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Study The Association Between Indothelin-1 And Visfatin with Insulin Resistance in Women with Poly Cystic Ovarian Syndrome

A Thesis

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

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" Study The Association Between Indothelin-1 And Insulin Resistance in Women with Poly Cystic Ovarian Syndrome"

We have examined the student **" Naba Haider Ali"** in its content and in our opinion; it meets the standard of a thesis for the degree of **"master in Clinical chemistry"** and it is adequate with **"Excellent"** degree.

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We certify that this M.Sc. thesis titled: -

Study The Association Between Indothelin-1 And Insulin Resistance in Women With Poly Cystic Ovarian Syndrome

Was prepared under our supervision at the College of Medicine/University of Karbala, as a partial fulfillment of the requirement for the Degree of of Master in clinical chemistry and Biochemistry

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Dedication

……..TO……

Gratitude for my support, strength and refuge, my mother and father.

…..TO…..

The companion of the path and the beloved of the soul

My husband(Samer).

…..TO….

My children Rayan and Reda

….TO….

Soulmate and heart lover my aunts(Eshraq andHala)

….TO….

My angel sister (Dr.Noor)and Saffana.

….TO…

the absent in his body, present in his soul

my grandmother(Layla) and second father(hussaien)

TO

My brothers(Mohammed.H,Ahmed,Ali ,Mohammed.A,Yossif)

And TO,

the pure hearts and loyal hands who assisted me in life

NABA

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In final all gratitude to all researchers hope to them all the best.

……..

Thanks for all…

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Summary

Background: Polycystic ovarian syndrome (PCOS), is a common heterogeneous endocrine disorder that affects women of growing age particularly at the early to the late reproductive stage (15–35 years) .

Endothelin-1 (ET-1) is an indicator of endothelial injury dysfunction and is elevated in women with androgen excess polycystic ovary syndrome (PCOS).(

Visfatin is an adipocytokine known as a pre-B cell colony-enhancing factor, 52kDalton protein expressed in a variety of tissues including adiposities, lymphocytes, bone marrow, liver, and muscle. Visfatin binds to insulin receptors and exhibits insulin-mimetic actions; therefore, it stimulates glucose uptake in adipocytes and muscle cells and suppresses glucose release from hepatocytes. It is now believed that visfatin action can be endocrine, paracrine, and autocrine as well. These autocrine effects of visfatin may play an important role in regulating insulin sensitivity in the liver

Aim of this study :

The aim was to evaluate serum ET-1 & visfatin levels
among women with PCOS and control to detect maybe these markers pre-indicators for PCOS . And is there a relationship between age and BMI.

Methods: This study is a case-control study involving (120 women) aged of them between (20-40)years and a BMI(of 20-35)kg/m². 60 PCOS patients. and 60 non-PCOS women as a control in child-bearing age at the Reproductive Fertility consultant of a gynecological and obstetric teaching hospital, BABILON. health directorate Iraq and College of Medicine, the University of Kerbela Iraq. The Rotterdam criteria-2003 was presumed to Patients with any 2 of the next 3 items that can be recognized in diagnosis: oligomenorrhea or amenorrhea, increase androgen levels. The controller group has regular menstruation, with normal ovaries as they

were detected by the gynecologist.PCOS cases were classified into three subgroups (A, B, C) according to infertility(normal which no infertility, primary infertility was 40, and secondary infertility was 20) .5ml of blood was drawn from women and put in a gel tube to measure serum ET-1 and Serum visfatin level (ng/ml) were assessed by ELISA, measure hormones(LH, FSH, F.testo) in second day of the cycle(CD2) by minividas, measure lipid profile (total cholesterol TC, high-density lipoprotein HDL, low-density lipoprotein LDL, very low-density lipoprotein VLDL and triglyceride TG) by colorimetric assay using a spectrophotometer, measure insulin by ELISA assay. and fasting blood sugar by maglumi device.

Results: The mean levels of biomarkers among infertility groups in women with PCOS showed that parameters significantly differed among the groups (p values <0.05). level of LH; Testosterone; TG; Insulin; W\H and LH/FSH ratio were shown to have a positively significant increase with secondary infertility cases. While FSH; HDL and fasting blood sugar decreased significantly in the same group. Endothelin-1 and visfatin were shown as diagnostic points for predicting PCOS cases compared to the control group. ET-1 increases in primary infertility than secondary, and **positive** relation between ET-1, visfatin and LH, F.testo, TG, FBS, IR,w/h . and negative relation with HDL. And there is a high positive relation between ET-1 and visfatin. The efficiency of the predicting value was assessed using the receiver operating characteristic (ROC) curve.

Conclusion: according to the observed data concluded that:

- Endothelin-1& Visfatin may be pre-indicator diagnostic markers for PCOs because increase in patient than control.

-Endothelin was increasing with age

-Visfatin was associated with abdominal obesity and insulin resistance as well as obesity, but not with metabolic syndrome and pre-diabetes Mellitus.

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List of Abbreviations	
4-AA	4-aminoantipyrine
5-DHT	5-DiHydroTestosterone
AE-PCOS	Androgen Excess Poly Cystic Ovary
ApoA	Apolipoprotein-A
ApoB	Apolipoprotein-B
AR	Androgen Recepter
BMI	Body Mass Index
CV	CardioVascular diseases
E2	Estrogen
ECE	ENDOTHELIN converting enzyme (ECE)
EDN1	Endothelin-1
ELISA	Enzyme-linked immunosorbent assay)
ET-1	Endothelin
F.TESTO	Cholesterol
FFA	Free fatty acids
FSH	Follicle-Stimulating Hormone
G-3-P	Glycerol-3-phosphate
GnRH	Gonadotropin-Releasing Hormone
H2O2	Hydrogen peroxidase
HA	Hyper Androgenism
hCG	human chorionic gonadotropin (hCG)
HDL	high-density lipoprotein
HRP	Horse radish peroxidase

IR	Insulin Resistance
LDL	Low-density lipoprotein
LH	Luteinizing Hormone
LH/FSH	Luteinizing Hormone / Follicle-Stimulating Hormone
LPL	Lipoprotein lipase
NAD+	nicotinamide adenine dinucleotid
NIH	National Institutes of health
OD	Optical Density
PCOM	Polycystic ovarian morphology
PCOS	polycystic ovary syndrome
SHBG	sex hormone binding globulin
T	Testosteron
TG	triglycerides
VISFATIN	serum visfatin
VLDL	Very-low-density lipoprotein
W\H	Waist/HIP
WHO	World Health Organization

CHAPTER ONE

Introduction and literature review

Introduction

Polycystic ovary syndrome (PCOS) is the most common gynecological disease in women at reproductive age, which is characterized by metabolic and endocrine abnormalities, as well as chronic inflammation (Lambertini *et al*.,2017; Seyyed Abootorabi *et al*., 2018).

The prevalence is generally considered to be between 6-20%, depending on the definition and the population studied (Azziz *et al*., 2016). PCOS is due to a combination of genetic, environmental, and endocrine factors, which are the main causes of female ovulatory infertility (Szczuko *et al*., 2017).

The most common risk factors for the progress of PCOS include a family history of PCOS, fast food diet habits, lack of physical exercise, body mass index, and waist circumference (Begum *et al*., 2017).

The clinical manifestations of PCOS are acne, amenorrhea or oligomenorrhea hirsutism, hyperinsulinemia, infertility, and mood disorders (**Yvette C** *et al*.,2015).

Women with PCOS are more likely to develop many metabolic and reproductive health complications that include dyslipidemia, abnormal glucose level, insulin resistance, miscarriage, gestational diabetes, mood disorders, hypertensive disorders, preeclampsia, prediabetes, type 2 diabetes, obesity, obstructive sleep apnea, cardiovascular disease, stroke, chronic kidney disease, renal failure, breast cancer, endometrium cancer and others (Escobar-Morreale,2018).

The diagnosis of PCOS is dependent on two of the following three findings (Rotterdam): intermittent or absent menstrual cycles, high circulating levels of testosterone (T) or hirsutism (Gorsic lk *et al*., 2019), and ultrasonographic findings of the ovary (12 or more small follicles that are between two and nine mm in diameter in both ovaries) (Kadri *et al*., 2021).

Other conditions that produce similar symptoms include Thyroid disease, hyperprolactinemia, and hyperlipidemia (Trent *et al*., 2020).

1.1. History of the disease

The earliest description of the polycystic ovary dates back to the 17th century(Azziz et al., 2016). Stein-Leventhal was first described Polycystic ovaries syndrome in 1935, as infertility, menorrhagia, hirsutism, enlarged ovaries (El Hayek et al.,2016; Mohammad et al., 2017), amenorrhoea, chronic anovulation, and obesity(Escobar- Morreale, 2018).

The history of diagnostic the PCOS begins as the following: In 1990, the National Institutes of Health suggested Hyperandrogenism and Oligo-anovulation as the two criteria and which are needed to diagnose PCOS(Fox, C.W., *et al*., 2019), which is the first formal attempt to classify PCOS.

In 2003, the Rotterdam criteria by the Rotterdam European Society of Human Reproduction and Radiology (ESHRE) and the American Society for Reproductive Medicine (ASRM), ESHRE/ASRM sponsored PCOS Consensus Workshop Group were published, which suggest two of the three following criteria (HA, ovulation dysfunction, and PCOM) (Anagnostis et al., 2018), which the second formal attempt to classify PCOS.

In 2006, the Androgen Excess and PCOS Society (AE-PCOS) concluded that PCOS should be based only on two criteria, that is clinical and/or biochemical hyperandrogenism with either polycystic morphology or clinical anovulation (Anagnostis et al., 2018), which is the third formal attempt to classify PCOS.

In 2012, the National Institutes of Health (NIH) Evidence-based Methodology Workshop published the last report for PCOS diagnosis which suggested two of three following criteria (HA, ovulation dysfunction, and PCOM) (Jungari et al.,2020).

1.2. Symptoms Of PCOS:

1. 2.1. Irregular Menstrual Cycles:

Irregular menstrual cycles as a result of ovulatory dysfunction are key symptoms of PCOS according to the Rotterdam criteria (Louwers and Laven, 2020). When ovulation does not occur, the uterine lining (called the endometrium) does not shed and regrow uniformly as it does during a normal menstrual cycle. Instead, the endometrium becomes thicker and may shed irregularly, which can result in heavy and/or prolonged bleeding. Irregular or absent menstrual periods can increase a woman's risk of endometrial overgrowth (called endometrial hyperplasia) or even endometrial cancer.

PCOS women typically have less than six to eight menstrual periods each year. Some women have normal cycles during puberty, which may become irregular if the woman becomes overweight (Barbieri and Ehrmann, 2018).

1.2.2. Weight gain:

About 80% of PCOS women gain weight (Kataoka etal., 2017). When the body stores fat more than is healthy, particularly in the midsection, it increases the chance of having severe chronic conditions such as diabetes, heart disease, and even endometrial cancer (Conway et al., 2014).

While gaining weight doesn't result in PCOS, management of weight may be difficult, but it's important to understand that losing only 2-10% of excess body fat can improve many PCOS- related symptoms .

1.2.3. Hirsutism :

Defined as the occurrence of terminal hair in a masculine on the face and/or body. It is one of the main characteristics of hyperandrogenism in PCOS . The incidence of hirsutism in PCOS women ranges between 60 and 80% (Mara Spritzer et al., 2016; Keen et al., 2017). The amount and distribution of hair

growth are determined by the androgens, particularly testosterone. Hirsutism in PCOS women is attributed to increasing circulatory levels of free testosterone and a more active form of testosterone, i.e., dihydrotestosterone , formed by the activity of 5α reductase on testosterone in the pilosebaceous gland. Hirsutism is the most consistent and reliable symptom used for evaluating clinical hyperandrogenism. hair is scored in nine parts of the body, which include the upper lip, chin, chest, upper and lower back, upper and lower abdomen, and upper and lower limbs (Ashraf et al., 2019).

1.2.4. Acne:

Regarded as a sign of hyperandrogenism and is included as an equivalent of hyperandrogenism in the diagnostic criteria of most PCOS guidelines (Teede et al., 2018). It is not surprising that PCOS is associated with acne due to the role of increasing levels of androgens in determining acne(Carmina, 2020).

1.2.5. Skin modification:

Insulin resistance and high insulin levels can cause thick, velvety skin patches that are darker in color than normal skin tone. Acanthosis nigricans is a skin condition that causes creases in the neck, groin, and breasts. Insulin resistance can also be indicated by skin tags (very small skin growths) in your armpits

1.3. Epidemiology

Polycystic ovarian syndrome is the most common endocrine disorder in reproductive-aged women worldwide. Depending on the diagnostic criteria , the prevalence ranges between 5% and 15%. Based on the disorder characteristics, with various metabolic and reproductive consequences (Leon *et al*., 2020).

Rotterdam criteria include a wider prevalence than the National Institute of Health 1990 Criteria and not accepted by all (Ning et al., 2013) with calls for them to be updated (Goodman et al., 2015). When the PCOS is diagnosed according to the Rotterdam criteria, the prevalence of PCOS is estimated to be about 4 to 21% .The PCOS prevalence is estimated to be around 4%–6.6 %, based on the report from the NIH workshop of 2012 (Lizneva et al., 2016).

According to population samples assessed in the United States, Western Europe, the Middle East, East Asia, and Australia, the prevalence of PCOS in females of reproductive age varies by geographic region, ranging from 1 to 19% (Merkin et al.,2016). PCOS can have a wide range of prevalence due to genetic and environmental factors. A lower socioeconomic status is also linked to poorer health, which can result in hormonal changes and/or activate a genetic predisposition to disease development. Inadequate healthcare conditions also lead to lower rates of accurate diagnosis and appropriate treatment (Merkin et al., 2016).

1.4. Etiopathogenesis of polycystic ovarian syndrome

The etiological factors associated with PCOS are not yet so clear and still under debate. There are different factors that could be contributes to the etiopathology of PCOS such as genetic, biochemical, environmental and immunological. Many genes have been shown to be crucial contributors to PCOS , however, to date, none of these factors could be implicated as the main cause(Glueck & Goldenberg, 2019; Khan et al., 2019; Shaaban et al., 2019) .show in figure(1-2).

