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

Association of Zinc Alpha 2 Glycoproteins and Heat Shock Protein 70 with Polycystic Ovarian Syndrome in Iraqi Infertile Women .

A Thesis

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

By

Lahieb Shakir Mahmood
B. Sc in Chemistry, Baghdad university-1997

Supervisor
Assist.Prof. Shaymaa Z. Nada
Department of Chemistry and Biochemistry
University of Kerbala

1443/4 A.H **2021/11A.D**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

((الْحَمْدُ لِلَّهِ الَّذِي هَدَانَا لِهَٰذَا وَمَا كُنَّا لِنَهْتَدِيَ لَوْلَا أَنْ هَدَانَا اللَّهُ))

صَدَقَ اللهُ العَلِيُّ العَظيم

سورة الاعراف اية (٤٣)

Supervisor Certification

I certify that this M.Sc. thesis entitled:-

Association of Zinc Alpha 2 Glycoproteins and Heat Shock Protein 70 with Polycystic Ovarian Syndrome in Iraqi Infertile Women.

Was prepared under my supervision in laboratories at Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala as a partial requirement for the Degree of Master in Clinical Chemistry.

Assist. Prof. Shaymaa Z. Nada
in Clinical Biochemistry

Assist. Prof. Rana Majeed Hameed, Ph. D.
Head of Chemistry and Biochemistry Department
College of Medicine – University of Kerbala

Dedication To

My family, for their time, loves and support.

Acknowledgments

First of all, I thanks GOD for giving me health, patience, strength and knowledge to complete this academic requirement.
My thanks extended to all patients who accepted to help me to perform my work and reach my final results .

I'd like to give my deepest thanks to my supervisor. ***Assist prof Dr. Shaymaa Z.Nada*** , for her guidance, encouragement and her mutual relationship with me & my colleagues, as she's leading us to further social and scientific progress.

Also I express my great thank to ***Dr.Hiba AL-Wazzan***. the consulted for this study.

I also express my great thanks to the head of department of chemistry and biochemistry in Collage of Medicine , University of kerbala ***Assist profDr.Rana Majeed hameed.***
I would like to express my gratitude and thanks to the Deanship of the College of Medicine and the University of Karbala for welcoming me and accepting me among its students.

At last, I like to thank my family for their love, time, support and belief in me.

Content

List of Content

Content

Content

Content

List of Figures

List of Figures

List of tables

Abbreviations

Abbreviations	Description
AA	Adrenal androgen
ACC	Acetyl-CoA carboxylase
ANOVA	Analysis of variance
ATGL	Adipose tissue triglyceride lipase
ATP	Adenosine triphosphate
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CVD	Cardiovascular Disease
DHEAS	Dehydroepiandrosterone sulfate
EDTA	Ethylenediaminetetraacetic acid
FAS	Fatty acid synthase
FBG	Fasting Blood glucose
FSH	Follicle stimulating hormone
GLUT4	Glucose transporter type 4
GnRH	Gonadotropin-releasing hormone
HbA1c	Glycated hemoglobin
HLS	Hormone-sensitive lipase
HOMA-IR	Homeostatic model assessment for insulin resistance
HSP	Heat shock proteins
IR	Insulin resistance
IRS-1	Insulin receptor substrate 1
KD	Kilo Dalton

Abbreviations

LH	Luteinizing hormone
LMF	Lipid-mobilizing factor
MHC	Major histocompatibility complex
NEFA	Non esterified fatty acids
OD	Optical density
PCOS	Polycystic ovary syndrome
r	Pearson's correlation coefficient
SHBG	Sex hormone binding globulin
TSH	Thyroid stimulating hormone
UCPs	Uncoupling proteins
USG	Ultrasonography
WAT	White adipose tissue
WHO	World Health Organization
ZAG	Zinc alpha 2 glycoprotein

Summary

Polycystic ovary syndrome (PCOS) is imbalance hormone due to high level of androgen (male hormone) in females; Signs including absence of evolution of eggs (pre mature) and ovarian cysts and irregular menstrual periods, infertility, excess hair growth on face (hirsutism) and acne . Excessive insulin in the body stimulates the ovaries to produce excess androgen and increase the risk of development of type 2 diabetes.

Zinc alpha 2 glycoprotein (ZAG) is a single chain polypeptide with a molecular weight of 40-43 KiloDalton that is present in the majority of bodily fluids. Reduced ZAG levels lead to a reduced rates of lipolysis and associated with fat accumulating.

Heat Shock Proteins (HSP) are molecular chaperones that play a key role in maintaining protein homeostasis and transport. Heat shock proteins with a molecular weight of approximately 70 kD (Hsp70) are involved in assisting protein folding, preventing protein aggregation and transporting proteins across membranes . Inflammation causes the release of Hsp70 into the blood., serum Hsp70 levels might provide a marker for inflammatory processes .

The objective of study to evaluate ZAG levels in sera of PCOS patients and their association with infertility in comparison to those in the controls group, study the association between ZAG level with hormonal biomarkers and insulin resistance, Assessment of HSP70 levels in PCOS patients' sera and compare them to those in the controls group, Investigate their relationship with hormonal biomarkers and insulin resistance.

A case-control study included 81 females, in reproductive age [18-45 years old] 50 of them were diagnosed with polycystic ovarian syndrome, while the other 31 were healthy controls which was divided in to three groups depending on fertility to primary infertility, secondary infertility for patients group and fertility for controls group the samples were collected from November 2020 till May 2021.

ZAG levels and HSP70 levels were measured by ELISA technique from Bioassay Lab company-china and biochemical variables BMI calculated weight divided on square height in meter (Kg/m^2); hormonal LH, FSH, testosterone and HbA1c were measured by fluorescence Immunoassay (Boditech –Korea), fasting blood glucose (FBG) level was measured by spectrophotometer (Liner-SPAIN), Fasting insulin by Cobas e411 (Roche-Germany).

The results of this study shows the mean of ZAG levels (µg/ml) were highly significant decrease in women with PCOS group as compare to controls group ($P<0.001$), and found that highly significant elevation in hormonal biomarkers that measured testosterone, LH, LH/FSH ratio and metabolic biomarkers FBG, fasting Insulin, HOMA-IR, HbA1c and BMI in patients group as compare to healthy groups while significant decrease in FSH in patients groups as compare to controls groups in $P\leq0.05$. Also there is negative significant correlation between ZAG level, BMI, insulin resistance and LH $p<0.001$. The results observed in this study after dividing the groups according fertility degree (primary infertility, secondary infertility , fertility) that the hormonal disorder and BMI in primary was higher than secondary infertility in patient groups. Also We found a significant decrease between groups primary infertility and secondary infertility in $P\leq0.05$ that's may be mean in lower ZAG level lead to increase infertility degree.

The mean of HSP70 levels (ng/ml) in patient group was significantly higher than that in the control group in ($P<0.001$). also found positive significant correlation between HSP70 and IR , F insulin in $P\leq0.05$, $p<0.001$ respectively.

the observed data conclusion that ZAG level is decreased in PCOS patient and had negative correlation with BMI, IR, LH and it decrease with abnormal hormones levels, so it effects the fertility, when it's decreased that leads to increase the degree of infertility. That mean levels of ZAG in primary infertility is lower than it's levels in secondary infertility **.**

HSP70 level is increased in PCOS patient and had positive correlation with IR, F insulin level. and a significant high levels of the all hormones and parameters including LH, total testosterone, FBG, F insulin and HbA1c were found in women with PCOS as compared with the control, except the FSH values were decreased .

The reduction in ZAG levels in the sera of women with PCOS increase the risk of metabolic disorder, and it's association with increase the risk of infertility.

The increase in HSP70 levels in sera of women with PCOS associated with increased risk of metabolic disorder and their complication in increase the infertility.

