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Role of Neudesin and Neuregulin-4 in the Biochemical Mechanism of Insulin Resistance Amongst Polycystic Ovary Syndrome Phenotypes

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala, in Partial Fulfillment of the Requirements for the Master Degree in Clinical Chemistry

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

﴿وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا﴾

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Supervisor Certification

we certify that this M.SC. thesis entitled:

(Role of Neudesin and Neuregulin-4 in the biochemical mechanism of insulin resistance amongst polycystic ovary syndrome phenotypes)

was prepared under my supervision at the College of Medicine/University of Kerbala as a Partial Fulfilment of the requirements for the degree of (Master) in clinical chemistry.

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Examining Committee Certification

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(Role of Neudesin and Neuregulin-4 in the Biochemical Mechanism of Insulin Resistance Amongst Polycystic Ovary Syndrome Phenotypes)

We have examined the postgraduate student (**Afnan Hayder Abbood**) in it M.Sc. thesis content and in our opinion; it meets the standard for the degree of master in (**Clinical Chemistry**)

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Dedication

To,

Whose love, encouragement, and prayers of day and night make me able to get such success, my family

Afnan

2023

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Summary

Polycystic ovarian syndrome is a syndrome of ovarian dysfunction that is characterized by the presence of cardinal features of hyperandrogenism and polycystic ovary morphology. Clinical or biochemical hyperandrogenism (HA), ovulatory dysfunction (oligo/amenorrhea) (OD), and/or polycystic ovarian morphology (PCOM) are the three criteria that have been used to characterize PCOS since the creation of the Rotterdam Consensus in 2003, PCOS presence of at least two out of three criteria. This definition results in several PCOS phenotypes, such as phenotype A (HA, OD, PCOM), phenotype B (HA, OD), phenotype C (HA, PCOM), and phenotype D (OD, PCOM).

Neudesin(NENF) and Neuregulin 4(NRG4) are adipokines members that synthesize adipose tissues, based on metabolic and cardiovascular dysfunctions, assessing abdominal obesity in patients with PCOS may be critical. Research on the NENF and NRG4 plays roles in metabolic regulation, which may be considered potential insulin resistance markers in some medical conditions, such as diabetes mellitus, obesity, and PCOS, which has recently gained interest. In spite of that the NENF, NRG4 with PCOS phenotype relationship is poorly represented in the literature. Therefore, the aim of this study would bring to light the role of Neudesin and neuregulin_4 as a biomarker for insulin resistance in females affected by different phenotypes of PCOS.

Methods: This study was designed as a case-control study, with a total of 210 participants including 140 female cases effect by different phenotypes of PCOS. Patient samples were collected from the reproductive fertility consultant of the Teaching Hospital of Obstetrics and Gynecology, Kerbala health directorate/ Iraq. serum hormonal levels and insulin concentration were determined by the electrochemiluminescence immunoassay “ECLIA” system (Cobas e 411, Roche Diagnostic, Germany). Fasting blood glucose was

determined by a clinical chemistry analyzer (Monarch 240, Biorex Diagnostic, United Kingdom). Serum lipid panel ((total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG)) were measured using fully automatic chemistry analyzer (SMART-120, Geno TEK, United States of America). Serum-free testosterone hormone was measured using a fully -auto chemiluminescence immunoassay analyzer ((MAGLUMI 600, Snibe Diagnostic, Germany). Elisa system was used for the detection of Neudesin and Neuregulin-4 protein level. Anthropometric Measurements (Visceral Adiposity Index (VAI), lipid accumulation product (LAP) and The Body Adiposity Index (BAI)) , Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) and Basal Metabolic Rate (BMR) values measurements were also evaluated.

As a result, classic PCOS (phenotype A) showed a high prevalence (49.28%). In phenotype A, NRG4 level was shown a significant increase compared to other phenotypes, while NENF level was highly significant in phenotype C, and it was twice risk factor as in phenotype B.

Results of the receiver operating curve (ROC) were shown that NRG4, has the best performance for predicting PCOS patients specially phenotypes A. BAI has a good performance for prediction group B. While LAP and BAI were the best predictions for phenotypes group C. NRG4 and NENF have a good performance for prediction group D.

In conclusion, the levels of NRG4 and NENF significantly increased in all PCOS phenotypes, the metabolic indices have a significant correlation with all phenotypes. Abdominal visceral obesity played a significant role in the development of metabolic changes, irrespective of the PCOS phenotypes.

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List of Abbreviations

Abbreviations	Full Nomenclature
AE	Androgen Excess
ASRM	American Society for Reproductive Medicine
AUC	Area Under Curve
BAI	Body Adiposity Index
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BMR	Basal Metabolic Rate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptors
ErbB	Epidermal Growth Factor Receptors
ESHRE	European Society for Human Reproduction and Embryology
FBG	Fasting Blood Glucose
FSH	Follicle-Stimulating Hormone
FT	Free Testosterone
GnRH	Gonadotropin-Releasing Hormone
HA	Hyperandrogenism
HDL	High-Density Lipoprotein
HI	Hyperinsulinemia
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
hs-CRP	High-Sensitivity C-Reactive Protein
IGF	Insulin-Like Growth Factor
IR	Insulin Resistance
LAP	Lipid Accumulation Product
LDL	Low-Density Lipoprotein
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone-Releasing Hormone
MAPKS	Mitogen-Activated Protein Kinase
MS	Metabolic Syndrome
NENF	Neudesin (Neuron-Derived Neurotrophic Factor)
NAFLD	Non-Alcoholic Fatty Liver Disease
NIH	National Institutes of Health
NRG4	Neuregulin 4
NRG	Neuregulin
OD	Ovarian Dysfunction
PCOM	Polycystic Ovarian Morphology
PCOS	Polycystic Ovarian Syndrome
PGRMC1	Progesterone Receptor-Membrane Component 1

PI3K	Phosphoinositide 3-kinase Signaling
ROC	Receiver Operating Curve
SHBG	Sex Hormone-Binding Globulin
SNS	Sympathetic Nervous System
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TSH	Thyroid Stimulating Hormone
VAI	Visceral Adiposity Index
WAT	White Adipose Tissue
WC	Waist Circumference
WHR	Waist Hip Ratio
WHtR	Waist to Height Ratio

List of Normal laboratory Value	
Parameters	Reference value
Luteinizing Hormone(LH)	1.5-8 m.lU/mL follicule phase
Follicular Stimulating Hormone(FSH)	2.9-12 m.lU/mL follicule phase
Prolactin	5-35 ng/mL follicule phase
Thyroid Stimulating Hormone (TSH)	0.27-4.2 ulU/mL
Progesterone	1.5-20 ng/mL luteal phase
Free Testosterone (FT)	<4.2 pg/ mL follicule phase
Total Cholesterol	0-200 mg/dl
Triglycerides	0-200 mg/dl
Low -Density Lipoprotein Cholesterol (LDL-C)	0-160 mg/dl
High -Density Lipoprotein Cholesterol (HDL-C)	40-80 mg/dl

Chapter one

Introduction

and

Literature Review

1. Introduction

The most common endocrine condition in females of reproductive age is polycystic ovarian syndrome (PCOS) with a global prevalence of 4 to 20% depending on the diagnostic criteria used to define the syndrome (**Deswal *et al*.,2020)**. The term "polycystic ovarian syndrome" does not entirely or accurately reflect the disorder's nature owing to its very wide range of clinical manifestations and related morbidities. Stein and Leventhal were the first to describe PCOS in 1935 as a gynecological disorder when seven females presented with menstrual disturbances, hirsutism (a condition of male pattern terminal hair growth in women), and bilateral polycystic ovaries **(Stein and Leventhal,1935)**.

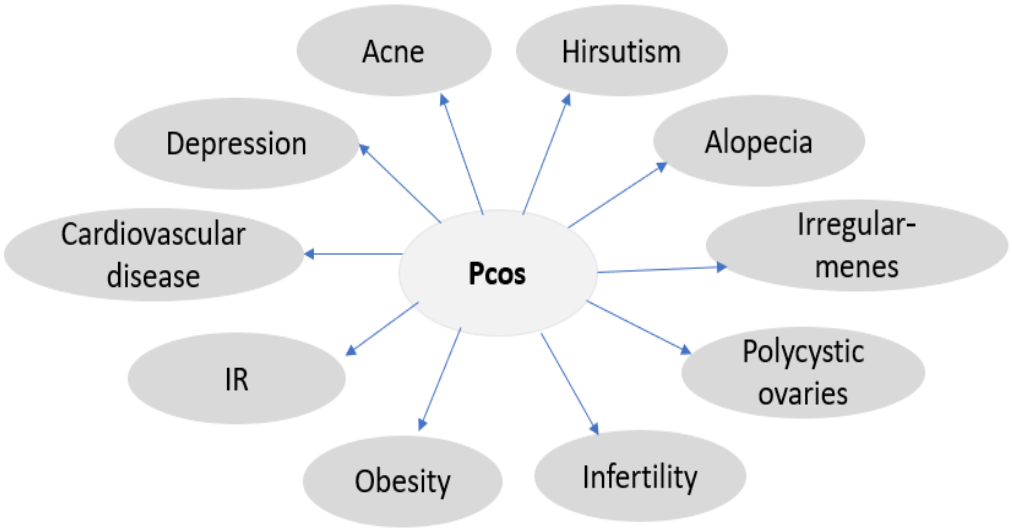
Polycystic ovary syndrome was formerly known as Stein-Leventhal syndrome, after the first two doctors to make the connection between polycystic ovaries and infertility. However, as more research was conducted and the disorder pathophysiology became clearer, the name of the disorder was changed to polycystic ovary syndrome **(Vaclav and Lunenfeld ,1990)**.

1.1. Clinical Manifestations of Polycystic Ovarian Syndrome

A heterogeneous condition marked by chronic ovulatory dysfunction and elevated androgen levels. PCOS females are also characterized by elevated serum levels of luteinizing hormone (LH), also have an abnormal ratio of luteinizing hormone (LH) to follicle-stimulating hormone (FSH) in their blood, which causes hyperandrogenism. **(Iervolino *et al*.,2021).**

Clinical manifestations include menstrual irregularities and indications of an excess of androgen**.** (e.g. hirsutism, acne, and alopecia) **(Teede *et al* .,2018a)** as shown in Figure (1.1) . Insulin resistance and hyperinsulinemia have the ability to develop endocrine and reproductive features of PCOS , nevertheless, hyperandrogenemia may affect insulin action directly and / or through many

changes that take place in different body sites especially muscle and adipose tissue **(Moghetti and Tosi, 2020)**. Long-term metabolic syndrome development, including type II diabetes mellitus(T2DM) and cardiovascular disease, is most common in females with polycystic ovarian syndrome. **(Dadachanji , Shaikh and Mukherjee,2018).**



Figure(1.1) : Clinical Manifestations(by auther)

IR= insulin resistance; PCOS= polycystic ovarian syndrome.

1.2. Etiopathogenesis of Polycystic Ovarian Syndrome

It has been reported that the etiopathogenesis of PCOS is a complex of genetic, environmental, and lifestyle interactions. The common features of PCOS are hyperandrogenism and insulin resistance (IR) **(Moghetti *et al*.,2020).** Elevated serum LH levels, biochemical evidence of hyperandrogenism, and increased insulin resistance are also frequent symptoms of this disorder**(Glueck and Goldenberg , 2019).**

The definition of this disorder as a syndrome is not a disease due to the presence of a cluster of symptoms that cannot be identified by a certain etiological

factor or as a certificated pathophysiological axis. In addition, it is believed that hormones act on most of the body tissues but at a variant rate which results in the variation of clinical features due to hormonal disturbances and metabolic consequences. **(Krug , Giles and Paganini ,2019).**

The pathophysiology of PCOS is unknown, but substantial evidence suggests that it is a multifactorial disorder in which interactions between endocrine, metabolic, genetic, and environmental factors intrinsic to each other operate in concert to produce a common outcome **Yau *et al*(2017)** as shown in figure (1.2) .

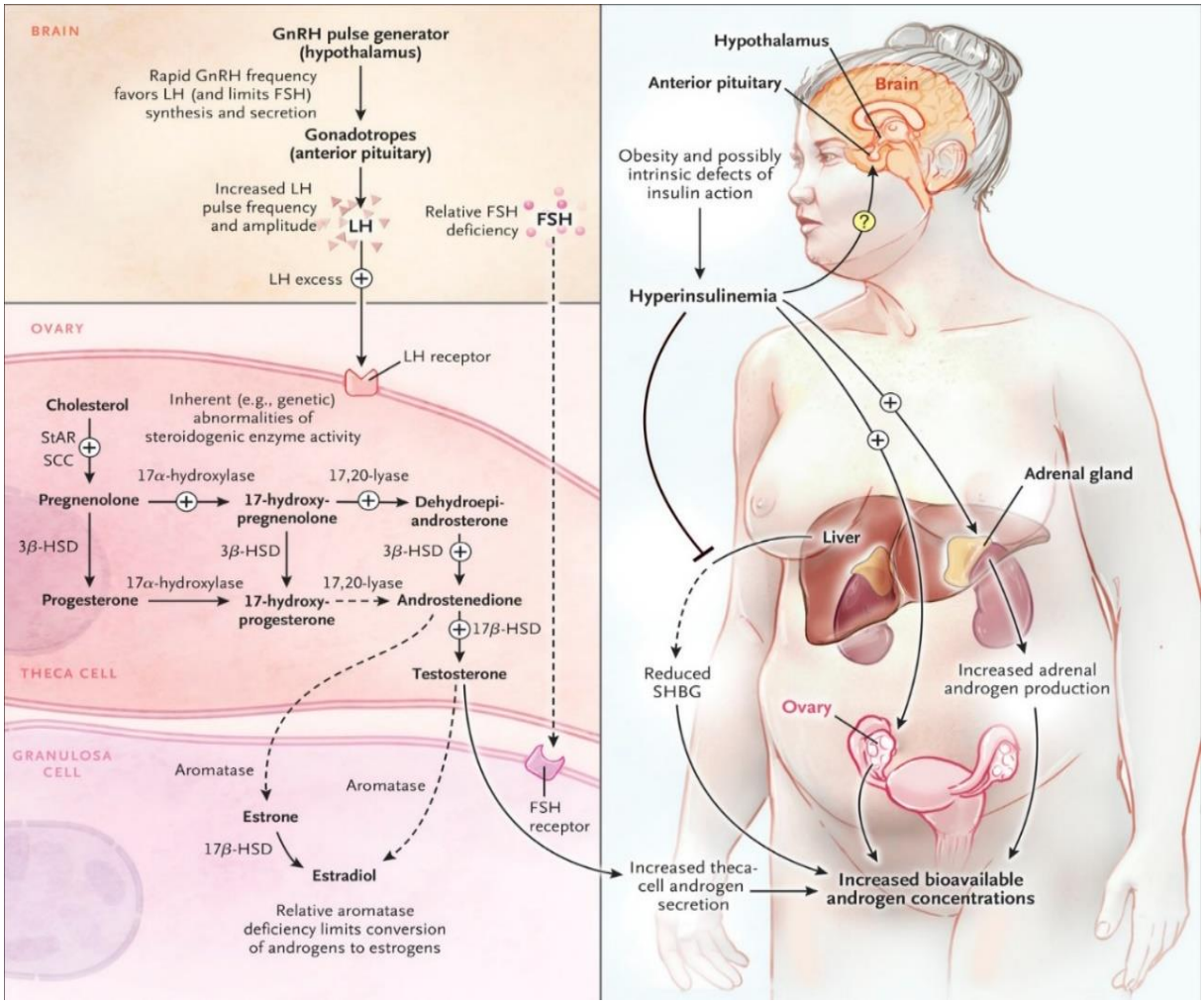


Figure (1.2): Pathophysiology of Polycystic Ovarian Syndrome.

3β-HSD=3-beta-hydroxysteroid dehydrogenase;17 β-HSD=17 beta-hydroxysteroid dehydrogenase ;FSH=follicle-stimulating hormone ; GnRH=gonadotropin-releasing hormone ;LH=luteinizing hormone ;SHBG=sex hormone-binding globulin; StAR =Steroidogenic acute regulatory; SCC=cholesterol side-chain cleavage. (Yau et al.,2017).

1.3. Prevalence of Polycystic Ovarian Syndrome

According to a study conducted in the Middle East and North Africa (MENA) region, the age-standardized point prevalence and incidence rate for PCOS were 37.9% (95% UI: 31.7 to 45.0) and 33.7% (95% UI: 27.7 to 40.3), respectively. In Iraq, the rate was 13.4%. **(Motlagh Asghari *et al*.,2022)**.

The woman with PCOS is the leading cause of infertility in Iraqi Arab females, accounting for approximately 46% of all infertility cases **(Saeed , AL-Mohaidi and Ismail, 2021)**.

The prevalence of PCOS appears to be on the rise as a result of modern lifestyle, diet, and obesity **Fattah *et al*(2021)**. The prevalence of PCOS among Chinese females of reproductive age reached 7.8% in 2020, a rise of approximately 65% over the previous decade **(Yang *et al*.,2022).** The prevalence of PCOS ranges from 5% to 20% worldwide **(Ntumy *et al*., 2019).**

Different prevalence estimates of PCOS were identified by various studies when three sets of diagnostic criteria known as National Institutes of Health (NIH), Rotterdam, and Androgen Excess and PCOS Society (AE-PCOS criteria) are applied. The highest prevalence is seen when the feature of polycystic ovaries is included in the diagnosis. The worldwide prevalence of PCOS across most of these studies has been relatively uniform between 5 and 10% defined by NIH criteria, 5 to 20% by Rotterdam criteria, and the prevalence ranges from 10-15% by AE-PCOS criteria **(Bozdag *et al*.,2016).**

The prevalence was reported to be higher than other Asian communities at 28.9% by the NIH criteria, 35.3% by the Rotterdam criteria, and 34.3% by the AE-PCOS criteria. These variations among the studies using the same diagnostic criteria across countries might be due to populations of different ethnicity/races, geographical factors, and differences in the characteristics of the study population **(Ganie *et al*.,2020).**

1.4. Hypothalamic Pituitary Ovarian Axis

In the hypothalamus, a deca-peptide called gonadotrophin-releasing hormone (GnRH) releases hormones in pulsation into the hypothalamic-hypophysial portal system **(Umayal *et al*., 2018).**

GnRH stimulates the release of gonadotrophins, LH, and FSH, from the anterior pituitary gland, the LH and FSH are dimeric glycopeptides secreted in pulses. This pulsatile release is necessary for the gonadotrophins to exert their physiological actions; FSH and LH bind to ovarian receptors, producing estrogen and progesterone. LH in the ovary that adheres to LH receptors on the theca-cells, which is essential for sexual development and reproduction **(Shaaban *et al*.,2019).**

The LH stimulates theca cells to convert available cholesterol into androgen, this androgen is then carried to the granulosa cells where FSH glycoprotein hormone binds to a receptor of FSH that stimulate the transformation of androgen into estradiol-β-17 using (aromatase) enzyme, normally folliculogenesis depends on the transformation of intra-ovarian androgens for estradiol synthesis. The effects of GnRH on LH and FSH release, in terms of the amounts secreted at different stages of the menstrual cycle, are strongly influenced by negative feedback control effects exerted by estradiol and progesterone (**Messinis , Messini and Dafopoulos ,2014)**as shown in figure (1.3) During a normal menstrual cycle,

females experience fluctuating levels of sex steroid hormones. Progesterone acts principally 7–10 days after ovulation, resulting in extensive physiological effects **(Arazi , Nasiri and Eghbali ,2019).**

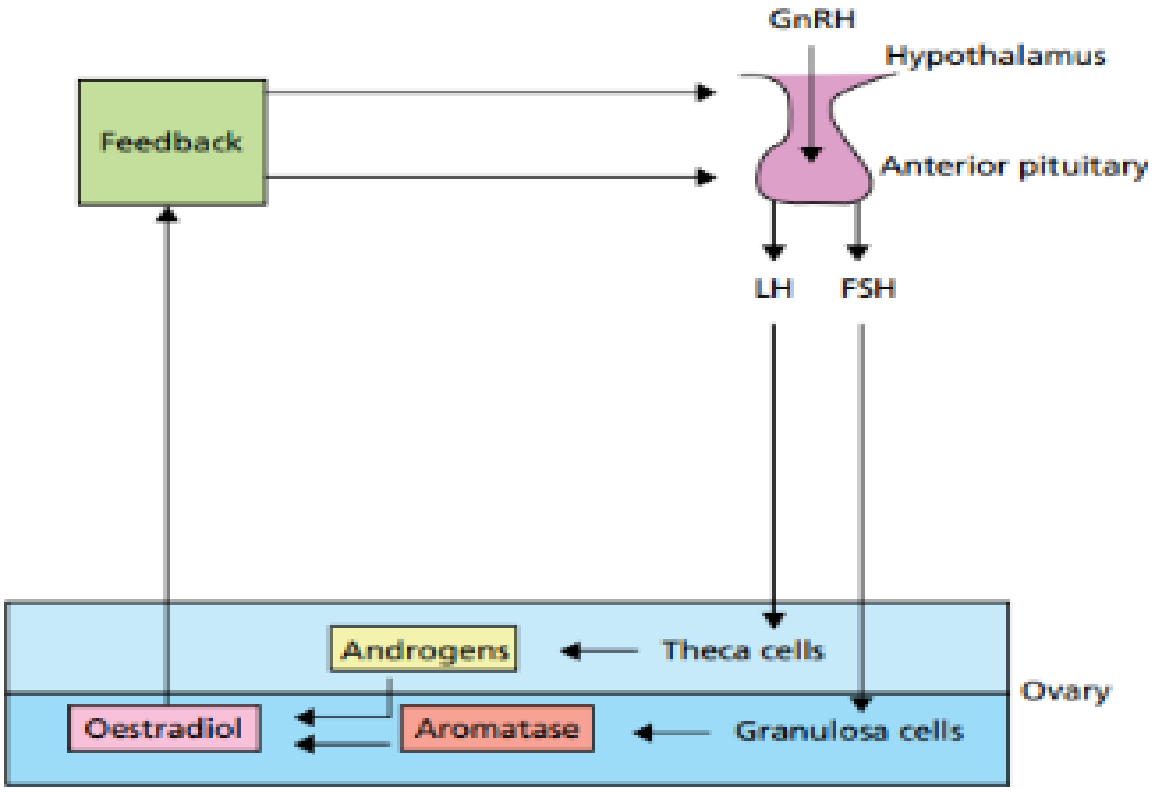


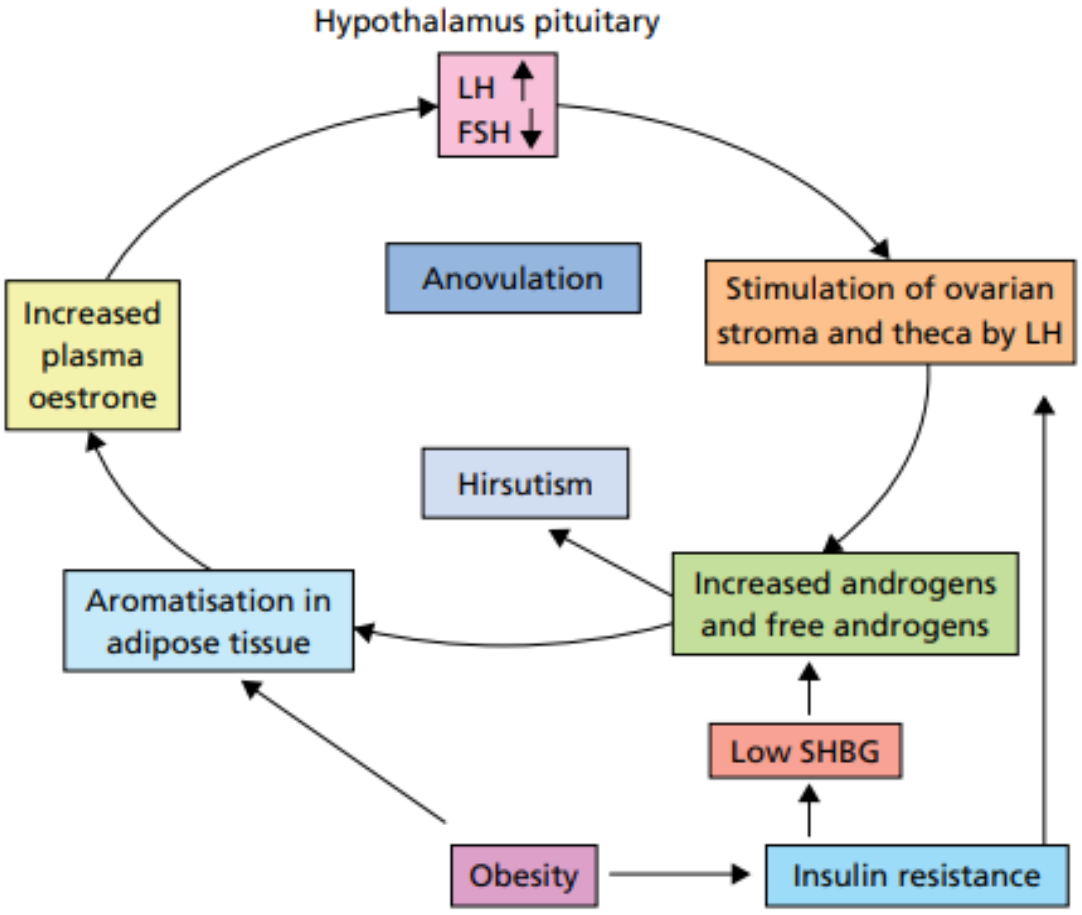
Figure (1.3): The Hypothalamic–Pituitary–Ovarian Axis (Rae, Crane and Pattenden, 2018).