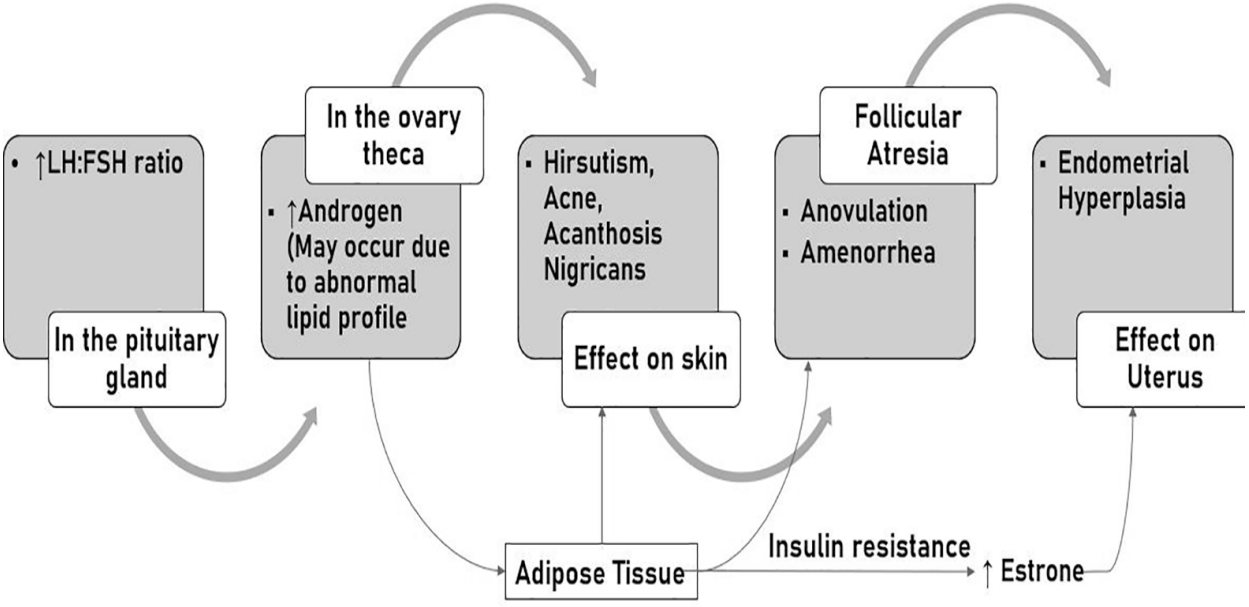


Figure (1-2): Etiopathogenesis of pcos (Corrie et al., 2021).

1.5-Type of Polycystic Ovarian Syndrome:

1.5.1- Insulin resistant PCOS:

Hyperinsulinemia is the root cause of excess androgens as insulin directly simulates the action of LH and raises the GnRH indirectly (M. Puttabyatappa and V. Padmanabhan *et al*., 2018) Insulin decreases the sex hormone binding globulin (SHBG), a main circulatory protein controlling the testosterone levels. So reduced SHBG would result in a raised level of free androgens that produce clinical

manifestations like hirsutism, alopecia, and acne (J. Roja *et al.*, 2014). Insulin resistance can cause dyslipidemia and patients with PCOS are at high risk for cardiovascular disease and diabetes (A.L. Rocha *et al*., 2019).

1.5.2 Post-pill PCOS

Post-pill PCOS occurs in some people after they stop taking the oral contraceptive pill. In this type, symptoms like acne, irregular periods, and excess hair growth were not present before starting the pill(Oguz *et al*;2021).

1.5.3 Adrenal PCOS

Impaired folliculogenesis is the result of surplus androgens that disrupt normal androgen synthesis. The excess androgens promote the development of primordial follicles and increase the antral follicles at the early gonadotropin stage (**R.L. Rosenfield and DA. Ehrmann., *et at*., 2016).**

The secretion of GnRH from the hypothalamus will activate the gonadotropin hormone release from the pituitary. Luteinizing hormone activates the LH receptor to promote androgen production in ovarian theca cells, and the follicular stimulating hormone acts on the FSH receptor simultaneously in the ovarian granulosa cells to transform the androgens to estrogens, which promote follicle growth (**S. Ashraf *et al*., 2019**).

It has been assumed that the dysregulation in the neuroendocrine system results in an imbalance of the hypothalamic-pituitary-ovarian axis leading to a surplus level of gonadotropin. The rise in the GnRH promotes the production of LH over FSH, resulting in a marked hormonal increase in the LH: FSH ratio in PCOS (**K.A. Walters *et al*, 2018).**

1.5.4.1-The Relationship between Hyperandrogenism and the Pathophysiology of PCOS.

Hyperandrogenism represents a chief attribute of PCOS as elevated androgen levels are the most constant feature, with the majority (−60%) of patients exhibiting hyperandrogenism (Rotterdam definition) (Livadas S.,*et al*.,2014) .Women with hyperandrogenic PCOS present with elevated levels of various androgens, including testosterone (T) and the pro-androgens androstenedione (A4) and dehydroepiandrosterone sulfate (DHEAS), as well as the enzyme required to convert pro-androgens to bioactive androgens, 3β-hydroxysteroid dehydrogenase (3β-HSD) in serum (Palomba S.,*et al*.,2014). Excess androgens can be induced by insulin resistance and hyperinsulinemia, as they cause a reduction in sex hormone binding globulin levels, which lead to a subsequent increase in free androgens and unfavourable metabolic profiles (Pappalardo M.A.,*et al.*,2017) The ovarian PCOS morphological traits of enlarged, multi-cystic ovaries and theca interstitial hyperplasia are reported in women who are subjected to high levels of androgens as a result of endogenous adrenal androgen hypersecretion in congenital adrenal hyperplasia ,or exogenous testosterone treatment in female-to-male transsexuals. Additionally, cultured human theca interna cells removed from PCOS ovaries exhibit higher androgen secretion that continues during long-term culture .These observations corroborate a role for androgens in the acquisition of the PCOS ovarian features.

1.5.4 Inflammatory PCOS

In inflammatory PCOS, chronic inflammation causes the ovaries to make excess testosterone, resulting in physical symptoms and issues with ovulation. Adipose tissue is recognized as an important site for excess production of pro-inflammatory mediators with subsequent chronic inflammation in women with PCOS as well as other insulin-resistant conditions such as obesity (Tilg H., *et al*., 2008).

Activation of inflammatory pathways in adipocytes has been found to impair triglyceride storage with increased release of free fatty acids, which could induce insulin resistance (Guilherme A., *et al*., 2008).

1.6. Types of Infertility

Infertility is a public health problem during the re. productive age, affecting about 10-15% of couples attempting to achieve pregnancy worldwide (Blaževičienė A, *et al*., 2014). Infertility is defined by the failure to achieve a natural pregnancy after 12 months or more of regular un. protected sexual intercourse (Gurunath S, *et al*., 2011). For many couples, the inability to bear children is a shocking tragedy leading to serious physical, social, psychological, and sexual dysfunction in their lives (Smith JF, *et al*., 2009). According to World Health Organization (WHO), the term primary infertility is used when a woman has never conceived and secondary infertility is the incapability to conceive in a couple who have had at least one successful conception in the past (Tabong PT, *et a*l., 2013).

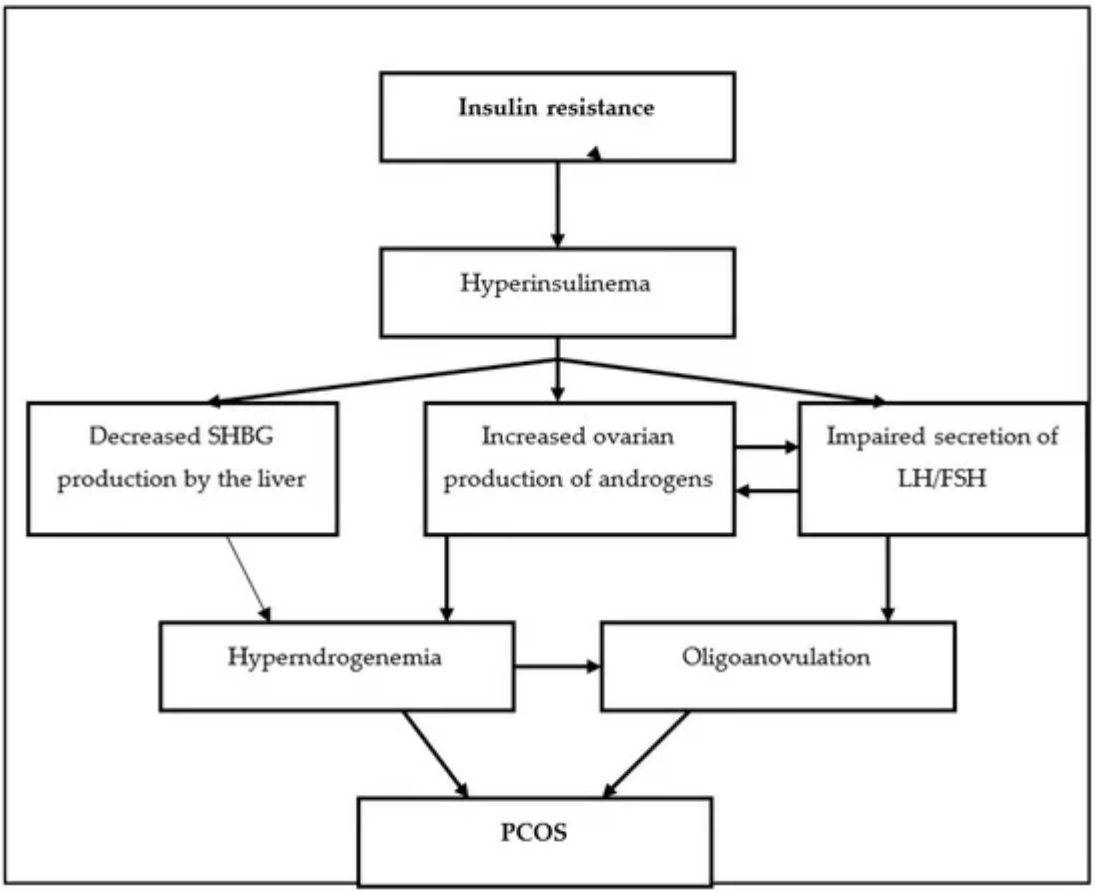
Infertility can be attributed to anomalies associated with either male or female reproductive systems or with both partners. Several factors can disturb the process of fertility at any step. For example, female infertility may be due to one or more reasons such as polycystic ovary syndrome (Lash MM, *et al*., 2008).

hormonal disorders (Kazerooni T, *et al*., 2003) premature ovarian failure, genital infection, endometriosis, fallopian tube obstruction, congenital uterine anomalies, uterine synechiae, or other medical complications (diabetes and thyroid disorders) (Benksim *et al*., 2018).

1.7 Resistance to Insulin and Polycystic Ovary Syndrome:

Insulin resistance is defined clinically as the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population (Lebovitz, Endocrinol, *et al.*, 2001).

In an insulin-resistant status, peripheral tissues have less sensitivity to the action of insulin; therefore, higher concentrations of the hormone than normal are required to maintain normoglycemia, resulting in the development of compensatory hyperinsulinemia (Philip Z., *et al*., 2020). Although not invariably present in PCOS, IR plays a central role in its pathogenesis, and it is a common finding in subjects with PCOS, both lean and affected by obesity (arreau A.M., *et al*., 2015) as shown in (Figure 1-3). It is estimated that between 50 and 90% of women with PCOS manifest insulin resistance (Venkatesan AM, *et al*., 2001). As a compensatory response to insulin resistance, hyperinsulinemia develops which in turn interacts synergistically with luteinizing hormone (LH) as a co-gonadotropin within ovarian theca cells (Franks S, *et al*., 1997). elevated insulin levels exert their effect on hormones to develop hormonal imbalances in PCOS. The levels of LH together with testosterone increase with an increase in the amount of insulin in these PCOS cases.



Figure(1,3)Role of insulin resistance in the development of polycystic ovarian syndrome (PCOS) (Zdravko Kamenov ,Antoaneta GatevaDepartment *et al* 2020)

1.8.Low Sex Hormone Binding Globulin(SHBG).

SHBG is a liver-produced globulin, its main function being to buffer the available sex hormone concentrations in the blood. Sex hormones are bound to SHBG non-covalently, similar to albumin, but unlike albumin SHBG-bound hormones are not bioavailable. This effectively creates a control mechanism on the bioavailability of sex hormones, particularly of androgens for their high affinity to SHBG (Hammond, 2011). Therefore, each mechanism or substance that modifies the hepatic production of SHBG, thereby changing its own concentration in blood,

finally impacts the bioavailability of sex hormones. It has been confirmed that insulin lowers SHBG concentrations in IR generally, and insulin-resistant PCOS specifically. Since SHBG normally binds T with a greater affinity than E2 (Mean, et al., 1977).the downstream effect of hyperinsulinemia tends to be hyperandrogenic. Furthermore, it has been shown that in obese women SHBG affinity for T is lowered, potentially having a compound effect on IR-caused hyperandrogenism (Del Mar Grasa, et al., 2017).Available data implies the existence of SHBG isoforms of different chain lengths, which presumably would have differing affinities to hormones as well. In addition, SHBG affects sex hormone activity by its direct modulation of sex hormone receptor pathways. Most of the research on this topic was motivated by the inquiry into a significantly higher incidence of autoimmune diseases in women, resulting in findings currently best explained by SHBG having an active membrane receptor (Fortunati, 1999).There are several candidates for an SHBG receptor, one well-studied option being megalin (Hammes, et al., 2005). Furthermore, there is also evidence of androgens themselves affecting the concentration of SHBG: One meta-analysis on androgenic therapy trials in post-menopausal women has shown that exogenous T therapy in post-menopausal women lowers SHBG (Marina, et al., 2020).

In PCOS, SHBG is generally lower than in non-PCOS women of similar age. Although its activity is heavily influenced by insulin and sex hormones, there is enough data to point to it being independently considered in the previously hypothesized PCOS CV disease risk calculation. One great advantage of SHBG in this regard is the relative stability of its concentration in the blood, as well as ease of measuring it. This notwithstanding, more research in this area is certainly necessary.(Pandurevic S,et al.,2021).

1.9. Dyslipidemia in women with polycystic ovary syndrome

Dyslipidemia is a very common metabolic abnormality in women with polycystic ovary syndrome (PCOS). Insulin resistance is a key pathophysiology of PCOS, thus dyslipidemia in women with PCOS may be consistent with those found in an insulin resistant state: decreased levels of high-density lipoprotein-cholesterol (HDL-C) and apolipoprotein (Apo) A-I, and increased levels of triglycerides (TG), ApoB and very low-density lipoprotein (**Kim, J. J., & Choi *et al* ,.2013).**

HDL encompasses several different classes of lipoproteins (HDL2 and HDL3) according to its density, metabolism and properties (**Warnick GR, *et al*., 2001).** HDL subclasses are known to differ in their capacity to confer cardio-protection, and HDL2 has been reported as the most anti-atherogenic HDL subtype. Thus decreased levels of HDL2 have been strongly associated with coronary heart disease, and HDL subclass profile has been investigated in women with PCOS (Talbott E, *et al*., 1995) recruited a total of 206 women with PCOS and 206 age-matched controls, and total HDL and HDL2 levels were significantly lower in women with PCOS than controls even after controlling for both age and BMI (Conway GS, *et al*., 1992) also found that even lean women with PCOS had reduced serum HDL and HDL2 concentrations compared to controls. These findings suggest that women with PCOS not only have low serum HLD-C levels, but also show alterations in HDL quality.