Chapter One

Introduction and Literature review

1. Introduction and Literature Review

1.1.Polycystic ovary syndrome

1.1.1. Definition of polycystic ovary syndrome

Polycystic ovary syndrome(PCOS) is hormonal disorder due to high level androgen (male hormone) and increased LH/FSH ratio in females of reproductive age, Signs including absence of evolution of eggs (pre mature), ovarian cysts, irregular menstrual periods, infertility, excess hair growth on face hirsutism and acne **(Legro, 2008 ; Legro, 2003**), that has evolved over the years from disease to syndrome.

PCOS is one of the most common causes of anovulatory infertility as high as 15% when the broader Rotterdam criteria are applied **(Ünlütürk, Sezgin and Yildiz, 2016)**. Other reasons for androgen excess that have been ruled out include certain types of congenital adrenal hyperplasia (which accounts for up to 9 percent of cases of androgen excess), and high prolactin. In other word PCOS is most common reproductive endocrinopathy, cause of PCOS is a group of genetic and environmental factors that are common pathologies, commonly associated with clinical symptoms of dyslipidemia, hirsutism, acne, and hyperandrogenism, as well as persistent infertility **(Sumathi *et al*., 2021).**

1.1.2.Types of polycystic ovary syndrome

According to various reasons PCOS can be classified in to four type :-

1. Insulin-resistant PCOS.
2. Inflammatory PCOS.
3. Adrenal PCOS .
4. Thyroid dysfunction in women with polycystic ovarian syndrome.

1-Insulin resistance means having high insulin hormone level. It's also called metabolic syndrome or pre-diabetes. The best way to test for insulin resistance is to measure the hormone insulin. In general, insulin resistance and compensatory hyperinsulinemia affect approximately 65-70% as a proportion of women with PCOS **(Marshall and Dunaif, 2012 ; Dumesic *et al*., 2019).**

2- Inflammatory PCOS:- in this type many markers elevated including C-reactive protein, interleukin-18, white blood count and increased oxidative stress **(Duică *et al*., 2021 ; Borthakur *et al*., 2020 ; Duleba and Dokras, 2012)**.

Since multiple pathogens such as Chlamydia, Pneumoniae and Helicobacter pylori are related to chronic inflammation and inflammation Cardiovascular disease **(Duleba and Dokras, 2012).**

Additional sources of chronic infectious processes associated to inflammation and cardiovascular risks involved pathogens include in periodontal disease(**Duleba and Dokras, 2012)**.

Chronic inflammation associated with chlamydial infections can lead to pathogenetic processes leading to PCOS by metabolic and hormonal disorders **(Morin-Papunen *et al*., 2010)**.

3- Adrenal PCOS:- adrenal androgen (AA) excess occupy ratio between 20 and 30% of patients with PCOS can be detectable by elevated dehydroepiandro-steron sulfate (DHEAS) levels **(Yildiz and Azziz, 2007 ; Nadir *et al*., 2018).** The differential diagnosis of adrenal hyperandrogenism in PCOS may include non-classic adrenal hyperplasia, adrenal androgen-producing tumors and Cushing's syndrome or heritability of adrenal androgen secretion **(Halimova *et al*., 2019**).

The adrenal cortex also accounts for about 25% of the circulating testosterone (T) levels. AA excess in PCOS may represent hypothalamic-pituitary alterations or adrenocortical steroidogenic abnormalities or abnormalities in the metabolism of cortisol in PCOS hepatic tissue (**Yildiz and Azziz, 2007).**

4-Thyroid dysfunction with PCOS:- that PCOS is associated with hypothyroidism (**Kedar, Rewatkar and Akare, 2019**).

The elevated level in thyrotropin-releasing hormone(TRH) in primary hypothyroidism leads to increased prolactin and thyroid stimulating hormone(TSH) as show in figure (1.1). Prolactin contributes toward polycystic ovarian morphology by inhibiting ovulation as a result of the change in the ratio of follicle stimulating hormone (FSH) and luteinizing hormone(LH) and increased dehydroepiandrosterone from the adrenal gland, increased TSH also contributes due to its spill-over effect on FSH receptors. Increased collagen deposition in ovaries as a result of hypothyroidism has also been suggested **(Ho *et al*., 2020 ; Chen *et al*., 2020 ; Singla *et al*., 2015).**

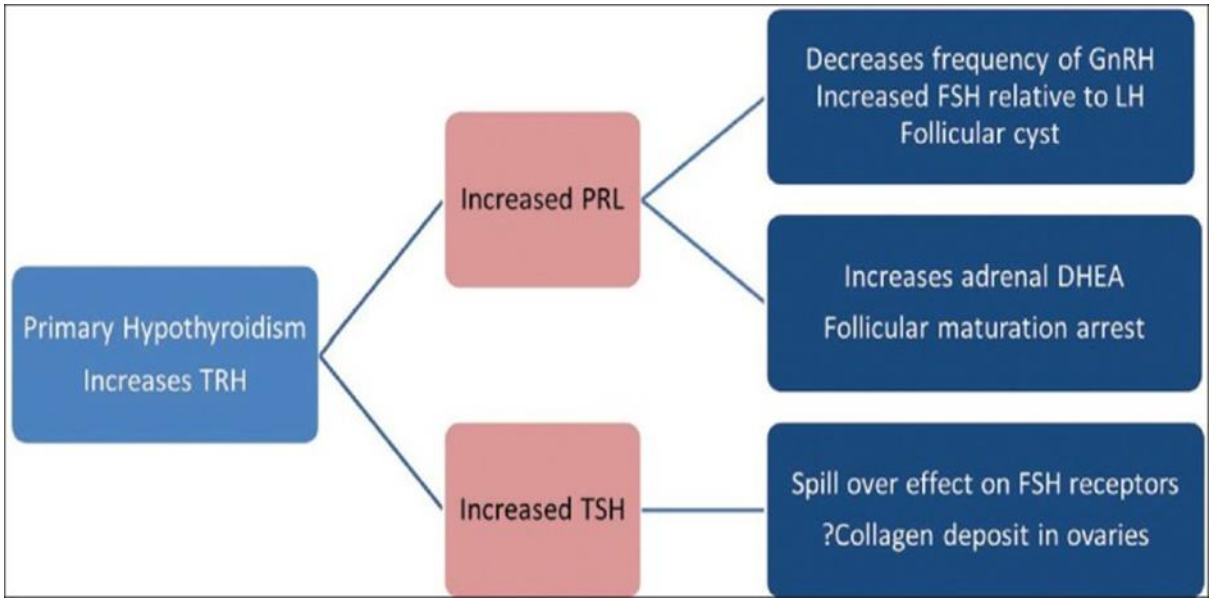

Fig 1.1: Pathophysiology of primary hypothyroidism in patients with polycystic ovaries (Singla *et al*., 2015)

1.1.3. Insulin resistance in the syndrome

The main type of PCOS is insulin resistance, basically meaning that their bodies' cells do not respond to the insulin hormone in the body, impaired sensitivity of cells to insulin signaling, resulting in failure to take up glucose. Because glucose is not used for energy generation, it is retained in the body, leading in weight gain (**Crook, 2013**). In this case people with insulin resistance may or may not go on to develop type 2 diabetes. In type 2 diabetes, there is probably enough insulin but the cells upon which it should act are not normally sensitive to its action.

Diabetes, obesity, metabolic syndrome, poor glucose tolerance, hypertension, inflammation, heart disease, and dyslipidemias are all examples of insulin resistance (IR). IR is complex and contains hereditary components, according to

population research, such as abnormalities in the insulin-signaling pathway (such as serine phosphorylation on insulin substrate or reduced signaling activation).IR is linked to mitochondrial abnormalities, oxidant overproduction, and fat accumulation **(Molina, Ferder and Manucha, 2015).**

Insulin resistance is diagnosed by blood glucose testing, insulin hormone level and testing of the level of glycosylated hemoglobin (glycohemoglobin or hemoglobin A1C) **(Crook, 2013).**

Excess insulin production from beta cell in pancreas leads to induce the ovaries to produce more androgen, resulting in oligo-ovulation (unusual ovulation) or anovulatory (the asbsence of ovulation) **(Sharif, 2019).** The detection rate of insulin resistance varies according to the insulin sensitivity index used **(Shahin *et al*., 2019)**.