GnRH=gonadotropin-releasing hormone; FSH=follicle-stimulating hormone; LH=luteinizing hormone.

The high prevalence of PCOS suggests that it may not be a single disease. Impaired conversion of androgens to estrogens in the ovary also leads to increased release of ovarian androgens. These androgens are then converted in adipose tissue by aromatase to oestrone, which inhibits FSH release and stimulates LH

secretion, so PCOS is characterized by elevations in LH and the LH/FSH ratio in many females. **(Ashraf *et al* .,2019).**

Due to increased LH stimulation, numerous follicles in the theca cells of ovaries become arrested in the preantral and antral stages, resulting in hyperplasia of theca cells and subsequent accumulation of follicular fluid forming cyst-like structures along the ovary's periphery, giving it a string-of-pearls appearance. Due to an increase in the number of follicles and the expression of key enzymes involved in androgen synthesis, an excessive quantity of androgens is produced **(Chaudhary *et al*.,2021)** as shown in figure (1.4)



Figure(1.4) : Biochemical, Metabolic, and Endocrine Changes in Polycystic Ovarian Syndrome (Rae, Crane and Pattenden, 2018).

1.5. Diagnostic Criteria of Polycystic Ovarian Syndrome

There are three sets of diagnostic criteria for PCOS that have been recommended for use in identifying premenopausal women with the syndrome in table (1-1)

Table(1-1) :The Common Diagnostic Criteria for PCOS

Parameter	NIH 1990	ESHRE/ASRM 2003	AE-PCOS 2006	NIH 2012 extension of ESHRE/ASRM 2003
Criteria	➢ HA ➢ OD	➢ HA ➢ OD ➢ PCOM	➢ HA ➢ 2. OD and/or PCOM	➢ HA ➢ OD ➢ PCOM
Limitations	2 of 2 Criteria required	2 of 3 Criteria required	2 of 2 Criteria required	2 of 3 Criteria required; and identification of specific phenotypes: A: HA + OD + PCOM B: HA + OD C: HA + PCOM D: OD + PCOM
Exclusion of related etiologies				

NIH= National Institutes of Health; ESHRE/ASRM= European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM); AE-PCOS= Androgen Excess and PCOS Society; HA= Hyperandrogenism; OD=ovarian dysfunction; PCOM= polycystic ovarian morphology (Lizneva *et al*., 2016a).

The National Institute of Child Health and Human Development of the US National Institutes of Health (NIH) conference first attempted to classify PCOS in April 1990. The NIH criteria require both clinical hyperandrogenism and/or biochemical hyperandrogenemia and chronic oligo-/anovulation to be present **(Hong *et al*.,2023).**

The second definition was based on the consensus, which met in Rotterdam, the Netherlands in 2003. The conference was partially sponsored by the European

Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), as a result of this meeting, ultrasound characteristics for polycystic ovarian morphology were added to the NIH 1990 definition, making it more complex. According to the ESHRE/ASRM2003, three characteristics are proposed; hyperandrogenism, ovarian dysfunction, and polycystic ovarian morphology (the presence of ≥20 follicles or any ovarian volume >10 ml) **(Chen and Pang, 2021).**

To be diagnosed with PCOS, a woman must exhibit two of the following three criteria, adrenal hyperplasia and hyperprolactinemia are two more possible causes of hyperandrogenism that need to be ruled out. **(Eshre and Group, 2004).**

In 2006, the Androgen Excess and PCOS Society (AE-PCOS) criteria were introduced. According to AE-PCOS criteria, hyperandrogenism is the primary cause of polycystic ovary syndrome ,therefore, a diagnosis needs to account for the presence of hyperandrogenism plus ovarian dysfunction and/or polycystic ovaries, reducing the number of phenotypic possibilities **(Azziz *et al* ., 2006).**

Due to controversies between diagnostic criteria, NIH sponsored Evidence-based Methodology Workshop (NIH and ESHRE/ASRM) on PCOS in 2012 recommended the wider Rotterdam/ESHRE/ASRM 2003 criteria together with the phenotyping specifications as 1) hyperandrogenism (Clinical and/or biochemical) plus ovulatory dysfunction (oligo- or anovulation); 2) hyperandrogenism plus Polycystic Ovary Morphology (PCOM); 3) hyperandrogenism plus ovulatory dysfunction plus PCOM; 4) ovulatory dysfunction plus PCOM Presently out of various diagnostic criteria of PCOS. The Rotterdam criterion is found to be more inclusive and most commonly used **(Fauser *et al*.,2012).**

1.5.1. Hyperandrogenism

Most females who have PCOS, also have a condition known as hyperandrogenism, which is crucial in making a diagnosis. It is one of the hallmark features of PCOS pathophysiology. Androgens are primarily produced by ovaries and adrenal glands; peripheral tissues including fat and skin also play a role in transforming androgens into more potent forms. Hyperandrogenism diagnosis can be based on clinical symptoms or excess serum androgen measurements **(Meek *et al*., 2013).**

Clinical hyperandrogenism most commonly manifests as hirsutism. To evaluate hirsutism, an observer should use the modified Ferriman-Gallwey scale, which asks for ratings on a scale ranging from 0 to 4 for nine different areas of the body (upper lip, chin, chest, upper and lower abdomen, thighs, upper and lower back, and upper arms). Measurements of excessive terminal hair growth are included in this method of measuring. The cutoff for hirsutism is a total modified Ferriman-Gallwey score of ≥4-6, depending on ethnicity**(Teede *et al*., 2018b)**. A less precise indication of hyperandrogenism is acne and alopecia. Biochemical hyperandrogenism is the term used to describe elevated serum androgen levels **(Mumusoglu *et al*.,2020).**

1.5.2. Ovarian Dysfunction

A common feature of PCOS usually presents as oligomenorrhea or amenorrhea, resulting from chronic oligo-ovulation/anovulation. Menstrual cycles are irregularly defined as having cycle durations of >35 days or having 8 menstrual cycles annually which are the clinical manifestation of ovulatory dysfunction. While amenorrhea means the absence of menstruation, more than 3 months post-menarche **(Mumusoglu and Yildiz,2020).**

Ovarian dysfunction is observed in approximately 70 to 80% of PCOS females. It is also reported that about 85 to 90% of women with oligomenorrhea will have PCOS and 30 to 40% of amenorrheic females will have PCOS **(Sirman and Pate , 2013).**

About 75% of PCOS patients present with anovulatory infertility making PCOS the most common cause of anovulatory infertility. Obesity further accelerates infertility where studies have shown that obese PCOS patients have more ovulation impairments and lower rates of pregnancy than the normal weight of PCOS women **(Costello *et al*.,2019).**

1.5.3. Polycystic Ovarian Morphology

While PCOM can be confirmed histopathological, ovarian cysts are detected clinically using ultrasonography, with criteria including the existence of 12 or more follicles in ovaries measuring between 2 and 9 mm in diameter, or an increase in ovarian volume of more than 10 c in at least one ovary. Classical features typical of PCOS ultrasound are the arrangement of follicles in the periphery of ovaries giving the image of a "string of pearls" **(Bozdag *et al*., 2016).**

1.6. Phenotypes of Polycystic Ovarian Syndrome

PCOS has been defined by the presence of at least two out of three criteria: clinical or biochemical HA, oligo- or amenorrhea (OM), and/or PCOM. This definition results in several PCOS phenotypes, such as phenotype A (HA, OM, PCOM), phenotype B (HA, OM), phenotype C (HA, PCOM), and phenotype D (OM, PCOM) investigated according to Rotterdam Criteria 2003**.(Spritzer *et al*., 2022).**

Phenotype A is considered classic, followed by Phenotype B as anovulatory, phenotype C as ovulatory, and much less frequently phenotype D as nonandrogenic**(Nikolayenkov *et al*., 2021)** as shown in table (1-2)

Table (1-2) : Comparative Characteristics of Polycystic Ovary Syndrome Phenotype (Nikolayenkov et al., 2021).

Phenotype variants	Phenotype A (Classic)	Phenotype B (anovulatory)	Phenotype C (ovulatory)	Phenotype D (nonandrogenic)
Hyperandrogenemia	Present	Present	Present	Absent
Oligo/anovulation	Present	Present	Absent	Present
Polycystic ovarian morphology	Present	Absent	Present	Present

Hyperandrogenemia, overweight, and severe menstrual irregularities in PCOS can be assumed as dependent predictors of metabolic disorders **(Zaeemzadeh *et al*.,2020; Xing *et al*.,2022)**. Thus, **Azziz (2018)** noted that the severity of menstrual irregularities correlates directly with insulin resistance (IR) level**(Amisi,2022)**.

According to the research, patients with PCOS phenotypes A and B have more severe menstrual abnormalities, hyperinsulinemia (HI) and IR are hallmarks of this phenotype, patients with this phenotypes are at a far higher risk of developing metabolic syndrome than phenotype D individuals. **(Afjal Hossain *et al* .,2021)**.

Excess body weight is more typical for women with androgenic phenotypes of PCOS, and it occurs in 54% with the anovulatory phenotype, 33% with classic phenotype,14% with the nonandrogenic phenotype and 11% in ovulatory phenotype **(Carmina and Lobo,2022)**.

Phenotype A patients are the most likely to be obese (86.0%), phenotype B (27.9%), phenotype C (46.6%), and phenotype D (38.8%) **(Nikolayenkov *et al* .,2021)**.

The highest prevalence of atherogenic hyperlipidemia was found in patients with phenotype A (>65.9%); Both high cholesterol and low levels of alphaproteinemia are further hallmarks of this syndrome **(Kim *et al* .,2014)**.

No significant changes were seen across the various PCOS phenotypes in the markers of carbohydrate metabolism, but in the androgenic phenotypes, especially the classical one, there is an increase in the amount of immunoreactive fasting insulin by 28.5%-34.3%. In the phenotype D , immunoreactive insulin levels increased by 14.3% **(Pandurevic *et al* .,2021)**.

Most studies found that patients with nonandrogenic phenotype D in PCOS do not have significant endocrine and metabolic disorders; consequently, the prevalence of the metabolic syndrome is low **(Carmina *et al* .,2019)**.

According to published data, phenotype A accounts for more than half of PCOS patients, while phenotypes B, C, and D each account for almost the same percentage. More than two-thirds of PCOS patients fall under phenotypes A and B **(Sachdeva *et al* ., 2019)**.

Numerous studies have shown that females with PCOS have an increased risk of cardiovascular disease and metabolic disorders compared to females in control groups**(Pandurevic *et al* .,2021).** The metabolic disorders of PCOS are mainly related to hyperandrogenism and compensatory hyperinsulinemia and occur dependently on obesity **(Barber and Franks,2021)**.

1.7. Risk Factors of Polycystic Ovarian Syndrome

The polycystic ovarian syndrome is associated with metabolic aberrations such as insulin resistance, obesity, and dyslipidemia contributing to an increased lifetime risk of developing cardiovascular diseases, type 2 diabetes mellitus, hypertension, **Osibogun, Ogunmoroti and Michos(2020)** and psychological features like anxiety, depression, poor self-esteem, eating disorders, and psychosexual dysfunction**(Derewianka-Polak *et al*.,2020)**.

Females with PCOS are also linked to pregnancy complications, including gestational diabetes, pre-eclampsia, fetal macrosomia, small-for-gestational-age neonates, and perinatal mortality. Consequently, the effects of this disorder are not restricted to the reproductive years but persist throughout life. There are health issues associated with a PCOS diagnosis, many of which are lifelong complications **(Bahri Khomami *et al*.,2019).**

1.7.1. Insulin Resistance

Insulin resistance is defined as a condition of a cell, tissue, or organism that requires an increased amount of insulin to obtain the appropriate response. The increased secretion of insulin by pancreatic β-cells leads to compensatory hyperinsulinemia. As long as insulin resistance is overcome by hyperinsulinemia, levels of glucose remain normal**(Freeman and Pennings,2022).**

As the β-cells decline in compensatory response, it results in relative or absolute insulin insufficiency with metabolic consequences. In addition to metabolic effects, insulin performs both mitogenic and reproductive actions. Insulin resistance is a distinctive feature of PCOS that occurs in 50-70% of PCOS women independent of obesity, though it's not universal and varies between PCOS clinical phenotypes **(Webb,2021)**. About 90% of obese PCOS females have

insulin resistance and its effect is additive to that of PCOS **(Barthelmess and Naz,2014).**

Clinically insulin resistance may present as Acanthosis nigricans, a skin lesion characterized by thickened, dark, velvety, hyperkeratotic, hyperpigmented, papillomatous skin patches around the skin of the neck, groin, antecubital fossae, in the axillae or areas under the breast and other skin folds **(Phiske,2014)**.

Insulin resistance and compensatory hyperinsulinemia are believed to play a crucial role in the pathogenesis of PCOS and are associated with a number of its phenotypic characteristics. Different explanations have been suggested to explain the development of insulin resistance in PCOS. Post-binding defects in insulin signaling caused by increased serine phosphorylation and decreased tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 influence metabolic pathways in both classical pathways (skeletal muscles and adipocytes) and ovaries **(Anagnostis , Tarlatzis and Kauffman , 2018).**

Reduced abundance of insulin receptor-β in omental adipose tissue, reduced GLUT4 (Glucose Transporter 4) in subcutaneous adipocytes contributing to reduced uptake of glucose, mitochondrial dysfunction, constitutive serine kinase activation in MAPK-ERK (Mitogen-Activated Protein Kinase/ Extracellular Signal-Regulated Kinases) pathway and genetic perturbation of insulin signaling in the central nervous system are additional factors which contribute to Insulin resistance is a common result of PCOS. **(Diamanti-Kandarakis and Dunaif,2012).**

In PCOS the central paradox is that insulin acts via its cognate receptor and promotes ovarian steroidogenesis amid insulin resistance toward glucose metabolism. This paradox can be attributed to the presence of systemic defect

disrupting the metabolic and not the mitogenic activity of insulin as observed in skin fibroblast of PCOS **(Corbould *et al*.,2006).**

Insulin acts directly on the ovaries and increases androgen production by stimulating a bifunctional enzyme cytochrome P450c17α. Insulin stimulates the production of androgens by acting synergistically with LH on ovarian theca cells. **(Morgante *et al*.,2020)**.

Another adverse effect of hyperinsulinemia on the ovaries of females with PCOS involves arresting the growth of ovarian follicles up to a size of 5 to 10 mm and preventing ovulation **(Kumariya *et al*.,2021).**

Insulin also acts as a co-gonadotropin that enhances the LH activity by stimulating the insulin and Insulin-like growth factor (IGF) ovarian receptors or indirectly intensifying the sensitivity to GnRH stimulation by increasing the amplitude of the LH serum pulse. Insulin can also increase ovarian androgen production by reducing the production of sex hormone-binding globulin (SHBG) in the liver, thereby increasing the bioavailable androgen levels **(Siemienowicz *et al*.,2021)**.

Hyperinsulinemia further alleviates the PCOS pathogenesis by reducing the production of insulin-like growth factor-1 binding proteins (IGFBP) in the liver and ovary, resulting in increased IGF-I availability, which in turn improves insulin activity both in the liver (further resulting in reduced SHBG levels) and ovaries; increases the ovarian IGF type 1 receptors and thus amplifies IGF-I and IGF-II ovarian action and accelerates the amplitude of GnRH stimulated LH pulse; resulting in PCOS characteristic features- hyperandrogenemia, oligo/anovulation, production of multiple cystic follicles in the ovaries and follicular atrophy **(Bremer and Miller,2008).**

Androgens in turn can lead back to insulin resistance by increasing the free fatty acid levels and by changing the structure and functionality of muscle tissues, perpetuating a vicious cycle of insulin resistance, hyperinsulinemia-hyperandrogenemia **Rojas *et al* (2014)** as in figure (1.5)

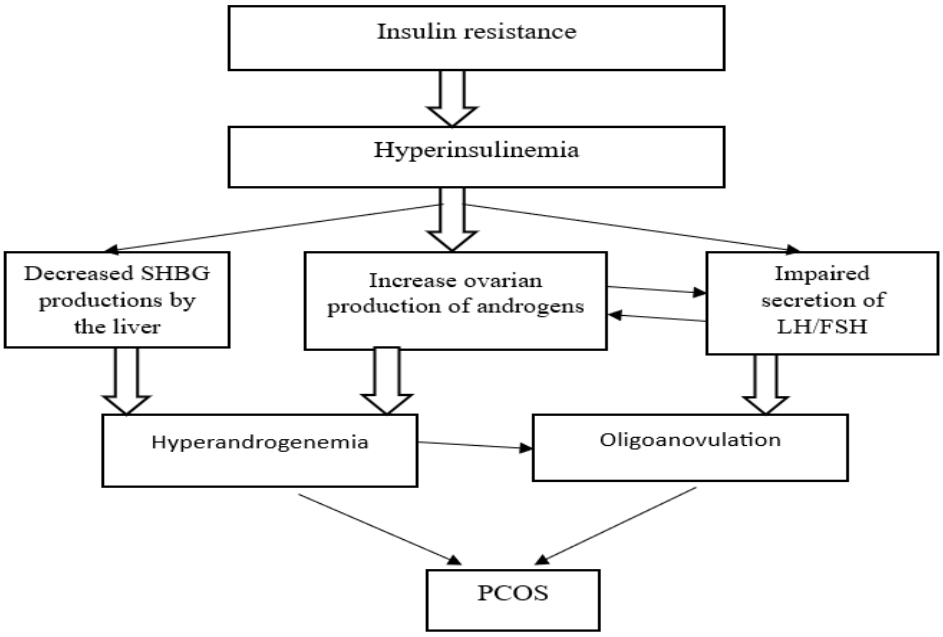


Figure (1.5) :Role of Insulin in the Pathogenesis of PCOS(Kamenov and Gateva ,2020).

1.7.2. Obesity

In developing countries ,approximately one-third of adults are obese and one-third are overweight. In 1980, 28.8% of Iraqi adults were obese. In contrast, 31.9% of Iraqi adults were obese in 2015. Rapid changes in socioeconomic status and demographics, as well as the adoption of an energy- and fat-rich diet and a sedentary lifestyle, are largely to blame for the rise in the prevalence of obesity. **(Chooi , Ding and Magkos ,2018)**.

Obesity is a leading risk factor for cardiovascular diseases and all-cause mortality, and its prevalence is on the rise, which has serious implications for

healthcare. Obesity is highly linked to PCOS and is related to cardiometabolic problems as well. It's a typical symptom for ladies with PCOS. Half of PCOS patients are overweight or obese, The females with a history of weight gain frequently report oligomenorrhea and hyperandrogenism, signifying the etiologic role of obesity in the subsequent development of the syndrome **(Rashid *et al*.,2022).**

There are also mechanisms, where PCOS development may lead to further weight gain that can impede efforts to achieve successful weight loss, thereby creating a vicious cycle that can be hard to overcome **(Barber *et al*.,2019)**.

Obesity, particularly visceral adiposity, which is common in obese and non-obese females with PCOS, exacerbates all metabolic and reproductive outcomes associated with PCOS. It may contribute to insulin resistance and subsequent hyperinsulinemia in women with PCOS. Obese PCOS patients appear to be at increased risk for metabolic disorders, which are characterized by elevated lipid profile, fasting glucose, and insulin resistance levels**(Barber *et al*.,2019).**

1.8. Visceral Adipose Tissue and Adipocytokines in PCOS

Fat tissue, which is often think of as a storage site for energy is a crucial endocrine tissue in the body. Adipokines, by definition, are cytokines secreted by adipose tissue. Accordingly, serum levels of adipokines usually associate with body fat mass and the severity of obesity is so involved in many critical physiological processes in the body, dysregulation of adipokines may lead to endocrine diseases. **Chen *et al* (2019)** modulate glucose and lipid metabolism, inflammation, and insulin sensitivity and, thus, might be involved in the pathogeneses of IR, T2DM, and Metabolic syndrome **(Cai *et al*.,2016).**

Adipokines are active hormones and other factors that are secreted by adipocytes. Adipose tissue can be divided into two distinct types: white and brown. White adipose tissue (WAT) is specialized for the storage of excess energy as triglycerides, whereas brown adipose tissue (BAT) dissipates energy as heat, thereby counteracting obesity. The regulation of adipose tissue function depends on the sympathetic nervous system (SNS), which plays a fundamental role in maintaining energy homeostasis in living organisms **(Lee ,Lee and Oh,2019).**

The SNS also modulates the development of obesity because it stimulates lipolysis in WAT and enhances heat production in BAT by activating adrenergic signaling **(Larabee, Neely and Domingos ,2020).**

1.8.1 Neudesin

Neudesin was first discovered to be a neurotrophic secreted protein in mouse embryos. **(Kimura *et al*.,2005).** Thereafter, it was, known as a GIG47 oncogene or the neuron-derived neurotrophic factor (NENF) **(Byerly *et al*.,2013).**

Human Neudesin is a secreted protein of 172 amino acids with a conserved cytochrome5-like heme/steroid-binding domain of _100 amino acids **(Kimura *et al*.,2008).**

Neudesin belongs to the membrane-associated progesterone receptor (MAPR) protein family, which also includes progesterone receptor-membrane component (PGRMC1), PGRMC2, and Neuferricin, all of which share the same distinctive cytochrome 5-like heme/steroid-binding domains as Neudesin. This quartet of proteins Different parts of the progesterone receptor membrane play important roles in processes like steroid and cholesterol production, medication metabolism, and cellular response. The ways they function are influenced by their interactions with cytochrome p450 (CYP) enzymes **(Ryu, Klein and Zanger ,2017).** Progesterone binding has only been reported for PGRMC1**. Petersen *et al* (2013)** and PGRMC1 may be a mediator of progesterone's quick actions**.** It was discovered that PGRMC1 was present in immortalized GnRH neurons (GT1-7 cells), and that it rapidly suppressed intracellular calcium fluctuations in GnRH neurons, hence suppressing GnRH and LH release**. (Bashour and Wray 2012).**

1.8.1.1 Structure of Neudesin

Nuclear magnetic resonance analysis showed that Neudesin, also known as GIG47, has an α-helice/β-strand structure with a β1- α1- β2- β3- α2- β4- α3- α4- β5- β6 topology .The heme/steroid-binding domain is located in the α2- β4- α3 topology. A homology modeling calculation with the known tertiary structure of 1TOG, a hypothetical protein of unknown function with cytochrome b5-like fold,

indicated the tertiary structure of Neudesin. A potential heme/steroid-binding hydrophobic pocket is visible between the α2 and α3 helices. Tyrosine residues 82 and 88 in this pocket are essential for heme-binding **(Han *et al* .,2012).**

1.8.1.2 Activity and Mechanism Action of Neudesin

Neudesin activates the mitogen-activated protein kinase and phosphoinositide 3-kinase signaling(PI3K) pathways. The phosphorylation of extracellular signal-regulated kinase (ERK)1/2 by Neudesin is inhibited by the pertussis toxin (PTX), an inhibitor of the Gi/Go-protein, indicating that its activity is mediated via activation of the $MAPK_S$ and PI3K pathways, which are potentially coupled with the Gi/Go-protein-coupled signaling pathway and has a role in neural cell differentiation, cell proliferation, and tumorigenesis. Neudesin is preferentially expressed in the central nervous system and the spinal cord, where it promotes neural cell differentiation and showed neurotrophic activity **(Ohta *et al*.,2015)** .