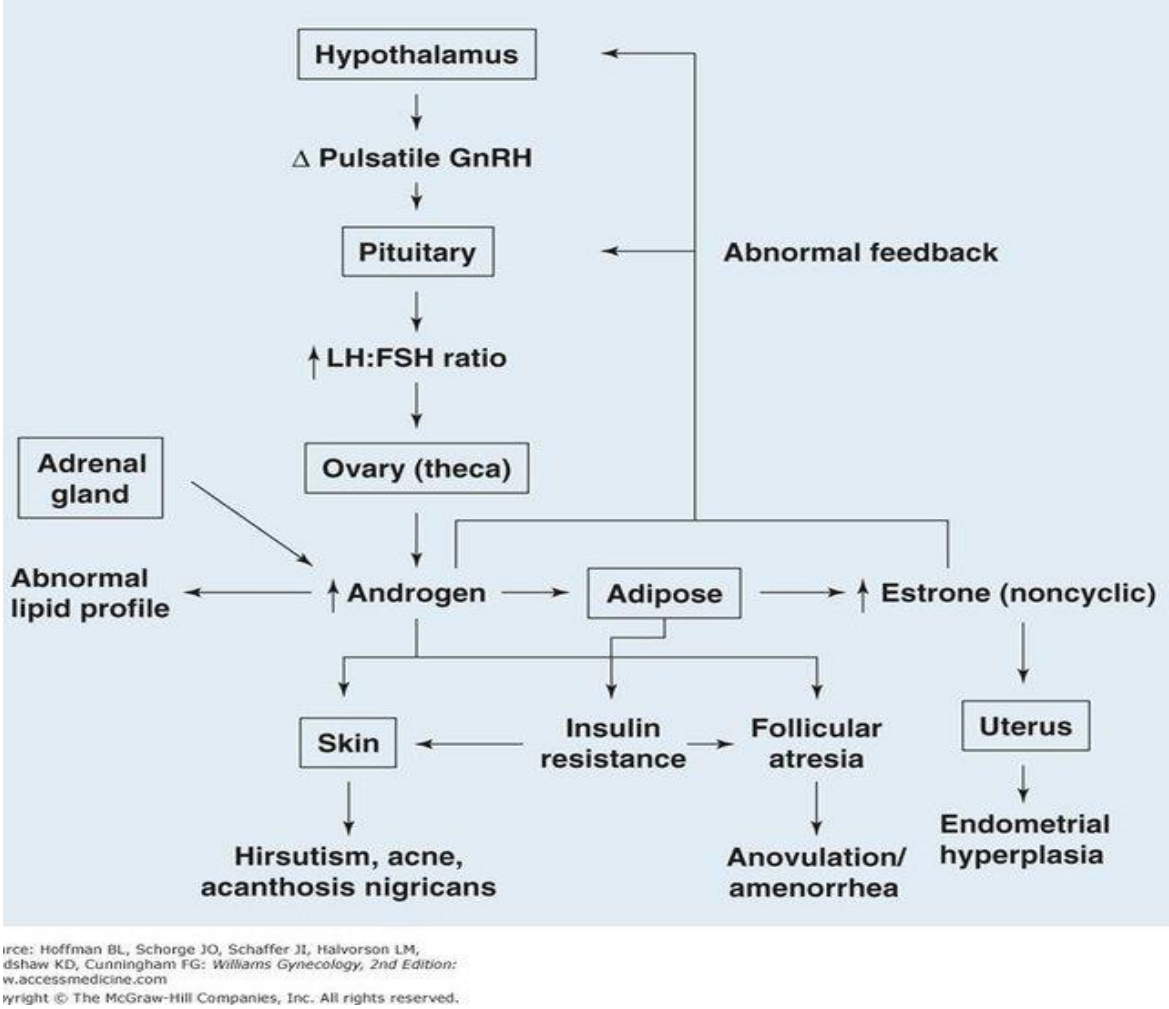
Many studies have reported that LDL-C is increased in women with PCOS which is usually not noted in insulin resistant states. The reason why LDL-C in also increased in women with PCOS in not clear yet, but increased LDL-C levels in women with PCOS may be related to hyperandrogenism or genetic factor. (Valkenburg O, *et al*., 2008). Some studies have investigated Apo A-I and Apo B

measurements in women with PCOS. Apo A-I levels were significantly lower in women with PCOS. However, there were no differences in Apo B levels between PCOS patients and controls. They also analyzed whether hyperandrogenism and obesity were independent predictors for the presence of a more atherogenic lipid profile in women with PCOS and found that free androgen index and BMI were independent predictors for serum ApoA-I levels in women with PCOS. They concluded that **ApoA-I** levels were significantly **lower** in women with PCOS without any difference in ApoB levels, and both obesity and hyperandrogenism contribute to these changes (Valkenburg O, *et al*., 2008) ApoC-I inhibits the uptake of TG-rich lipoproteins via hepatic receptors and has been reported to increase postprandial serum lipid level as is common in coronary artery disease patients. In women with PCOS. (**Huang S, *et al*., 2010)** .

evaluated the role of ApoC-I level and assessed relationships between ApoC-I and clinical features of PCOS.

1.10. Hyperandrogenism:

PCOS is characterized by an increased frequency of gonadotropin-releasing hormone (GnRH) pulsatility that selectively increases luteinizing hormone (LH) secretion. LH stimulates multiple steroidogenic enzymes in the theca cells of the ovary, leading to theca cell hyperplasia and increased testosterone production. Because of relative follicle-stimulating hormone (FSH) deficiency, testosterone is not completely aromatized and degraded by the granulosa cells. (Sarah A, et al.,2022).show in figure(1-4).



Figure(1,4).Pathophysiology of PCOS, (Williams gynecology, 2012).

The increased levels of testosterone feedback on the hypothalamus, decrease the ability of estradiol and progesterone to slow down GnRH pulse frequency(Diamanti-Kandarakis E, et al .,2012). The ovaries do not appear to be the primary abnormality in PCOS since they can respond promptly to changes in gonadotropin secretion; ovulation occurs in response to the surge in the secretion of FSH stimulated by clomiphene citrate. Furthermore, weight reduction in obese patients reduces estrone and insulin levels, normalizes gonadotropin secretion, and regulates menstrual cycles in women with PCOS(Benito E, *et al*,.2020)

1.11-. Estrogen-Progesterone Imbalance

In healthy pre-menopausal women, estradiol (E2) is the most potent circulating estrogen, and most of E2 comes from the activity of aromatase in granulosa cells of the ovary by conversion of thecal T. This makes E2 very dependent on cyclical changes in the ovary during the menstrual cycle, and also on age-related transformations. they start to fluctuate wildly and then practically disappear from circulation with menopause. This coincides with the aforementioned increase in CV disease in women. This association sparked questions about the potential beneficial effect of hormonal replacement therapy (HRT) in reducing CV events in women in the menopausal period. However, no clinical trial to date has shown a benefit through either CV disease risk makers like carotid intima-media thickness (cIMT) or CV events incidence (Miller, *et al*., 2019).

The genomic effects of estrogens are initiated by their binding to the estrogen receptors (ERs). Estrogens are ligands for two different cytoplasmic receptors: ER- α and ER- β. They are highly homologous, but they regulate gene expression differently. Different experiments have shown various combinations of ER- α and ER- β -related effects, depending on the tissue affected. For example, several studies used the model of VSMC proliferation, and they found that even though both are protective by themselves, only when both are present is VSMC proliferation regulated normally (Hogg, *et al*., 2012).

Interestingly, ER- α and ER- β expression also changes independently during the menstrual cycle and the occurrence of ovulation—ER- in vascular endothelium was 30% lower in the early compared to late follicular phase, and post-menopausal women also continuously had 33% lower expression than pre-menopausal women in postovulatory phase (Gavin, *et al*., 2009).

There are no data on vascular ER- β in humans specifically, but measurements in myometrium do show an increase in ER- β and a decrease in ER- α in post-menopause when compared to pre-menopause.

In PCOS there are still conflicting accounts on if and how exactly ER expression is different. Alterations of ER- α and ER- β expression in ovaries and endometrium are well supported by data (Wang, *et al*., 2010).

More research is needed, but this difference in the receptor makeup could explain some differences in PCOS concerning the general population in terms of estrogen effects on CV risk, even without major differences in circulating hormones.

On the other hand, the increased level of LH, frequently found in PCOS, could have a protective effect on CV risk. The ratio between LH and follicle-stimulating hormone (FSH) concentration in the early follicular phase is normally < 1, or favoring FSH; in PCOS, inversion is often seen, with or without menstrual or ovulatory dysfunctions. While FSH receptors are specific to gonads, the LH receptor is expressed in various tissues throughout the body, blood vessels included.

It was demonstrated in animal and cell studies that LH/human chorionic gonadotropin (hCG) receptor activation has a biphasic vasodilating effect in vivo: The first peak is mediated by LH receptors themselves, while the following peak is mostly secondary to LH/hCG receptor-induced increase in estradiol concentration (Ziecik, *et al*., 2007).

PCOS is characterized by menstrual cycle abnormalities and, frequently, anovulation, leading to lower monthly average progesterone levels, altered estrogen receptor activity, and increased circulating LH. However, anovulatory and/or irregular cycles are neither enough to diagnose PCOS, nor do all PCOS women have them. LH, specifically to FSH (LH/FSH ratio), has been notably shown to only be elevated in less than 50% of PCOS patients (Banaszewska, *et al*., 2003).

Furthermore, the incidence of menstrual irregularity, as well as clinical characteristics of hyperandrogenism, fall with age (Gunning, *et al*., 2017).

Therefore, in an attempt to evaluate CV risk in PCOS, it is plausible that the estimate of the menstrual cycle and concomitant hormonal changes need to also be considered, other than the presence of increased LH levels.

There is also evidence of estrogen-progesterone interaction in the vasculature, mediated by endothelin-1 (ET-1) (Tan, *et al*., 2003).

In women, both estrogens and progesterone activity lower ET-1 concentrations, and it is notably the lowest during the late follicular and luteal phase of the cycle when estradiol and progesterone levels are at their highest (Polderman, *et al*., 2000).

In anovulatory cycles, there is no progesterone spike, and the luteal phase constitutes a smaller part of oligomenorrheic cycles anyway, so the compounding effect is a very big difference in estrogen/progesterone ratios between PCOS and non-PCOS women.

Furthermore, one study has shown that even in the early follicular phase of eumenorrheic cycles, ET-1 is four times higher in PCOS compared to controls, and when stratifying by obesity classes the difference remains (Diamanti-Kandarakis, *et al*., 2001).

Therefore, overexpression of ET-1, in part mediated by estrogen/progesterone alterations, could be one of the mechanisms increasing CV risk in PCOS.

1.12.Endothelin-1(ET-1)

Endothelin 1 (ET-1) is a potent vasoconstrictor peptide that is also expressed in neurons. The peptide comprises 21 amino acids with two intrachain disulfide linkages and was first isolated from the culture supernatant of porcine aorta endothelium cells in 1988 (M. Yanagisawa, 1988).Endothelin derives from "big endothelin" a prepropeptide that is cleaved by endothelin-converting enzymes to produce mature endothelin (R. M. Kedzierski and M. Yanagisawa, 2001).ECE is found on the endothelial cell membrane. ET-1 formation and release are stimulated by angiotensin II (AII), antidiuretic hormone (ADH), thrombin, cytokines, reactive oxygen species, and shearing forces acting on the vascular endothelium. ET-1 release is inhibited by prostacyclin and atrial natriuretic peptide as well as by nitric oxide.

Endothelin has attracted intensive interdisciplinary interest because of its unique profile as an endothelium-derived vasoactive factor with a powerful and characteristically long-lasting vasopressor activity. Thus, whereas the cellular mechanism of endothelin action appears to be similar to classic vasoconstrictor substances such as angiotensin II and norepinephrine, the slow time course of the regulatory mechanisms of its biosynthesis and secretion resembles more that of inflammatory cytokines. These characteristics make this family of small peptides unique within the realm of intercellular mediators with cardiovascular relevance (M. Yanagisawa, 1994).

Endothelin is expressed in three isoforms called ET-1, ET-2, and ET-3, with slightly different amino-acid sequences and different distributions in various tissues. Accordingly, three different genes encoding the endothelins have been identified in the human, rat, and pig genomes (Inoue. A, 1989).

Furthermore, three ET receptor subtypes called ETA, ETB, ETC have been described.

ET-1 is present in the aqueous humor at concentrations several times higher than in plasma, presumably because it is secreted by the ciliary epithelium and not derived from plasma (Lepple-Wienhues, 1992).

The vascular endothelium modulates local vascular tone by releasing relaxing factors such as nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factors as well as the potent vasoconstrictor peptide endothelin-1 (R. F. Furchgott and J. V. Zawadzki, 1980).

Endothelin-1 is one of the several circulating molecules of endothelial injury products. Endothelin-mediated vascular tone and metabolic function are abnormal in obesity and diabetes (Taupe et al. 2002; Cardillo et al. 2004; Lteif et al. 2007; Mundy et al. 2007). Endothelin also plays a role in adipogenesis and lipolysis (Bhattacharya and Ullrich 2006; van Harmelen et al. 2008), thus endothelin blockade may be particularly feasible for preventing cardiovascular and renal disease in patients and may also be suitable for treating obesity and its associated complications, such as insulin resistance (Berthiaume et al. 2005). Moreover, endothelin contributes to glycemic control and glucose uptake (Wu-Wong et al. 1999; Rachdaoui and Nagy 2003; Said et al. 2005) and the development of type 1 diabetes (Ortmann et al. 2005), to play a role in the early events of endothelial dysfunction, and has been used as a marker of abnormal vascular reactivity (Yanagisawa M, 1989).

Elevated endothelin-1 (ET-1) levels have been reported in some insulin-resistant states such as obesity (**Ferri C, *et al*., 1997**), diabetes mellitus (DM) (**Hopfner RL, *et al*., 1999)**, and hypertension.

Studies estimating ET-1 levels in women with PCOS preliminary results by Paradisi (**Paradisi G. *et al*., 1998**) was shown abnormal endothelium vascular response in obese PCOS (PCOS-OB) women.