High androgen level has a role in the deposition of abdominal fat and type 2 diabetes mellitus **(Rajkhowa *et al*., 2009 ; Sharif, 2019 ; Condorelli *et al*., 2017)** .

The primary defects in PCOS patients are both in the body relative LH/ FSH level and insulin resistance **(Sharif, 2019)**. Patients in PCOS have increase adipocyte ,less lipoprotein lipases activity and reduced lipolysis **(de Medeiros, Rodgers and Norman, 2021)**.

Additionally the two major hormones were Luteinizing hormone(LH) and Follicle stimulating hormone(FSH) are pituitary hormones that govern fundamental changes in the body for reproduction .

FSH is usually higher than LH at the beginning of the menstrual cycle. However, in PCOS, this is reverse, with hypersecretion of LH at first, rather than an increased FSH, since the hypothalamic release of gonadotropin-releasing hormone (GnRH) is sometimes abnormal. There is an increase in LH, which releases an overload of androgen substrates, leading to higher in testosterone level, which is correlated to extensive body and facial hair (**Sharif, 2019**).

The ecological factors are affected of PCOS through altering one's lifestyle Increased body weight and dietary saturated fat intake for example, defective metabolic and reproductive disorders.. Obesity has been given to be related to worsen metabolic and ovulatory dysfunction linked to PCOS and weight loss has been found to return ovulation and enhance hyperandrogenism and hirsutism (**Merkin *et al*., 2016**).

Inheritance factors and genetic for PCOS show a strong familial relationship, A woman's chances of developing PCOS are usually between 30 and 50 percent (**Sharif, 2019**).

PCOS has been linked to a number of genes controlling insulin action and androgen production **(Sharif, 2019**).

Compensatory hyperinsulinemia increases the androgen pool by acting directly on theca ovarian cells and indirectly by inhibiting hepatic production of SHBG and insulin-like growth factor binding protein 1(IGFBP-1), Excess androgens induce visceral adipose tissue (VAT) to produce FFAs, which adds to insulin resistance as show in figure (1.2). These occurrences act in tandem to keep PCOS at bay **(Shaikh, Dadachanji and Mukherjee, 2014)**.

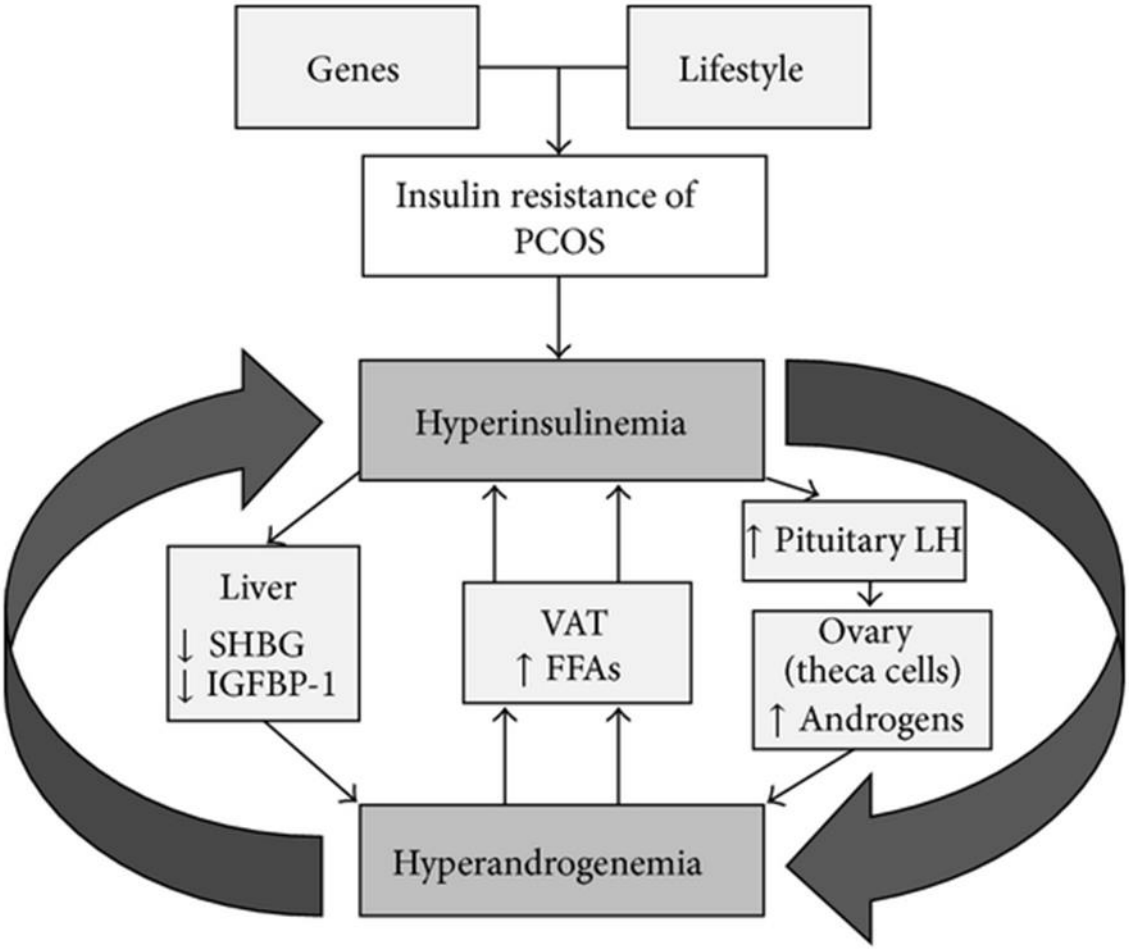

Fig 1-2 : The vicious cycle between hyperinsulinemia and hyperandrogenemia in PCOS development:. (**Shaikh, Dadachanji and Mukherjee, 2014**)

FFAs: Free Fatty Acids; IGFBP-1: Insulin-like growth factor binding protein 1 ; LH: Luteinizing Hormone ; SHBG :Sex Hormone Binding globulin ; VAT : Visceral Adipose Tissue.

Lipid disorders are obtain in PCOS-affected women. PCOS has a variety of lipid abnormalities, including lower concentrations of high density lipoprotein, elevated triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL.C), and also relatively high lipoprotein concentration, all of which increase the risk of cardiovascular disease (**Liu *et al*., 2019).**

According to a recent study, application of diet of high -fat is stimulate metabolic and ovarian conversion present in PCOS, thus found that effected hyperlipidemia on the hormonal profile (**Liu *et al*., 2019**).

Due to ovarian abnormalities, an increased number of polycystic follicles, and an elevation in follicular layer thickness, infertility is correlated to dyslipidemia in PCOS. Dyslipidemia affects up to 70% of PCOS patients. Obese women had a larger ratio of IR diagnoses than females with a normal BMI, indicating a relation between IR and dyslipidemia **(Liu *et al*., 2019 ; Rojas *et al*., 2014).**

Aerobic exercise training intervention improved cardiorespiratory fitness, and cardio metabolic health of overweight/obese women with PCOS (**Costa *et al*., 2018)**. Therefore, it is that chronic inflammation may constitute another aspect of the PCOS pathophysiology related hyper-inflammation ,as has also been suggested for diabetes **(Kyrou *et al*., 2020**).

1.1.4. Disease progression in polycystic ovarian syndrome

The progression of the syndrome can be summarized development of the diseases associated with the cyst to :-

1-The skin symptoms of PCOS, such as hirsutism and acne, should be recognized during the physical examination. When the amount of free testosterone in a woman's body rises from 1 to 2%, hirsutism might appear, Excessive hair growth on the face, neck, chest, and lower abdomen is stimulated by abnormally high blood androgen levels **(Archer and Chang, 2004 ; Martin *et al.*, 2008**).