Neudesin mRNA expression has also been documented in other tissues, including adipose tissue, heart, lungs, and kidney. Neudesin can influence appetite control in the hypothalamus or the anxiety-like behavior controlled by the dentate gyrus of the hippocampus **(Byerly *et al*.,2013).**

1.8.1.3. Neudesin and Polycystic Ovarian Syndrome

The increased GnRH pulse frequency is attributed to the loss of negative feedback inhibition by progesterone which may be due to decreased progesterone levels or decreased effects because of hyperandrogenemia **(Szeliga *et al* .,2022).**

Membrane-associated progesterone receptors are a group of four proteins with a similar heme-binding domain related to cytochrome b5 (a membrane-bound hemoprotein that functions as an electron carrier for microsomal cytochrome P450 monooxygenase systems) **(Ryu *et al*.,2017).**

Although PGRMC1 (which binds progesterone) and NENF share structural features, there is no data about the interaction between neudesin and progesterone **(Kimura *et al*.,2013).**

It is proposed that may act as a binding protein for lipophilic progesterone and hold it on the cell surface in the extra-cellular environment and this complex may act on the unknown cell surface progesterone receptor to exert a rapid effect. The data are limited on the evaluation of membrane-associated progesterone receptors in patients with PCOS, the study by Bozkaya *et al.* in 2020, which revealed a decrease in NENF levels in PCOS, comprised the first attempt to investigate a putative link between PCOS diagnosis and serum neudesin concentration. In study performed by Bozkaya in 2020 , the level significantly decreases between the study and control group, and a positive correlation between neudesin and progesterone was noted in the affected individuals. An insignificant difference in the neudesin level was observed between the PCOS group with and without insulin resistance **(Bozkaya *et al*.,2020).**

In the study performed by Yasar *et al.* in 2021, levels of progesterone and insulin were positively linked with neudesin levels, which were shown to be lower in the PCOS group. **(Yasar *et al* .,2021)**.

In 2022 another study performed by Kruszewska *et al.* the lower neudesin concentration in PCOS females with insulin resistance was observed **(Kruszewska, Laudy-Wiaderny and Kunicki, 2022).**

1.8.2. Neuregulin Family

In the early 1990s, several groups isolated proteins encoded by this gene NRG. These were named Neu differentiation factor (NDF) **Wen *et al* (1992)**, heregulin (HRG) **Holmes *et al* (1992)** glial growth factor (GGF), acetylcholine receptor-inducing activity(ARIA) ,sensory and motor nerve-derived factors. Later, it was proposed that this family's members be included in the term "neuregulin" **(Fischbach and Rosen,1997).**

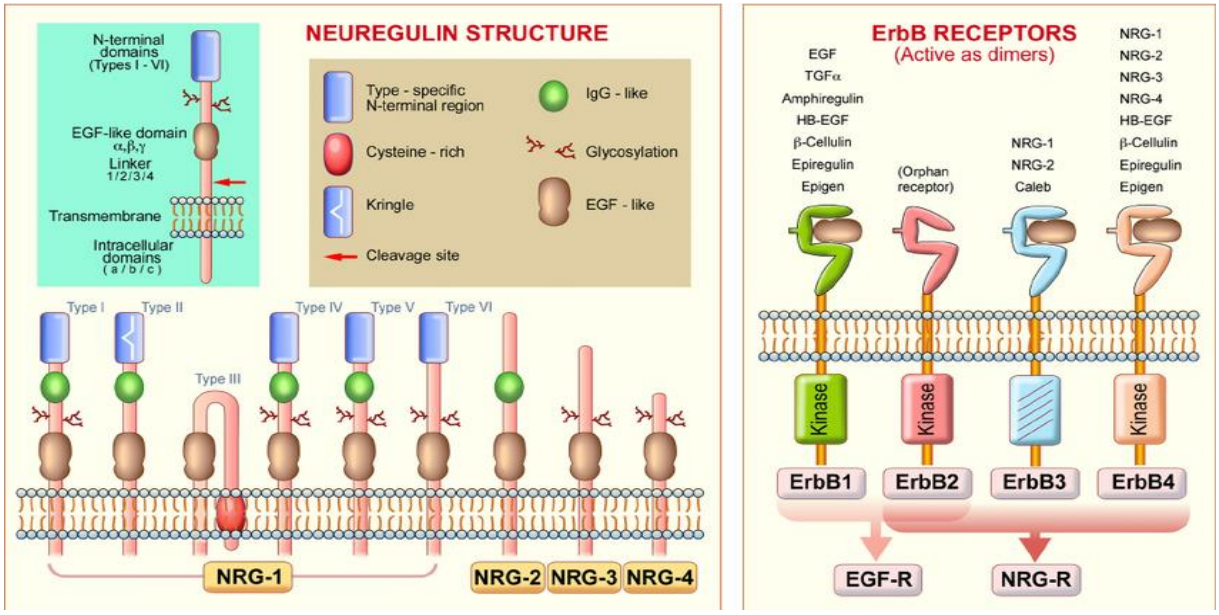


Figure (1.6): Neuregulin (NRG) Structure and Binding to ErbB Receptors(Guma *et al*.,2010).
NRG= neuregulin; EGF=epidermal growth factor; ErbB= The epidermal growth factor family of receptor tyrosine kinases; HB-EGF= heparin-binding EGF-like growth factor.

Neuregulin (NRG) growth factor belongs to a complex family of proteins that are structurally related to epidermal growth factor receptors (EGFR), also known as ErbB receptors. Four NRG genes have been identified (neuregulin-1 to-4) as shown in figure (1.6) **(Worthington *et al*.,2010).**

All EGF family members are ligands of ErbB receptors, albeit with distinct specificities and affinities, with ErbB3 and ErbB4 being the NRG-binding receptors. NRG-1 isoforms have been classified into types I–VI based on differences in the NH2-terminal distal region. Thus, isoforms initially known as acetylcholine receptor-inducing activity, heregulin, and Neu differentiation factor belong to the type I isoforms, have an immune-globulin-like domain; glial growth factor belongs to the type II isoforms, which have a kringle domain; and sensory and motor nerve-derived factor belongs the to type III isoforms, have a cysteine-rich domain. Structural variations in the COOH-terminal site of the EGF domain and the linker site (1, 2, 3, and 4), as well as in the length of the intracellular region (a, b, and c), NRG isoforms are expressed mainly by cells of endothelial, mesenchymal, and neuronal origin and are thus critical for the proliferation, survival, migration, and differentiation of several cell types, including epithelium, nerve, cardiac, and skeletal muscle **Guma *et al*(2010)** serve as an adipokine that is synthesized in many tissues, especially in brown adipose tissue (BAT) **Tutunchi *et al*(2019**) have an effect on insulin sensitivity and cardiometabolic risk as shown in Figure (1.8) **(Guma *et al*.,2020).**

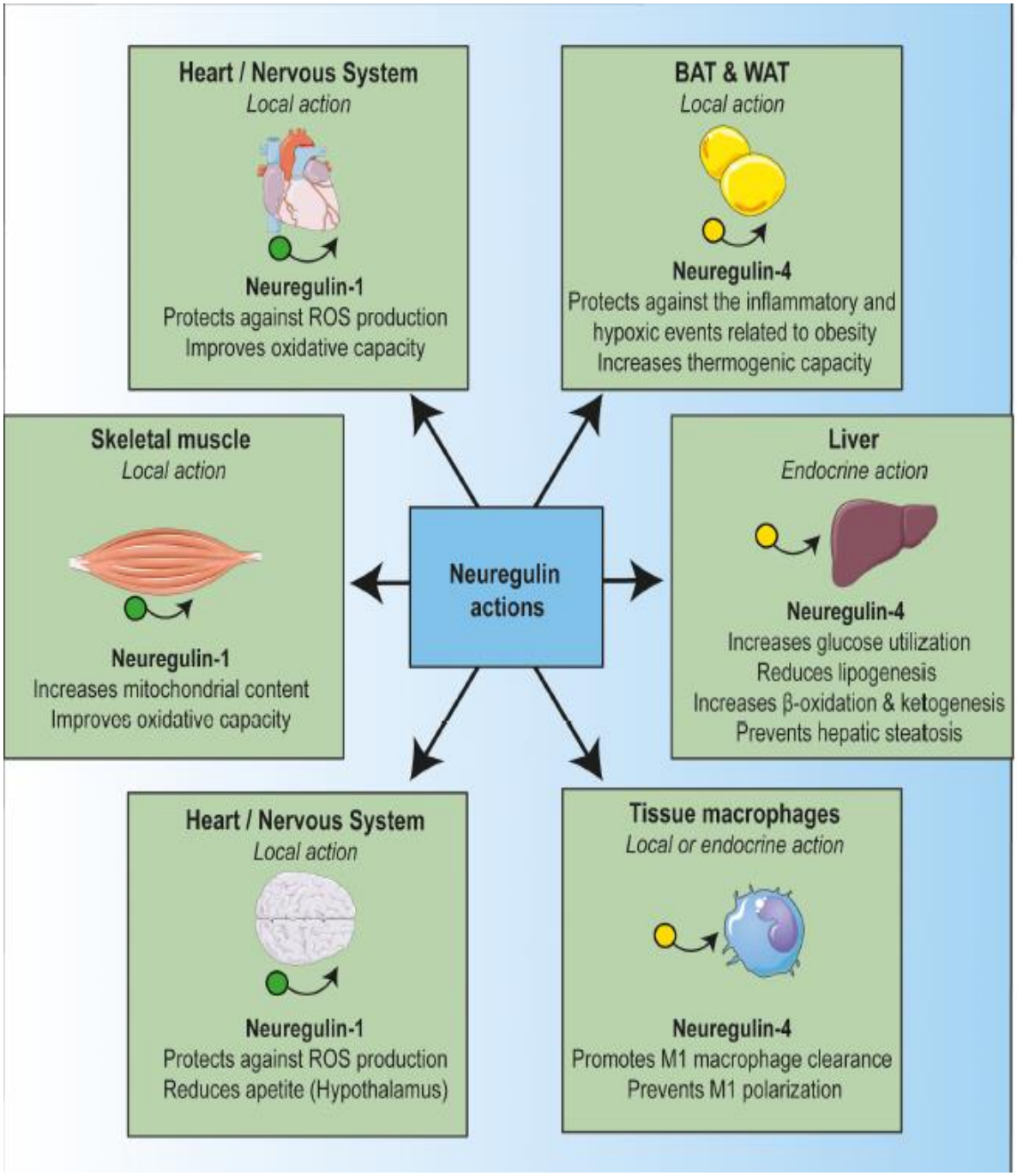


Figure (1.7): Protective Actions of Neuregulin on Different Tissue (Guma *et al*.,2020).

1.8.2.1. Neuregulin 4

Neuregulin 4 is a secreted water-soluble protein that has been found in the circulation. In addition to its activation of epidermal growth factor receptors (EGFR, also known as ErbB receptors), NRG4 has been detected in a variety of organs. Its expression is highest in brown adipose tissue **(Pfeifer,2015).**

A member of the epidermal growth factor (EGF) family of extracellular ligands and binds specifically to ErbB4 receptors initiating cell-to-cell signaling via tyrosine phosphorylation **Guma *et al* (2010)**and acts as an autocrine, paracrine, or endocrine signal by releasing the EGF-like domain after photolytic cleavage also acting on distal tissues with special incidence on the liver**(Jiang *et al*.,2016).** Neuregulin 4 is a small protein with a transmembrane domain and a short extracellular portion containing an EGF-like domain, which is flanked at the C-terminal by a proteolytic site that, upon cleavage, releases the EGF domain, which binds specifically to ErbB receptors **(Chen *et al* .,2023).**

1.8.2.2 Functions of Neuregulin 4

Neuregulin 4 has many biological functions, including inhibiting apoptosis and inflammation and promoting neurite outgrowth **(Rosell *et al*.,2014).** NRG4 has been shown to affect the development of obesity and metabolic disorders including type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) in animals and humans **(Wang *et al*.,2018).**

Decreased Nrg4 levels may lead to IR, T2DM, and Metabolic syndrome (MS) development. A recent study demonstrated that Nrg4 mRNA levels in subcutaneous and visceral adipose tissues were significantly lower in patients with impaired glucose tolerance or T2DM than in normal individuals **(Tutunchi *et al*.,2019).**

In the liver, Nrg4 protects against the deleterious effects of a high-fat diet (HFD) by inhibiting de novo lipogenesis **Diaz-Saez *et al* (2021)** and activating fatty acid oxidation and ketogenesis. Additionally, Nrg4 downregulates the expression of proinflammatory cytokines in WAT **Shi *et al* (2021)** and the liver **De Munck *et al* (2021)**, and induces angiogenesis in adipose tissue **(Scheja and Heeren, 2019).**

1.8.2.3 Neuregulin 4 and polycystic ovarian syndrome

Neuregulin 4 is one of the adipokines members that its synthesis and secretion from adipose tissues, it acts to activate the epidermal growth factor receptor (EGFR). The EGFR has an important role in the healthy luteinizing hormone-releasing hormone (LHRH) function that is responsible for female normal pubertal development because any deficiency in EGFR can cause failed LHRH secretion **(Ayoob, 2022).** The hypothalamic-pituitary-gonadal axis and the LHRH are required for healthy pubertal development. It has been established that ErbB receptors are crucial to the normal functioning of LHRH. An inverse correlation was found between the level of NRG4 and the risk of metabolic syndrome with insulin resistance **(Cao and Hu, 2021).**

Numerous studies reveal that NRG 4 binds to ErbB receptors and indirectly alters its hormonal environment**. (Heim *et al*., 2020)**. NRG4 levels were found to be significantly higher in females with PCOS. There was also a significant correlation between HOMA-IR, high-sensitivity C-reactive protein (hs-CRP), and NRG4. Elevation of NRG4 levels in PCOS may be associated with increased insulin resistance and low-grade chronic inflammation (**Eken *et al* .,2019)**. The recent study performance by KRUSZEWSKA *et al*. in 2022 confirm elevation of NRG4 level in PCOS patients that have insulin resistance **(Kruszewska, Laudy-Wiaderny and Kunicki, 2022).**

1.9. Importance of the study, Implications and Contribution to the Knowledge Gap

Many studies have implicated the importance of adipokines in PCOS, **Yasar *et al* (2021)** requested a further study that should be conducted to learn more about the progesterone influence in PCOS patients and the neuroendocrine functions of neudesin. Also**, Kruszewska *et al* (2022)** confirmed the need for more research to determine the functions of these markers, their role in the etiology of PCOS, and whether or not quantifying their levels would be useful in clinical settings. **Bozkaya *et al* (2020)** indicated in their study that Hormonal irregularities in PCOS were linked to low neudesin levels. Neudesin levels were inversely related to the likelihood of developing PCOS. More research is needed to determine the role of neudesin in the pathophysiological processes that contribute to PCOS. **Afjal Hossain *et al*(2021)** confirmed that all women with PCOS should be screened for metabolic abnormalities, especially those with hyperandrogenic phenotypes, which can have serious consequences for their health. Furthermore, a study by Sachdeva *et al*(2019) identified that Patient prognosis and treatment decisions for infertility caused by polycystic ovary syndrome (PCOS) can be improved by identifying and treating certain phenotypes

1.10. Aim of Study

The current study aimed at:

1-Estimating the level of Neudesin and Neuregulin 4 in PCOS phenotypes.

2-Examing the metabolic indices related to PCOS phenotypes.

3-Studying the correlation of serum Neudesin and Neuregulin 4 and metabolic indices with PCOS phenotypes.

4-Investigating the diagnostic preferences of Neudesin, Neuregulin 4 and metabolic indices with PCOS phenotypes using ROC analysis.

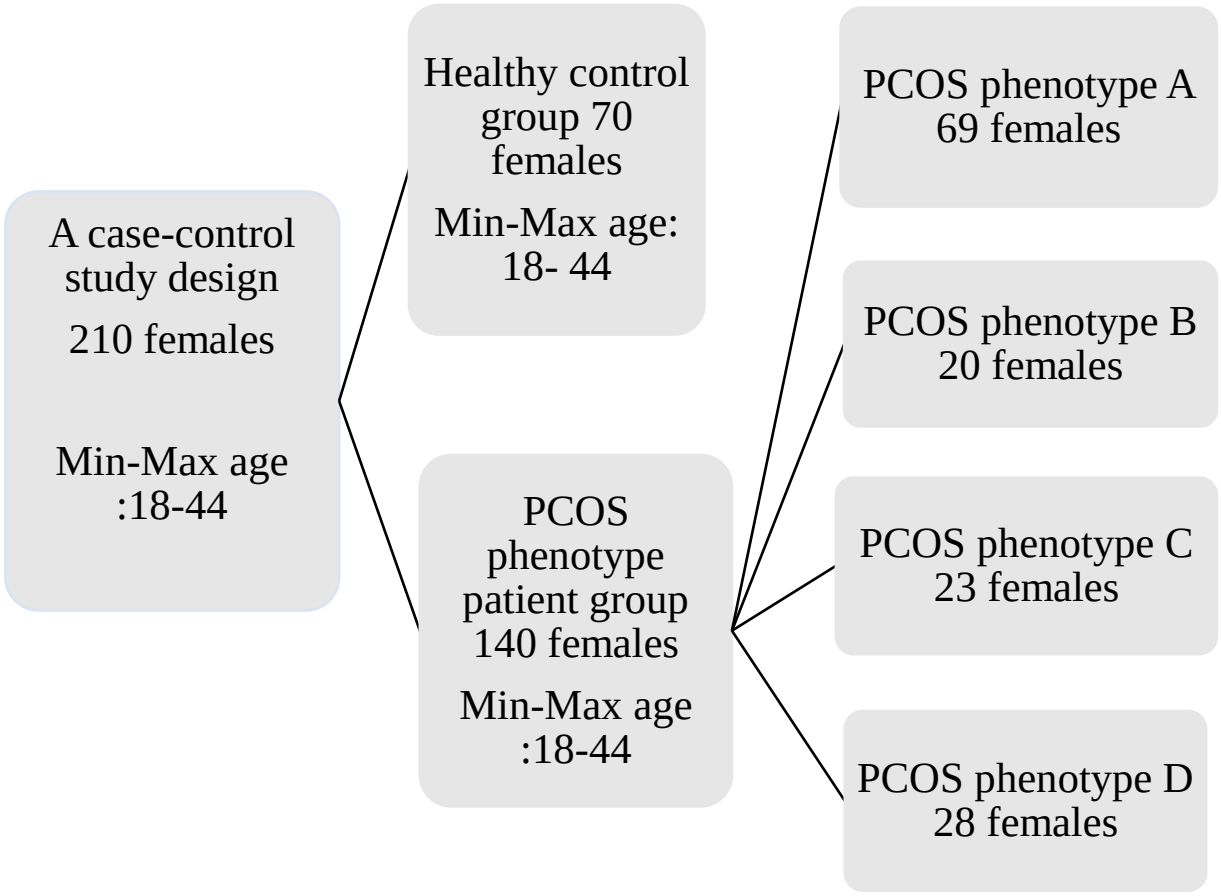
Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Study design and ethical approval

A case-control study design was done for a total of 210 subjects. The study was conducted throughout the period from Sep. 2022 to June 2023. College of Medicine, University of Kerbala, and Kerbala Health Directorate have validated the study's ethical approval. The approval was also taken from the administration of the gynecological and obstetric teaching hospital, also from the patient after explaining the purpose of the study.



Scheme of The Study

2.2. Subjects

2.2.1. Patients

The biochemical reports of 140 PCOS cases fitting the inclusion criteria (according to Rotterdam criteria) of reproductive age (18- 44 years) females were collected from the reproductive fertility consultant of the teaching Hospital for Obstetrics and Gynecology, Kerbala health directorate, Iraq, and infertility outpatient clinic.

The sample size was determined using the formula of calculating equation based on the latest reported prevalence. An interview was conducted to collect the participant's history and demographic information

A questionnaire was designed to get the data of the patient which included the age, weight, height, waist circumference was measured in the standing position, waist circumference, menstrual regularity, fertility, hirsutism, acne, alopecia, having any chronic disease, medication history, smoking status, duration of diagnostic and duration on starting treatment as shown in the appendix.

The measurements of clinical characteristics and some metabolic incised included: Hirsutism assessment, body mass index (BMI), waist-to-hip ratio (WHR), body adiposity index (BAI), visceral adiposity index (VAI), lipid accumulation product (LAP), and basal metabolic rate (BMR)

❖ **Inclusion criteria**

The Rotterdam criteria-2012 was assumed to be PCOS females (N=140) with ages reached between (18 - 44 years).

It is possible to diagnose patients with any two of the subsequent three conditions: clinical or biochemical hyperandrogenism (HA), which is clinically manifested as acne, hirsutism assessed by the Ferriman–Gallwey scale, and male-pattern hair loss are commonly observed while the biochemical used to describe elevated serum androgen levels, oligo- or amenorrhea (OM) is irregular and inconsistent menstrual blood flow in females the length of menstrual cycle greater than 35 days or four to six menstrual cycles in a year, and/or polycystic ovarian morphology (PCOM) (ovarian volume greater than 10 ml on ultrasonography, and more than 12 follicles measuring 2 to 9 mm in diameter) into four phenotypes**(Freeman, Burks and Wild, 2022).**

Phenotype A PCOS was diagnosed in patients presenting with all three characteristics of the syndrome (chronic anovulation, hyperandrogenism, and polycystic ovaries). Phenotype B PCOS was diagnosed in patients presenting with (hyperandrogenism and chronic anovulation) but no polycystic ovaries, Phenotype C-PCOS was diagnosed in patients presenting with (hyperandrogenism and polycystic ovaries) in the presence of ovulatory cycles while Phenotype D PCOS was diagnosed in women presenting with (chronic anovulation and polycystic ovaries**(Nikolayenkov *et al*., 2021)**) .

❖ **Exclusion criteria**

Female with chronic diseases (cushing syndrome ,nonclassical adrenal hyperplasia, autoimmune disease, diabetes mellitus, thyroid disease, hyperprolactin, cardiovascular disease, hypertension, chronic liver failure, chronic renal failure, and malignant diseases) were excluded. Females who have

a history of medication (as lipid-lowering agents, contraceptive pills, ovulation stimulation, corticosteroids, antidiabetic and antihypertensive medications) were excluded also.