On the cardiac vascular risk side, there are many biochemical markers (hyperandrogenism, estrogen/progesterone imbalance, IR, compensatory hyperinsulinemia, and low SHBG) that tell us that PCOS should be regarded as a high-risk state. The non-correspondence of alterations of mediators of CV risk, early atherosclerotic processes, and CV events in PCOS later in life could be a product of lacking clinical data (prospective longitudinal studies) with adequate cohorts that are followed long enough, the extreme heterogenicity of the cohorts of PCOS included in the studies, or of their inadequate phenotypization in terms of mediators of CV risk, in part due to the inadequacy of the methods used to measure it. In the meantime, the understanding of the underlying biochemical processes of CV risk in PCOS shifts attention to specific subgroups of the PCOS population which potentially carry most of the risk for CV events. (**Srdan Pandurevic, *et al*.,2021)**

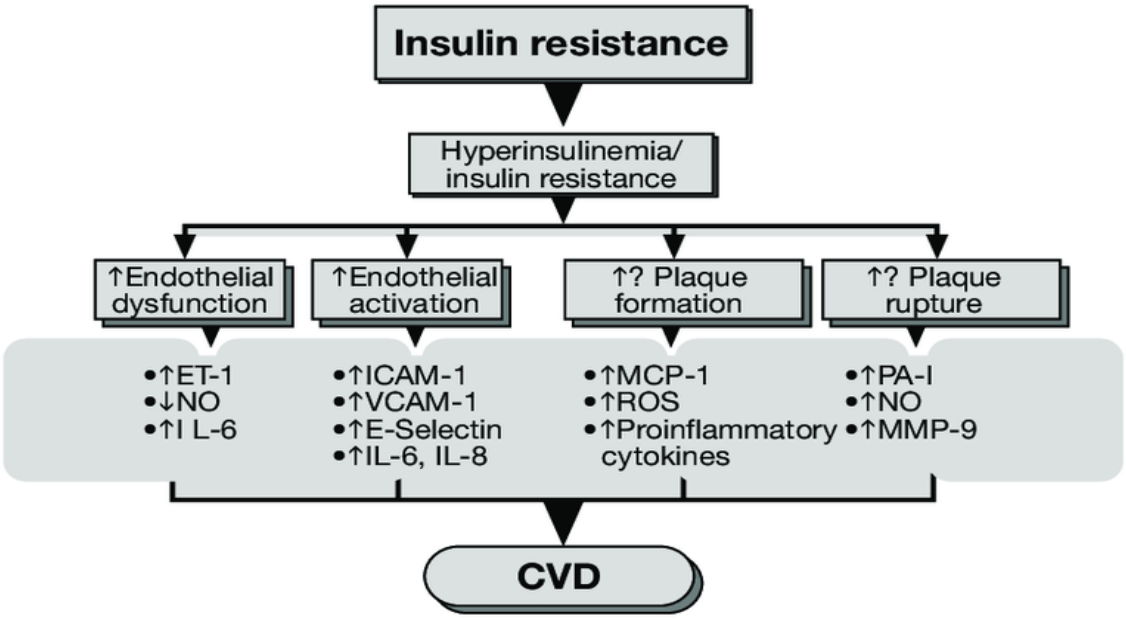
Since Abdominal adiposity and obesity are often present in PCOS. Mounting evidence indicates that adipose tissue is involved in innate and adaptive immune responses. Continuous release of inflammatory mediators such as cytokines, acute phase proteins, and adipokines perpetuates the inflammatory condition associated with obesity in women with PCOS, possibly contributing to insulin resistance and other long-term cardiometabolic risk factors. (**Ojeda-Ojeda, *et al*., 2013)**

1.13-Insulin resistance and endothelial function

The coexistence of insulin resistance and hyperinsulinemia with endothelial dysfunction and

elevated ET-1 levels is supported by in vitro and in vivo studies(**Ferri C *et al* .,1995**) . In diabetic patients, it has been shown that plasma ET-1 levels were rapidly increased during euglycemichyperinsulinemic clamps **(Ferri C *et al* ., 1995**). women with PCOS had hyperinsulinemia and reduced glucose utilization and exhibited elevated ET-1 levels. This may be indicative of the involvement of hyperinsulinemia and insulin resistance in the abnormal endothelial status,

manifested in this study by the elevated ET-1 levels. the negative correlation between glucose utilization and ET-1 levels indicates the interaction of metabolic aberrations and endothelial status. Thus, therapeutic interventions that increase insulin sensitivity may offer an additional benefit, possibly by protecting and/or restoring the endothelial barrier.show in figure(1-5).



Figure(1,5): The role of insulin resistance/hyperinsulinemia in the atherogenesis process in polycystic ovary syndrome. As shown in figure, insulin implicates in all stages of atherogenesis process: endothelial dysfunction, endothelial activation, plaque formation and plaque rupture through up-and down-regulation of adhesion molecules, inflammatory cytokines, vasoactive substances, fibrinolytic factors. ET-1: endothelin 1; NO: nitric oxide; IL: interleukin; ICAM-1: intercellular adhesion molecule-1; VCAM-1: soluble vascular cell adhesion molecule-1; MCP-1: monocyte chemotactic protein-1; ROS : reactive oxygen species; PA-I: ???; MMP-9: matrix metaloproteinase-9; CVD: cardiovascular disease.

(E. Kassi *et al* .,2008)

1.14-Visfatin

Visfatin, termed of pre-B-cell colony-enhancing factor or nicotinamide phosphoribosyltransferase, is an adipocytokine that is predominantly produced in visceral adipose tissue (**Shi KL,*et al*., 2016**).

Visfatin is an adipokine identified in 2004 (**Fukuhara A, *et al*,.2005**) and thus named for the suggestion that it would be predominantly produced and secreted in visceral fat. Visfatin is highly preserved across animal evolution. It has a molecular weight of 52 KDa and its gene encodes 491 amino acids. It is identical to the pre-B cell colony-enhancing factor (PBEF), described in 1994 as a cytokine produced by lymphocytes, acting on lymphocyte maturation and inflammatory regulation.

Visfatin was also soon recognized as the formerly described Nicotinamide phosphoribosyltransferase (Nampt), the limiting enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis. In addition to being produced in human leukocytes and adipose tissue, visfatin is also expressed in human and animal hepatocytes and muscles (**Costford SR, *et al*.,2010**) (**Garten A et al .,2010**). and in animal adipocytes, kidneys, and heart(**Krzysik-Walker SM *et al* .,2008**). Visfatin was found to be released predominantly from macrophages rather than from adipocytes in visceral adipose tissue. In this regard, there is sufficient evidence to consider that visfatin is expressed by the macrophages infiltrating adipose tissue and is produced in response to inflammatory signals (**Varma V, *et al*,.2007)** it is now believed that visfatin actions can be endocrine, paracrine, and autocrine as well. These autocrine effects of visfatin may play an important role in regulating insulin sensitivity in the liver (**Skop V, et al,.2009**) Visfatin is involved in the pathogenesis of various metabolic disorders; increased plasma concentrations of visfatin have been reported in an individual with obesity, as well as in patients with gestational diabetes mellitus (GDM) or metabolic syndromes (**Vejrazkova D, *et al*., 2017**).

Visfatin functions as an immunomodulatory cytokine involved in inflammatory responses and is a factor associated with obesity, inflammation, and insulin resistance (**Sun L, *et al*.,2017).**

Visfatin serves important roles in the induction of insulin resistance (**Hosseinzadeh-Attar MJ,*et al*., 2016**).

Numerous previous studies indicated that visfatin was associated with inflammation (**Pattou F,*et al*.,2010**); however, the underlying molecular mechanisms remain unknown. Furthermore, visfatin functions as the rate-limiting enzyme of NAD+ biosynthesis and converts nicotinamide into nicotinamide mononucleotide, a key NAD+ intermediate (**Stromsdorfer KL, *et al*.,2016**).

In addition, visfatin-mediated NAD+ biosynthesis in adipocytes is an important physiological regulator of the metabolic function of adipose tissue and whole-body (**Stromsdorfer KL,*et al*., 2016**).

Although still controversial, visfatin was reported to exert insulin-mimetic or sensitizing effects; similar to insulin, visfatin increases lipogenesis, enhances glucose uptake of myocytes and adipocytes, and inhibits hepatocyte glucose release, consequently contributing to glucose and lipid metabolism (**Kim HS,*et al*., 2014).**

1.15-Insulin-Like Function:

Insulin once secreted from B-cells of the pancreas into circulation, binds to the insulin receptors of insulin-sensitive targets, leading to internal signaling. These things are well-known facts. Hence when we say insulin is like the ACtivity of visfatin it means to say it has its journey in showing its function the same as that of insulin. The binding affinity of visfatin/PBEF/Nampt to the IR (insulin receptor) was found to be similar compared with that of insulin(**Fukuhara A *et al* .,2005**). Many

studies have demonstrated increased levels of visfatin in diabetes mellitus(**HAIDER DG., *et al* .,2005**). However, an experiment conducted on a cohort of obese patients did not show any correlation between PBEF/visfatin to glucose infusion(.**BERNDT J., *et al* .,2005**). Whether visfatin binds to the IR remains controversial. But they still stand up to their conclusions Whether visfatin binds to insulin receptors and exerts its insulin-mimetic activity is still a controversy, but recent research has shown that Nampt/visfatin-mediated systemic NAD+ biosynthesis is necessary for β cell function, suggesting that visfatin helps in regulation of glucose homeostasis(**REVOLLO JR., *et al*.,2007**).

Aim of study

1-To investigate serum ET-1 & visfatin levels as predators for PCOS among women with PCOS and control.

2-Is their a relation ship with groups(age and BMI).

Conclusion

This study was concluded that:

1. ET1 and visfatin may be predator for PCOS.

2. ET1 increase with age.

The level of vesfstin on obese patient more than over weight

CHAPTER TWO

Materials and Methods

2-Materials and Methods

2.1- Study design:

This study is a case-control study involving (120 women) aged of them between (20-40)years and a BMI(of 20-35)km/m². 60 PCOS patients. and 60 non-PCOS women as a control at the reproductive fertility consultant of a gynecological and obstetric teaching hospital, BABILON. health directorate Iraq and College of Medicine, the University of Kerbela Iraq. The Rotterdam criteria-2003 was presumed to Patients with any 2 of the next 3 items that can be recognized in diagnosis: oligomenorrhea or amenorrhea, increase androgen levels, ovarian volume > 10mL on U/S, and follicles ≥12 with diameter 2-9 mm (Park et al., 2022). The control group has regular menstruation, with normal ovaries as they were detected by the gynecologist . PCOS cases were classified into three subgroups (A, B, C) according to infertility(normal which no infertility, primary infertility was 40, and secondary infertility was 20) .5ml of blood was drawn from women and put in a gel tube to measure serum ET-1 and Serum visfatin level (ng/ml) were assessed by ELISA, measure hormones(LH, FSH, F.testo) in cycle day two(CD2) by minividas, measure lipid profile (total cholesterol TC, high-density lipoprotein HDL, low-density lipoprotein LDL, very low-density lipoprotein VLDL and triglyceride TG) by colorimetric assay using a spectrophotometer, measure insulin by ELISA assay. and fasting blood sugar by maglumi device.

2 .1.2. Descriptive variable of subjects:A special form has been created for each subject's data and demographic information, which includes age, BMI, history of infertility, ultrasound , w/hratio, marriage status, phone number, and address, number of baby.

2.1.3-Ethical issue:

All participants have given their variable permission for their information and samples to be included in the study. The information about each case collected from the study (patients and controls) were taken with ethical considerations.The question aire was structured into different sections. The socio demographic characteristics of the participants were obtained and represented by the Baseline characteristics. Additionally, participants were asked to report their history with chronic conditions.

2.2- Instruments:

In this chapter, materials, instruments and tools were described and listed in Table (2.1)

Table 2.1: The instruments used in the study

Instrument	Suppliers
Centrifuge	HETTICH/ Germany
Deep Freeze	COOLTECH/ China
ELISA system	UNO/HUMAN/ Germany
Incubator	Heraeus/ Germany
MAGLUMI	GERMANY
MINIVIDAS	FRENCE
Roller Mixer	China
Spectrophotometer	GERMANY

2.3-Tools, materials and Kits

The Tools, materials and kits with their supplier which were used in this study are listed in table (2.2) .

Table 2.2: Tools and materials used in the study

Tools and Materials	Suppliers
Eppendroff Tubes	China
Gel tubes	China
Gilson Micro-tips, 100µl	China
Gilson Tips,1000µl (blue)	China
Gloves	China
Micropipette(10-100 µl)	DRAGON LAB/ USA
Syringe	China

Table 2.3 Kits which are used in this study

Chemicals kits	Suppliers
Cholestrol	Spain
Endothelin 1(ET1)	China
Follicle stimulating hormone(FSH)	Mannheim /Germany
Free Testosterone(FT)	Mannheim/ Germany
Glucose	Bio Lab/ France
High density lipoproten(HDL)	Spain
Insulin (IN)	Mannheim/ Germany
Luteinizing hormone (LH)	Mannheim/ Germany
Triglyceride(TG)	Spain
visfatin	China

2.4 Inclusion and Exclusion criteria:

2.4.1 Patients Criteria

All patients were subjected to the full clinical history, clinical examination, and relevant laboratory investigations. with no thyroid disorder and DM. The diagnosis of the PCOS clinical conditions was established according to the age from (20-40)years , BMI(20-35)km/m², fasting to estimate lipid profile ,sugar and insulin ,no history of obesity ,problem with menstrual cycle,CD2.

2.4.2 Patients Exclusion criteria:

Patient excluded who above 40years ,BMI above 35km/m²,history of obisty, who not fasting, who not CD2,not married, with history of heart disease,thirod disorder.

2.4.3 Control Criteria:

Control group of an apparently healthy 60 subjects were chosen from well-known volunteers' participants. Blood samples were drawn from the volunteers, participants had no history of heart diseases. The ages of the participants were also convergent in the whole study group. Demographic information of the participants was also collected through the self- reported technique (student questionnaire).

2.5-Study variables :

Serum vesfitin-1, Endotheline-1,Blood Sugar, Insulin level, Age, smoking state, Lipid profile, sex hormones, BMI, waist and Hip measurements.

2.6- Measurement and Data collection

2.6.1-Blood Collection and Storage

Blood samples were collected from Obstetrics and Gynecology Hospital. Five mls of blood samples were drown by venipuncture using 5ml disposable syringes, blood was left for (15 min) at room temperature in gel tube. Serums were separated by centrifuging for 10 minutes at approximately 4000 xg. Serum samples were aliquot into two Eppendorf and store at -20°C to avoiding multiple freezing-thawing

cycles and used for the further measurement. Blood collection tubes were be disposable, non-pyrogenic, and non-endotoxin.

2.7- Methods:

2.7.1- Measurement of Human Endothelin 1 levels in serum by using Sandwich-ELISA Technique:

Principle : Enzyme-Linked Immunosorbent Assay (ELISA). The plate was been pre-coated with Human EDN1 antibody. EDN1 present in the sample was added and binds to antibodies coated on the wells. And then biotinylated Human EDN1 Antibody was added and binds to EDN1 in the sample. Then Streptavidin-HRP was added and binds to the Biotinylated EDN1 antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was then added and color develops in proportion to the amount of Human EDN1. The reaction was terminated by addition of acidic stop solution and absorbance was measured at 450 nm.