Because the sebaceous glands on the face are larger than those on other parts of the body, acne tends to appear first on the face. However, acne lesions on the neck, chest, and upper back affect up to 50% of women with hyperandrogenism. **(Archer and Chang, 2004).**

2- Early pregnancy losses or miscarriage **(Palomba *et al*., 2015),** Women with PCOS at an elevated risk of pregnancy problems (gestational diabetes, preterm birth, and pre-eclampsia) aggravated by obesity **(Archer and Chang, 2004 ; Palomba *et al*., 2015).**

3- Dyslipidemia**:** disorders of lipid metabolism-cholesterol and triglycerides **(de Medeiros *et al*., 2021 ; Palomba *et al*., 2015)**.

4- Blood pressure that may be too high.

5- Cardiovascular disease **(Blagojevic *et al*., 2017)** .

6- Strokes **(Palomba *et al.*, 2015)**.

7- Infertility **(Palomba *et al*., 2015)**.

1.1.5. History of discovery and diagnosis criteria of PCOS

Stein and Leventhal were the first to linked amenorrhea, obesity, and hirsutism to polycystic ovaries , and therefore PCOS condition was first known as Stein–Leventhal syndrome (PCOS) **(Waseem *et al*., 2021**), then in 1935, Vallisneri described a married, infertile female with pure white ovaries the size of immature ova. Except in the 1990, during a National Institute of Health (NIH)-sponsored symposium on the disease, were formal diagnostic criteria for PCOS created and extensively utilized. Despite the fact that it is now widely recognized as being complicated and somewhat inherited, a number of possible genes have been postulated. Insulin resistance has been reported in a large number of PCOS women, Especially those with androgen excess, despite the fact that it is not a diagnostic criterion **(Szydlarska, Machaj and Jakimiuk, 2017).**

The guidelines laid by a team of professionals in a Rotterdam conference in 2003 (Sponsored PCOS Consensus Workshop Group of the Rotterdam ESHRE/ASRM) are mandatory. The "Rotterdam criteria" that followed added the size and shape of the ovary as assessed by ultrasonography in to the diagnostic criteria **(Szydlarska, Machaj and Jakimiuk, 2017).**

The following are examples of the different names for the same syndrome that have been discovered in the literature: polycystic ovary disorder, polycystic ovary syndrome, functional ovary androgenism, chronic anovulation, syndrome of polycystic ovarian, hyper androgenic, ovarian dysmetabolic syndrome and sclerotic polycystic ovary syndrome **(Szydlarska, Machaj and Jakimiuk, 2017)**.

According to that, two out of the three criteria listed below must be present in order to diagnose PCOS :-

1- Physical examination and medical history (menstrual periods, lack of ovulations, unwanted hair growth and weight changes).

2- Laboratory investigations:- serum tests for female as well as :-

   a) Female hormones Ratio of LH/FSH is higher example 2:1 or more.

   b) Lipid such as Cholesterol and triglycerides .

   c) Blood sugar level.

   d) Androgen level .

3- Ultrasonography (USG): Ultrasonography (USG) can detect enlarged ovaries and cysts (increased ovarian volume more than 10 cm3). According to **(Legro, 2008).** After exclusion other pathologies distinguished by hyperandrogenism, such as hypercortisolemia that is adrenocorticotropic hormone-dependent or independently, thyroid gland diseases, adrenal gland tumors or ovary tumors that produce androgens, and the impact of medicine **(Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome, 2004**).

Protein and enzyme markers are frequently used in the diagnosis and monitoring of PCOS patients in order to avoid the onset of complications like CVD. Insulin resistance and the development of PCOS have been found to be significantly linked to adipose tissue proteins **(Sharif, 2019).**

So that this study will examine new proteins and assess the extent of their relationship and relevance to PCOS and the possibility of treatment which are:-

1- Zinc alpha 2 glycoprotein .

2- Heat shock protein 70 .

1.2. Zinc alpha 2 glycoprotein

Zinc alpha 2 glycoprotein (ZAG) is a soluble protein that was originally extracted from human plasma. , ZAG was initial isolated from human plasma in 1961 (**Schmid and Bürgi, 1961 ; Ullah *et al*., 2020**), and represents approximately for 0.2% of total serum protein **(Russell and Tisdale, 2009**). The protein is generated by the liver (**Clerc *et al*., 2015**). subsequently purified in 1961 **(Hassan *et al*., 2008**).

ZAG was named by the fact that it tended to precipitate with zinc salts. It has one tight and fifteen loosely zinc-binding sites **(Lau *et al*., 2019**) its electrophoretic mobility in the area of the α_2 globulins, and its carbohydrate content of 12-18% **(Bagherani, 2012 ; Sanchez, Lopez-Otin and Bjorkman, 1997)**, that is represented by the Alpha-2-glycoprotein 1, zinc-binding (AZGP1) gene in humans.

The molecular weight of a single chain polypeptide is 40-43 KD that can be detected in the majority of human fluids, including serum, saliva, perspiration, breast milk, seminal plasma, cerebrospinal fluid, and urine **(Archer and chang,2004**).

According to finding the Zn-α2-gp gene is expressed in liver and normal breast, but not in placenta and thyroid (**Freije *et al*., 1991**).

1.2.1. Structure of Zinc alpha 2 glycoprotein

ZAG included of a single polypeptide chain of 276 amino acid residues which contain 8 tryptophan residues and has a pyroglutamyl residue in the amino terminus **(Araki *et al*., 1988).** ZAG and antigen-presenting molecules such as class I major histocompatibility complex (MHC) molecules are both similar in the sequence and structural levels **(Bagherani, 2012)** . Because ZAG protein is not membrane bound like other MHC molecules, its function is different **(Bagherani, 2012).**

The ZAG gene, which is found on chromosome 7q22.1, has four exons and three introns **(Hassan *et al*., 2008).**

ZAG has four half-cystine residues in its amino acid sequence. The location of cysteine and disulfide pairing as measured by biochemical and structural research indicates the formation of two bonds: one between residues 101 and 164 and the other between residues 203 and 258 **(Hassan *et al*., 2008).** The presence of a glycan chain on a3 domains suggested that it had a functional role in cell signaling and binding to various cell surface receptors. **(Hassan *et al*., 2008).**

Four *N*-glycosylation sites have been detected on ZAG **(Clerc *et al*., 2015).**. numerous glycoforms of ZAG were produced by changing the Asparagine (Asn) residues to glutamine, seven different ZAG glycoforms resulted from single and combination mutations **(Romauch, 2020)** .

1.2.2.Biochemical roles of Zinc Alpha 2 Glycoprotien

The function of ZAG include the following :-

1- As a lipid-mobilizing factor (LMF) generated by cachexia stimulate tumors was similar in amino acid sequence to ZAG. When fed a high fat diet, ZAG-deficient mice gain weight. ZAG has been reported to stimulate lipolysis in white adipose tissue (WAT) via activating adenylyl cyclase in a GTP-dependent mechanism **(Russell and Tisdale, 2009)**. Limiting of body fat and increases serum free fatty acid levels, by increased intracellular cAMP levels activate hormone-sensitive lipase **(Bing *et al*., 2004)..**

2- ZAG enhance glucose up take in cell **(Russell and Tisdale, 2009**). Insulin signaling proteins were stimulated, such as cell membrane glucose transporter type 4 (GLUT4) and phosphorylation of insulin receptor substrate 1(IRS-1), were significantly increase in the skeletal muscle when used ZAG as therapy **(Gao *et al*., 2018)** .

3- ZAG may regulate the production of many other adipokines, because recent study found that ZAG promotes the expression of adiponectin **(Bing *et al*., 2004).** This improves their insulin sensitivity in peripheral organs **(Severo *et al*., 2019).**

4- ZAG increases expression of uncoupling proteins-1 and -3 (UCPs) in brown adipose tissue (BAT) and white adipose tissue (WAT) with a 0.4 C^{o} increase in body temperature (**Russell and Tisdale, 2009)** .