2.2.2. Control

Seventy healthy females were selected as a control group, their ages ranging between (18-44 years). They have a regular menstruation period, with normal ovaries as they were observed by the gynecologists. The questionnaire for the control group was the age, weight and height, hip and waist circumferences, and menstrual regularity with fertility and hirsutism. Not complaining from kidney , liver, thyroid, cancer, strokes, acute or chronic inflammatory disease, alcoholics, rheumatoid arthritis, autoimmune disease, patients with type 1 and 2 diabetes mellitus, and no history of contraceptive drugs.

2.2.3. Blood Collection and Storage of Samples

Disposable syringes were used for blood collection (5 mL). Blood samples were collected from PCOS patients and control groups by vein puncture after an overnight fast of 12hr, during the early follicular phase (cycle day 2 – 4) for evaluation of FSH, LH, Prolactin, TSH, Free testosterone and NRG4. Where NENF and progesterone are in the med luteal phase (cycle day 20-21). Each sample was put in a tube containing 5 milliliters of blood in gel tubes. Then, it stayed for 15 to 20 minutes at room temperature for clotting. Then it was centrifuged for 5 to 10 minutes at 2000 xg. Serum was divided into 5 parts and put in Eppendorf tubes then stored at -35 ˚C till the examination of the biomolecules in this study.

2.2.4. Kits

The kits used in this study are summarized in table (2-1).

Table (2-1): Kits Used in This Study and Their Suppliers

NO.	Kits	Company and Country
1	Fasting blood glucose	Biorex/ UK
2	Elecsys Follicular Stimulating Hormone kit	Roche/Germany
3	Free Testosterone Hormone kit	Snibe/Germany
4	HDL Kit	Giesse/Italy
5	Human Neuregulin 4 (NRG4) Elisa kit (Cat: ELK3451)	Elk Biotechnology/china
6	Human Neudesin Neurotrophic factor (NENF)Elisa kit (Cat: ELK 3900)	Elk Biotechnology /China
7	Elecsys Insulin Hormone Kit	Roche/Germany
8	LDL Kit	Giesse/Italy
9	Elecsys Luteinizing Hormone kit	Roche/Germany
10	Elecsys Prolactin Hormone kit	Roche/Germany
11	Elecsys Progesterone Hormone kit	Roche/Germany
12	TG Kit	Giesse/Italy
13	Total cholesterol Kit	Giesse/Italy
14	Elecsys TSH Hormone kit	Roche/Germany

2.2.5. Instruments and Lab Equipment

The instruments and laboratory tools used in this study are summarized in table (2-2).

Table (2-2): Instruments and Lab Equipment Used in This Study

No.	Instruments and Lab Equipment	Company and country
1	SAMRT -120, chemistry analyzer	Geno TEK/ USA
2	Centrifuge	Kokusan /Japan
3	Monarch 240, Clinical chemistry analyzer	Biorex /UK
4	Cobas, e 411 analyzer	Roche / Germany
5	Deep freezer	Lab Tech/Korea
6	Eppendorf tubes	Mheco/ China
7	ELISA system	Bio Tek /USA
8	Gel tube	Mheco/ China
9	Gilson Tips, 1000µl (blue)	Mheco/ China
10	Gilson Micro-tips, 100µl	Mheco/ China
11	Maglumi 600	Snibe/Germany
12	Micropipette variable volumes	HUMAN Humapette /Germany

2.3. Methods

2.3.1. Hirsutism Assessment

The modified Ferriman-Gallwey scoring system was used to determine whether a person has hirsutism. Nine different body parts as seen in figure (2.1) [the upper lip, chin, chest, upper and lower abdomen, thighs, upper and lower back, and upper arms] are scored from 0 to 4, a score of 0 represents the absence of terminal hair growth, and a score 4 represents extensive growth. Depending on the patient's race, a total mFG score ≥ of 4-6 is used to define hirsutism**. (Mumusoglu and Yildiz, 2020).**

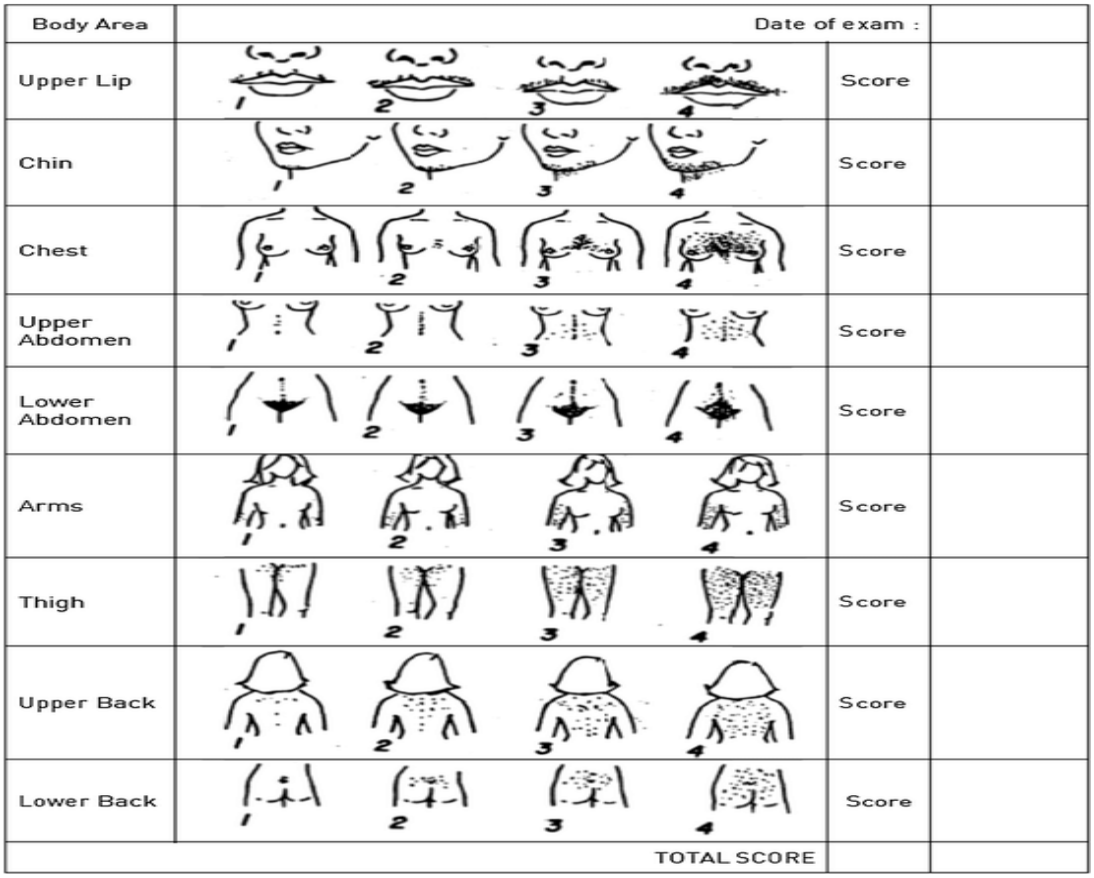


Figure (2.1): The Modified Ferriman-Gallwey Score (Khan et al., 2019).

2.3.2. Body Mass Index Measurement

Obesity was categorized using the body mass index (BMI) according to the traditional World Health Organization (WHO) which was calculated from the following equation: BMI (Kg/m²) = Weight (kg) / Height (m^2).

The ranges of (BMI) were categorized into groups **Namjou *et al*(2021),** see table (2-3).

Table (2-3): Body Mass Index.

Weight status	BMI(Kg/m^2)
Underweight	≤ 18.5
Normal weight	18.5-24.9
Overweight	25-29.9
Obese	≥ 30

2.3.3. Waist Hip Ratio Measurement

The Waist Hip Ratio (WHR) ≥ 0.85 is the diagnostic standard for abdominal obesity in women according to WHO **(Lee *et al*., 2022).**

The formula is:WHR= waist (cm) / hip (cm)

2.3.4 Anthropometric Measurements

*Visceral Adiposity Index (VAI) : Indicator of adipose distribution.

*lipid accumulation product (LAP):An index reflect lipid accumulation .

*The Body Adiposity Index (BAI) : An index for evaluating body composition in epidemiologic studies or clinical practice, especially for identifying overweight or obesity in individuals

Table (2-4): Formulas of Anthropometric Measurements

Anthropometric measurements	References
BAI %= hip circumference (cm)/height (m)$^{1.5}$ – 18	**(Li *et al*., 2016)**
VAI (Female) = [WC (cm)/ (36.58+ (1.89 × BMI)] × [Triglycerides (mmol/l)/0.81]× [1.52/HDL (mmol/l)]	**(Amato *et al*. ,2014)**
LAP (Female) = (WC in cm – 58) × Triglycerides in mmol/L	**(Ahn *et al*., 2019)**
BAI: body adiposity index; , LAP: lipid accumulation product and VAI:visceral adiposity index	

2.3.5. Basal Metabolic Rate

Basal Metabolic Rate (BMR): The number of calories required to keep your body functioning at rest, also known as your metabolism.

The Mifflin-St Jeor for Basal Metabolic Rate (BMR) has used the following equation: BMR (Female):[(10 × weight in kg) + (6.25 × height in cm) - (5 × age in years) – 161] **(Mifflin *et al*., 1990)**

2.3.6. Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

A surrogate marker of insulin resistance was used from the following equation:

HOMA-IR =[fasting blood sugar (mg/dl) x fasting insulin (µU/ml) /405]

(Bahadur *et al*.,2021)

2.4. Determination of Hormones Levels

Luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid stimulating hormone (TSH), prolactin hormone, Progesterone hormone, and insulin hormone levels were measured by the electrochemiluminescence immunoassay “ECLIA” system **(Cobas e 411, Roche Diagnostic, Germany).**

General Assay Principle:

A completely automated, random access, software-controlled system for immunoassay analysis is the Roche Diagnostic Cobas e411 Immunoassay System **(Black and da Silva Costa, 2018).** The system offers six test principles: a bridging principle to identify antibodies in the sample, a sandwich principle (one or two steps) for larger analytes, and a competitive principle for extremely small analytes **(Nivedhita and Brundha, 2020)**. Applying voltage to the immunological complexes connected to the streptavidin-coated microparticles causes chemiluminescent reactions that result in the emission of light from the ruthenium complex to begin electrically **(Toboc *et al*., 2018).**

Procedure:

1. Assay cups and assay tips were loaded in the instrument (cobas e411).

2. Regents of each parameter (LH, FSH, TSH, Prolactin, Progesterone, and insulin), loading in reagent rack.

3. Calibration and control of each reagent were made.

4. The serum sample was loaded into the sample rack then the instrument automatically began analysis.

2.4.1. Determination Luteinizing Hormone Level

A. Test Principle

The sandwich principle of the Elecsys LH level assay employs two monoclonal antibodies specifically directed against human LH.

B. working solutions and Reagents

The label on the reagent rack pack reads LH.

M Microparticles with 0.72 mg/mL streptavidin coating.

R1 Anti-LH-Ab~biotin: Mouse monoclonal anti-LH antibody that has been biotinylated 2.0 mg/L in TRIS (Trisaminomethane) buffer at pH 8.0.

R2 Anti-LH-Ab~Ru: Mouse monoclonal anti-LH antibody labeled with ruthenium complex at a concentration of 0.3 mg/L in TRIS buffer at a pH of 8.0.

C. Procedure

1. First incubation: A sandwich complex was created from 20 µL of the sample, a monoclonal LH-specific antibody that has been biotinylated, and a monoclonal LH-specific antibody that has been labeled with ruthenium complex.

2. Second incubation: Following the addition of microparticles coated in streptavidin, the complex was linked to the solid phase by an interaction between biotin and streptavidin.

3. The measurement cell receives the reaction mixture by aspiration, and the microparticles were magnetically attracted to the electrode's surface there. Pro Cell/Pro Cell M was then used to eliminate any remaining unbound materials. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.4.2. Determination Follicular Stimulating Hormone Level

A. Test principle

The sandwich principle of the Elecsys FSH level assay employs two monoclonal antibodies specifically directed against human FSH.

B. working solutions and Reagents

The label on the reagent rack pack reads FSH.

M Microparticles with 0.72 mg/mL streptavidin coating.

R1 Anti-FSH-Ab- biotin: Mouse monoclonal FSH antibody that has been biotinylated 0.5 mg/L in MES (morpholino)ethane sulfonic acid buffer at pH 6.0.

R2 Anti-FSH-Ab- Ru: Mouse monoclonal anti-FSH antibody labeled with ruthenium complex at a concentration of 0.8 mg/L in MES buffer at a pH of 6.0.

C. Procedure

1. First incubation: A sandwich complex was created from 40 µL of the sample, a monoclonal FSH-specific antibody that has been biotinylated, and a monoclonal FSH-specific antibody that has been tagged with a ruthenium complex.

2. Second incubation: The complex was bonded to the solid phase after the addition of microparticles coated in streptavidin through the interaction of biotin and streptavidin.

3. The measurement cell receives the reaction mixture by aspiration, and the microparticles were magnetically attracted to the electrode's surface there. Pro Cell/Pro Cell M was then used to eliminate any remaining unbound materials. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.4.3. Determination Thyroid Stimulating Hormone Level

A. Test principle

The Elecsys TSH assay employs monoclonal antibodies specifically directed against human TSH.

B. working solutions and Reagents

The reagent rack pack is labeled as TSH.

M Microparticles with0.72 mg/mL Streptavidin-coating .

R1 Anti-TSH-Ab~biotin: mouse monoclonal anti-TSH antibody has been Biotinylated 2.0 mg/L; phosphate buffer 100 mmol/L, pH 7.2.

R2 Anti-TSH-Ab~Ru(bpy): mouse/human Monoclonal anti-TSH antibody labeled with ruthenium complex 1.2 mg/L; phosphate buffer 100 mmol/L, pH 7.2.

C. Procedure

1. First incubation: A sandwich complex was created from 50 µL of the sample, a monoclonal TSH specific antibody has been biotinylated, and a monoclonal TSH specific antibody that has been tagged with a ruthenium complex.

2. Second incubation: The complex was bonded to the solid phase after the addition of microparticles coated in streptavidin through the interaction of biotin and streptavidin.

3. The measurement cell receives the reaction mixture by aspiration, and the microparticles were magnetically attracted to the electrode's surface there. Pro Cell/Pro Cell M was then used to eliminate any remaining unbound materials. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.4.4. Determination Prolactin Hormone Level

A. Test principle

Two monoclonal antibodies designed specifically against human prolactin were used in the Elecsys Prolactin Level Assay sandwich principle.

B. Reagents - working solutions

The label on the reagent rack pack reads "PRL II."

M Microparticles with 0.72 mg/mL streptavidin coating.

R1 Anti-Prolactin-Ab- biotin: Mouse monoclonal anti-prolactin antibody that has been biotinylated, 0.7 mg/L in phosphate buffer at pH 7.0.

R2 Anti-Prolactin-Ab- Ru(bpy): Mouse monoclonal anti-prolactin antibody labeled with ruthenium complex at a concentration of 0.35 mg/L in phosphate buffer at a pH of 7.0.

C. Procedure

1. First incubation: A initial complex was formed by 10 µL of material and a monoclonal prolactin-specific antibody that has been biotinylated.

2. Second incubation: An interaction between streptavidin and biotin causes a sandwich complex to develop with the addition of a monoclonal prolactin-specific antibody tagged with a ruthenium complex and microparticles coated in streptavidin.

3. The measurement cell receives the reaction mixture by aspiration, and the microparticles were magnetically attracted to the electrode's surface there. Pro Cell/Pro Cell M was then used to eliminate any remaining unbound materials. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.4.5. Determination Progesterone Hormone Level

A. Test principle

The determination of progesterone is utilized in fertility diagnosis for the detection of ovulation and assessment of the luteal phase.

B. Reagents - working solutions

The reagent rack pack is labeled PROG III.

M Microparticles with 0.72 mg/mL Streptavidin-coating.

R1 Anti-progesterone-Ab~biotin: recombinant/ sheep monoclonal anti-progesterone antibody that has been Biotinylated 30 ng/mL, phosphate buffer 25 mmol/L, pH 7.0.

R2 Progesterone-peptide~Ru(bpy): vegetable origin Progesterone coupled to a synthetic peptide labeled with ruthenium complex, 2 ng/mL; phosphate buffer 25 mmol/L, pH 7.0.

C. Procedure

1. First incubation: By incubating the sample (20 µL) with a progesterone-specific biotinylated antibody, immunocomplexes are formed, the amount of which is dependent upon the analyte concentration in the sample.

2. Second incubation: After the addition of streptavidin-coated microparticles and a progesterone derivative labeled with a ruthenium complexa), the stillvacant sites of the biotinylated antibodies become occupied, with the formation of an antibody hapten complex. The entire complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

3. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode.

Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

2.4.6. Determination Insulin Hormone Level

A. Test principle

The Elecsys Insulin assay employs two monoclonal antibodies which are specific for human insulin.

B. Reagents - working solutions

The reagent rack pack is labeled INSULIN.

M Microparticles with 0.72 mg/mL Streptavidin-coating.

R1 Anti-insulin-Ab~biotin: Mouse monoclonal anti-insulin antibody that has been Biotinylated 1 mg/L; MES buffer 50 mmol/L, pH 6.0.

R2 Anti-insulin-Ab~Ru(bpy): Mouse Monoclonal anti-insulin antibody labeled with ruthenium complex 1.75 mg/L; MES buffer 50 mmol/L, pH 6.0.

C. Procedure

1. First incubation: Insulin from 20 µL sample, a biotinylated monoclonal The insulin-specific antibody, and a monoclonal insulin-specific antibody labeled with a ruthenium complexa) form a sandwich complex.

2.Second incubation: After the addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

3. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode.

Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

2.5. Determination Free Testosterone Hormone Level

The quantitative determination of free testosterone in human serum using a fully -auto chemiluminescence immunoassay analyzer(MAGLUMI 600 , Snibe Diagnostic , Germany).

A. solutions and reagents

Table (2-5): Working Solutions and Reagents for Free Testosterone Hormone Assay

Reagent	Content	Concentration
Magnetic microbeads	Magnetic microbeads coated with test antigen, containing BSA, NaN_3	2.5 mL
Calibrator low	Test antigen, containing BSA,NaN_3	2.5mL
Calibrator high	Test antigen, containing BSA,NaN_3	2.5 mL
Buffer	containing BSA,NaN_3	7.5mL
ABEI Label	Anti-test monoclonal antibody labeled with ABEI, containing BSA, NaN_3	11.5mL
Internal quality control	Test antigen, containing BSA, NaN_3	2.0mL

BSA: Bovine serum albumin and NaN_3: Sodium azide.

B. Principle of the test

1)The sample, ABEI labeled with anti-test monoclonal antibody and magnetic microbeads coated with purified test antigen are mixed and incubated, forming antibody-antigen complexes.

2)After precipitation in the magnetic field, decant the supernatant, and then perform a wash cycle.

3)The reagent 1+2 are added to initiate a chemiluminescent reaction .

4) The light signal is measured by a photomultiplier as relative light units (RLUs) which is inversely proportional to the concentration of free testosterone in the sample .

C. Procedure and preparation of the reagent

1)Resuspension of the magnetic microbeads takes place automatically when the kit is loaded successfully .

2)To ensure proper test performance, strictly adhere to the operating instructions of MAGLUMI 600 series fully -auto chemiluminescence immunoassay analyzer.

D. Calculation

The analyzer automatically calculates the free testosterone concentration in each sample by means of a calibration curve which is generated by a 2-point calibration master curve procedure, the result is expressed in pg/mL.

2.6. Determination of Lipid Profile Levels

Total cholesterol, Low-density lipoprotein, High-density lipoprotein, and Triglyceride were measured by a fully automatic chemistry analyzer (SMART-120, Geno TEK, USA).

2.6.1. Determination of Total Cholesterol Level

Quantitative determination of Total cholesterol in serum was performed based on **(Trinder, 1969; Bishop, 2020).**

A schematic representation of the reaction is shown in the following equations:

Cholesterol ester $\xrightarrow{CHE}$ Cholesterol + fatty acids

Cholesterol $\xrightarrow{CHOD}$ 4-Cholesten-3-one + H_2O_2

H_2O_2 + Phenol + 4-AAP $\xrightarrow{POD}$ colored complex + H_2O

A. solutions and reagents

Table (2-6): Working Reagents for Total Cholesterol Assay

Reagent	Content	Concentration
Reagent (A)	Buffer	100mmol/L
	4-AAP	1mmol/L
	CHE	300U/L
	CHOD	300U/L
	POD	1500U/L
	Derivative of phenol	1mmol/L
Standard	cholesterol	200mg/dl

4-AAP: 4 aminoantipyrine ;CHE:cholesterol esterase; CHOD:cholesterol oxidase and POD:peroxidase.

B. Principle

1-Esterified cholesterol is hydrolyzed into free cholesterol and fatty acid by cholesterol esterase (CHE).

2-Cholesterol oxidase (CHOD) oxidizes the free cholesterol into cholestene-3-one with the formation of hydrogen peroxide.

3-In the of peroxidase (POD), hydrogen peroxide reacts with a derivative of phenol and 4-amino antipyrine to produce a color, the color intensity directly proportional to the total cholesterol concentration in the sample .

C. Preparation of reagent and procedure

Liquid reagent, bring to room temp.(15-25ºC) before use

Table (2-7): Procedure for Total Cholesterol Assessment.

Content	Blank	Sample	Standard
Reagent (A)	1000 µL	1000 µL	1000 µL
Water	10 µL		
Sample		10 µL	
Standard			10 µL

After addition, the solutions were mixed and incubated at least for 5 min at 37 ºC and the absorbance of samples and standards was measured at wavelength 510 nm

D. Calculation

The total cholesterol concentrations in samples calculated according to the equation were shown below:

Conc. (mg/dL) = Abs. of sample /Abs. of standard ×200 (standard value mg/dL).

2.6.2. Determination of Low-Density Lipoprotein Cholesterol Level

Quantitative determination of LDL cholesterol in serum .

A. solutions and reagents

Table (2-8): Working Reagents for Low-Density Lipoprotein Assay

Reagent	Content	Concentration
Reagent (A)	Good Buffer HDAOS	20Mm 1mM
Reagent (B)	Good Buffer Cholesterol esterase Cholesterol oxidase Peroxidase 4-AAP	20Mm 5.0U/mL 1.0U/mL 15U/mL 3.0U/mL

4-AAP: 4 aminoantipyrine and HDAOS:N(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline.

B. Principle

1- The protecting reagent binds to LDL and protects LDL from enzyme reactions when the sample mix with the reagent (A). The non-LDL lipoprotein [Chylomicrons (CM), VLDL, and HDL] react with cholesterol esterase and cholesterol oxidase, hydrogen peroxide produced is decomposed by catalase.

2-The hydrogen peroxide produced a color complex when reagent (B) is added, the enzymatic reaction was conducted solely on the LDL fraction. The oxidase condensation with HDAOS and 4-AAP in the presence of peroxidase.

C. Preparation of reagents and procedure

Liquid reagent, bring to room temp.(15-25 ºC) before use

Table (2-9): Procedure for Low-Density Lipoprotein Assessment

Content	Blank	Sample	Calibrator
Reagent (A)	300µl	300µl	300µl
Water	4µl		
Sample		4µl	
Calibrator			4µl
Reagent (B)	100µl	100µl	100µl

Mix, incubate at 37 ºC for 5 minutes, read the absorbance of a blank sample (Abx) against blank reagent then add reagent (B) Mix, incubate at 37 ºC for 5 minutes, read the absorbance of the sample(Ax), and calibrator(Ac) against blank reagent at wavelength 600nm.

D. Calculation

The Low-density lipoprotein concentration in samples is calculated according to the equation as shown below:

LDL (mg/dl) = (Ax-Abx)/(Ac-Abc)*calibrator value.

LDL-C = total cholesterol - (HDL-cholesterol + TG/5).

2.6.3. Determination of High-Density Lipoprotein Cholesterol Level

Quantitative determination of HDL cholesterol **(Trinder, 1969)**.

