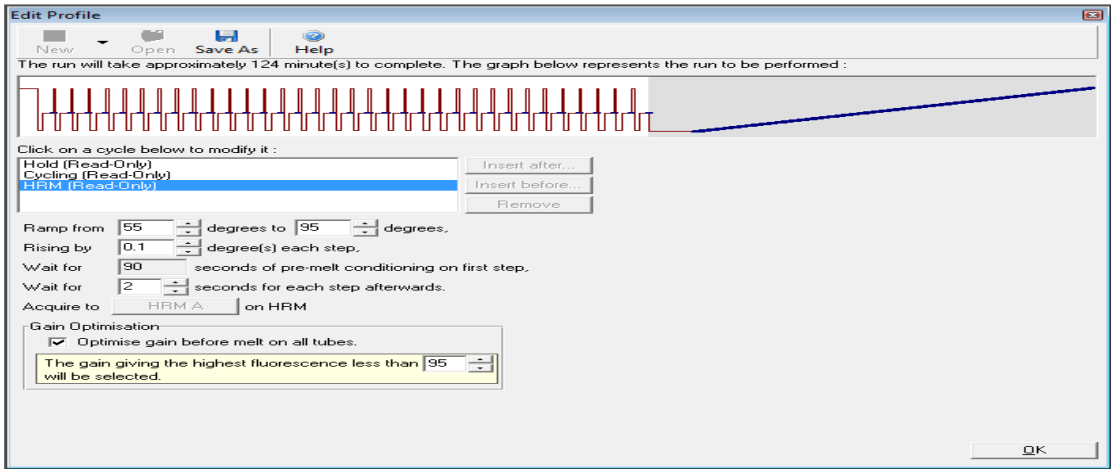
Confidence Threshold	% 90
Normalisation Region 1	71.42-72.97
Normalisation Region 2	91.47-92.47
Sample Page	Page 1

No.	Colour	Name	Genotype	Confidence %
3		6	Mutant AA	72.87
4		7	Wild GG	77.33
5		10	Hetero GA	84.22
6		11	Wild GG	82.33
7		12	Hetero GA	60.29
8		15	Mutant AA	100.00
9		16	Wild GG	77.33
10		17	Hetero GA	100.00
11		18	Hetero GA	47.86
12		20	Wild GG	77.33
13		49	Wild GG	100.00
14		50	Hetero GA	53.85
15		51	Hetero GA	62.42
16		52	Wild GG	72.70
17		54	Wild GG	77.01
18		55	Hetero GA	33.04
19		56	Wild GG	77.33
20		58	Hetero GA	94.74
21		59	Wild GG	33.49
22		83	Hetero GA	42.48
23		87	Wild GG	75.30
24		110	Wild GG	77.33
25		101	Wild GG	76.86
26		8	Mutant AA	58.62
27		105	Hetero GA	65.06
28		111	Wild GG	77.33
29		112	Wild GG	77.33
30		113	Hetero GA	62.55
31		114	Wild GG	38.58
32		118	Wild GG	92.22
33		120	Hetero GA	41.69