5- Fertilization. ZAG is 6 times more abundant in human seminal fluid than in human serum, indicating that it plays a function in fertilization **(Hassan et al., 2008 ; Bagherani, 2012).**

6- The plasma ZAG may action as a carrier protein in the nephritogenic renal glycoprotein (**Hassan *et al*., 2008).**

7- Recent research has shown that this protein (ZAG) has a metastatic and prognostic function in a variety of cancers, including prostate, breast, lung, colorectum, and liver carcinomas **(Liu *et al*., 2018).**

Points 1, 2, 3 and 4 can be summarized by the following mechanics Zinc is necessary for zinc-2-glycoprotein to bind to fatty acids and 3-adrenergic receptors, as well as for its structure. Thus, adipokine suppresses lipogenesis, enhances glucose receptor translocation, increases adiponectin production, and promotes thermogenesis in brown adipose by promoting lipolysis via crucial enzyme control as show in figure (1.3) **(Severo *et al*., 2019).**

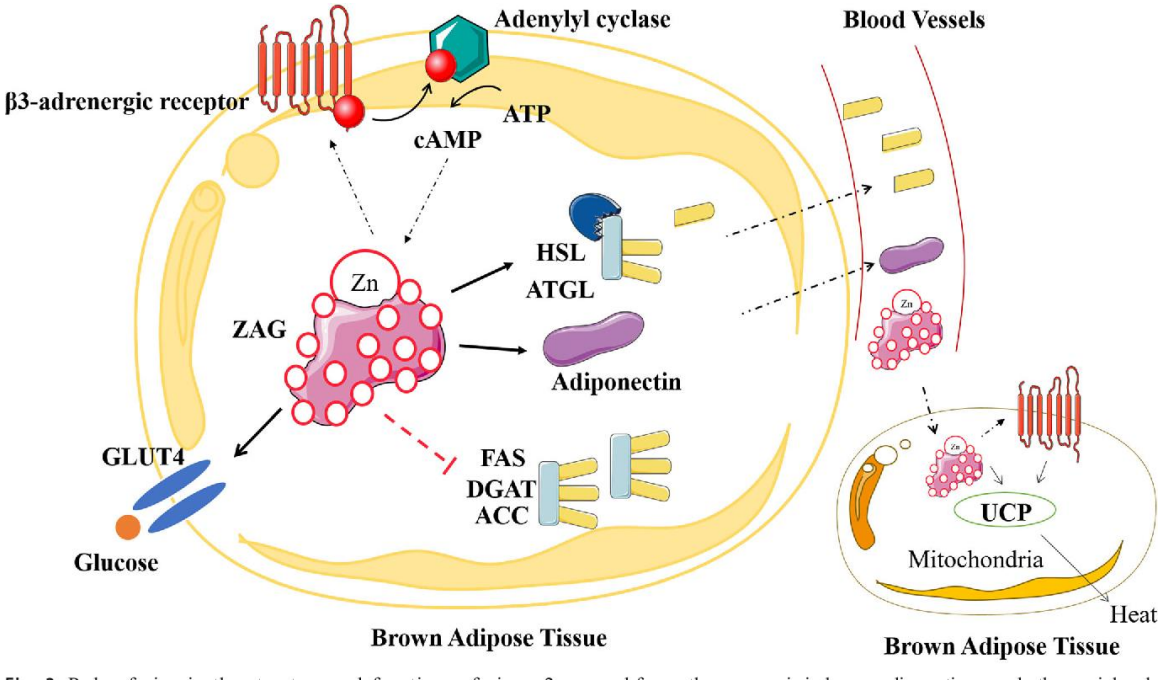

Fig 1.3 : Zinc's role in the building and function of zinc-2 glycoprotein (Severo *et al.,* 2019) .

ACC : acetyl-CoA carboxylase, ATGL : adipose tissue triglyceride lipase, ATP : adenosine triphosphate, cAMP : cyclic adenosine monophosphate, DGAT : diglyceride glycerol acyltransferase, FAS : fatty acid synthase, GLUT4 : glucose transporter type 4 .

ZAG is an anti-obesity protien that works by inducing a brown fat-like phenotype in white adipocytes **(Xiao *et al*., 2018).** ZAG improved mitochondrial activity and lipid catabolism in WAT browning **(Xiao *et al*., 2018).** According to ZAG, the traditional method of activating beige fat is to stimulate sympathetic neurons, which release noradrenaline (NE), which then acts on b-adrenergic receptors, particularly the b3-adrenoreceptor **(De Jong *et al*., 2017).**

Additionally, activation of protein kinase A (PKA) facilitates phosphorylation of adipose tissue triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) which leading to TG breakdown and release of NEFA **(Pagnon *et al*., 2012).** Their actions were achieved by lipogenic enzyme suppression and increased production of lipases in fatty tissue, such as hormone-sensitive lipase (HLS) and adipose tissue triglyceride lipase (ATGL) **(Xiao et al., 2018)**,

In endometrial fatty tissue, lipogenic enzymes including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and diglyceride-glycerol acyltransferase (DGAT) are suppressed **(Severo *et al*., 2019).**

The management of lipid synthesis and lipolysis-related enzymatic by ZAG alters weight &body mass index (BMI) **(Xiao *et al*., 2018).**

The involvement of ZAG in fatty tissue homeostasis leads to the question about whether ZAG and dysglycamia are linked.

1.3. Heat shock proteins

Heat shock proteins (Hsps) are a group of highly homologous chaperone proteins which are activated in response to the environment, physical, and chemical stressors, limiting the effects of damage and facilitating cellular recovery **(Hoter and Naim, 2019** ; **Beere, 2004)**. They were initially reported in relationship to heat shock, but it is now recognized that they may also be produced during other stressors such as cold exposure **(Matz *et al*., 1995**). Ultraviolet (UV) light, heavy metals, oxidative stress, some drugs, nutrient deficiency, chronic inflammatory diseases, viral infections **(Ikwegbue *et al*., 2017** ; **Cao *et al*., 1999 ; Yang *et al*., 2020)** as well as during wound healing and tissue remodeling **(Atalay *et al*., 2009).**

HSPs are present in almost every living organism, as bacteria to humans **(Cao *et al*., 1999** ; **Li and Srivastava, 2004).**

The molecular weight of heat-shock proteins is used to name them. Hsp60, Hsp70, and Hsp90 (the most extensively researched HSPs), for example, are heat shock proteins with sizes of 60, 70, and 90 KD, respectively **(Buttacavoli *et al*., 2021 ; Li and Srivastava, 2004)**. The inducible Hsp expression was controlled by heat shock transcription factors (HSFs) **(Hoter and Naim, 2019** ; **Pirkkala *et al*. , 2001**).

The small 8-kilodalton protein called ubiquitin, which marks proteins for degradation, in addition has characteristics of a heat shock protein (**Datta, Rahalkar and Dubey, 2017**). Small heat shock proteins (sHsps) are ubiquitous molecular chaperones which prevent denaturing proteins from aggregating irreversibly **(Haslbeck and Vierling, 2015).**

In 1962, F. Ritossa was found the heat shock response **(Pirkkala *et al*., 2001** ; **Vostakolaei *et al*., 2020**). The enhanced production of a few proteins immediately after exposure cells to a stress such as heat shock was first

identified in 1974 **(Schlesinger, 1990 ; Datta, Rahalkar and Dubey, 2017**). Hsps have a necessary function in stress-induced damage protection **(Pirkkala, Nykänen & Sistonen, 2001**). play an important function in protein transport and homeostasis.

DnaK, HscA (Hsc66), and HscC (Hsc62) are components of the Hsp70 family. All of these proteins have a similar domain design and a same principle of action adopted **(Radons, 2016**).

1.3.1.Structure of heat shock protein 70

The human Hsp70 includes 641 amino acid residues and a molecular weight of 70,052 Dalton **(Saibil, 2013**). The Hsp70 proteins have three major functional domains:-

1- N-terminal ATPase domain – links ATP (Adenosine triphosphate) and hydrolyzes to ADP (Adenosine diphosphate). ~44 Kilodalton nucleotide-binding domain (NBD) **(Saibil, 2013)**.