A schematic representation of the reaction is shown in the following equations:

HDL + LDL + VLDL + CM → HDL + (LDL+VLDL+CM)

HDL $\xrightarrow{\text{CHE.CHOD}}$ Fatty acids $+H_2O_2$

H_2O_2 + 4-AAP + HDAOS → colored complex $+H_2O$

A. solutions and reagents

Table (2-10): Working Reagents for High-Density Lipoprotein Assay

Reagent	Content	Concentration
Reagent (A)	Good Buffer	100mmol/L
	Polianions	1mmol/L
	4-AAP	4mmol/L
Reagent (B)	Cholesterol esterase	800U/L
	Cholesterol oxidase	500U/L
	Peroxidase	1500U/L
	HDAOS	1mmol/L
	Detergent	4mmol/L

4-AAP: 4 aminoantipyrine and HDAOS:N(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline.

B. Principle

1- The first phase of specific polianions blocks the interfering lipoprotein (LDL, VLDL, and chylomicrons) .

2- A specific surface-active agent inhibits the coloration of VLDL, LDL, and chylomicrons in the second phase .

3-The intensity of color produced is directly proportional to the HDL .

C. Preparation of reagents and procedure

Liquid reagent, bring to room temp.(15-25 ºC) before use

Table (2-11): Procedure for High-Density Lipoprotein Assessment

Content	Blank	Sample	Calibrator
Reagent (A)	300µL	300µL	300µL
Water	4µL		
Sample		4µL	
Calibrator			4µL
Reagent (B)	100µL	100µL	100µL

Mix, incubate at 37 ºC for 5 minutes, read the absorbance of a blank sample (Abx) against blank reagent then add reagent (B) Mix, incubate at 37 ºC for 5 minutes, read the absorbance of the sample(Ax), and calibrator(Ac) against blank reagent at wavelength 600nm.

D. Calculation

The high-density lipoprotein concentration in samples is calculated according to the equation as shown below:

HDL -C (mg/dl)= (Ax-Abx)/(Ac-Abc)*calibrator value.

2.6.4. Determination of Triglycerides Level

Quantitative determination of triglyceride **(Fossati and Prencipe, 1982).**

A schematic representation of the reaction is shown in the following equations:

Triglyceride $\xrightarrow{\text{LPL}}$ Glycerol + Fatty acids

Glycerol + ATP $\xrightarrow{\text{GK}}$ Glycerol-1-phosphate + ADP

Glycerol-1-phosphate + O_2 $\xrightarrow{\text{GPO}}$ Dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-AAP + TOOS $\xrightarrow{\text{POD}}$ colored complex + H_2O

A. solutions and reagents

Table (2-12): Working Reagents for Triglycerides Assay

Reagent	Content	Concentration
Reagent (A)	Good Buffer	100mmol/L
	Magnesium chloride	15mmol/L
	ATP	4mmol/L
	4-AAP	1mmol/L
	TOOS	0.1mmol/L
	LPL	2500U/L
	POD	1800U/L
	GK	1000U/L
	GPO	5500U/L
Standard	Glycerol	200mg/dl

4-AAP:4 aminoantipyrine ;TOOS: N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt ; LPL :lipoproteinlipase ; POD :peroxidase; GK :glycerol kinase and GPO: glycerol phosphate oxidase.

B. Principle

1-Free fatty acids and glycerol the product of hydrolyzed triglycerides by lipoproteinlipase.

2-The glycerol participates in a series of coupled enzymatic reactions, the H2O2 is generated due to the involvement of glycerol kinase (GK) and glycerol phosphate oxidase (GPO).

3- The hydrogen peroxide reacts with TOOS and 4-AAP to form a colored complex**.**

C. Preparation of reagent and procedure

Liquid reagent, bring to room temp.(15-25 ºC) before use

Table (2-13): Procedure for Triglycerides Assessment

Content	Blank	Sample	Standard
Reagent (A)	1000 µL	1000 µL	1000 µL
Water	10 µL		
Sample		10 µL	
Standard			10 µL

After addition, the solutions were mixed and incubated at least for 5 min at 37 ºC and the absorbance of samples and standards were measured at wavelength 546 nm.

D. Calculation

The concentrations of triglycerides in samples were calculated according to the equation below:

TG in (mg/dL) = A (Sample)/ A (Standard) x 200 (standard value) mg/dL.

2.7. Determination of fasting blood glucose (FBG)

Determined by a clinical chemistry analyzer(Monarch 240, Biorex Diagnostic, United Kingdom).

A. Principle

Enzymatic indicator test based on the Trinder reaction quantified by the formation of a pink quinoneimine dye. In this reaction, glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed is catalyzed by peroxidase and reacts with phenol and 4-aminoantipyrine to form the dye indicator.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} H_2O_2 + \text{Gluconic acid}$$

$$H_2O_2 + \text{Phenol} + 4\text{Aminoantipyrine} \rightarrow 4H_2O + \text{Quinoneimine}$$

B. solutions and reagents

Table (2-14): Working Reagents for Fasting Blood Glucose Assay

Reagent	Content	Concentration
Glucose reagent	Glucose Oxidase	>15U/mL
	Peroxidase	>1U/mL
	4-aminoantipyrine	0.7mmol/L
	Phenol	11mmol/L
	MOPS Buffer	0.05mol
	Phosphate Buffer	0.025mol

MOPS=3-(N-morpholino) propane sulfonic acid .

C. Procedure

Table (2-15): Procedure for Fasting Blood Glucose Assessment

Compound	Reagent Blank	Standard/ sample
Standard/sample		10µL
Glucose Reagent	1mL	1mL

Mix and incubate for 10 min at 15-25 ºC in the water bath and then the absorbance was measured at 500 nm.

D. Calculation

The concentrations of fasting blood glucose in samples were calculated according to the equation below:

Glucose conc.(mg/dl)=Sample abs./Standard abs. *Standard conc.

2.8. Enzyme-Linked Immunosorbent assay (ELISA) tests

Enzyme-Linked Immunosorbent Assay system (ELISA) was done using a sandwich method to determine the concentrations.

2.8.1. Detection of Human Neudesin Neurotrophic Factor (NENF) ELISA Kit

A- Principle

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Neudesin Neurotrophic Factor(NENF). Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to Neudesin Neurotrophic Factor(NENF). Next, Avidin conjugated to Horseradish

Peroxidase (HRP) is added to each microplate well and incubated. After the TMB substrate solution is added, only those wells that contain Neudesin Neurotrophic Factor(NENF), biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a color change. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of (450nm ± 10nm). The concentration of Neudesin Neurotrophic Factor(NENF) in the samples is then determined by comparing the optical density of the samples to the standard curve.

B-Kit components

Table (2-16): Working Reagents for Neudesin Neurotrophic Factor Assay

Reagents	Quantity
Pre-coated Microplate	12 strips x 8 wells
Standard (lyophilized)	2
Standard Diluent Buffer	20mL
Biotinylated Antibody (100×)	120 µL
Biotinylated Antibody Diluent	12mL
Streptavidin-HRP (100×)	120 µL
HRP Diluent	12mL
Wash Buffer (25×)	20mL
TMB Substrate Solution	9mL
Stop reagent	6mL
Plate Covers	2
Instruction manual	1

HRP:Horseradish Peroxidase and TMB:3,3,5,5-Tetramethylbenzidine.

C. Reagent Preparation

1. Bring all kit components and samples to room temperature (18-25°C) before use.

2. If the kit will not be used up at one time, only take out strips and reagents for the present experiment, and save the remaining strips and reagents as specified.

3. Dilute the 25x wash buffer into 1x working concentration with double steaming water.

4. **Standard working solution**-Reconstitute the Standard with 1.0mL of Standard Diluent, kept for 10 minutes at room temperature, and shake gently(not to foam). The concentration of the standard in the stock solution is 10 ng/mL. Please prepare 7 tubes containing 0.5mL Standard Diluent and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 10 ng/mL, 5ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, 0.32 ng/mL, 0.16 ng/mL, and the last EP tubes with Standard Diluent is the blank as 0 ng/mL. To guarantee the experimental result's validity, please use the new standard solution for each experiment.

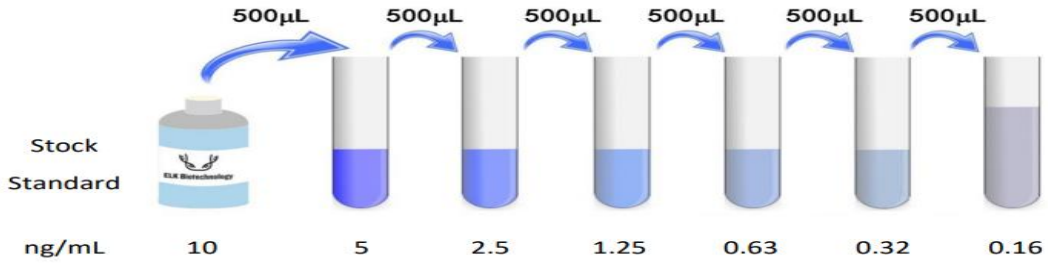


Figure (2.2): Serial Dilution Method for Neudesin Standard.

5. **Biotinylated Antibody and Streptavidin-HRP**: Briefly spin or centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6**. TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial

D. Procedure

This test was done according to the manufacturer's instructions and as the following:

1. Determined wells for diluted standard, blank, and sample. Prepared 7 wells for standard and 1 well for blank. Added (100 µL) each of the standard working solutions,or 100 µL of samples into the appropriate wells. Covered with the Plate sealer. Incubated for 80 minutes at 37°C.

2. Removed the liquid from each well. Aspirated the solution and wash with (200 µL) of 1× Wash Solution to each well and let it sit for 1-2 minutes. Removed the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Washed 3 times. After the last wash, removed any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

3. Added (100 µL) of Biotinylated Antibody working solution to each well, covered the wells with the plate sealer, and incubated for 50 minutes at 37°C.

4. Repeated the aspiration, and wash process a total of 3 times as conducted in step 2.

5. Added (100 µL) of Streptavidin-HRP working solution to each well, covered the wells with the plate sealer, and incubated for 50 minutes at 37°C.

6. Repeat the aspiration, and wash process a total of 5 times as conducted in step 2.

7. Added (90 µL) of TMB Substrate Solution to each well. Covered with a new Plate sealer. Incubated for 20 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid turn blue with the addition of the TMB Substrate Solution.

8. Added (50 µL) of Stop reagent to each well. The liquid turn yellow with the addition of the Stop reagent. Mixed the liquid by tapping the side of the plate. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop reagent is done the same as that of the TMB Substrate Solution.

9. Removed any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at (450 nm) immediately.

E. Calculations

A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and a best-fit curve was drawn through the points on the graph .These calculations can be best done with computer-based curve-fitting software and the best-fit line can be determined by regression analysis. The result was expressed in (ng/mL) as in an appendix.

2.8.2 Detection of Human Neuregulin 4 (NRG4) ELISA Kit

A- Principle

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Neuregulin 4(NRG4). Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to Neuregulin 4(NRG4).

Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After the TMB substrate solution is added, only those wells that contain Neuregulin 4(NRG4), biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a color change. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of Neuregulin 4(NRG4) in the samples is then determined by comparing the optical density of the samples to the standard curve.

B-Kit components

Table (2-17): Working Reagents for Neuregulin 4 Assay

Reagents	Quantity
Pre-Coated Microplate	12 strips x 8 wells
Standard (Lyophilized)	2 vials
Biotinylated Antibody (100×)	120 µL
Streptavidin-HRP (100×)	120 µL
Standard/Sample Diluent Buffer	20 mL
Biotinylated Antibody Diluent	12 mL
HRP Diluent	12 mL
Wash Buffer (25×)	20 mL
TMB Substrate Solution	9 mL
Stop Reagent	6 mL
Plate Covers	2 pieces

HRP:Horseradish Peroxidase and TMB: 3,3,5,5-Tetramethylbenzidine.

C. Reagent Preparation : As mentioned in 2.8.1.C

D- Procedure

1. Determined wells for Diluted Standard, Blank, and Sample. Prepared 7 wells for Standard and 1 well for Blank. Added (100 µL) each of Standard Working Solution or 100µL of samples into the appropriate wells. Covered with the Plate sealer. Incubated for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Removed the liquid from each well. Aspirated the solution and wash with (200 µL) of 1×Wash Solution in each well and let it sit for 1-2 minutes. Removed the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, removed any remaining

Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

3. Added (100 µL) of Biotinylated Antibody Working Solution to each well, covered the wells with the plate sealer, and incubated for 50 minutes at 37°C.

4. Repeated the aspiration, and wash process a total of 3 times as conducted in step 2.

5. Added (100 µL) of Streptavidin-HRP Working Solution to each well, covered the wells with the plate sealer, and incubated for 50 minutes at 37°C.

6. Repeated the aspiration, and wash process a total of 5 times as conducted in step 2.

7. Added (90 µL) of TMB Substrate Solution to each well. Covered with a new Plate sealer. Incubated for 20 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid turn blue with the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

8. Added (50 µL) of Stop Reagent to each well. The liquid turn yellow with the addition of Stop Reagent. Mixed the liquid by tapping the side of the plate. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

9. Removed any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at (450 nm) immediately.

E. Calculation :As mentioned in 2.8.1.E

2.9.Statistical Analysis:

Information from the questionnaire from all participants was entered into a data sheet and was assigned a serial identifier number. Multiple entries were used to avoid errors. The data analysis for this work was generated using The Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA,) and the Real Statistics Resource Pack software (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020). Descriptive statistics were performed on the participants' data of each group (Mean ± SD)and Med (Min-Max). Values were illustrated by n (%) for categorical. The distribution of the data was checked using the Shapiro-Wilk test as numerical means of assessing normality . The Kruskal Wallis test was used to compare between the groups were P-value 0.05 statically significant and Dunn's test post hoc test ,were the data not normally distribution .

The association between the analyzed factors was estimated using odds ratios (ORs) and a 95% Confidence Interval Range which was calculated by a non-conditional logistic regression.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p-values <0.05 (two-sided) were considered to be statistically significant.

The optimal threshold with high specificity and sensitivity for PCOS phenotypes was detected using receiver operating characteristic (ROC) analysis.

Chapter Three

Results

3. Result

3.1. Demographic and Clinical Characteristics

There were a total of 210 females in this research, seventy samples were collected from normal participants as a healthy control, and other 140 PCOS. Patients were divided into subgroups (A, B, C, and D) based on Rotterdam Criteria. The demographic, laboratory, and metabolic indices of the study group's population were summarized in table (3-1).

Table (3-1): The Demographic Characteristics, Metabolic Indices and Biomarker Levels in PCOS Patients and Controls.

	(Mean ± SD)	Median (Min-Max)	(Mean ± SD)	Median (Min-Max)
Demographic Parameters				
Age (years)	(24.66±4.89)	24(18-38)	(27.60±4.10)	28(21-43)
BMI(Kg/m^2)	(28.98±5.4)	28.6(19.4-41.6)	(23.64±1.96)	23.6(19.2-29)
WHR	(0.84 ±0.05)	0.84(0.70-0.95)	(0.79±0.05)	0.78(0.72-0.94)
Hormonal Parameters				
LH(m.lu/mL)	(9.60±4.04)	8.94(2.12-21.70)	(4.40±1.15)	4.40(2.14- 6.94)
FSH(m.lu/mL)	(5.76±1.53)	5.50(2.90- 10.32)	(8.64±1.53)	8.74 (5.73-11.58)
LH/FSH	(1.79±0.79)	1.68(0.45- 4.06)	(0.51±0.12)	0.49(0.31- 0.76)
Prolactin (ng/mL)	(17.46±6.66)	16.83(5.31-30)	(15.81±5.53)	15.80(4.96-25)
TSH(ulU/mL)	(2.24±0.78)	2.26(0.63-4.05)	(2.30±0.48)	2.26(0.80- 3.20)
Progesterone (ng/mL)	(1.68±3.17)	0.60(0.16-16.61)	(14.95±3.03)	14.86(9.18-19.58)
FT(pg/ mL)	(1.96±1.05)	1.70(0.55-4.90)	(0.83±0.44)	0.91(0.12-1.52)
Lipid Profile				
Cholesterol (mg/dl)	(158.49±29. 63)	161(91-237)	(156.41±22.4 4)	161(110-198)
Triglycerides (mg/dl)	(86.41±28.5 5)	84(35-170.58)	(74.43± 15.13)	75.93(40-112)

LDL (mg/dl)	(88.66±26.7)	86.7(39.4-158.78)	(70.59±16.3)	71(40-99.60)
HDL (mg/dl)	(54.98±7.11)	55(40.31-74)	(65.83±4.86)	64.56(58-77)

Insulin Resistance Index				
HOMA-IR	(3.17±0.94)	3.10(1.09-5)	(1.38±0.33)	1.29(1.02-2.40)
Proposed Biomarkers for PCOS Phenotypes				
NRG4 (ng/mL)	(8.87±3.03)	8.90(2.83-17.39)	(7.09±4.11)	5.40(2.40-9.643)
NENF (ng/mL)	(1.24±0.59)	1.08(0.37-2.81)	(0.79±0.37)	0.70(0.34-1.82)
Metabolic Indices				
VAI	(1.19±0.49)	1.14 (0.33-2.41)	(0.81 ± 0.21)	0.79 (0.30-1.27)
LAP	(23.7±14.81)	20.09 (3.30-79.04)	(17.45 ± 11.7)	13.20 (5.10-56.70)
BMR	(1485.4±142.34)	1465.28 (1148.6-1997.8)	(1412.67±106 .16)	1377.5 (1253.3-1695.5)
BAI	(33.74±6.73)	33.09 (13.86-56.17)	(29.79 ± 6.37)	27.95 (19.76-47.06)

Table Note: Data expressed as mean ± standard deviation (SD) and median (interquartile range); BAI: body adiposity index; BMI: body mass index; BMR: basal metabolic rate; WHR: waist hips ratio; FT: free testosterone; FSH: follicle-stimulating hormone; LAP: lipid accumulation product; LH: luteinizing hormone; LDL: low-density lipoprotein; HDL: high-density lipoprotein; HOMA-IR: homeostasis model assessment of insulin resistance; TSH: Thyroid-stimulating hormone; NRG4: Neuregulin4 NENF: Neudesin; VAI: Visceral Adiposity Index.

The analysis data for the hirsutism score (modified Ferriman-Gallwey) was shown that about (75%) of the participants were having hirsutism, Also the present and absent of acne were estimated to be 57.85%, and 42.14% respectively, as well as alopecia was present in 83.57% and while absent in 16.42% , as shown in figure (3.1)

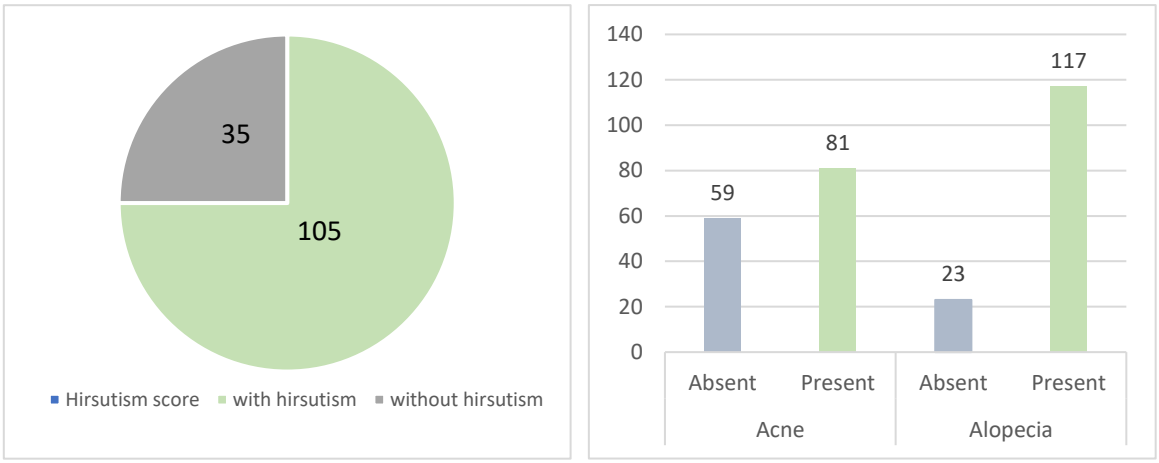


Figure (3.1): Descriptive of the Demographic Characteristics of the Study Patients (N=140).

The analysis of ovulatory dysfunction was revealed that about 117(83.5%) of the PCOS group were have oligo-amenorrhea that represents groups (A, B, and D) of patients, while 23(16.5%) have regular menstrual group (C). The investigation of ovarian morphology showed that 120 have PCO in groups (A, C, and D) of patients and only 20 participants were having normal morphology as in group (B) as presented in figure (3.2)

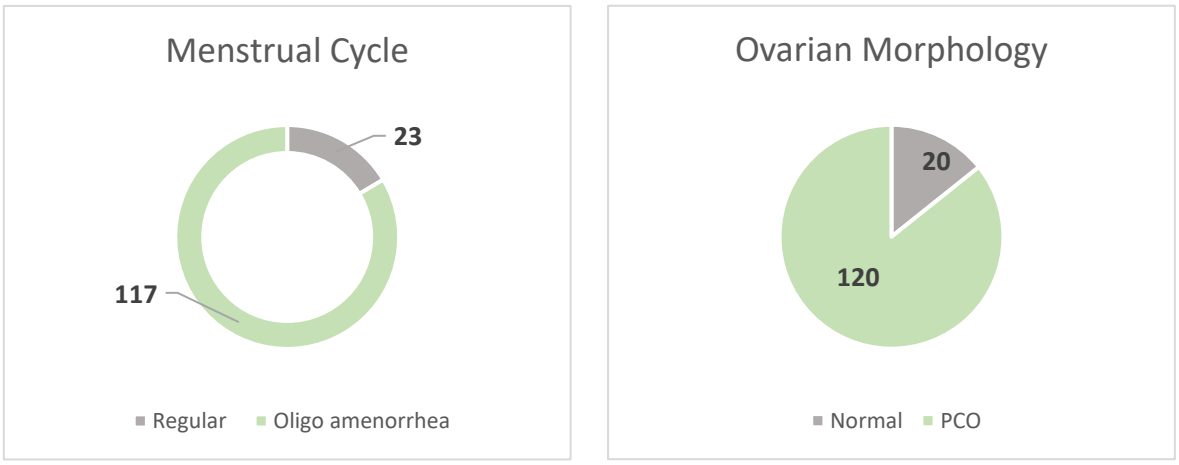


Figure (3.2): Descriptive of the Demographic Characteristics of the Study Patients (N=140).

Smoking is an important modifiable risk factor, a complex mixture of chemical substances contained in cigarette smoke can exert a composite effect on different targets such ovary and uterus. It was examined, most of the patients (57.14%) were confirmed to be passive smokers, while (37.14%) were non-smoking, and the remaining were active as summarized in figure (3.3)

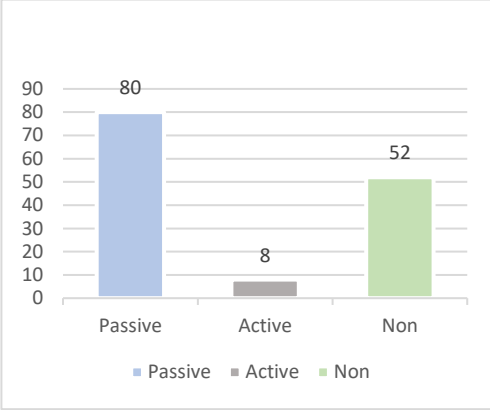


Figure (3.3): Descriptive of the Demographic Characteristics of the Study Population (N=140).