2.7.2-Reagents :

The Measurement of Human Endothelin 1 method reagents were shown in Table (2.4):

Components	Quantity (96T)
Standard solution	(640ng/L) 0.5ml x1
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop solution	6ml x1
Substrate solution A	6ml x1

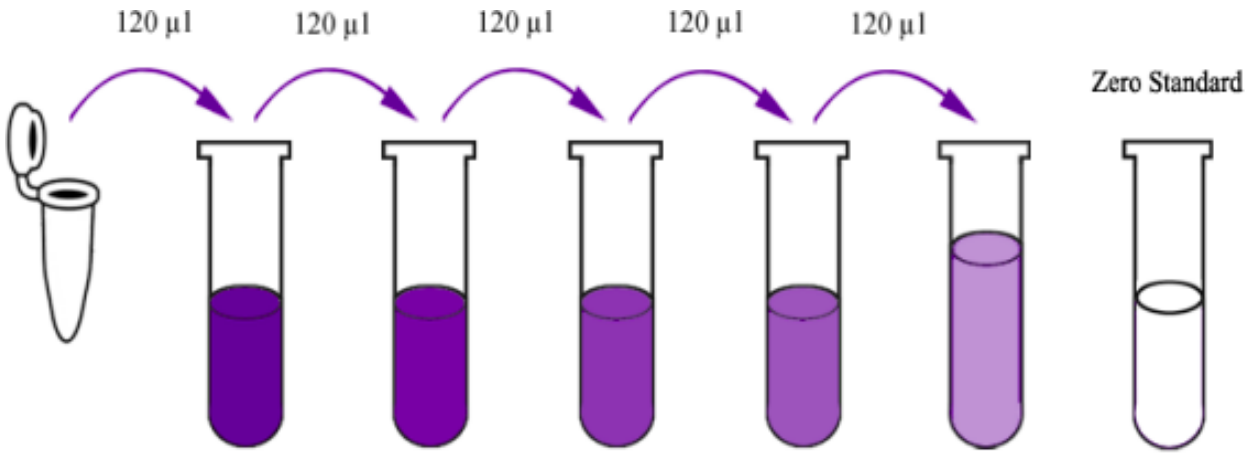
Substrate solution B	6ml x1
Wash buffer Concentrate (25x)	20ml x1
Biotinylated Human EDN1 antibody	1ml x1
User instruction 1	1
Plate sealer	2 pics

2.7.3-Reagent Preparation

All reagents be at room temperature before use. Standard Reconstitute the 120ul of the standard (640ng/L) with 120ul of standard diluent to generate a 320ng/L standard stock solution. the standard was sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (320ng/L) 1:2 with standard diluent was produce 160ng/L, 80ng/L, 40ng/L and 20ng/L solutions. Dilution of standard solutions suggested are as follows in**Table (2:5)**

320ng/L	Standard No.5	120ul Original standard + 120ul Standard diluent
160ng/L	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
80ng/L	Standard No.3	120ul Standard No.4 + 120ul Standard diluent
40ng/L	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
20ng/L	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

Preparation procedure steps of standard were summarized in the **Figure (2-1)** below



2.7.4-Assay Procedure

1. Prepared all reagents, standard solutions and samples as instructeded. By Bring all to room temperature before use. The assay was performed at room temperature.

2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should was stored at 2-8°C.

3. we added 50ul standard to standard well.

4. we added 40ul sample to sample wells and then added 10ul Human EDN1 antibody to sample wells, then added 50ul streptavidin-HRP tosample wells and standard wells . Mix well and was Covered the plate with a sealer. Incubateed to 60 minutes at 37°C.

5. Removed the sealer and was washed the plate 5 times with wash buffer. Soaked wells with 300ul wash buffer for 30 seconds to 1 minute

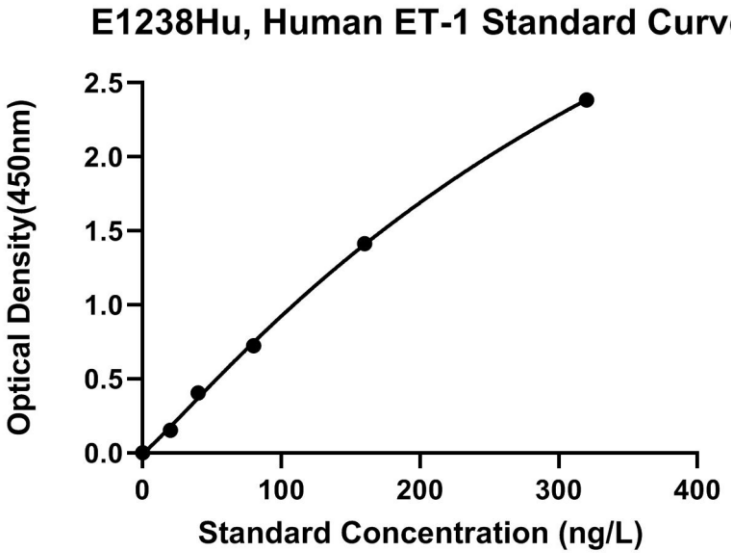
6. Added 50ul substrate solution A to each well and then added 50ul substrate solution B to each well. Incubateed plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. Added 50ul Stop Solution to each well, the blue color will change into yellow immediately.

8. Determined the optical density (OD value) of each well using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.7.5-Calculation of Result

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.



2.8- Measurement of Human Visfatin levels in serum by using Sandwich-ELISA Technique:

Principle : Human Visfatin ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Human Visfatin in serum (Human Visfatin concentration is low in normal serum/plasma, and may not be detectable in this assay), plasma and cell culture supernatants. This assay employs an antibody

specific for Human Visfatin coated on a 96-well plate. Standards and samples are pipetted into the wells and Visfatin present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human Visfatin antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of Visfatin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2.8.1-Reagents :

The **Measurement of Human Visfatin levels** method reagents were shown in **Table (2.6)**:

Component	**Size / Description**	**Storage / Stability After Preparation**
Visfatin Microplate (Item A)	96 wells (12 strips x 8 wells) coated with anti-Human Visfatin.	1 month at 4°C*
Wash Buffer Concentrate (20X) (Item B)	25 ml of 20X concentrated solution.	1 month at 4°C
Standard Protein (Item C)	2 vials of Human Visfatin. 1 vial is enough to run each standard in duplicate.	1 week at -80°C
Detection Antibody Visfatin 2 vials of biotinylated anti-Human Visfatin. Each (Item F)	vial is enough to assay half the microplate.	5 days at 4°C

HRP-Streptavidin (Item G).	200 µl 250X concentrated HRP-conjugated streptavidin.	Do not store and reuse.
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) buffer solution.	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid.	N/A
Assay Diluent A (Item D)	30 ml of diluent buffer, 0.09% sodium azide as preservative.	N/A
Assay Diluent D (Item K)	15 ml of 5X concentrated buffer.	1 month at 4°C
Assay Diluent B (Item E)	15 ml of 5X concentrated buffer.	1 month at 4°C

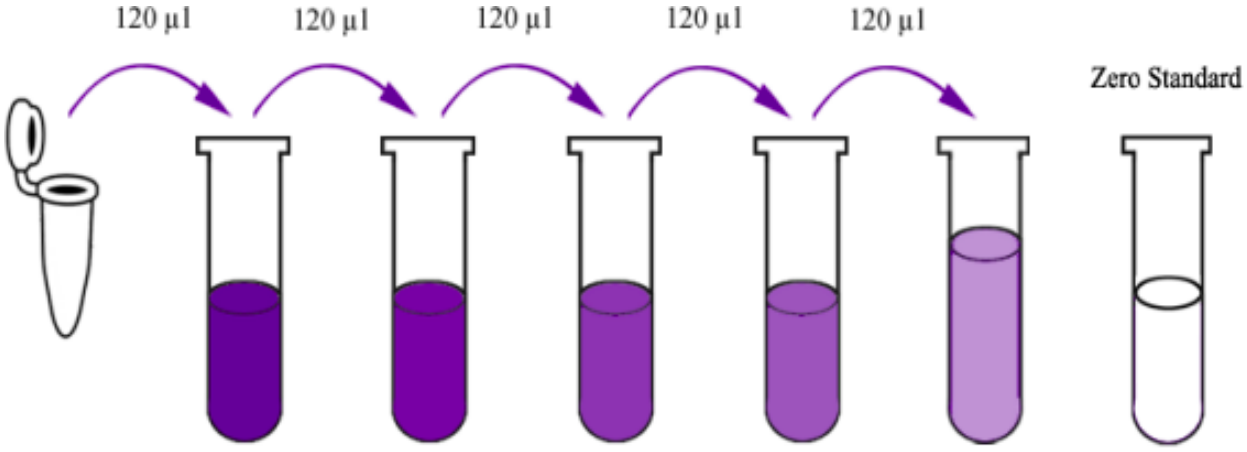
2.8.2-REAGENT PREPARATION

1. Brought all reagents and samples to room temperature (18 - 25oC) before use.
2. Assay Diluent D (Item K) and Assay Diluent B (Item E) was diluted 5-fold with distilled water before use.
3. Sample dilution: Assay Diluent A (Item D) was used for dilution of serum and plasma samples. 1X Assay Diluent D (Item K) was used for dilution of cell culture supernatant samples. The suggested dilution for normal serum/plasma has 2 fold.
4. Preparation of standard: Briefly spin a vial of Item C.was Add 400 µl Assay Diluent A for serum 1x Assay Diluent D (for cell culture medium, Assay Diluent D was diluted 5-fold with distilled water before use into Item .C vial

prepared a 300 ng/ml standard. Dissolve the powder thoroughly by a gentle mix. Pipette 270 µl Assay Diluent A or 1x Assay Diluent D into each tube. Used the 300 ng/ml standard solution to produce a dilution series (shown below). Mixd each tube thoroughly before the next transfer. Assay Diluent A or 1x Assay Diluent D serves as the zero standard (0 ng/ml).

5. The Wash Concentrate (20X) (Item B) contains visible crystals, warm to room temperature and mixed gently until dissolved. Diluted 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
6. Spined the Detection Antibody vial (Item F) . Added 100 µl of 1X Assay Diluent B (Item E) into the vial to prepared a detection antibody concentrate. Pipetted up and down to mix gently .The detection antibody concentrate was diluted 80-fold with 1X Assay Diluent B (Item E) and useded in step 5 of Part VI Assay Procedure.

spined the HRP-Streptavidin concentrate vial (Item G) and pipetted up and down to mix gently before used, as precipitates may form during storage. HRP-Streptavidin concentrate was diluted 250-fold with 1X Assay Diluent B (Item E). Preparation procedure steps of standard were summarized in the **Figure (2-2)** below



2.8.4- ASSAY PROCEDURE

Brought reagents and samples to room temperature (18 - 25oC) before use. Added 100 µl of each standard and sample into appropriate wells. Covered wells and incubateed for 2.5 hours at room temperature with gentle shaking.

1. Discarded the solution and washed 4 times with 1X Wash Solution. Washed by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette . Complete removal of liquid at each step was essential to good performance. After the last wash, removeed any remaining Wash Buffer by aspirating . Invert the plate and blot it against clean paper towels.
2. We Added 100 µl of 1X prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
3. Discard the solution.
4. Added 100 µl of prepared Streptavidin solution to each well. Incubate for 45 minutes at room temperature with gentle shaking.
5. Discarded the solution.
6. We Added 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
7. Added 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

2.8.5-Calculation of Results

Calculated the mean absorbance for each set of duplicated standards, controls and samples, and subtract the average zero standard optical density. Ploted the standard curve using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

2.9- Measurement of Lipid profile levels in Human serum .

2.9.1- Method for Quantitation Serum total cholesterol level (TC):

Enzymatic colorimetric method was used to measure concentration of serum total cholesterol, This method for the measurement of total cholesterol in serum involves the use of three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and peroxidase (POD). In the presence of the former the mixture of phenol and 4-aminoantipyrine (4-AA) are condensed by hydrogen peroxide to form a quinoneimine dye proportional to the concentration of cholesterol in the sample. The absorbance of Total cholesterol was measured at the wavelength 500 nm. Reference values for serum adults 50-199 mg/dL (1).

2.9.2- Method for Quantitation Serum Triglyceride level(TG):

Enzymatic colorimetric method was used to measure concentration of serum triglyceride, The method was based on the enzymatic hydrolysis of serum or plasma triglyceride to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL). The glycerol is phosphorylated by adenosin triphosphate (ATP) in the presence of glycerolkinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). G-3-P is oxidized by glycerophosphate oxidase (GPO) to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide. A red chromogen is produced by the peroxidase (POD) catalyzed coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogen peroxide (H2O2), proportional to the concentration of triglyceride in the sample. The absorbance of Triglyceride was measured at the wavelength 500 nm. Reference values for serum adults 0-149 mg/dL (2) .
And VLDL calculated byFriede wald's equation**.**

$$VLDL = \frac{TG}{5}$$

2.9.3- Method for Quantitation Serum High Density Lipoprotein level(HDL):

Enzymatic colorimetric test was used to measure concentration of serum high density lipoprotein. This technique used a separation method based on the selective precipitation of apoliprotein B-containing lipoproteins (VLDL, LDL and (a)Lpa) by phosphotungstic acid/MgCl2, sedimentation of the precipitant by centrifugation, and subsequent enzymatic analysis of high density lipoproteins (HDL) as residual cholesterol remaining in the clear supernatant. The absorbance of HDL was measured at the wavelength 500 nm. Reference values for serum adults 40-60 mg/dL (3).

2.9.4- Method for Quantitation Serum Low Density Lipoprotein level(LDL):

Enzymatic colorimetric test was used to measure concentration of serum low density lipoprotein. This technique used a separation method based on the specific precipitation of low-density lipoproteins (LDL) by polyvinyl sulfate in whole serum, sedimentation of the precipitant by centrifugation, and subsequent test as residual cholesterol of the rest of lipoproteins (VLDL+ HDL) remaining in the clear supernatant. LDL-cholesterol was calculated by substracting the supernatant cholesterol fractions from the total cholesterol of the sample. The absorbance of LDL was measured at the wavelength 500 nm. Reference values for serum adults 0-100 mg/dL optimal, 100-129 mg/dl above optimal, 130-159 mg/dl borderline high (4).

2.10- Hormonal Analysis

2.10.1-Method for Quantitation Serum Luteinizing Hormone level(LH):

Human LH (Luteinizing Hormone) ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human LH in serum, plasma, and cell culture supernatants. This assay employs an antibody specific for human LH coated on a 96-well plate. Standards and samples are pipetted into the wells and LH present in a sample was bounded to the wells by the immobilized antibody. The wells are washed and biotinylated anti- human LH antibody is added. After washing away unbound biotinylated antibody, HRP- conjugated streptavidin was pipetted to the wells. The wells are again washed, a TMB substrate solution was added to the wells and color develops in proportion to the amount of LH bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color was measured at 450 nm.