2-Substrate binding domain (SBD)– was composed of a 15 Kilodalton β sheet subdomain and a 10 Kilodalton helical subdomain **(Rosenzweig *et al*., 2019).** SBD seems to have a groove that affinity neutral, hydrophobic amino acid residues **(Caza *et al.*, 2016)**. The two HSP70 domains are connecting by the linker (13aa) **(Albakova *et al.*, 2020).**

3- The C-terminal domain, which is rich in alpha helical structure, functions as a 'lid' for substrate binding domain. When a Hsp70 protein binds to ATP, the lid opens, allowing peptides to bind and release relatively quickly. When ADP binds to Hsp70 proteins, the lid closes and peptides are tightly bound to the substrate binding doman **(Mayer, 2010).**

Figure(1.4) was shown Hsp70 Structure and Domain Organization **(Rosenzweig *et al*., 2019 ; Mayer *et al*., 2001).**

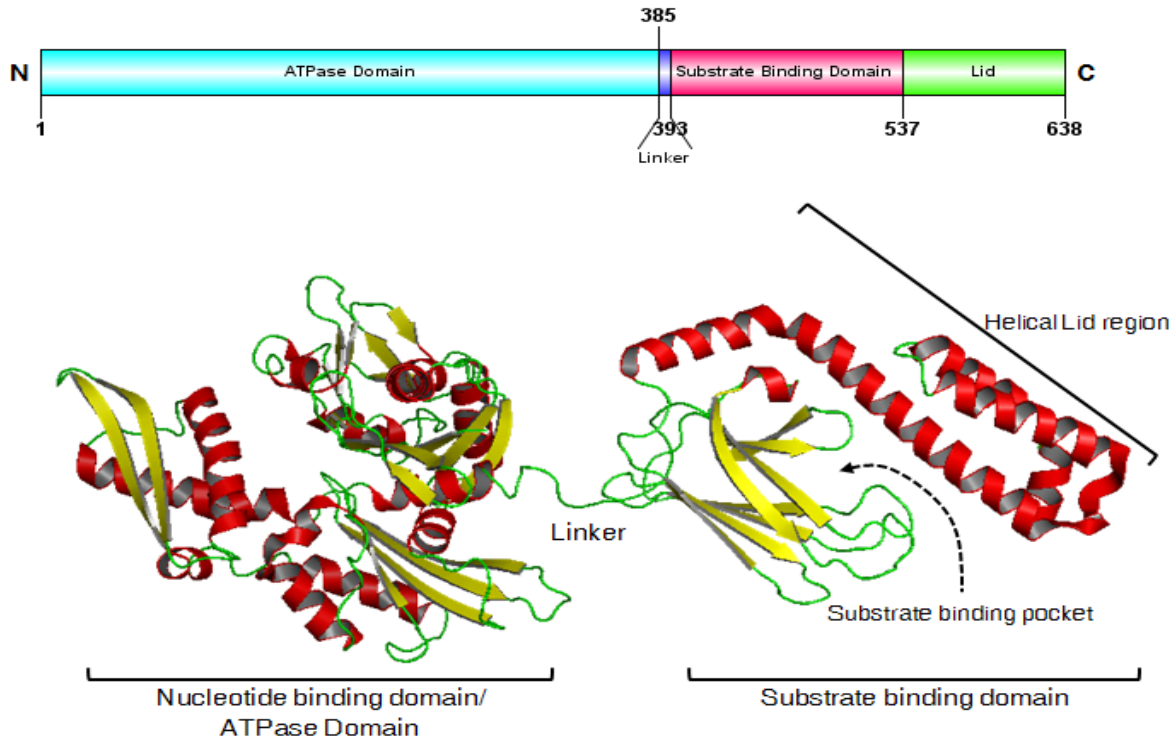

Fig 1-4: Hsp70 structure and domain organization (Rosenzweig *et al*., 2019).

The structure of the Hsp70 and Hsp70-related protein families' domains. The ATPase domain (385 residues, light blue), a short linker, and the substrate-binding domain, which is split into the fl-sheet (150 residues, purple) and the ct-helical subdomains (100 residues, gradient) are shown schematically.

1.3.2. Function and mechanism of HSP 70

The Hsp70 system interacts with prolonged peptide segments of proteins and also partly folded proteins to produce protein aggregation in key pathway, causing activity to be deregulated (**Vostakolaei *et al*., 2020).**

Heat shock protein 70 (HSP70) interacts with several co-chaperones and nucleotide exchanges factors and plays an essential function in protein quality which control increases survival by saving cells from environmental stressors

(Hartl, Bracher and Hayer-Hartl, 2011 ; Radons, 2016). As a result, it plays an important role in protein homeostasis and transport **(Breuninger and Erl, 2014)**.

Hsp70 also helps in protein transmembrane transportation by stabilizing them in a partly folded state. It has also been shown to be phosphorylated. Hsp70 molecular chaperones play important roles ensure the effective translocation process of proteins across membranes on both sides of the endoplasmic reticulum and mitochondria **(Craig, 2018**) which regulates a variety of its functions (**Gao and Newton, 2002).**

They are essential in protein–protein interactions, such as folding and facilitating in the development of correct protein conformation (shape) and the avoidance of unwanted protein aggregation. By facilitating in the stabilization of partly unfolded proteins **(Borges and Ramos, 2005**).

"Molecular chaperones are one of the most important cell defense mechanisms against protein aggregation and misfolding" **(Ciechanover and Kwon, 2017 ; Borges and Ramos, 2005)**.

Hsp70 suppresses apoptosis directly **(Albakova *et al*., 2020** ; **Beere *et al*., 2000**). Because of its anti-apoptotic action, Hsp70 functions as a "friend" of cancer formation. As a result, researchers are presently experiment to enhance cancer therapy treatments through the use of Hsp70 inhibitors **(Hoter, Rizk and Naim, 2019) .**

On the other hand, found the Hsp70 protein on the surface of cancer cells may sensitize tumor cells to cytotoxic attacks by natural killer (NK) cells and trigger a particular anti-tumor response. As a result, it appears that the intracellular characteristics of Hsp70 aid in the growth of cancer cells, but the extracellular Hsp70 may be recognized by immune system cells and therefore help in the generation of appropriate immunotherapy **(Murphy, 2013 ; Albakova *et al*.,**

2020). As a result, it appears to be an effective therapeutic target in human cancers **(Vostakolaei *et al*., 2020).** "HSP70 is transported to the plasma membrane by binding to other proteins" **(Albakova *et al*., 2020)**.

HSP70 and HSP47 were found to be expressed in the dermis and epidermis after laser irradiation (**Sajjadi, Mitra and Grace, 2013).** HSP70 may biochemically describe the thermal damaging zone in which cells are target for destruction, allowing for accurate and precise tissue ablation and promoting fast wound healing after laser-based surgery **(Sajjadi, Mitra and Grace, 2013).**

Hsp70 is found in the peripheral circulation of healthy people, even if Hsps are considered as intracellular proteins with molecular chaperone and cyto protective activities **(Pockley, Shepherd and Corton, 1998) .**

Elevated serum Hsp70 concentrations have also been associated to ovarian damage **(Narayansingh *et al*., 2004).** Serum or plasma Hsp70 levels have also been found to be elevated in a variety of chronic diseases T2DM **(Nakhjavani *et al*., 2010)**, cardiovascular diseases (CVD) **(Zhang *et al*., 2010)** and cancer **(Vostakolaei *et al*., 2020)**. The association between circulating Hsp70 and PCOS is not well understood.

"Serum extracellular eHSP70 concentrations are positively correlated with insulin resistance" **(Krause *et al*., 2014),** "markers of inflammation, such as C-reactive protein, monocyte count, and TNF-α" (**Krause *et al*., 2015).** Chronic exercise(training) were suppress eHSP70 levels (**Krause *et al*., 2015).** HSP70 may give important information regarding endothelial dysfunction in cardiovascular disorders, as well as promoting the release of inflammatory cytokines and the generation of reactive oxygen species (**Costa-Beber *et al*., 2020**).

Diabetes DM lead in to significantly higher blood HSP70 levels **(Nakhjavani *et al.*, 2010)**. because of hyperglycemia and the oxidative stress damage and inflammation caused by hyperglycemia.