The screening of data for the status of the study population that most patients (37.14%) were married without a child (primary infertility), (26.42%) were single, and (22.85% and13.57%) were married with one child and married with two children(secondary infertility) respectively as shown in figure(3.4)

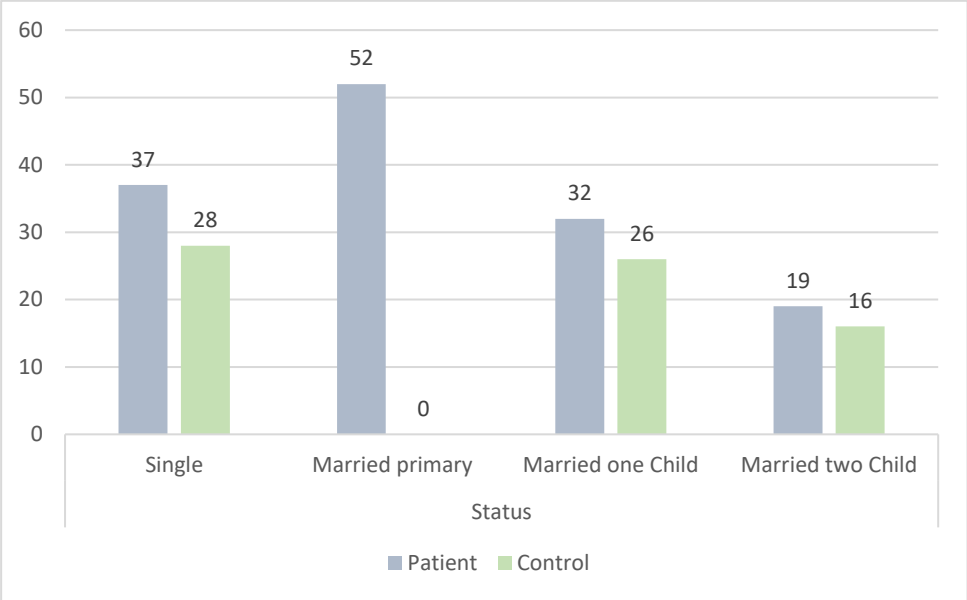


Figure (3.4): Descriptive of the Demographic Characteristics of the Study Population (N=210).

3.2 Difference Between the Level of Parameters with Age in the Patient and Control Groups for Group 1 (18 - 27) Years and Group 2 (28-38) Years

Age is the most important determining factor affecting female fertility because fertility declines rapidly after 35 years of age. In the current study, the mean ± SD age of the cases was 24.66± 4.89 . The age range of participants was within (18-35) years old, (67.14%) of the patient was within (18- 27) year, while (32.86%) of the patient were within the age range (28- 38) years, figure (3.5). Diagnosis of PCOS mostly starts in the first year after marriage in females with infertility. Polycystic ovarian syndrome is reported to be more prevalent in younger (<35 years) than older females. The following figures illustrated the mean level of biochemical in the Patients and control groups according to Age. In the age range of (18- 27), all parameters NENF, NRG4, VAI, LAP, BMR, and BAI were increased significantly in patients compared to control, with p-value < 0.05. But the range of age groups (28-38) years just in NENF, VAI, and BMR were highly statistically significant in patients compared with control groups, with p-value < 0.05, as shown in figure (3.5).

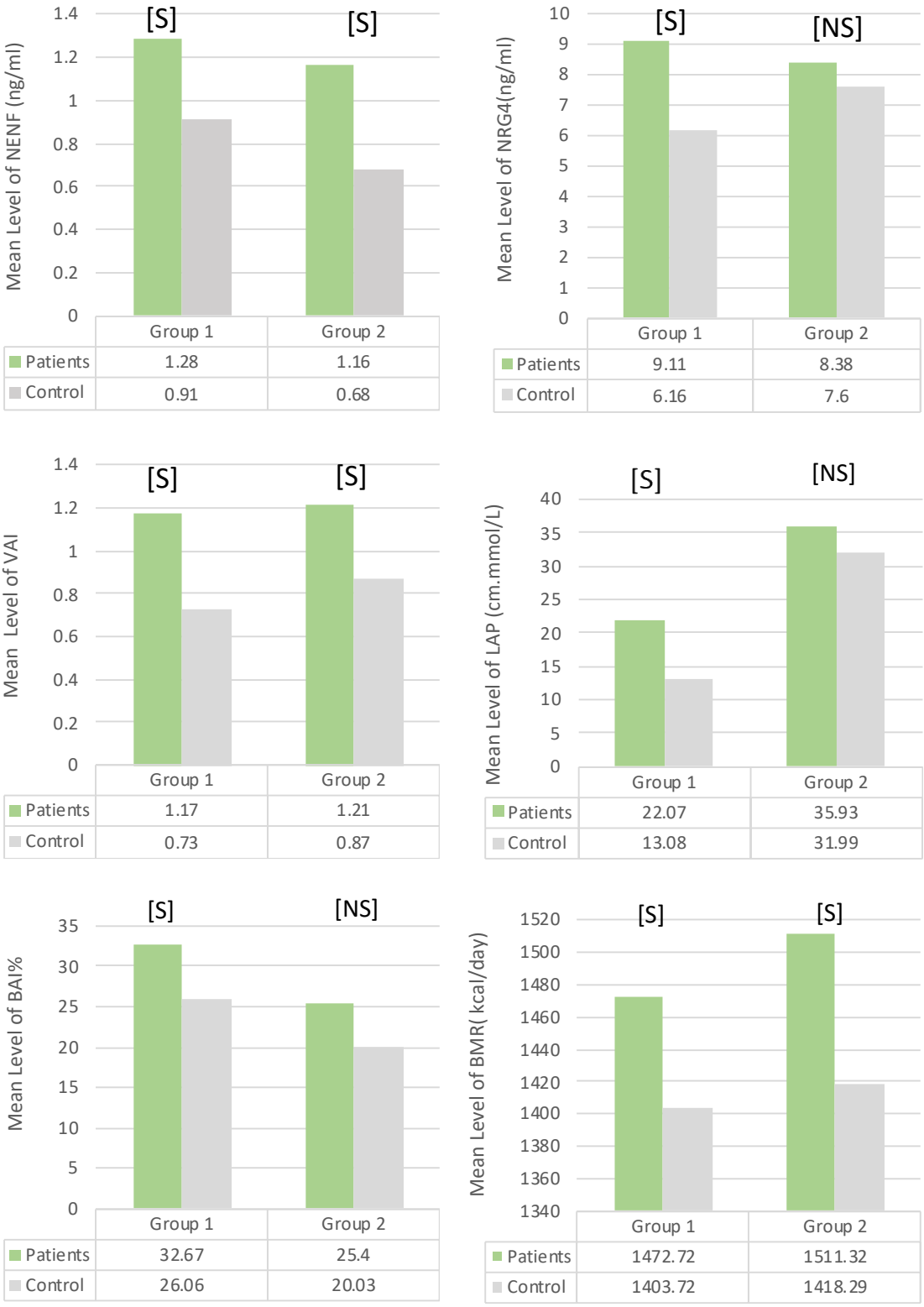


Figure (3.5): Difference Between Mean Levels of Parameters with Age in PCOS patients and control groups

3.3 Examination of the Levels of NENF, NRG4 , VAI, LAP, MBR, and BAI for PCOS Patients Group Compared to the Control Group

Generally, patients with the polycystic ovarian syndrome showed an increasing range level of the NENF, NRG4, VAI, LAP, BMR, and BAI in comparison to healthy populations. Results indicated a statistically significant variation in NENF, NRG4, VAI, LAP, BMR, and BAI levels among groups, The means level was presented in Fig (3.6). The mean levels of NENF, NRG4, VAI, LAP, BMR, and BAI patients were (1.24±059) , (8.87±3.03) (1.19±0.49), (23.70±14.81), (1485.40±142.34) and (33.74±6.73), and which were significantly higher than for control group ($p \leq 0.001$).

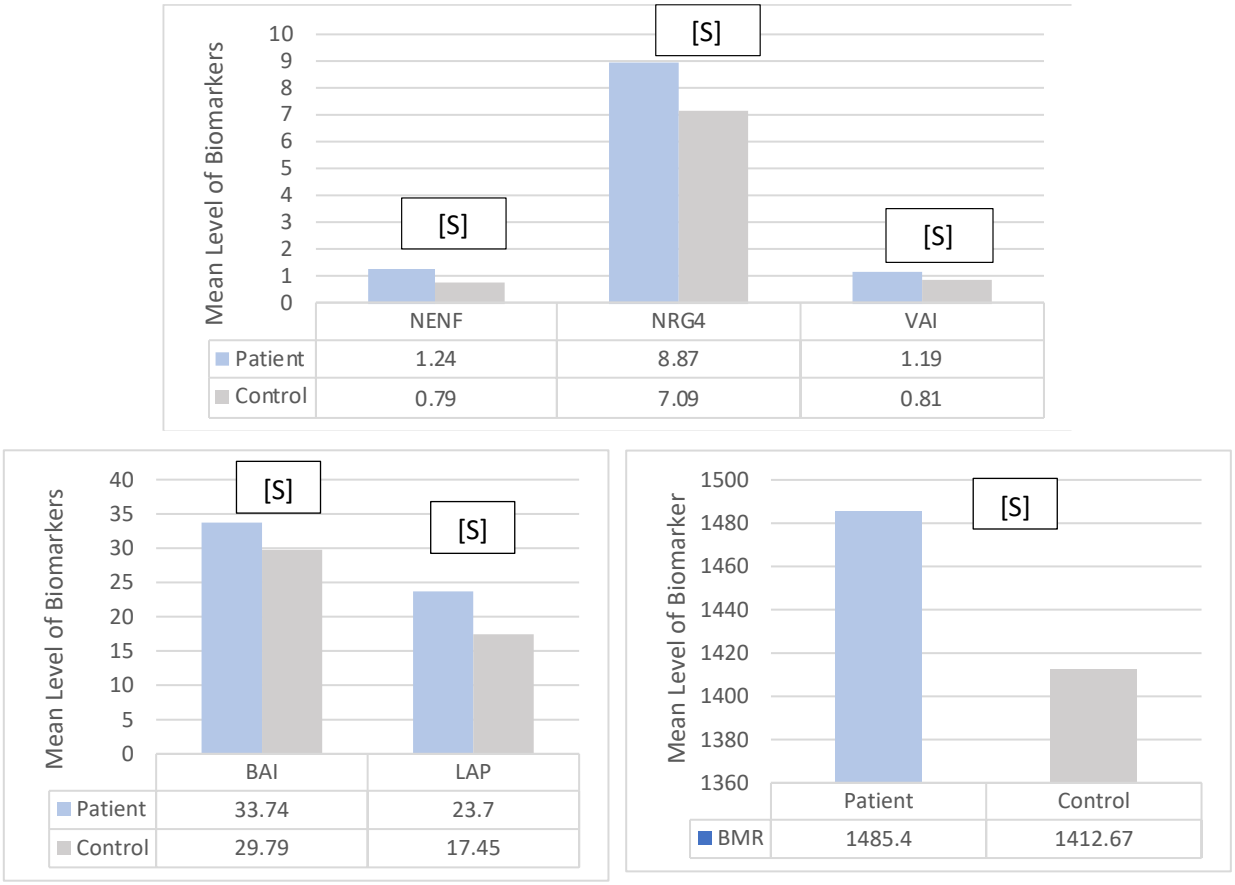


Figure (3.6): The Distribution of Serum Levels of NENF , NRG4, VAI, LAP, BMR, and BAI in the PCOS Patients Group Compared to the Control Group.

3.4. Distribution of Various PCOS Phenotypes

In this research, the classic PCOS phenotype (phenotype A) was the most prevalent, encompassing all three characteristics: HA, OD, and PCOM. It had a prevalence of 49.28% (69 females). The prevalence of phenotypes (B, C, and D) were 14.28% (20 females), 16.42% (23 females), and 20% (28 females) and the demographic and laboratory for phenotypes as shown in table (3-2).

Table (3-2): The Demographic Characteristics, Laboratory, in PCOS Phenotypes and Control .

	Phenotype A (N:69) Med(Min-Max)	Phenotype B (N:20) Med(Min-Max)	Phenotype C (N:23) Med(Min-Max)	Phenotype D (N:28) Med(Min-Max)	Control (N:70) Med(Min-Max)
Age(years)*	23 (18-38)	23 (18-38)	25 (18-35)	24 (18-37)	28 (21-43)
BMI(Kg/m^2)*	30.2 (19.5-41.6)	26.5 (20-36.2)	28.2 (20.8-35.4)	26.7 (19.4-36.3)	23.6 (19.2-29)
WHR*	0.85 (0.77-0.95)	0.85 (0.76-0.91)	0.85 (0.75-0.93)	0.84 (0.702-0.94)	0.78 (0.72-0.94)
LH(m.lu/mL)*	10.86 (5.8-21.7)	9.6 (4.5-21)	7.9 (6.09-15.2)	5.4 (2.1-6.8)	4.40 (2.14- 6.94)
FSH(m.lu/mL)*	5.64 (2.9-10.3)	5.3 (3.3-7.6)	5.1 (3.4-7.4)	5.8 (3.9-8.4)	8.74 (5.73-11.58)
LH/FSH*	2.06 (1.6-4.06)	1.9 (1.3-3.8)	1.7 (1.1-3.4)	0.8 (0.45-0.9)	0.49 (0.31- 0.76)
TSH(ulU/mL)	2.26 (1.1-4.05)	2.3 (1.4-4)	2.3 (0.8-3.8)	1.9 (0.6-4)	2.26 (0.80- 3.20)
Pro(ng/mL)	17.87 (5.31-30)	13.9 (5.3-30)	12.5 (6.2-30)	18.3 (5.8-30)	15.80 (4.96-25)
F.T(pg/ mL)*	2.5 (0.7-4.9)	1.7 (0.7-3.2)	2.1 (1-4.5)	1.5 (0.55-3.1)	0.91 (0.12-1.52)
Prog.(ng/mL)*	0.59 (0.16-5)	0.69 (0.33-2.26)	0.53 (0.26-16.61)	0.49 (0.2-2.59)	14.86 (9.18-19.5)
HOMA-IR*	3.51 (2.2-5)	2.85 (1.5-4.3)	3.3 (1.9-4.8)	2.4 (1.09-3.7)	1.29 (1.02-2.40)
LDL-C(mg/dl)*	95 (50-159)	76 (41-157.5)	83 (52-123.8)	85.6 (39.4-136.8)	71 (40-99.60)
HDL-C(mg/dl)*	55 (45-70)	50.5 (40.3-66)	54 (42.8-68)	54.5 (42-74)	64.56 (58-77)
TG(mg/dl)*	91 (50-167)	75.1 (44-170.5)	81 (40-163)	66.5 (35-114.5)	75.93 (40-112)
TC(mg/dl)*	167 (94-237)	140.5 (91-205)	154 (123-198)	159.5 (106-230)	161 (110-198)
NENF(ng/mL)*	1.28	1.03	1.30	1.27	0.70

	(0.39-2.67)	(0.37-2.38)	(0.36-2.81)	(0.47-2.6)	(0.34-1.82)
NRG4(ng/mL)*	9.6 (4.4-17.3)	6.7 (2.9-12.9)	7.9 (2.8-14.5)	7.6 (3.3-14.3)	5.40 (2.40-9.643)
VAI*	1.25 (0.62-2.41)	1.05 (0.6-2.2)	1.13 (0.43-2.29)	0.82 (0.33-2.02)	0.79 (0.30-1.27)
LAP*	21.6 (4.25-77.1)	19.5 (3.3-43.8)	20.9 (7.7-79.04)	14.18 (3.8-47.3)	13.20 (5.1-56.7)
BAI*	43.9 (23.9-56.2)	31.9 (25.9-47.8)	32.8 (25.1-44.8)	30.5 (13.9-50)	27.95 (19.76-47.06)
BMR*	1500 (1236.2-1997.8)	1399 (1148.6-1646.4)	1432.8 (1304.1-1645.8)	1427.4 (1235.5-1732.7)	1377.5 (1253.3-1695.5)

*** $p<0.05$ considered significantly different**

3.5. Examination of The Level of NENF, NRG4, VAI, LAP, BMR, and BAI for the PCOS Phenotype Group Compared to the Control Group

Dunn's test for multiple comparisons was used to determine exactly which groups are different the significance level is 0.05. The NRG4 level in comparing phenotypes vs. phenotypes (B-A), control(Co) vs. phenotypes (Co-A) were significant. As well as, NENF levels were nonsignificant in phenotypes vs. phenotypes, while significant in comparison control to phenotypes (Co-A and Co-D) . VAI were significant in comparing phenotypes vs. phenotypes (D-A) , were significant control vs. phenotypes (Co-A and Co- C). LAP in phenotypes vs. phenotypes (D-A) and control vs. phenotypes (Co-A) were significant .BAI in comparing phenotypes vs. phenotypes non significent,while control vs. phenotypes (Co-A) were significant. BMR were nonsignificant in comparing phenotypes vs. phenotypes while control vs. phenotypes was significant (Co-A) .as showed in figure(3.7)

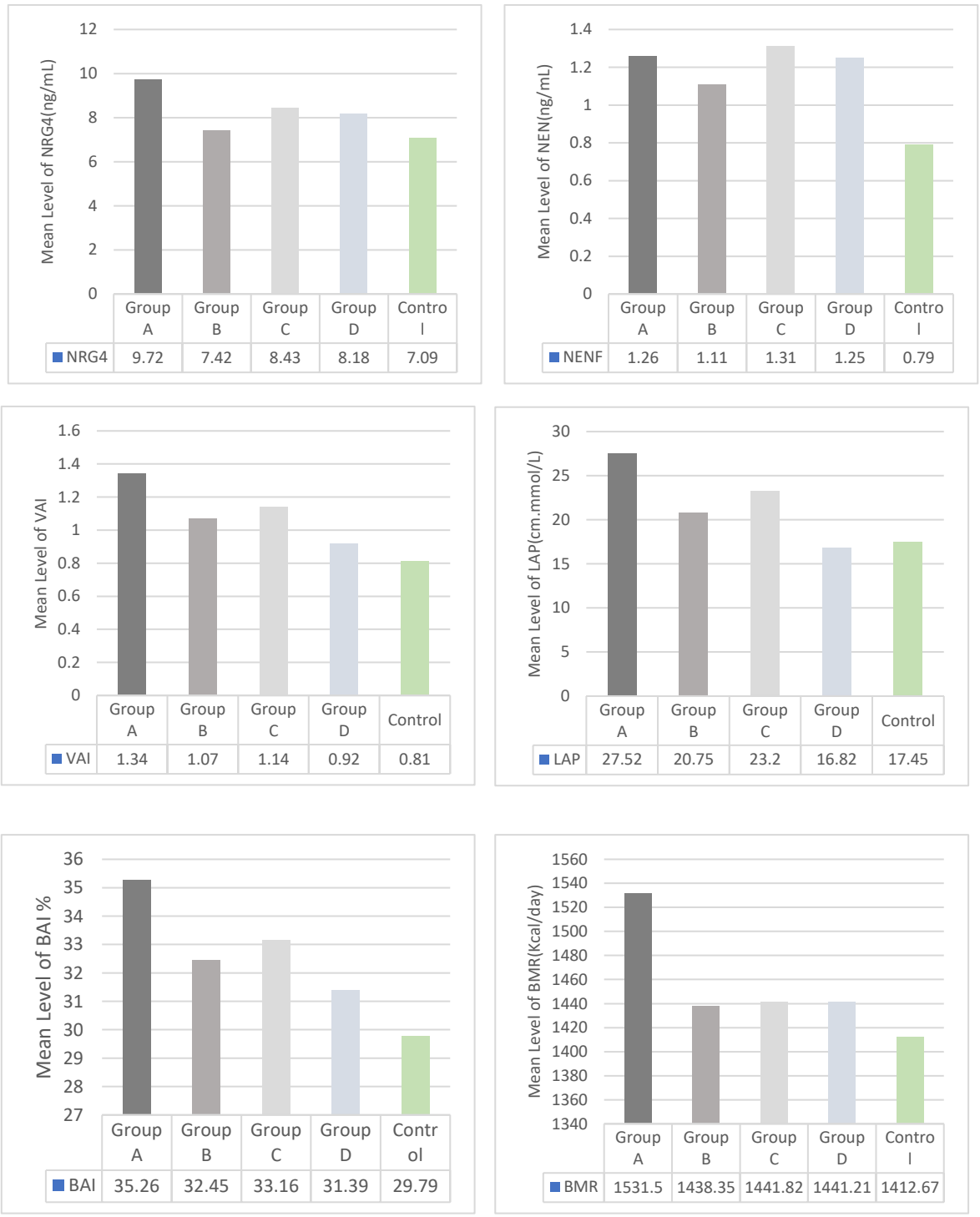


Figure (3.7): Mean Differences in Serum Level of parameters (NENF, NRG4 VAI, LAP, BMR, and BAI) for PCOS Phenotype Group Compared to Control Group.

3.6 Study the Correlation of the Measured Parameters in PCOS patient group

Considering the important role of the measured parameters in PCOS patients, the Spearman rank test analysis of such patients was used to show the response relationship between parameters. The correlation study demonstrated many significant relationship among the measured parameters. P values were < 0.001 Also all the measured lipid profiles demonstrated high positive significant results to each other, as shown in figure (3.8)

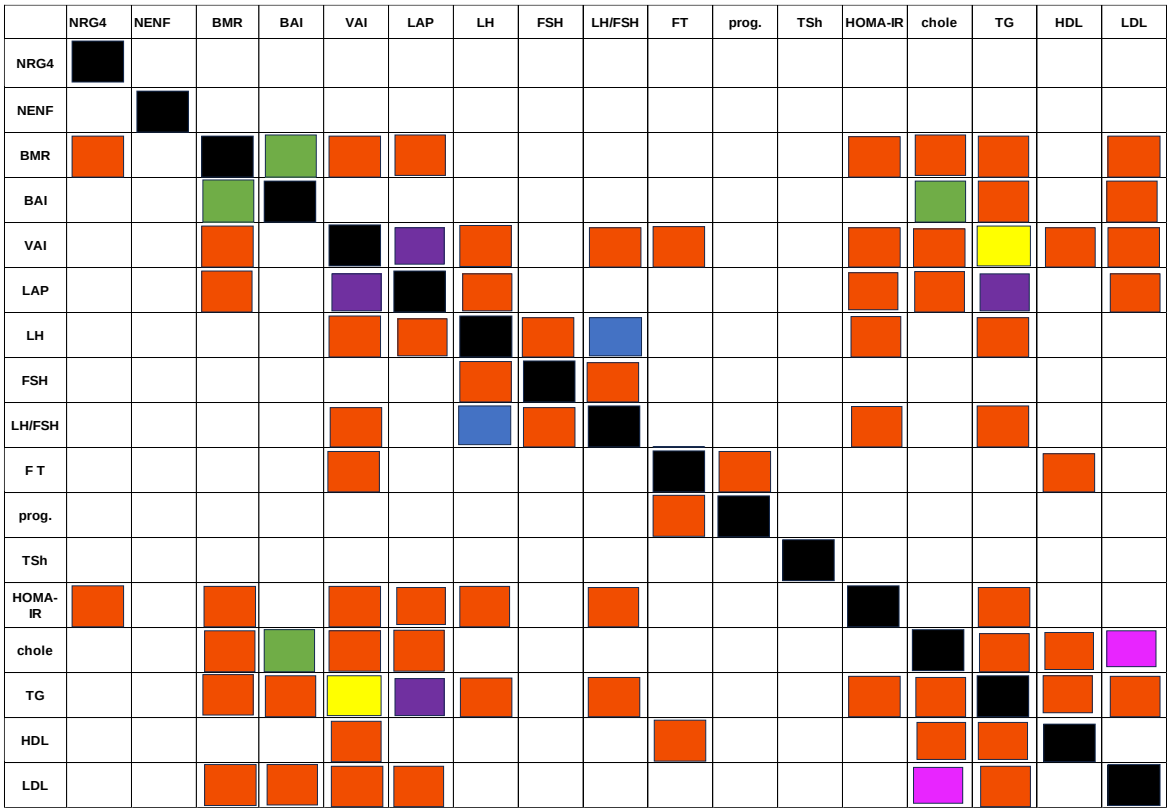


Figure (3.8): Heatmap of Patient Analysis of the Spearman Rank Test. Data express White boxes show the absence of a correlation (p>0.05), whereas colored boxes indicate statistically significant direct and indirect correlations. The following relationship is indicated by the degree of intensity of the color: Yellow (r=0.9); Pink (r=0.8); blue(r=0.7); purple (r=0.6); green (r=0.5), and Red (r=0.4). BAI: body adiposity index; BMR: basal metabolic rate; Chole: Cholesterol ; FT: free testosterone; FSH: follicle-stimulating hormone; LAP: lipid accumulation product; LH: luteinizing hormone; LDL: low-density

lipoprotein; HOMA-IR: homeostasis model assessment of insulin resistance; HDL: high-density lipoprotein; TSH: Thyroid-stimulating hormone; TG: Triglyceride NRG4: Neuregulin4 ; NENF: Neudesin; Prog: progesterone;VAI: Visceral Adiposity Index.