2.10.2- Method for Quantitation Serum Follicular Stimulating Hormone level(FSH):

Human FSH (Follicular Stimulating Hormone) ELISA kit was an in vitro enzyme- linked immunosorbent assay for the quantitative measurement of human FSH in serum, plasma and cell culture supernatants. This assay employed an antibody specific for human FSH coated on a 96-well plate. Standards and samples are pipetted into the wells and FSH present in a sample was bounded to the wells by the immobilized antibody. The wells was washed and biotinylated anti-human FSH antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells was washed again, a TMB substrate solution was addeded to the wells and color develops in proportion

to the amount of FSH bound. The Stop Solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

2.10.3- Method for Quantitation Serum Testosterone Hormone level:

Testosterone test kit was a solid phase competitive ELISA. The samples, working Testosterone-HRP Conjugate and anti-Testosterone-biotin solution was added to the wells coated with streptavidin. Testosterone in the patient's serum competes with the Testosterone enzyme (HRP) conjugate for binding sites. Unbound Testosterone and testosterone enzyme conjugate was washed off by washing buffer. Upon the addition of the substrate, the intensity of color was inversely proportional to the concentration of Testosterone in the samples. A standard curve was prepared relating color intensity to the concentration of the Testosterone.

2.10.4- Method for Quantitation Insulin level:

Sandwich immunoluminometric assay was used an anti-Insulin monoclonal antibody to label ABEI, and use another monoclonal antibody to label FITC. Sample, Calibrators or Control with ABEI Label, FITC Label and nano magnetic microbeads coated with sheep anti-FITC are mixed thoroughly and incubated at 37°C, forming a sandwich; after sediment in a magnetic field, decant the supernatant, then cycle washing it for 1 time. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as RLU within 3 seconds and is proportional to the concentration of Insulin present in samples.

2.11- Method for quantitative Glucose levels

Principle:- Glucose was oxidized by glucose-oxidase (GOD) to gluconate and hydrogene peroxide according to the following equation(Barham and Trinder 1972)

$$Glucose + O_2 + H_2O \xrightarrow{GOD} H_2O_2 + Gluconate$$

$$2H_2O_2 + Phenol + 4\ Amino_{antipyrine} \xrightarrow{peroxidase} 4H_2O + Quinonimine$$

2.11.1-Reagents:-

1- Reagent 1 (Buffer):- Consist of 100 mmol/L of phosphate buffer pH7.5 and 0.75 mmol/L Phenol.

2-Reagent 2 (Enzymes):- Consist of ≥15 KU/L of glucose oxidase, ≥1.5 KU/L of peroxidase and 0.25mmol/L of 4-amino-antipyrine.

3- Reagent 3 (Standard):- Consist of 100 mg/dl or 5.55 mmol/L of glucose.

2.11.2-Preparation of Reagents:-

Working reagents was prepared by adding the substance containing reagent 2 in the vial (enzymes) to the vial of reagent 1 containing reagent 2 in the vial (Buffer). To complete the dissolving of all components, the mixture is mixed gently.

The tube was permitted to stand at a temperature (37 ºC) for 5 minutes after addition, then the absorbance was read at 500 nm, using a 1 cm light path cuvette.

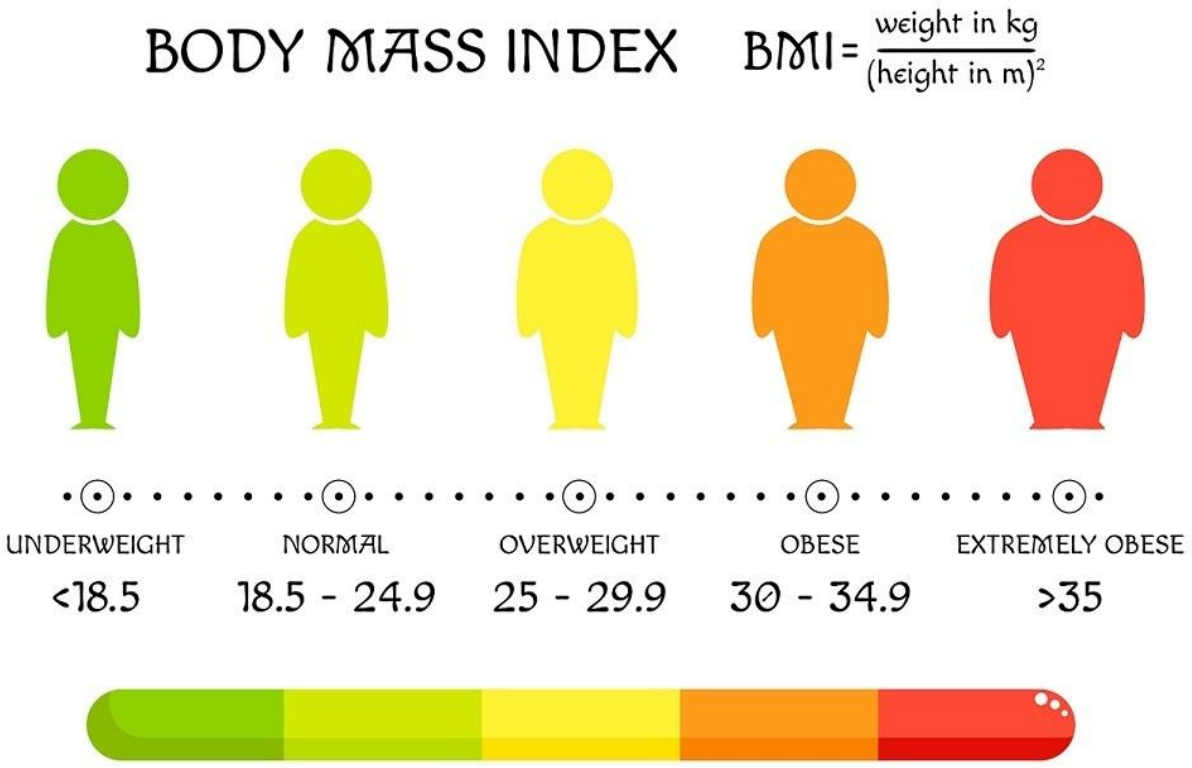
2.11.3-Calculation:-

$$Glucose\left(\frac{mg}{L}\right) = \frac{A.test}{A.STD} * Conc.of\ STD(\frac{100mg}{dl})$$

Reference Values:- Fasting serum glucose concentration between 75-115 mg/dl.

2.12- Body Mass Index Calculation(BMI):

Body Mass Index is a simple calculation using a person's height and weight. The formula is BMI = kg/m^2 where kg is a person's weight in kilograms and m^2 is their height in meters squared.



CHAPTER THREE

Results and discussion

3.Results and discussion

3.1 Demographic and clinical characteristics:

The clinical demographic characteristics of patients & control groups were summarized in Table (3.1).

Table illustrated the mean age of participants which was within the age group of (20-40) years old and BMI(20-35)kg/m². The patients group were divided into three subgroups based on types of fertility (A,B,C) (normal which no fertility , primary infertility was 40, secondary infertility was 20) .

The frequencies of the Age groups, BMI groups and fertility types among the study groups were shown in Figures (3.1); (3.2); and (3.3).

Table 3.1: Descriptive of the Demographic and of the study population (n= 120)

Variable		Control N(60)	Patient N (60)
Age (Years)	20 - 26 Years	30	29
	27 - 32 Years	20	22
	More than 33 Years	10	9
BMI Category (km/m²)	Normal weight(19-25)	18	11
	Over weight(25-30)	22	24
	Obese(30-35)	20	25
Types of infertility	A	60	0
	B	0	40
	C	0	20

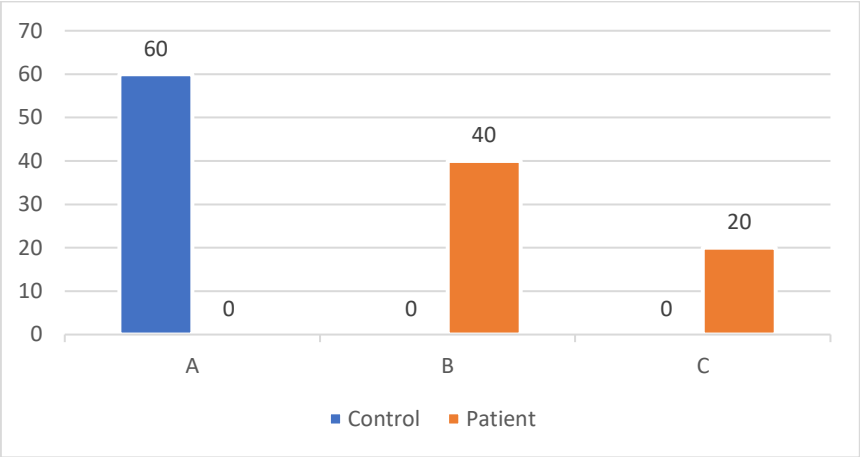


Figure (3.1): Frequencies of fertility types of participants in the study groups (A=normal, B=primary infertility, C=second infertility)

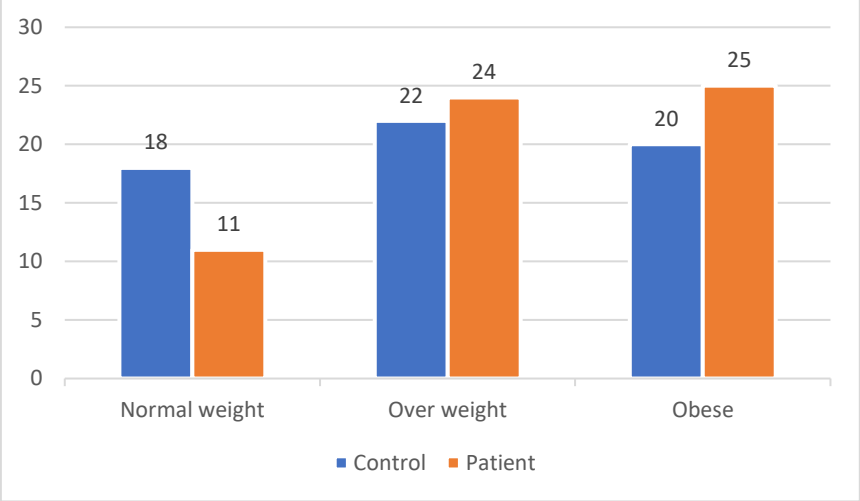


Figure (3.2): Frequencies of BMI groups of participants Between Control and Patient.

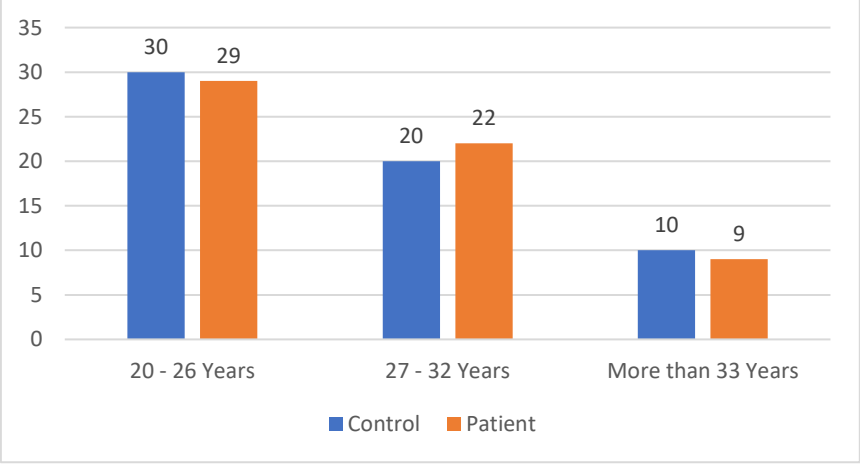


Figure (3.3): Frequencies of Age groups of participants Between Control and Patient.

3.2 Examination the mean levels of biomarkers in women with PCOS compared to control group

Polycystic ovary syndrome (PCOS) was hypothesized to result from functional ovarian hyperandrogenism (FOH) due to dysregulation of androgen secretion.

Table 3.2: Mean levels of biomarkers in women with PCOS compared to control group

biomarkers Mean ± 2SD	**Study groups**		P value
	Control (N= 60)	**Patient (N= 60)**	
LH (mIU/ml)	4.87±1.88	9.03±7.25	**<0.001[S]**
FSH (mIU/ml)	6.71±2.06	5.42±2.96	**0.002[S]**
F.TESTO (pg/ml)	0.96±0.35	1.81±0.78	**<0.001[S]**
TC. (mg/dl)	148.58±37.46	173.01±53.07	**0.017[S]**
TG (mg/dl)	108.87±22.58	145.47±66.24	**<0.001[S]**
HDL (mg/dl)	44.17±7.78	37.22±9.40	**<0.001[S]**
VLDL (mg/dl)	21.75±4.47	29.19±13.51	**0.002[S]**
LDL (mg/dl)	82.17±41.89	106.53±47.43	**0.009[S]**
FBS (mg/dl)	95.69±8.28	91.77±26.60	**0.004[S]**
ET1 (ng/ml)	54.37±6.31	92.31±62.49	**<0.001[S]**
VISFATIN (ng/ml)	10.48±3.15	25.11±43.33	**<0.001[S]**
IR	3.46±1.54	2.85±1.91	**0.006[S]**
W\H	0.86±0.09	0.92±0.19	**0.011[S]**
LH/FSH	0.74±0.22	1.73±0.87	**<0.001[S]**
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant			

Results of table (3.2) were indicated a significant increased levels of LH hormone, Testosterone, LH/ FSH ratio, endothelin and visfatin, TG,LDLthe mean levels of the parameters in patients' group were 9.03; 1.81;1.73; 92.31 and 25.11,145.47,106,53 respectively.

The pathophysiology of PCOS is still unresolved. Recent studies suggest that the brain is an important regulator as well as an affected organ in PCOS. The brain containing several receptors (androgen, estrogen and progesterone) and neurons with their neurotransmitters produces an increased pulse frequency of gonadotropin. As a result, an elevated luteinizing hormone (LH) secretion compared to follicle-stimulating hormone (FSH) is observed among patients with PCOS (**Coutinho EA, et al., 2019**). This altered LH-FSH ratio is responsible for increased ovarian androgen production. On the other hand, hyperandrogenemia reduces the negative feedback of estrogen to the hypothalamus leading to the increased pulse frequency of LH. This cycle is produced that is responsible for several clinical manifestations of PCOS including hyperandrogenism **(Ashraf S, et al., 2019**).The normal gonadotrophin axis is disturbed in PCOS women, therefore LH levels increase, and FSH levels decrease, leading to a reversal of the LH/FSH ratio(**Balen AH, *et al*.,2003**).

*Obesity, insulin resistance, and dyslipidemia are PCOS-related morbidities and were found to be correlated with the LH/FSH ratio (**Aug, et al., 2020**). Also, IR plays an important role in the development of PCOS, in which the insulin response increases resulting in hyperinsulinemia as a unique feature in PCOS (**Qi Liu *et al., 2019).***

Patients were also shown increased lipid profile parameters and the waist/ hip ratio, the mean levels of the lipid panel were: TC (173.01); TG (145.47); VLDL

(29.19); LDL (106.53), and the W/H ratio was (0.9), while in the control group: TC (148.58); TG (108.87); VLDL (21.75); LDL (82.17) and the W/H ratio was (0.92).