Research aims and objectives

The aims of this study are to:

- Investigate circulating zinc-α2-glycoprotein (ZAG) concentrations in women with PCOS and compare them to those in the control group its association with the infertility.
- Observe characteristics of serum ZAG and its relationship with endocrine and metabolic indicators of PCOS patients.
- Assess the relationship between ZAG, androgen excess and insulin resistance in women with PCOS
- Attempted to examine the association between circulating heat shock protein 70 (Hsp70) concentrations and PCOS
- Find out the role of HSP70 in the pathogenesis of PCOS by assess the levels of HSP70 in PCOS patients' sera and compare them to those in the control group.
- Assess the role of ZAG and HSP70 in different infertility groups.
- Investigate the correlation among ZAG, HSP70, hormones and inflammatory factors such as dysglycemia, insulin resistance, insulin level.

The most important questions that were researched are:

1-Is ZAG level related to polycystic ovarian syndrome?

2-Does ZAG level affected with infertility and insulin resistance on PCOS patients?

3-Can ZAG be considered as anew indicator and treatment monitoring in PCOS?

4- Is HSP70 consider as new marker for investigate inflammatory for PCOS and study it's related to degree of infertility in PCOS?

5- Can be consider serum HSP70 and ZAG levels as predictive risk factor for PCOS ?

Chapter two

Materials and Methods

2- Materials and Methods

2.1. Subjects and study design

A case-control study was carried out at Al-Batoul Women and Children Hospital / Wasit in the city of Kut. All samples were collected from November 2020 till May, 2021.

The study was executed for 81 female Iraqi individuals their age ranged (18-45) years old within reproductive age and 50 of them diagnosed with polycystic ovarian syndrome and 31 as healthy control group

2.1.1.Inclusion criteria

Thirty one apparently healthy women in reproductive age with normal cycles between (18-45) years old, were taken as a control group. Each one had a history of regular 26 to 32 day menstrual cycles, absence of hirsutism and other manifestations of hyperandrogenism , and thyroid dysfunction. This group was checked by gynecologist. And fifty women with PCOS (all of them were newly diagnosed with PCOS according to Rotterdam criteria by gynecologist) were taken as patients group.

Permission was taken from all subjects to participate in this study furthermore, each person who contributed in this study underwent full history, age, height, weight to calculate their body mass index (BMI), other diseases, and gynecological history, family history of disease, Primary or secondary infertility. The number of children and the age of the last child, the presence of coarse hair on the body and face, acne, waist circumference, Menstrual regularity and presence of other diseases.

2.1.2.Exclusion criteria

None of these subjects were alcoholics or chronic smokers. Patients suffering from diabetes mellitus, hyperprolactinaemia , congenital adrenal hyperplasia, thyroid disoders, Cushing's syndrome, hypertension, hyperlipidemia, take birth control pills or any origin other than polycystic ovary syndrome and patient with malignancies were excluded from the study.

2.1.3.Collection of sample

Fasting venous blood samples were collected from each case during 2^{nd} – 5^{th} day of the menstrual cycle (early follicular phase) for those of normal cycle . For patients with anovulation or oligomenorrhea blood samples were collected regardless of duration of the cycle.

1. About 6 ml of blood samples were obtained from veins of patients having polycystic ovarian syndrom and healthy control subjects

2. The blood samples were divided into two parts :

a) The first 2.5 milliliters of blood were collected in EDTA tubes to obtain plasma for the measurement of glycated hemoglobin HbA1c.

b) The second half, 3.5 milliliters of blood, was placed into a gel tube and kept at room temperature for 15 minutes. Following coagulation, the serum was separated by a centrifuge 4000 R per M for 10 min serum hormones Testosterone, Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Blood glucose level (fasting), and insulin level were measured immediately using suitable methods.

ii. The remainder were kept in deep freeze at -80 degrees Celsius. until assayed for zinc alpha 2 glycoprotien and heat shock protein 70. They were measured using enzyme-linked immune sorbent assay (ELISA) kits.

2.2. Instruments

All the instruments and tools which are used in this study are shown below in table 2.1

Table 2.1: The instruments and equipment's are used in this study

Instruments and Equipment's	**Company & Country**
Centrifuge	Hettich (Germany)
Deep Freezer	Samsung (Korea)
Disposable test tube	Meheco (China)
EDTA tube	Dia tech (USA)
Gel tube	Afco (Jordan)
ELISA system(reader, washer, printer)	Austria
Micropipette Iso 9001 (100-1000 µl)	Slamed (Germany)
Micropipette Iso 9001 (50-200 µl)	Slamed (Germany)
Pipette Tips (Yellow & Blue)	China
spectrophotometer	Apel - Japan
test-tube-rack	Philippines
Oven	Germany
I-chromA	Biditech MED Korea
Cobas e411	Germany

2.3. The chemicals and kits

The chemicals and kits that used in the study were of the highest purity and are listed in table (2.2) below with their suppliers:-

Table 2.2 : The chemicals and kits used in this study

Chemical\Kit	Company-Origin
Zinc Alpha-2-Glyco protein	Bioassay lab-china (Cat. No E3056Hu)
Heat shock protein 70	Bioassay lab-china (Cat. No E1813Hu)
FSH Kit	Boditech I-CHROMA -Korea
LH Kit	Boditech I-CHROMA -Korea
testosterone kit	Boditech I-CHROMA -Korea
Insulin Kit	Cobas e 411 Germany
HbA1c Kit	Boditech I-CHROMA -Korea
blood glucose Kit	LINEAR CHEMICALS - SPAIN

2.4. Methods

2.4.1. Body Mass Index

Body mass index (BMI) was evaluated according to World Health Organization (WHO) standard . To calculate BMI the mathematical formula based on persons weight and height, with BMI equal to weight in kilogram divided by Height in square meter.(It is defined as a person's weight in kilograms divided by the square of the person's height in meters (kg/m2) . WHO. Regional office of Europe. body mass index_BMI **(Body mass index - BMI, 2019) .**

Shown in Table 2.3. Nutritional status for different BMI states .

The equation below expresses on body mass index:-

BMI = weight (kg)/ height (m^2)

Table 2.3. Nutritional status

BMI	Nutritional status
Below 18.5 kg/m2	Underweight
18.5–24.9 kg/m2	Normal weight
25.0–29.9 kg/m2	Pre-obesity
30.0_ 34.9 kg/m2	obesity class I
35.0_39.9 kg/m2	obesity class II
above 40 kg/m2	obesity class III

2.5. Determination of zinc alpha 2 glycoprotein

2.5.1. Principle

This sandwich kit is for the accurate quantitative detection of human Zinc-Alpha-2-Glycoprotein (also known as ZAG) in serum. Itis an Enzyme-Linked Immunosorbent Assay (ELISA) **(BT LAB, 2020).**

1- The plate has been pre-coated with human ZAG antibody.
2- ZAG present in the sample is added and binds to antibodies coated on the wells.
3- And then biotinylated human ZAG Antibody was added and binds to ZAG in the sample.
4- Then Streptavidin-HRP (Horse Radish Peroxidase) was added and binds to the Biotinylated ZAG antibody.
5- After incubation unbound Streptavidin-HRP was washed away during a washing step.
6- Substrate solution then added and color develops in proportion to the amount of human ZAG.
7- The reaction terminated by the addition of acidic stop solution and absorbance was measured at 450 nm. The OD value proportional to the concentration of Human ZAG .Calculation of the concentration of Human ZAG in the samples compared to the OD of the samples to the standard curve.

2.5.2. Kits components of ZAG kit

Table 2.4: List of components of ZAG kit

No	Components	Quantity
1	Standard Solution (960µg/ml)	0.5ml x1
2	Pre-coated ELISA Plate	12 * 8 well strips x1
3	Standard Diluent	3ml x1
4	Streptavidin-HRP	6ml x1
5	Stop Solution	6ml x1
6	Substrate Solution A	6ml x1
7	Substrate Solution B	6ml x1
8	Wash Buffer Concentrate (25x)	20ml x1
9	Biotinylated human ZAG Antibody	1ml x1
10	User Instruction	1
11	Plate Sealer	2 pics
12	Zipper bag	1 pic

Material Is Needed But Isn't Getting It

• Incubator set to 37°C + 0.5°C.