3.7: Study the Association of Parameters with PCOS Phenotypes Group

Multinominal logistic regression was performed to analyze the association of the NRG4, NENF , VAI, LAP, BMR, and BAI with the PCOS phenotype group. It was found that all the parameters were highly significant differences in PCOS phenotypes group , while only BMR was significant in group A. NRG4 was represented as a risk factor in Phenotype A followed by phenotype (C, D and B). on the other hand, NENF considered as twice as risk significance factor in phenotype B, then in phenotype (A and D). VAI consider a fifteen-time risk in phenotype A than in phenotype D. LAP ,BMR, and BAI were highly risk in phenotype A as shown in table (3.3).

Table (3-3): The Multinomial Logistic Regression of PCOS Phenotype Groups with Parameters.

Variable	Groups	OR (Lower – upper)	P value
NRG4			
	Group A	1.26(1.13-1.40)	**<0.001[S]**
	Group B	1.03(0.88-1.20)	**0.023[S]**
	Group C	1.13(0.98-1.30)	**0.001[S]**
	Group D	1.10(0.96-1.27)	**0.004[S]**
	Control Group	1[a]	-
NENF			
	Group A	8.75(2.98-25.62)	**0.003[S]**
	Group B	5.56(1.56-19.87)	**0.008[S]**
	Group C	10.06(3.01-33.61)	**<0.001[S]**
	Group D	8.54(2.63-27.67)	**<0.001[S]**
	Control Group	1[a]	-
VAI			
	Group A	45.99 (12.99-162.77)	**<0.001[S]**
	Group B	12.40 (2.71-56.67)	**<0.001[S]**
	Group C	18.13 (4.28-76.75)	**<0.001[S]**

	Group D	3.64 (0.80-16.51)	**0.005[S]**
	Control Group	1[a]	-
LAP			
	Group A	1.05 (1.02-1.09)	**<0.001[S]**
	Group B	1.02 (0.98-1.07)	**<0.001[S]**
	Group C	1.04 (1.00-1.08)	**<0.001[S]**
	Group D	0.99 (0.95-1.03)	0.07[NS]
	Control Group	1[a]	-
BMR			
	Group A	1.007(1.004-1.010)	**0.003[S]**
	Group B	1.002 (0.998-1.006)	0.356[NS]
	Group C	1.002 (0.998-1.006)	0.273[NS]
	Group D	1.002 (0.998-1.006)	0.250[NS]
	Control Group	1[a]	-
BAI			
	Group A	1.14 (1.07-1.21)	**<0.001[S]**
	Group B	1.07 (.99-1.16)	**0.008[S]**
	Group C	1.09 (1.01-1.18)	**<0.002[S]**
	Group D	1.04 (0.97-1.12)	**0.05[S]**
	Control Group	1[a]	-

p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant

1[a]: reference category is Control

3.8. Receiver Operating Characteristic (ROC) Analysis

3.8.1. ROC Curve and AUC Analysis for the Adipokines and Metabolic Indices for PCOS Patients Group Compared to the Control group

ROC curve and AUC analysis of the NRG4, NENF, VAI, LAP, BMR, and BAI were performed. Results of the receiver operating curve (ROC) showed that NRG4, NENF VAI, LAP, BMR, and BAI have a good performance for prediction PCOS patients, data are presented in figures (3.9) and table (3-4).

For NRG4 levels: (sensitivity = 70%, specificity 90%) at a level = 0.6, NENF levels (sensitivity 76.40%, specificity 70%) at a level = 0. 464, VAI levels (sensitivity 65.7%, specificity 85%) at a level = 0.507, LAP levels (sensitivity 80.70%, specificity 65%) at a level = 0.457, BMR levels (sensitivity 51.4%, specificity 90% at a level = 1456.54, and BAI levels (sensitivity 70.7%, specificity 90%) at a level = 29.9, the p-values of the AUC were <0.05 and highly statistically significant. results confirmation of the Sensitivity & Specificity was confirmed using Youden's J statistics to the parameters.

Table(3-4): AUC, Optimal Threshold, Sensitivity and Specificity of Proposed Marker Obtained by The Receiver Operating Characteristic Curves PCOS Patients Group.

Variable	AUC	P value	Sensitivity %	Specificity %	Cut off	Youden index	CI %
NRG4	0.799	<0.001	70%	90%	0.6	7.37	0.71-0.88
NENF	0.741	<0.001	76.40%	70%	0.46	0.80	0.65-0.82
VAI	0.780	<0.001	65.70%	85%	0.50	0.89	0.71-0.84
LAP	0.741	<0.001	80.70%	65%	0.45	12.73	0.65-0.82
BMR	0.733	<0.001	51.4%	90%	1456.54	0.41	0.65-0.81
BAI	0.818	<0.001	70.7%	90%	29.9	0.60	0.75-0.88

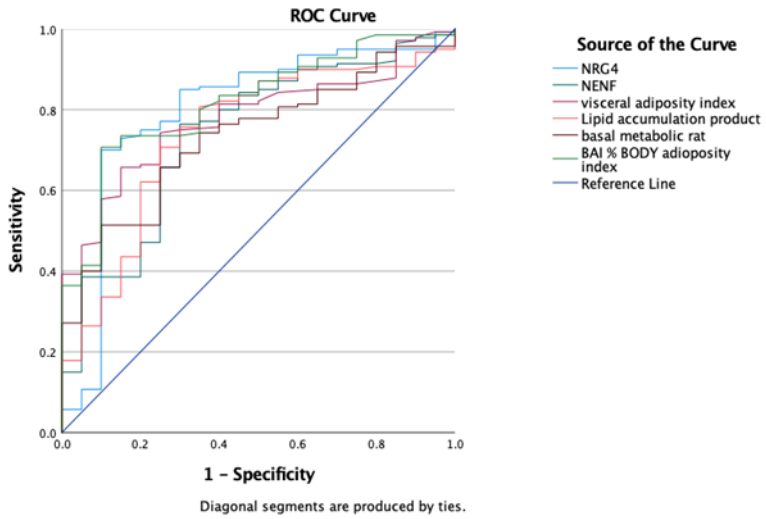


Figure (3.9): ROC Curve Analysis of NRG4, NENF, VAI, LAP, BMR and BAI Levels in PCOS Patients.

3.8.2.ROC curve and AUC analysis for the adipokines and metabolic indices in PCOS Phenotype A

ROC curve and AUC analysis for the NRG4, NENF, VAI, LAP, BMR and BAI for the phenotypes A compared to the control group were performed. Results of the receiver operating curve (ROC) curve and AUC analysis showed that NRG4 was shown a good performance for prediction phenotypes A, data are presented in figures (3.10) and table (3-5). The NRG4 levels show a sensitivity = 90%, specificity 89.7% at a level = 7.68, the p-values of the AUC were <0.05 and highly statistically significant.

Table (3-5): ROC Curve Showing Sensitivity and Specificity of NRG4, NENF, VAI, LAP, BMR and BAI in PCOS Phenotype A .

Variable	AUC	P value	Sensitivity %	Specificity %	Cut off	Youden index	CI %
NRG4	0.86	<0.001	90%	89.7%	7.68	0.79	0.77-0.95
NENF	0.77	<0.001	82.6%	69.2%	0.806	0.51	0.67-0.86
VAI	0.85	<0.001	71%	89.7%	1.045	0.60	0.78-0.92
LAP	0.80	<0.001	79.7%	74.4%	15.72	0.54	0.71-0.89
BMR	0.83	<0.001	81.2%	76.9%	1420.21	0.58	0.75-0.90
BAI	0.84	<0.001	76.8%	89.7%	30.01	0.66	0.77-0.92

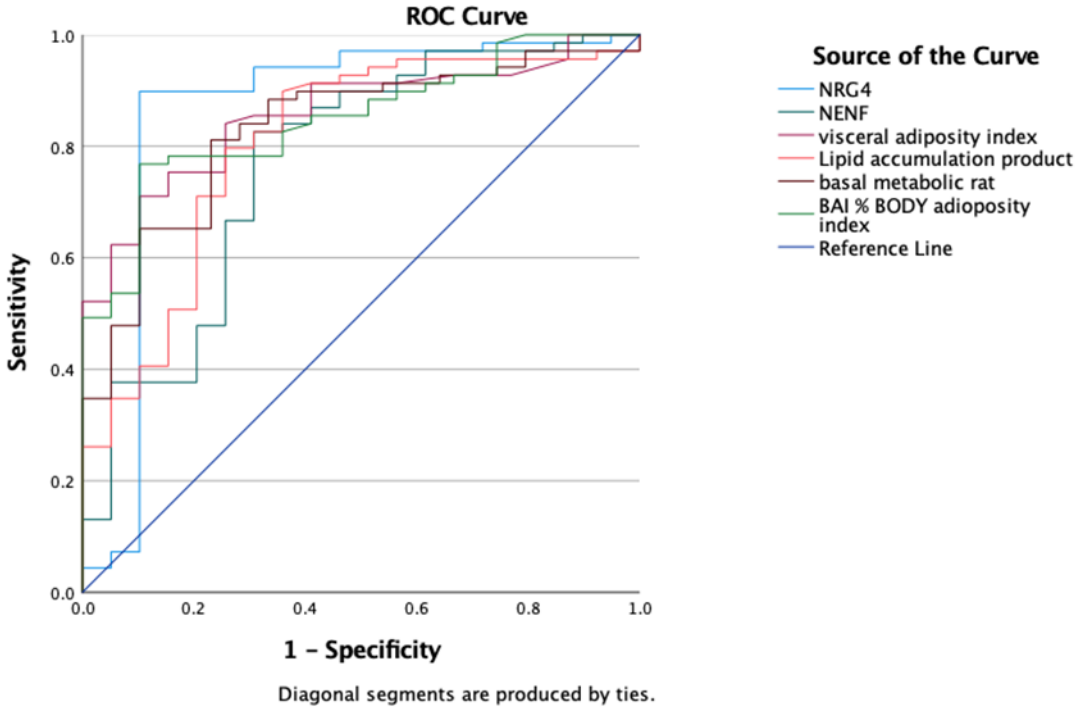


Figure (3.10): ROC Curve Analysis of NRG4, NENF, VAI, LAP, BMR, and BAI Levels in PCOS Phenotype A.

3.8.3.ROC curve and AUC analysis for the adipokines and metabolic indices in PCOS Phenotype B

ROC curve and AUC analysis of the NRG4, NENF VAI, LAP, BMR and BAI for Patients group B compared to control group were performed. Results showed that BAI have a good performance for prediction group B, data are presented in figures (3.11) and table (3-6).

BAI levels showed a sensitivity 75%, specificity 85% at a level = 29.69, the p-values of the AUC were <0.05 and highly statistically significant.

Table (3- 6): ROC Curve Showing Sensitivity and Specificity of NRG4, NENF, VAI, LAP, BMR and BAI in PCOS Phenotype B.

Variable	AUC	P value	Sensitivity %	Specificity %	Cut off	Youden index	CI %
NRG4	0.66	0.038	70%	70%	5.91	0.4	0.50-0.82
NENF	0.70	0.011	75%	70%	0.82	0.45	0.56-0.84
VAI	0.73	0.003	70%	75%	0.81	0.45	0.59-0.88
LAP	0.71	0.008	80%	70%	13.97	0.5	0.55-0.86
BMR	0.61	0.158	35%	99%	1553.33	0.34	0.44-0.77
BAI	0.80	<0.001	75%	85%	29.69	0.6	0.68-0.92

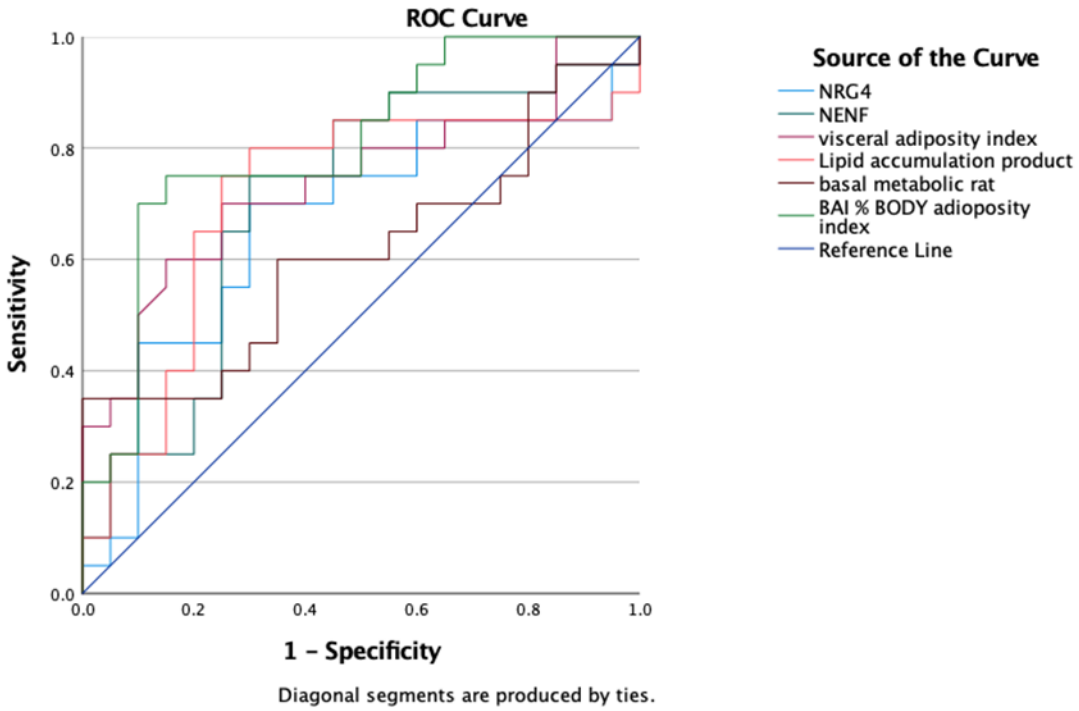


Figure (3.11): ROC Curve analysis of NRG4, NENF, VAI, LAP, BMR, and BAI Levels in PCOS Phenotype B.

3.8.4.ROC curve and AUC analysis for the adipokines and metabolic indices in PCOS Phenotype C

ROC curve and AUC analysis for the NRG4, NENF ,VAI, LAP, BMR and BAI for Patients group C compared to the control group were performed. Results showed that LAP, and BAI were the best prediction for phenotypes group C, data are presented in figures (3.12) and table (3-7).

LAP levels (sensitivity 82.6%, specificity 70%) at a level = 14.11, and BAI levels (sensitivity 78.3%, specificity 90%) at a level = 29.9, the p-values of the AUC were <0.05 and highly statistically significant.

Table (3-7): ROC Curve Showing Sensitivity and Specificity of NRG4, NENF, VAI, LAP, BMR and BAI in PCOS Phenotype C.

Variable	AUC	P value	Sensitivity %	Specificity %	Cut off	Youden index	CI %
NRG4	0.74	0.001	60.9%	85%	6.95	0.54	0.61-0.87
NENF	0.64	0.066	34.8%	95%	1.44	0.29	0.48-0.79
VAI	0.77	<0.001	69.6%	85%	0.91	0.54	0.64-0.9
LAP	0.74	0.001	82.6%	70%	14.11	0.52	0.60-0.87
BMR	0.67	0.019	65.2%	70%	1408.23	0.35	0.53-0.81
BAI	0.85	<0.001	78.3%	90%	29.9	0.68	0.76-0.95

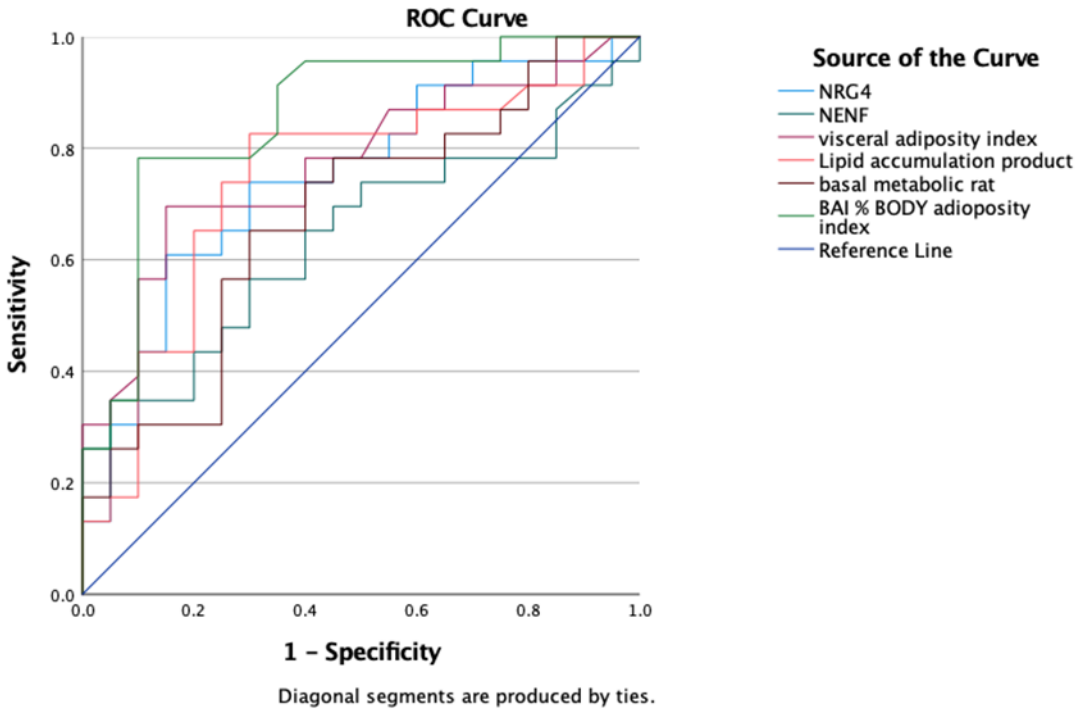


Figure (3.12): ROC Curve Analysis of NRG4, NENF, VAI, LAP, BMR and BAI Levels in PCOS Phenotype C.

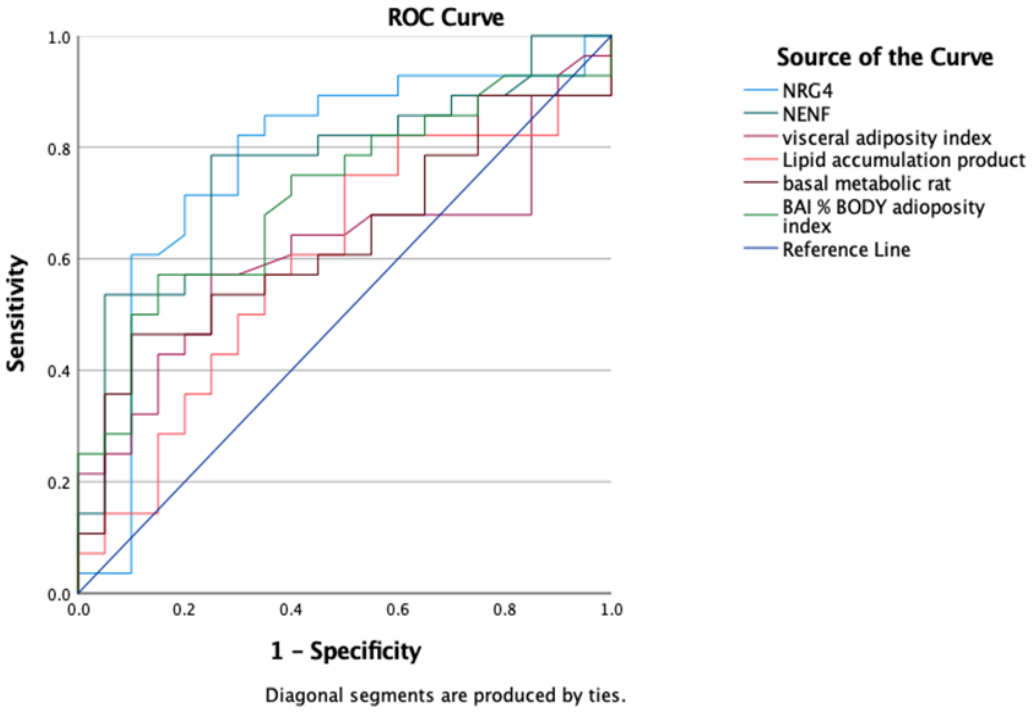
3.8.5. ROC curve and AUC analysis for the adipokines and metabolic indices in PCOS Phenotype D

ROC curve and AUC analysis for the NRG4, NENF VAI, LAP, BMR and BAI for Patients group D compared to the control group were performed. Results showed that NRG4, NENF have a good performance for prediction group D, data are presented in figures (3.13) and table (3-8).

For NRG4 levels: (sensitivity = 82.1%, specificity 70%) at a level = 5.85, NENF levels (sensitivity 78.6%, specificity 75%) at a level = 0.93, the p-values of the AUC were <0.05 and highly statistically significant.

Table (3-8): ROC Curve Showing Sensitivity and Specificity of NRG4, NENF, VAI, LAP, BMR and BAI in PCOS Phenotype D

Variable	AUC	P value	Sensitivity %	Specificity %	Cut off	Youden index	CI %
NRG4	0.77	<0.001	82.1%	70%	5.85	0.52	0.65-0.89
NENF	0.76	<0.001	78.6%	75%	0.93	0.53	0.64-0.88
VAI	0.61	0.116	57.1%	75%	0.80	0.32	0.46-0.76
LAP	0.59	0.178	75%	50%	9.85	0.25	0.45-0.73
BMR	0.63	0.062	46.4%	90%	1456.54	0.36	0.49-0.77
BAI	0.71	0.003	57.1%	85%	29.6	0.42	0.58-0.84



Figure(3.13): (ROC) Curve Analysis of NRG4, NENF, VAI, LAP, BMR and BAI Levels in PCOS Phenotype D.

Chapter four

Discussion

4. Discussion

In this study, the examination of the Neudesin and Neuregulin 4 in different phenotypes of PCOS was performed. In the present study, it was identified that the classic (Phenotype A) was the most frequent, subsequent to the non-hyperandrogenic (Phenotype D), followed by the ovulatory (Phenotype C), and lowest frequency, the anovulatory (Phenotype B) which is consistent with findings from another research **(Lizneva *et al*., 2016 b; Tavares and Rego Barros, 2019).**

In NRG4 level showed an increased in all PCOS phenotypes in comparison to healthy women specially in age range (18-27) years, as presented in figure (3.5).The present study showed a significant increase in PCOS patients compared to the control as in figure (3.6). As well as the most risk from the other phenotype due to the phenotype A consider the classic one has all feature of diagnostic criteria with a high risk of obesity, T2DM, IR ,coronary heart disease and other MS **(Polak *et al*., 2020)**, followed by Phenotype (C, D, and B) respectively in table (3.3), were in the ROC the more sensitive 90% and specific 89.7% it Phenotype A in figure (3.10) and table (3.5).