7. Thermo profile for SNP rs4889 HRM







8. Genotyping step for SNP rs4889HRM

www.qiagen.com

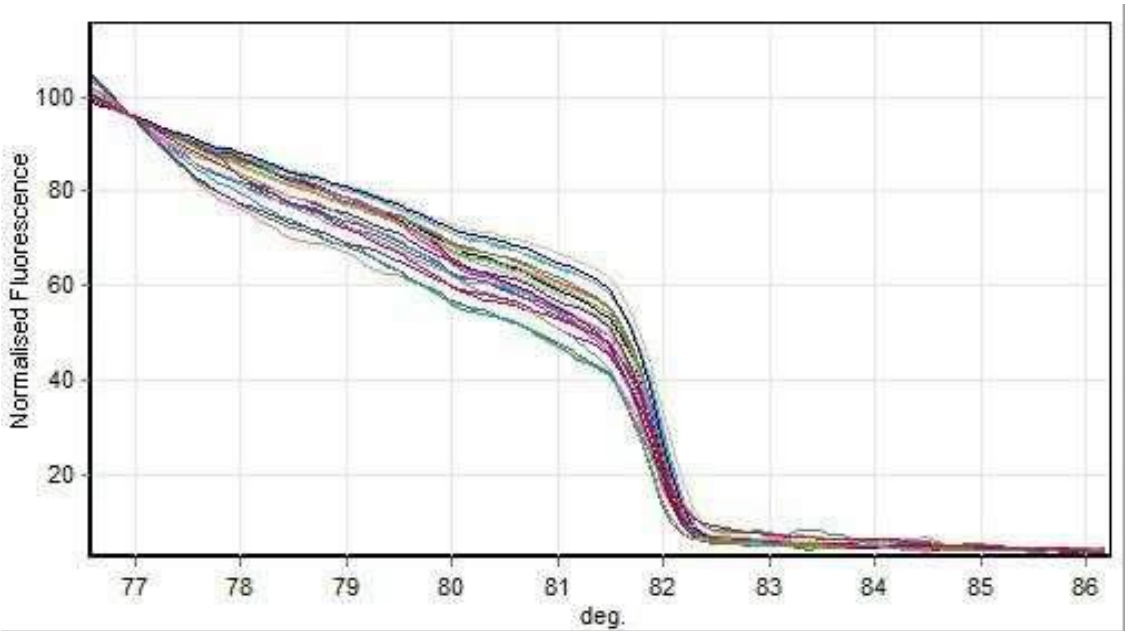
HRM Report

Experiment Information

Run Name	Noor Hala SNP rs89-3
Run Start	02/02/2022 11:50:52 ص
Run Finish	02/02/2022 01:56:06 م
Operator	
Notes	
Run On Software Version	Rotor-Gene 2.1.0.9
Run Signature	The Run Signature is valid.
Gain Green	5.33
Gain HRM	1.67

HRM Analysis Information

Confidence Threshold	%
Normalisation Region 1	79.93-80.74
Normalisation Region 2	93.27-94.08
Sample Page	Page 1



No.	Colour	Name	Genotype	Confidence %
3		6	Hetero GA	89.32
4		7	Mutant AA	84.97
5		10	Hetero GA	97.64
6		11	Hetero GA	90.48
7		12	Hetero GA	87.72
8		15	Mutant AA	90.71
9		16	Hetero GA	89.64
10		17	Hetero GA	94.58
11		18	Mutant AA	90.43
12		20	Hetero GA	94.69
13		49	Wild GG	95.07
14		50	Hetero GA	89.42
15		51	Hetero GA	96.77
16		52	Hetero GA	92.63
17		54	Hetero GA	89.55
18		55	Wild GG	91.44
19		56	Wild GG	92.33
20		58	Hetero GA	100.00
21		59	Hetero GA	95.05
22		83	Wild GG	91.09
23		87	Mutant AA	100.00
24		110	Wild GG	94.74
25		101	Hetero GA	94.76
26		8	Hetero GA	97.08
27		105	Hetero GA	95.69
28		111	Wild GG	93.54
29		112	Wild GG	92.27
30		113	Wild GG	92.75
31		114	Wild GG	100.00
32		118	Wild GG	96.69
33		120	Wild GG	93.68

9. Certificate for PCR


وزارة التعليم العالي والبحث العلمي

جامعة البصرة / كلية العلوم / قسم التحليلات المرضية

شـــهادة مشـــاركة

نـوّيد مشـــاركة

نور جمال طالب

في الورشة العلمية الافتراضية وتحت عنوان

Critical Parameters in qRT- PCR

يوم الاربعاء الموافق 06/10/2021 في كلية العلوم/ قسم التحليلات المرضية في جامعة البصرة وعليه زود بهذه الشهادة ... مع التقدير

عميد كلية العلوم

أ.م.د. موفق فاضل جبر الشهوان

رئيس قسم التحليلات المرضية

أ.د. عواطف حميد عيسى المحيسن