* Lipid abnormalities are found in women affected by PCOS. A recent study showed that mild hypercholesterolemia is frequently encountered in women with PCOS (**V. Pergialiotis *et al*., 2018**). Different lipid patterns are present in PCOS, including low levels of high-density lipoprotein cholesterol (HDL-C), high triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C), and significantly higher lipoprotein concentrations (**A. Ghaffarzad at al., 2016)**.The high levels of(TC) may be due to the dependence of the body on energy alternative sources cross consumption of stored fat results in a decrease in blood plasma or may be caused by a defect in the absorption of TC ***(Eckardstein A, et al,2002),*** also increase TC levels related with decrease absorption of fatty acids by adipose tissue and with low HDL-C levels, insulin resistance and risk of atherosclerosis (**Terasawa, *et al*, 2000**).

*The increase in TGs levels may result from a high oxidation rate in the body and the decreased lipoprotein lipase activity and these decreases lead to a defect in lipids levels in the blood (**Qi Liu, *et al***). reported that hyperlipidemia patients show low HDL-C levels and this agreement with the study HDL-C inhibits LDL-C oxidation through an antioxidant enzyme known as HDL-C associated paraoxonase (**Nofer j R,.*et al*, 2002**).Hyperlipidemia catalyzes the accumulation of oxidized LDL-C in arteries walls (**Jan Borén ,*et al*, 2020**). Decrease in LPL activity is associated with an increase in plasma triglycerides (TG) and decrease in high density lipoprotein (HDL) cholesterol (**Tsutsumi K, *et al*., 2003**).

This study show endothelin-1 elevated in women with PCOS, and elevated Visfatin . Elevated endothelin-1 (ET-1) levels have been reported in some insulin-resistant states such as obesity(**Ferri C *et al* .,1997**).The elevation of atherogenic molecules endothelin-1(ET-1) was demonstrated a contributes to an increased risk

for cardiovascular disease in this group.Since women with PCO syndrome display increased levels of oxidative stress (**Victor VM ,*et al*., 2009**). ET-1 has been also shown to enhance oxidative stress (**Paravicini TM *et al*., 2006**).It has been reported that insulin resistance is a major driver of endothelial dysfunction (**Muniyappa R,*et al*., 2007**), this could be a mechanism partly accounting for the reported association with increasing ET-1.

Elevated (ET-1) levels have been reported in some insulin-resistant states such as obesity (**Ferri C, *et al*,.2005**).This study show that the more the BMI (obesity) more visfatin levels and a positive correlation between circulating visfatin levels and insulin resistance. Visfatin was shown to be increased in females with obesity (visceral obesity)(**FUKUHARA A, *et al*;.2005**).Adipose tissue is implicated in the secretion of several hormones such as visfatin called adipocytokines, that are involved in energy homeostasis and metabolism (**A.H.Berg *et al*.,2002**).The difference in fat distribution in patients with PCOS may result in changed adipose tissue function and adipocytokines levels (**A. Fukuhara, *et al*., 2005**). There is an overall consensus about the action of visfatin, due to relevant endocrine, paracrine and autocrine functions. These autocrine effects of visfatin may play an important role in regulating insulin sensitivity in the liver (**Skop V, *et al*., 2009).**In agreement with our results, Chang et al. detected a positive correlation between circulating visfatin levels and insulin resistance (**Y.-H. Chang *et al*., 2011**).

Visfatin induces the production of inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α in human leukocytes. Its plasma level increases during chronic inflammatory conditions such as obesity (**E. Adghate, *et al*., 2008**).

Table (3.3) Mean levels of biomarkers among fertiality groups in women with PCOS and control.

Biomarkers Mean ± 2SD	Study Groups			P Value
	A (N= 60)	B (N= 40)	C N= 20	
LH	4.87±1.89	7.62±3.25	8.15±4.37	**<0.001[S]**
FSH	6.71±2.06	5.26±2.06	3.19±1.72	**0.002[S]**
F.TESTO	0.96±0.35	1.74±0.73	2.11±0.95	**0.011[S]**
T.C	148.58±37.47	168.61±39.74	161.78±59.58	**0.012[S]**
TG	108.87±22.58	142.77±64.34	135.29±52.88	**<0.001[S]**
HDL	44.17±7.79	37.39±9.91	36.64±6.79	**<0.001[S]**
VLDL	21.75±4.47	28.67±13.21	27.05±10.57	**<0.001[S]**
LDL	82.17±41.90	102.03±37.35	97.97±54.27	**0.01[S]**
FBS	95.69±8.28	92.25±28.68	89.64±14.84	0.515[NS]
INSULIN	14.47±6.04	12.54±6.30	11.10±5.16	0.42[NS]
ET1	54.37±6.31	81.32±29.75	65.44±17.09	**<0.001[S]**
VISFATIN	10.48±3.16	16.00±5.08	15.72±4.88	**0.021[S]**
IR	3.46±1.54	2.93±2.06	2.48±1.02	0.96[NS]
W\H	0.86±0.09	0.92±0.20	0.94±0.15	0.07[NS]
LH/FSH	0.74±0.23	1.52±0.62	2.33±1.35	**<0.001[S]**
Studdent ANOVA was [S] significant, [NS] non-significant, A=normal, B=primary infertility, C=secondary infertility				

In table (3.3), the mean levels of biomarkers among patient sub groups in women with PCOS was also measured, results were showed that all parameters were significantly differed among the groups. The mean levels of LH; Testosterone; TG; Insulin; W\H and LH/FSH ratio were shown a positively significant increasing with secondary infertility cases. While FSH; HDL and fasting blood sugar were decreased significantly in the same group.

On the other hand, Total cholesterol; VLDL; LDL; ET1 and VISFATIN levels were significantly increased in infertility cases .The most common cause of female infertility is ovulation disorders, and the most common non-ovulatory cause is polycystic ovary syndrome (PCOS). PCOS is a complex hormonal and metabolic disorder characterized by oligomenorrhea or amenorrhea, hyperandrogenism, and infertility **(Zhou J, *et al*., 2017)**.

The main pathophysiological components of PCOS are gonadotropic dysfunction and insulin resistance, which are often associated with high body mass index (BMI) note in table (3.3) (**Krynytska IY, *et al*., 2018**). PCOS is associated with irregular gonadotropin secretion, increased steroid hormone secretion and frequency, and a high LH/FSH ratio, which leads to an increase in androgen synthesis and prevents normal follicle development(**R.L. Rosenfield, *et al*.,2016**) . An increase in gonadotropin-releasing hormone up regulates transcription of the LH β-subunit through the FSH β-subunit, which leads to an increase in the LH/FSH ratio in PCOS patients (**Park CH, *et al*., 2016**). Experimental exhibited ovarian changes, such as an increase in the number of cystic follicles and increased granulosa cell degeneration, with thin granulosa cell walls and a thicker surrounding layer of theca cells(**Franks S, *et al* .,2008**). suggesting that infertility is associated with dyslipidemia in PCOS. abnormal lipid metabolism can promote the pathophysiology of hyperandrogenism, IR, oxidative stress, and infertility in PCOS.

In case of Primary and secondary infertility, Lipid disorders are hypothesized to play a role in female reproduction. The synthesis of steroid hormones in reproductive tissues occurs in thecal and granulosa cells, and utilizes cholesterol as the substrate for steroidogenesis. HDL-C and LDL-C both play important roles in the transport of cholesterol to ovarian tissue (**Huang Q, *et al*.,2019**). Altered endometrial lipid levels may impair endometrial receptivity and early embryo implantation **(Li J,*et al*., 2019**) note in table (3.3).

Many studies have reported associations between serum lipids and reproductive outcomes in subfertile women. In one prospective cohort study that included couples attempting pregnancy, increased serum free cholesterol concentrations in both men and women led to reduced fecundity (**Schisterman EF. *et al* .,2005**). In couples with prior pregnancy loss, higher serum TC and TG were associated with less spontaneous pregnancy (**Pugh SJ,*et al*., 2017**). Another study in women undergoing *in vitro* fertilization (IVF) suggests that HDL within the follicular fluid may play protective roles in the health of the human oocyte by reducing oocyte fragmentation. It has also been reported that follicular fluid HDL had an antioxidative function and was associated with normal oocyte fertilization **(Nagy RA, *et al*.,2019)** note in table (3.3) **.**

There is a clear positive relationship between level of visfatin level and infertility in women. Regarding the mechanism by which visfatin affect women's fertility. Normal level is needed for normal reproductive function in women, impaired level is associated with increased incidence of PCOs and endometriosis which reduce fertility in women. the positive correlation between serum visfitin, BMI, and insulin contributes significantly to the reproductive, neuroendocrine abnormalities associated. High visfatin level increase incidence of women's infertility, visfatin level increase in PCO when associated with IR or DM **(Hussein Mohamed Hussein,*et al*., 2018).**

3.3-Correlation among Serum Visfatin & Endothelin-1 levels and biomarkers among women with PCOS:

Considering the important role of the measured biomarkers in the progression of PCOS cases, Serum Endotheline-1 levels was positively related to the VISFATIN; Testosterone; TG; VLDL; and W/H ratio, the p values were <0.001,; FBS and IR Also only HDL negatively significant corelated to the level of Endotheline-1 as presented in Table (3.4).

Table (3.4): Correlation coefficients between mean levels of Endotheline-1 and biomarkers among women with PCOS

ET1		
Biomarkers	***Correlation coefficient (r)***	***P Value***
LH	0.5	**<0.001 [S]**
FSH	0.1	0.582[NS]
F.TESTO	0.5	**<0.001[S]**
T.C	0.1	0.2217[NS]
TG	0.3	**0.025[S]**
HDL	-0.3	**0.006 [S]**
VLDL	0.2	**0.044[S]**
LDL	0.1	0.126[NS]
FBS	-0.3	**0.001[S]**
VISFATIN	0.6	**<0.001[S]**
IR	-0.3	**0.007[S]**
W\H	0.3	**0.015[S]**
LH/FSH	0.2	0.057[NS]
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant		

Endothelin-1 (ET-1) is an indicator of endothelial injury and dysfunction and is elevated in women with androgen excess polycystic ovary syndrome . Plasma endothelin-1 [ET-1], an important indicator of endothelial injury, is increased in women with PCOS. Secreted through the basolateral compartment of end- othelial cells, ET-1 has vasoconstrictor actions on the vascular smooth muscle in the peripheral circulation via two primary receptor subtypes, ETAR and ETBR. Testosterone is integral to the development of end- othelial dysfunction and hypertension there is a clear androgen-induced EC signalling defect observed , This suggests a global endothelial dysfunction state, which has been suggested for women with PCOS and correlated to the early development of cardiovascular disease. **(Usselman,*et al*., 2019)**.

In this study indothelin-1 inhibit FSH.

Endothelin-1 inhibits LH,FSH, and hCG stimulated progesterone secretion by granulosa cells, suggestive of a function for this peptide in the control of premature luteinization**(Bridges PJ,*et al*.,2011**)**.**

Increased levels of plasma ET-1 have been observed in pathophysiological states including obesity and insulin resistance.**(Kahn SE,*et al*.,2006**)**.**

Table (3.5): Correlation coefficients between mean levels of Visfatin and biomarkers among women with PCOS.

Biomarkers	VISFATIN	
	Correlation coefficient (r)	***P Value***
LH	0.3	**<0.001 [S]**
FSH	-0.1	0.577[NS]
F.TESTO	0.4	**<0.001[S]**
T.C	0.1	0.260[NS]

TG	0.4	**<0.001[S]**
HDL	-0.4	**<0.001[S]**
VLDL	0.3	**0.001[S]**
LDL	0.1	0.163[NS]
FBS	-0.2	**0.016[S]**
ET1	0.6	**<0.001[S]**
IR	-0.1	0.228[NS]
W\H	0.3	**0.006[S]**
LH/FSH	0.1	0.621[NS]
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant		

Robust connection between visfatin with, sex hormones, and IR parameters in PCOS pathophysiology **(Shatha Rouf Moustafa, *et al*., 2022)** serum visfatin were found to be correlated with free testosterone levels suggesting a possible role of visfatin in the pathogenesis of PCOS **(Pepene, *et al*.,2011)**.Serum visfatin levels was fairly related to the ET-1, and LH hormones levels,TG,HDL,and VLDL,the p values were <0.001. correlation of visfatin with FBS ,IRand HDL were negative presented in Table (3.5).

*Visfatin binds to the insulin receptor at a site differ from that of insulin and causes hypoglycemia by stimulating glucose utilization in adipocytes and myocytes and reducing glucose release from liver(**mostafa Ahmed,*et al*.,2019**).

visfatin has arole in pcos and correlated positively and it deregulated by obisty.

*Visfatin is a cytokine with a physiological effect in reducing the level of plasma glucose.(**Asia Ali, *et al*.,2022)**

*serum visfatin has an important role of pathogenesis of PCOS. A study revealed that serum visfatin levels were significantly positive correlated with PCOS clinical

and biochemical phenotype as well as cardio-metabolic factors; TC, LDL, FBG, FSI, . **(Nearmeen M. Rashad, *et al*.,2018**)

A study conducted by Kowalsk and colleagues demonstrated a positive correlation with markers of hyperandrogenism, suggesting that obesity may deregulate visfatin expression and that other factors may be involved in this process (**Kowalska, et al., 2007**).

. plasma concentration of visfatin increases along with rising body mass index.

Table 3.6 : The mean differences of the ET-1 levels among Age groups in patient and control

ET-1	20 - 26 Years		27 - 32 Years		More than 33 Years	
	Patients	**Control**	**Patients**	**Control**	**Patients**	**Control**
Mean±SD	69.24±16.79	53.29±4.65	107.83±22.9	53.60±7	128.72±40.35	59.12±6.95
Correlation Coffecient r	0.95		0.76		0.81	
P value	**<0.001[S]**		**<0.001[S]**		**<0.001[S]**	

Results were indicated that there was a significant difference in the ET-1 levels in all age groups, p value <0.001.mean ET-1increase with aging.

Table 3.7 : The mean differences of the VISFATIN levels among age groups in patient and control

VISFATIN	20 - 26 Years		27 - 32 Years		More than 33 Years	
	Patients	Control	Patients	Control	Patients	Control
Mean±SD	13.71±4.41	10.86±3.53	41.68±4.54	11.09±2.12	21.32±6.41	8.14±2.23
Correlation Coffecient r	-0.2		-0.3		0.8	
P value	0.53[NS]		0.24[NS]		**0.002[S]**	

while visfatin levels were shown only a significant difference in the groups when the age was more than 33 Years, p value 0.002.table (3.7)

negative correlation between visfatin levels and age(**Anna Ashraf S , *et al* .,2019).**

Table 3.8 : The mean differences of the ET-1 levels among BMI groups in patient and control.