There is no recently published study about NRG4 in PCOS Phenotypes. Our study observed the correlation between NRG4 and HOMA-IR is compatible to previse study by **Temur *et al* in 2017**. NRG4 is one of the members of the adipokines family that is synthesized and secreted by adipose tissues. It acts to activate the epidermal growth factor receptor (EGFR), because EGFR deficiency can result in ineffective LHRH secretion, the EGFR plays a crucial role in the healthy function of luteinizing hormone-releasing hormone (LHRH), which is responsible for normal female pubertal development **(Ayoob, 2022).** Adipose tissue is an adynamic endocrine organ that plays a central role in the regulation of energy metabolism. White adipose tissue stores energy in the form of

triacylglycerols, whereas brown adipose tissue burns fatty acids to maintain body temperature.NRG4 is a member of the epidermal growth factor-like family of proteins expressed in brown adipose tissue, which functions as a brown-fat-enriched endocrine factor. NRG4 exerts a protective effect against obesity, type 2 DM, and non-alcoholic fatty liver disease **(Eken *et al* .,2019).**

In 2017, Temur *et al.* reported higher serum levels of NRG4 in PCOS positive correlations between NGR4 level and FBG, insulin, HOMA-IR, and High-Sensitivity C-Reactive Protein (hs-CRP), were observed in the study group HOMA-IR, and hs-CRP proved to be independent markers associated with NRG4(**Temur *et al*., 2017).** The study by Kurek Eken *et al*. in 2019 showed that serum NRG4 level was independently related to BMI through multiple regression analysis **(Kurek Eken *et al*., 2019)**.

Obesity was the most influential factor in NRG4 secretion in PCOS patients, and weight management was the key to resolving metabolic abnormalities and fertility issues associated with PCOS **(Cao and Hu, 2021).** Hyperandrogenism, HI, and IR are all metabolic diseases that both obesity and PCOS share in common**,** A meta-analysis highlights the significance of weight intervention and lifestyle adjustment in the treatment of adolescent PCOS. **(Li *et al*., 2017).**

The researches performed by Cao et al in 2021 had similar results to the Kurek Eken et al study in increase level of NRG4. Furthermore, the study demonstrated that even a brief (one-year) lifestyle intervention consisting of a change in diet, an increase in physical activity, and the avoidance of sedentary behavior can result in NRG4 levels comparable to those of healthy controls from the start of the study **(Cao and Hu, 2021).** The recent study in 2022 by KRUSZEWSKA *et al* confirms the elevation of NRG4 levels in PCOS patients that have insulin resistance **(Kruszewska *et al*.,2022).**

In this study, resulting a significant increase in neudesin levels in all age groups as in figure (3.5). In comparing the PCOS patients group and the control show a significant increase as in figure (3.6). In this study is that circulating NENF levels were considerably increased in all PCOS Phenotypes subjects compared to healthy group, as well as Neudesin levels displayed an association considering the most significant and risk increase in Phenotype C, Phenotype A, and Phenotype D, while the less one is Phenotype B as presented in table (3.3), the difference in result between Phenotypes due to assessment of NENF, a membrane-associated progesterone receptor, in PCOS patients has insufficient data.

ROC analysis shows that Phenotype A more sensitive at 82.6% and specificity at 69.2%. There is no recently published study on the NENF in PCOS Phenotypes. Although neudesin was discovered just over twenty years ago, it is still largely unexplored and is only now the subject of metabolism-related research. there is only two recently published study about neudesin as related to PCOS.

Neudesin may act as a binding protein for lipophilic progesterone and continue to hold it on the cell surface in the extracellular environment. This complex may exert a rapid effect on the unidentified cell surface progesterone receptor. In conjunction with a relative deficiency of neudesin, it is hypothesized that progesterone cannot exert a negative feedback effect on GnRH, which may contribute to the pathogenesis of PCOS **(Yasar *et al* .,2021)**.

However, it is uncertain whether decreased neudesin contributes to the pathogenesis of PCOS or is a consequence of the disorder. Neudesin is predominantly expressed in neurons in numerous tissues, including the brain, adipose tissue, heart, and lungs. PGRMC1 was detected in immortalized GnRH neurons (GT1-7cells) and rapidly inhibited intracellular calcium fluctuations in

GnRH neurons, thereby inhibiting GnRH and LH release decreased FSH levels contribute to the pathogenesis. Nonetheless, the precise etiology is unrevealed.

The study performed by Bozkaya *et al* (2020), comprised the first attempt to investigate a putative link between PCOS diagnosis and serum neudesin concentration ,the NENF levels were decreased in PCOS patients. In this study, the level decreased significantly between the study and control groups and positive correlation between neudesin and progesterone was noted in the affected individuals. An insignificant difference in the neudesin level was observed between the PCOS group with and without insulin resistance **(Bozkaya *et al*.,2020).**

In the study performed by Yilmaz Yasar *et al*(2021), the neudesin level was found to be lowered in the PCOS group and was positively correlated with progesterone and insulin levels **(Yasar *et al* .,2021).** The difference between our study and previse is that the neudesin level was measured in the recent study in the follicular phase while our study was in the med luteal, because it is a membrane-associated progesterone receptor and the peak of progesterone 6 to 8 days after ovulation specifically on the day (20-21) of menstrual cycle were measured**.** The result was inconsistent with other research that reported that NENF decrease in the PCOS patient compared to the control group.

Due to insulin having a gonadotropin-stimulating effect, IR may be the defining characteristic of PCOS's metabolic and reproductive processes. Insulin not only increases adrenal and ovarian steroid production, but it also increases pituitary LH secretion. Hyperandrogenism is also associated with IR **(Wang *et al*., 2023).** It is essential to detect IR as early as possible in PCOS patients and to provide treatment to enhance insulin sensitivity. Common predictors of IR, such as BMI, WHR, WC, WHtR, and other traditional parameters. Recently proposed

anthropometric indicators, such as the visceral adiposity index (VAI), lipid accumulation product (LAP), and body adiposity index (BAI), exhibit high precision when identifying visceral adiposity. In addition, research indicates that they may be reliable predictors of IR, MS, T2DM, and cardiovascular in PCOS **(Androulakis *et al*., 2014; Nascimento *et al*., 2015, Wang *et al*., 2023**).

Recent research has shown that the VAI, LAP, and BAI are more sensitive and reliable than anthropometric parameters in predicting insulin resistance (IR) , evaluating diagnostic accuracy and comparison of numerous adiposity, cardiometabolic, and insulin resistance indices to determine the most accurate predictor of insulin resistance and MS risk in polycystic ovary syndrome women of reproductive age because they incorporate the anatomical basis and pathophysiological changes related to fat accumulation **(Rashid *et al*., 2020)**.Asian populations are more likely to manifest fat accumulation and insulin resistance than Western populations **(Du *et al*., 2015).** The androgen production and metabolic clearance of PCOS patients with visceral adiposity are modified. Increases andogen promote lipolysis and enhances the outflow of free fatty acids and IR. Visceral obesity has an important effect on androgen and IR metabolism **(Wang *et al*., 2023)** .

This study found that the LAP and VAI scores were more accurate indicators of IR and have a positive correlation with HOMA-IR,in the study performed by Davut Sakiz *et al* in 2022 that have a positive correlation of HOMA-IR with (VAI , LAP, and BAI). The difference in result could from ethnic (**Davut Sakiz *et al*., 2022**), while the VAI, LAP, and BAI have significant correlation with metabolic parameters as in figure (3.8), in comparing between two age groups the VAI was significant in both age groups while LAP and BAI are significant in (18-27) years as in figure (3.5). The VAI, LAP, and BAI were significant in the PCOS group in

comparison to the healthy group in figure (3.6). According to VAI, LAP, and BAI, visceral adipose tissue increased in PCOS Phenotype A, as determined by our study. Based on these values patients with PCOS phenotype A had a greater risk of abdominal adiposity.

Observed the result of phenotype A most significant and high risk in association, Phenotype C, phenotype B, and less phenotype D in VAI shown in table (3.3). The recent study by Bil *et al* in 2016 the most significant was Phenotype B **(Bil *et al*., 2016),** Variations in the ethnicity, age, BMI, and dietary patterns of study participants may account for VAI threshold differences among various study **(Ehsani *et al*., 2016).**

This funding for LAP is significant in Phenotypes (A, B, and C), with the more significant risk in Phenotype A as shown table (3.3). A previous study by Bozic-Antic et al in 2016 that Phenotype B **(Bozic-Antic *et al* ., 2016).** this difference as being due to ethnic composition.

In this study, the funding that all PCOS Phenotypes is significant in BAI, while Phenotype A is riskier and more significant than the other Phenotypes. There is no research published on the BAI in different phenotypes, while the research in the PCOS patients' group and control considered the BAI a good indicator in the detection of cardiovascular risk and insulin resistance in PCOS. **(Gonulalan and Sackan, 2021; Davut Sakiz *et al*., 2022).** In the investigation by Johnson *et al* BAI was found to be far better than BMI in predicting adult adiposity, BMI does not distinguish between fat and muscles and it was suggested that it is the most accurate predictor of insulin resistance **(Johnson *et al*., 2012;).**

In 2020 study performed by Yesil *et al* that BAI is not a much better index than Bioelectric Impedance Analysis (BIA) and skin fold thickness in determining body fat %, BAI is a simple index that calculates the percentage of adipose tissue

in the body without requiring an assessment of body weight. In the absence of more complex or costly methodologies, such as skinfold thickness or BIA, BAI may therefore serve as an alternative predictor of body fat **(Yesil, Kose, and Ozdemir, 2020).**

This study phenotype A is more significant in VAI, LAP, and BAI more accurate of IR, MS, T2DM, and cardiovascular in PCOS due to it being the classic Phenotype with a high risk of obesity, T2DM, IR, coronary heart disease, and other metabolic disorders **(Polak *et al*., 2020)**.

In this result, all new metabolic indices indicate the risk of PCOS in predicting metabolic disturbance and insulin resistance associated with PCOS. These results are supported by the study conducted Davut Sakiz *et al* in 2022 that anthropometric measures may be useful in predicting the development of subclinical atherosclerosis and IR in females with PCOS. Similar results were obtained by the study conducted by Marzena Jabczyk *et al* in 2023 the majority of the analyzed anthropometric indices may be useful in evaluating metabolic disorders, particularly glucose and insulin abnormalities in women with PCOS **(Davut Sakiz *et al*., 2022;Marzena Jabczyk *et al*., 2023).**

The result of this study is that the VAI is more predicted to insulin resistance in all phenotypes, the research by De Medeiros *et al* in 2020 Since VAI was the most accurate predictor of MS in both obese and normal-weight PCOS women. It can be used clinically to screen for MS risk and initiate treatment sooner in PCOS patients, especially those who are obese**(De Medeiros *et al*., 2020).**

Women with PCOS are more likely to be obese, and this has been linked to a variety of adverse metabolic, cardiovascular, endocrine, reproductive, and mental health consequences. When examining associations between nutrients and health, it is essential to accurately measure diet and energy intake **(De Lorgeril *et al*.,**

2013). Obesity could be considered a confounding factor in patients with PCOS. Weight loss has been shown to have a positive effect on fertility and metabolic profile **(Wojciechowska *et al*., 2019).**

A calorie-restricted diet has been suggested for patients with PCOS who are overweight, but weight maintenance is difficult and the majority of subjects regain weight, putting them at risk for weight cycling **(Strohacker, Carpenter, and McFarlin, 2009).**

In this study, the result that basal metabolic rate (BMR) is significant in different age groups figure (3.5), while an increase in the PCOS patients group compared to the control group in figure (3.6) .To our knowledge, BMR in women with PCOS has not been extensively studied these results had been supporting by the study De Giuseppe *et al* in 2018 resulting Although PCOS patients had a significantly higher mean BMR than controls(1658.7 ± 201.1 kcal vs 1359.2 ± 103.7 kcal; $P < 0.0001$, respectively) **(De Giuseppe *et al*., 2018).**

The BMR had a positive correlation with the VAI, LAP, BAI, HOMA-IR, and metabolic parameters. It is significant in all phenotypes while the high risk and more significant in phenotype A. A recent study by Ilic *et al* in 2015 resulted in the phenotype (B and C) having higher BMR, their findings demonstrated a correlation between body composition and androgenic status. Consequently, phenotype B exhibited the most altered structure of body composition **(Ilic *et al*., 2015)**. This research measured BMR by bioelectrical impedance in our study by the Mifflin-St Jeor equation. Consequently, based on the findings of this study, women with PCOS consume less energy and should reduce their caloric intake to maintain normal body weight.

Chapter Five

Conclusions and Recommendations

5. Conclusions and Recommendations

5.1. Conclusions

- The levels of NRG4 and NENF significantly increase in all phenotypes compared to the healthy control. In phenotype A, NRG4 level was shown a significant increase compared to other phenotypes. While NENF level was highly significant in phenotype C compared to other phenotypes.
- The metabolic indices shown a significance differences with all phenotypes, while only the BMR was significant in phenotype A .
- Results were indicated a significant correlation between the NRG4 and HOMA-IR, significant VAI and LAP with LH, HOMA-IR, Cholesterol, TG, and LDL. BAI significant with cholesterol, TG, and LDL, BMR significant with HOMA-IR, cholesterol ,TG and LDL .
- Based on ROC, phenotype A was the most sensitive to the mean level of the proposed biomarkers.

5.2. Recommendations

- Study other adipokines such as (Xenopsin-related peptide, Xenin-25,) that might be involved in metabolic abnormalities, including obesity, dyslipidemia, hyperinsulinemia, and insulin resistance in females affected by PCOS
- Investigating the gene polymorphism of NRG4 and NENF in different PCOS phenotypes
- 3- Large-scale studies are needed to draw a definitive conclusion on metabolic indices that predict the risk of PCOS phenotypes.

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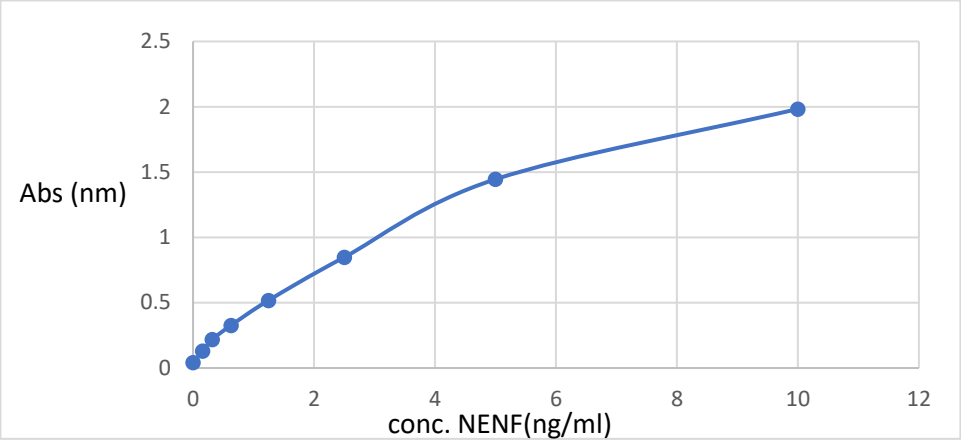
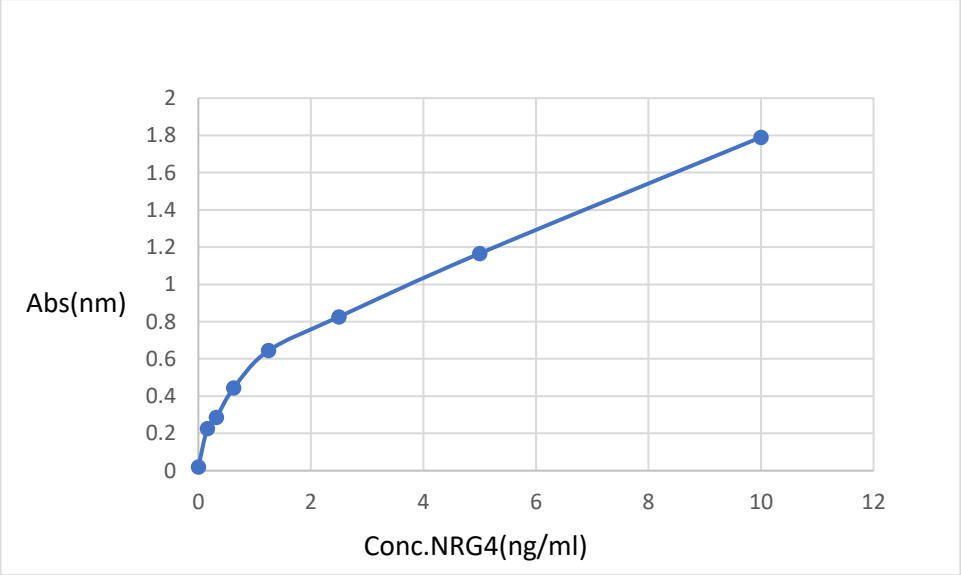
Appendix

Appendices



Figure(1): Standard Curve of the Human Neudesin which is plotted by OD Vs. the scale of a known concentration..The original concentration is calculated by multiplying the dilution factor.



Figure(2): Standard Curve of the Human Neuregulin 4 which is plotted by OD Vs the scale of a known concentration..The original concentration is calculated by multiplying the dilution factor.

Questionnaire

Name:…….. Sample no:

Age:….. Phone no:

Wight:……

Hight: ….

Waist:……

Hips:……..

Clinical features: 1) hirsutism … 2) acne... 3) alopecia….

Menstrual irregularities: A) regular menstruation……
B)oligomenorrhea……

TVS : A) normal ovary……. B)PCO ovary……

Disease: A) DM……. B) HT…….. C) Kidney…..
D) Liver …… E) thyroid …….

Drug history:

Lipid profile: I) HDL II) TG III) LDL

Hormones: 1) LH (2-4days) 2) FSH (2-4days) 3) Prolactin 4)free testosterone

5) progesterone (21days)

Fasting blood glucose:

Fasting insulin:

BP:

Smoking: A) passive : B) active

Duration of diagnostic : Duration on starting treatment:

الخلاصة

متلازمة تكيس المبايض هي متلازمة خلل وظيفي في المبيض تتميز بوجود السمات الأساسية لفرط الأندروجين وتشكل المبيض متعدد الكيسات. فرط الأندروجين السريري أو الكيميائي الحيوي (HA) ، ضعف التبويض (قلة / انقطاع الطمث) (OD) ، و / أو مورفولوجيا المبيض المتعدد الكيسات (PCOM) هي المعايير الثلاثة التي تم استخدامها لتوصيف متلازمة تكيس المبايض منذ إنشاء إجماع روتردام في عام 2003 ، متلازمة تكيس المبايض وجود اثنين على الأقل من ثلاثة معايير. ينتج عن هذا التعريف العديد من الأنماط الظاهرية لمتلازمة تكيس المبايض ، مثل النمط الظاهري A،(HA, OD، PCOM) النمط الظاهري B، (HA , OD)، النمط الظاهري C، (HA, PCOM)، والنمط الظاهري D، (PCOM, OD) .

NENF و NRG4 هما عضوان شحميان يصنعان الأنسجة الدهنية ، بناءً على الاختلالات الأيضية والقلب والأوعية الدموية ، وقد يكون تقييم السمنة البطنية لدى مرضى متلازمة تكيس المبايض أمرًا بالغ الأهمية. يلعب البحث على NENF و NRG4 دورًا في تنظيم التمثيل الغذائي ، والذي يمكن اعتباره علامات محتملة لمقاومة الأنسولين في بعض الحالات الطبية ، مثل داء السكري والسمنة ومتلازمة تكيس المبايض ، والتي اكتسبت اهتمامًا مؤخرًا. على الرغم من أن العلاقة بين النمط الظاهري NENF و NRG4- متلازمة تكيس المبايض ممثلة بشكل ضعيف في الأدبيات. لذلك ، فإن الهدف من هذه الدراسة من شأنه أن يسلط الضوء على دور Neudesin و neuregulin_4 كمؤشر حيوي لمقاومة الأنسولين في الإناث المصابات بأنماط ظاهرية مختلفة من متلازمة تكيس المبايض.

الطريقة: تم تصميم هذه الدراسة كدراسة حالات وشواهد، مع ما مجموعه 210 مشاركًا بما في ذلك 140 حالة أنثوية تتأثر بأنماط مختلفة من متلازمة تكيس المبايض. تم جمع عينات المرضى من استشاري الخصوبة التناسلية في المستشفى التعليمي لأمراض النساء والولادة ، مديرية صحة كربلاء / العراق. تم تحديد مستويات الهرمونات في الدم وتركيز الأنسولين عن طريق نظام المقايسة المناعية بالتوهج الكهرومغناطيسي "ECLIA" (Cobas e 411، Roche Diagnostic ، ألمانيا). تم تحديد نسبة الجلوكوز في الدم أثناء الصيام بواسطة محلل كيمياء سريري (Monarch 240، Biorex Diagnostic ، المملكة المتحدة). تم قياس لوحة الدهون في الدم ((الكوليسترول الكلي (TC) ، كوليسترول البروتين الدهني عالي الكثافة (HDL-C) ، البروتين الدهني منخفض الكثافة (LDL) والدهون الثلاثية (TG)) باستخدام محلل كيميائي أوتوماتيكي بالكامل (SMART-120، Geno TEK ، الولايات المتحدة الأمريكية). تم قياس هرمون التستوستيرون الخالي من المصل باستخدام محلل المقايسة المناعية ذات التلألؤ

الكيميائي التلقائي بالكامل ((MAGLUMI 600، Snibe Diagnostic، Germany). تم استخدام نظام Elisa للكشف عن مستوى بروتين Neudesin و Neuregulin-4. تم حساب قيم VAI و LAP وBAIو HOMA-IR و BMR.

النتيجة ، أظهر متلازمة تكيس المبايض الكلاسيكية (النمط الظاهري A) التي تتضمن جميع الميزات الثلاث: فرط الأندروجين ، والدورات غير المنتظمة ، و PCOعلى الموجات فوق الصوتية انتشارًا كبيرًا (49.28 ٪). في النمط الظاهري A ، أظهر مستوى NRG4 زيادة كبيرة مقارنة بالأنماط الظاهرية الأخرى ، بينما كان مستوى NENF ذو أهمية عالية في النمط الظاهري C ، وكان عامل خطر مضاعف كما في النمط الظاهري B. نتائج منحنى تشغيل المستقبل (ROC) أظهرت أن NRG4 ، لديه أفضل أداء للتنبؤ بمرضى متلازمة تكيس المبايض وخاصة الأنماط الظاهرية A. BAI لديه أداء جيد لمجموعة التنبؤ B. بينما كان LAP و BAI أفضل تنبؤات لمجموعة الأنماط الظاهرية C .NRG4 و NENF لديهما أداء جيد لمجموعة التنبؤ D.

الاستنتاج ، زادت مستويات NRG4 و NENF بشكل ملحوظ في جميع الأنماط الظاهرية لمتلازمة تكيس المبايض ، وللمؤشرات الأيضية علاقة معنوية مع جميع الأنماط الظاهرية. لعبت السمنة الحشوية في منطقة البطن دورًا مهمًا في تطور التغيرات الأيضية ، بغض النظر عن الأنماط الظاهرية لمتلازمة تكيس المبايض.

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دور Neudesin و Neuregulin-4 في الآلية الحيوية لمقاومة الأنسولين بين الأنماط الظاهرية لمتلازمة تكيس المبايض

رسالة ماجستير

مقدمة الى مجلس كلية الطب في جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في

(الكيمياء السريرية)

من قبل

افنان حيدر عبود

بكالوريوس صيدلة/ كلية اليرموك الجامعة (2015-2016)

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