ET-1	Normal weight		Over weight		Obese	
	Patients	Control	Patients	Control	Patients	Control
Mean±SD	74.38±21.8	54.87±3.1	93.57±22.76	52.951±8.4	95.42±35.7	55.24±5.27
Correlation Coffecient r	0.86		0.42		0.5	
P value	**<0.001[S]**		0.06[NS]		**0.02[S]**	

BMI were classified to a sub-group to investigate their effect on the levels of biomarkers, ET-1 levels were demonstrated a significant difference in the normal wight and obese when compared patients to control group, p values were < 0.001, 0.02 respectively. Table(3.8)

positive correlation between obesity and plasma endothelin-1 (ET-1) levels**.(Weil BR.,*et al* .,2011)**..

Table 3.9 : The mean differences of the VISFATIN levels among BMI groups in patient and control.

VISFATIN	Normal weight		Over		Obese	
	Patients	**Control**	**Patients**	**Control**	**Patients**	**Control**
Mean±SD	14.21±5.24	10.10±2.4	20.7844±4.01	9.813±2.8	34.60±5.82	11.41±3.78
Correlation Coffecient r	-0.2		-0.1		0.3	
P value	0.06[NS]		**0.05[S]**		**0.04[S]**	

Visfatin levels were correlated positively with body mass index(table3.9)

3.4 Associated factors of alteration biomarkers levels with PCOS cases compared to control group

Univariate odds ratio (OR) with 95% confidence intervals (CI) for potential biomarkers were evaluated for their association with PCOS patients, On multivariate conditional logistic regression analysis (Table 3.10).

Table (3.10): Associated factors of Dependent Variable in PCOS Patients Compared to control group

Study Groups (PCOS/ Healthy control)		
Biomarkers	OR (95% CI)	p value
LH	3.130 (1.537 - 6.372)	0.002 [S]
FSH	0.230 (0.093 - 0.566)	0.001 [S]
F.TESTO	46.700 (1.156 – 1. 886)	0.042 [S]
ET1	1.079 (0.907 - 1.284)	0.390 [NS]
VISFATIN	1.36(1.208 – 1.532)	<0.001[S]
IR	0.995 (0.878-1.129)	0.941[NS]
W\H	20.210 (1.298-3.663)	0. 32[S]
LH/FSH	1915.829 (102.301 – 378.346)	<0.001[S]
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant		

It was found that serum levels of LH, testosterone, ET1 and W\H ratio were to be dependent risk factors for PCOS cases since they were shown an associated with higher odds of PCOS outcome. For LH (OR: 3.130; 95% CI: 1.537 - 6.372), for Testosterone (OR: 46.7; 95% CI: 1.156 – 1.886), For ET- 1 (OR: 1.079; 95% CI: (0.907 - 1.284), for W/H ratio (OR: 20.21; 95% CI: (1.298-3.663) and for LH/FSH ratio (OR: 1915.82; 95% CI: (102.301 – 378.346), while FSH and IR were shown an associated with lower odds of outcome in PCOS patients.

3.5 Analysis the optimal diagnostic points for predicting PCOS cases by Receiver operating characteristics (ROC) curve

Results of the receiver operating curve (ROC) and AUC analysis for the VISFATIN and Endotheline-1 as possible diagnostic parameters for prediction PCOS Patients are presented in Table (3.11) .

Table (3.11) Receiver operating characteristics (ROC) curve analysis of Visfatin and Endotheline-1 levels in PCOS

Test Variable	AUP	P value	Sensitivity %	Specificity %	Youden index	Cut-off points	CI (95%)
ET-1	0.84	<0.001	85%	80%	0.65	55.975	0.754-0.913
VISFATIN	0.80	<0.001	65%	86.7|%	0.284	13.085	0.784-0.918

Endotheline-1 was shown more appropriate optimal diagnostic points for predicting PCOS cases compared to control group (sensitivity = 85%, specificity = 80%) at a level = 55.975 ng/dl. Youden's J statistics of the parameters in Figures (3.7) & (3.8) confirm these results.

Numerous studies have reported that endothelial dysfunction is commonly associated with PCOS (Diamanti-Kandarakis E, et al., 2006)

Results of this study confirmed the association of serum ET-1 levels to PCOS cases.

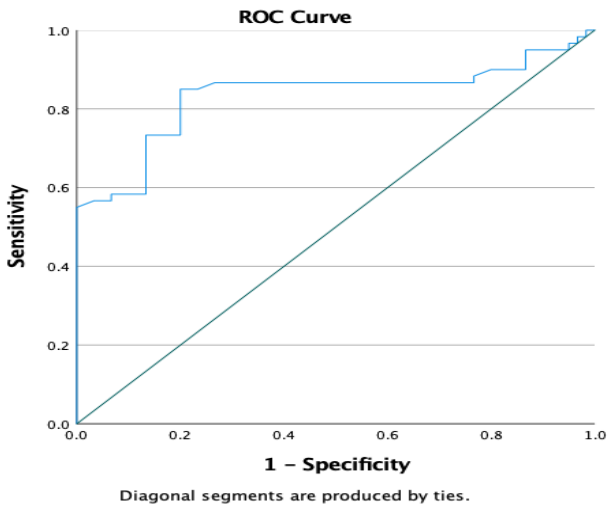


Figure (3.4) ROC curves for Endotheline-1 in PCOS patients to analyse the optimal diagnostic points for predicting cases compared to control group.

The results showed relatively good sensitivity and specificity. For VISFATIN the AUP was 0.851 while for Endotheline-1 was 0.85.

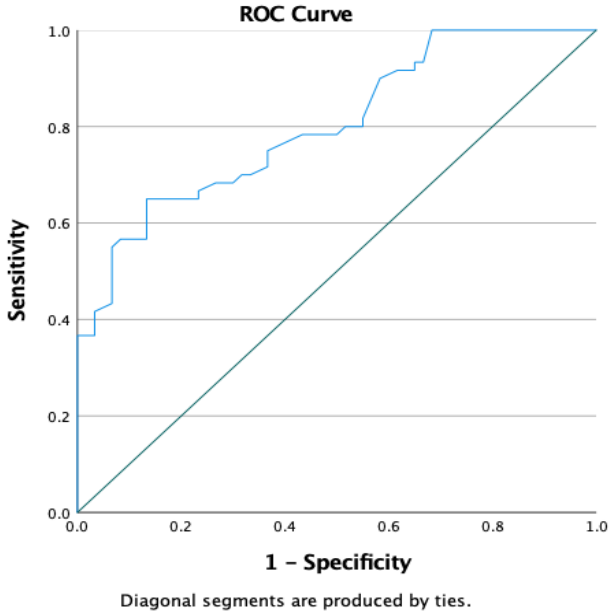


Figure (3.5) ROC curves for Visfatin in PCOS patients to analyse the optimal diagnostic points for predicting cases compared to control group.

To the best of our knowledge, this is an detailed study about the analysis of the optimal diagnostic points for predicting advanced complication PCOs case

Chapter Four

Conclusion

&

Recommendations

4.1 Final conclusion

This study was concluded that:

- Endotheline-1 and visfatin was shown diagnostic points for predicting PCOS cases compared to control group.
- Endothelin-1 increase with age.
- study show that more BMI (obesity) more the visfatin levels .
- Visfatin was associated with abdominal obesity and insulin resistance as well as obesity, but not with metabolic syndrome and pre-diabetes mellitus.

4.2 -Recommendations

- Future research should clarify the interdependent roles of Agess and ET-1 and their cardiovascular implications for women with PCOS.
- the role of visfatin in PCOS remains inconclusive, which might result from the different experimental approaches used in the previously mentioned studies. However, further studies are needed to elucidate its actions in PCOS and the underlying molecular mechanisms

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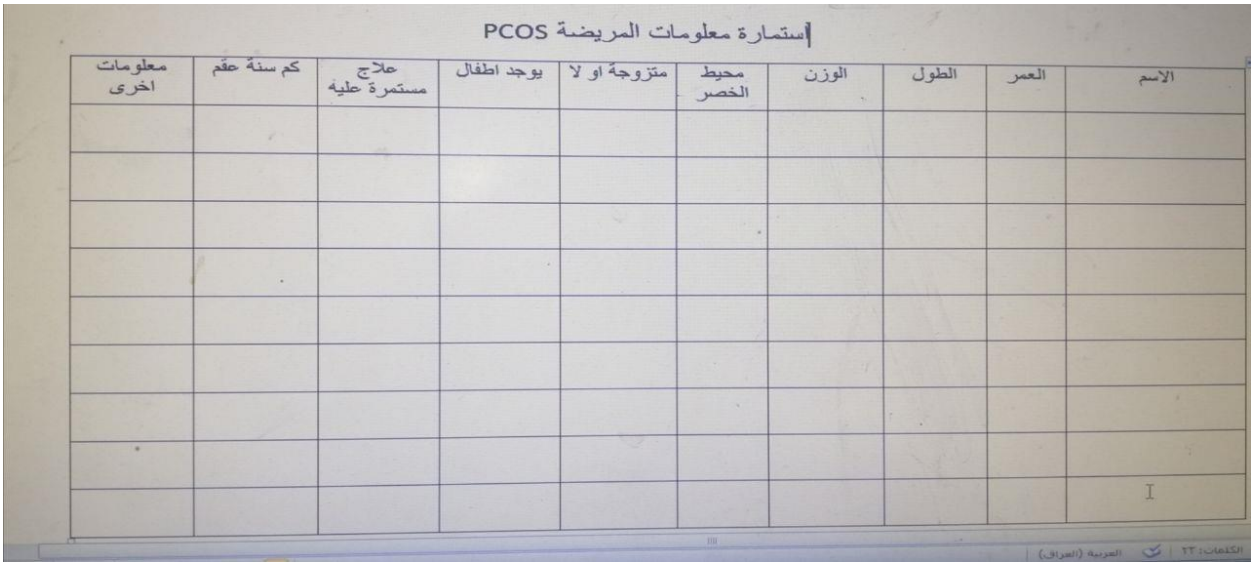
Appendix

Appendix I



استمارة معلومات المريضة PCOS

الاسم	العمر	الطول	الوزن	محيط الخصر	متزوجة او لا	يوجد اطفال	علاج مستمرة عليه	كم سنة عقم	معلومات اخرى

Appendix II

مخلص

متلازمة تكيس المبايض ، هي اضطراب الغدد الصماء الشائع الذي يصيب النساء في سن الإنجاب ويعتبر عامل خطر للعقم.

يعتبر الاندوثلين مؤشرًا على خلل وظيفي في إصابة بطانة الأوعية الدموية ويزداد عند النساء المصابات بمتلازمة المبيض المتعدد الكيسات الزائدة في الأندروجين (متلازمة تكيس المبايض).

فزفاتين هو أديبوتوكين له تأثير يشبه الأنسولين ويرتبط ارتباطًا وثيقًا بكمية الدهون الحشوية.

الهدف من هذه الدراسة:

كان الهدف هو تقييم مصل الاندوثلين ومستوى فزفاتين بين النساء المصابات بمتلازمة تكيس المبايض والتحكم في الكشف قد يكون هذا مؤشر مسبق لمتلازمة تكيس المبايض.

الطريقة: دراسة 120 امرأة مصنفة حسب مؤشر كتلة الجسم (20-35) كجم / م 2 والعمر (20-40) سنة.

تم تشخيص 60 امرأة بمرض متلازمة تكيس المبايض من قبل الدكتورة أنفال الكعبي هي طبيبة نسائية وبواسطة السونار و 60 امرأة تتمتع بصحة جيدة.

تم تصنيف حالات متلازمة تكيس المبايض أيضًا إلى ثلاث مجموعات فرعية (أ ، ب ، ج) التي تسببت في العقم (طبيعي حيث لا يوجد عقم ، كان العقم الأولي 40 ، وكان العقم الثانوي 20). قياس المصل الاندوثلين ومستوى مصل فزفاتين (نانوغرام / مل) تم تقييمها بواسطة الاليزا. قياس الهرمونات (LH، FSH، F.testo) في ثاني يوم الدورة عن طريق minividas. قياس مستوى الدهون (الكولسترول الكلي ، بروتين دهني عالي الكثافة HDL ، البروتين الدهني منخفض الكثافة LDL ، البروتين الدهني منخفض الكثافة VLDL والدهون الثلاثية TG) عن طريق القياس اللوني الفحص باستخدام مقياس الطيف الضوئي. قياس الأنسولين عن طريق مقايسة الاليزا والسكر عن طريق جهاز maglumi.

النتائج:

تتضح متلازمة تكيس المبايض عن طريق ارتفاع مستويات الأندروجين المنتشرة وانخفاض استراديول. علاوة على ذلك ، كانت التغييرات في لوحة ملف تعريف الدهون مرتبطة أيضًا بالتوازن في تكوين الستيرويد. أظهرت المستويات المتوسطة من المؤشرات الحيوية بين مجموعات العقم عند النساء المصابات بتكيس المبايض أن جميع المعلمات اختلفت بشكل كبير بين المجموعات (قيم p <0.05). مستوى LH ؛ التستوستيرون. TG. الأنسولين. أظهرت نسبة W \ H و LH / FSH زيادة معنوية إيجابية مع حالات العقم الثانوية. بينما FSH ؛ انخفض HDL وسكر الدم الصائم بشكل ملحوظ في نفس المجموعة. باستخدام تحليل ROC.

تم عرض نقاط تشخيص الاندوثلين و فزفاتين للتنبؤ بحالات متلازمة تكيس المبايض مقارنة بمجموعة التحكم. تم تقييم العلاقة بين العلامات البيوكيميائية ودراسة الحالة. تم تقييم كفاءة قيمة التنبؤ باستخدام منحنى خاصية تشغيل المستقبل (ROC).

استنتاج:

- قد يكون الاندوثلين و فزفاتين علامات تشخيص مسبقة لمرض تكيس المبايض.

- كان إندوثيلين يتزايد مع تقدم العمر

- قد يكون لفرط شحميات الدم علاقة بزيادة مستويات هرمون فيزفاتين.

- يرتبط فزفاتين بسمنة البطن ومقاومة الأنسولين بالإضافة إلى السمنة ، ولكن ليس مع متلازمة التمثيل الغذائي ومرض السكري.

- قد يكون لفيسفاتين تأثير على تطور مقاومة الأنسولين مؤشر كتلة الجسم (السمنة) أكثر مع زيادة مستويات الفيسفاتين.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الطب

فرع الكيمياء والكيمياء الحياتية

دراسة العلاقة بين إندوثيلين -1 وفيسفاتين مع مقاومة الأنسولين لدى النساء المصابات بمتلازمة تكيس المبايض.

رسالة ماجستير

إلى مجلس كلية الطب/ فرع **الكيمياء والكيمياء الحياتية** / جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير

من قبل

نبا حيدر علي

.بكالوريوس تحليلات مرضية /كلية التقنيات الصحية والطبية /كوفة 2015

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