• Paper that is absorbent.

• Pipettes with precision and disposable pipette tips

• Make sure the tubes are clean.

• Water that has been deionized or distilled.

Filte microplate reader with 450 10nm wavelength

2.5.3.Reagent preparation of ZAG kit

1- Before using any reagents, bring them to room temperature.
2- The 120µl of standard (960g/ml) has been reconstituted with 120µl of standard diluent to produce a 480µg/ml standard stock solution.
3- Before producing dilutions, we should let standard sit for 15 minutes with moderate agitation.
4- Duplicate standard points were prepared by diluting the standard stock solution (480µg/ml) 1:2 with standard diluent to provide 240µg/ml, 120µg/ml, 60µg/ml, and 30µg/ml solutions.
5- The zero standard is standard diluent (0 µg/ml). Any leftover solution has been frozen at -20°C and should be used within one month.
6- Wash Buffer has been installed. 20ml washed and diluted
7- Standard solution dilution was produced as follows :-

480µg/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
240µg/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
120µg/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluen
60µg/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
30µg/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

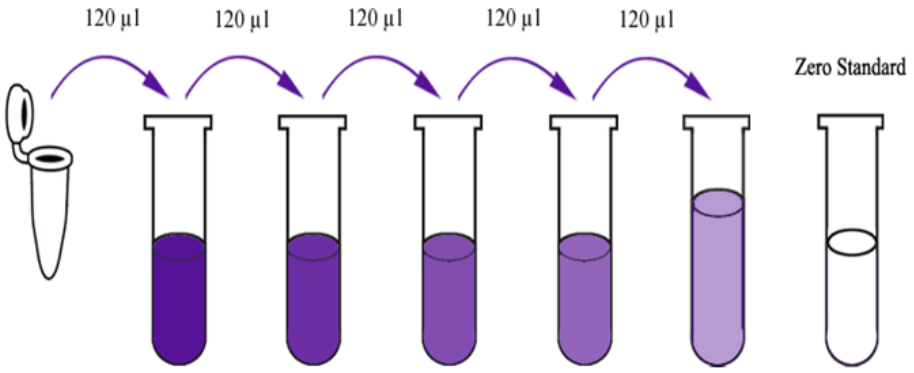

Fig 2.1: Standard ZAG dilution.

2.5.4. Calibration Curve of Human ZAG

Table 2.5: Calibration Curve of Human ZAG

Concentration	Optical density (OD)
0	0.095
30 µg/ml	0.129
60 µg/ml	0.269
120 µg/ml	0.47
240 µg/ml	1.007
480 µg/ml	1.751

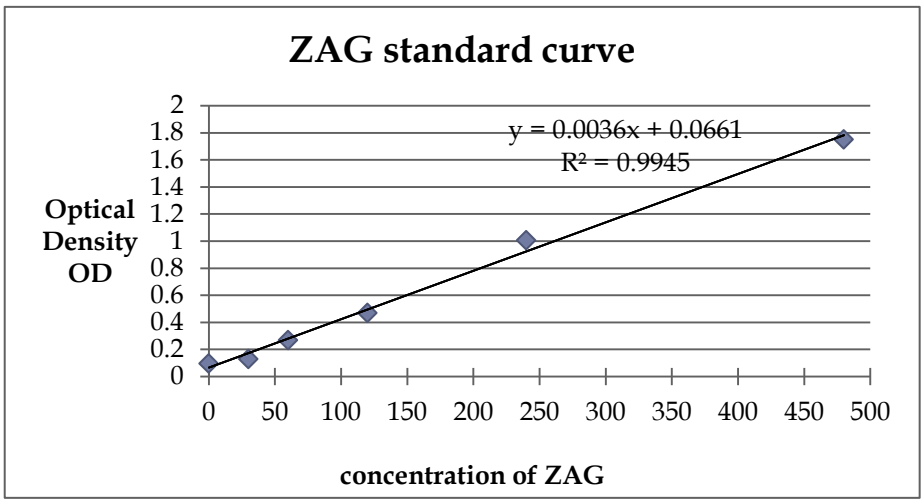

Fig 2.2: Calibration curve of human ZAG

2.5.5.Assay Procedure

1-All reagents, standard solutions, and samples were thoroughly examined and everything prepared just as directed. Before use, all reagents should be brought to room temperature. The test was carried out at room temperature.

2- After determining the number of strips necessary for the assay, the strips were placed into the frames for usage. Unused strips were kept at 2-8°C.

3- A standard well of 50 µl standard has been added. Because the standard solution contains biotinylated antibody, we did not add antibody to standard well.

4. 40µl samples was added to sample wells, followed by 10µl anti-ZAG antibody, followed by 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix thoroughly. I used a sealer to cover the plate. Incubated at 37°C for 60 minutes.

5- The sealer has been removed, and the plate has been washed 5 times with wash buffer. For each wash, wells were soaked in at least 0.35 ml wash buffer for 30 seconds to 1 minute. All wells were aspirated and washed 5 times with wash buffer, overfilling wells with wash buffer, for automated washing. The plate was blotted with paper towels.

6- 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. Incubated the plate for 10 minutes in the dark at 37°C with a clean sealer.

7. After adding 50µl Stop Solution to each well, the blue color turned to yellow immediately.

8. Within 10 minutes after applying the stop solution, the optical density (OD value) of each well was measured using a microplate reader set to 450 nm.

2.6. Determination of Heat shock protein 70(HSP70)

2.6.1. Principle

This sandwich kit is an Enzyme-Linked Immunosorbent Assay for the reliable quantitative detection of human Heat Shock Protein 70 (also known as HSP-70) in serum (ELISA) **(BT LAB, 2020)**.

1. Human HSP-70 antibody has been pre-coated on the plate.
2. HSP-70 was introduced to the sample and bound to antibodies coated on the wells.
3. Finally, a biotinylated human HSP-70 antibody was applied to the sample and bound to HSP-70.
4. The Streptavidin-HRP antibody was then added and coupled to the Biotinylated HSP-70 antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step.
5. The substrate solution was then added, and the color developed in accordance to the amount of human HSP-70 present.
6. The reaction was stopped by adding an acidic stop solution, and the absorbance at 450 nm was determined.

2.6.2. Kits of HSP70 components:-

Table 2.6 : List of components of HSP70 kits.

No	Components	Quantity
1	Standard Solution (96ng/ml)	0.5ml x1
2-	Pre-coated ELISA Plate	12 * 8 well strips x1
3-	Standard Diluent	3ml x1
4-	Streptavidin-HRP	6ml x1
5-	Stop Solution	6ml x1
6-	Substrate Solution A	6ml x1
7-	Substrate Solution B	6ml x1
8-	Wash Buffer Concentrate (25x)	20ml x1
9-	Biotinylated human HSP-70 Antibody	1ml x1
10	User Instruction	1
11	Plate Sealer	2 pics
12	Zipper bag	1 pic

Material Is Needed But Isn't Provided :-

- Incubator set to 37°C + 0.5°C.
- Paper that is absorbent.
- Pipettes with precision and disposable pipette tips
- Clean the tubes.
- Water that has been deionized or distilled.
- Microplate reader equipped with a 450 10nm wavelength filter.

2.6.3. Preparation of reagents

All reagents were brought to room temperature before use.

1-Standard has been reconstituted by combining 120µl of standard (96ng/ml) with 120µl of standard diluent to produce a 48ng/ml standard stock solution.

2-Before producing dilutions, the standard was allowed to sit for 15 minutes with moderate agitation.

3- Duplicated standard points were created by diluting the standard stock solution (48ng/ml) 1:2 with standard diluent to obtain solutions at 24ng/ml, 12ng/ml, 6ng/ml, and 3ng/ml..

4- As the zero standard (0 ng/ml), standard diluent was used. The leftover solution was stored at -20°C and utilized within a month.

5- Diluted 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. When crystals develop in the concentrate, they are gently mixed until they are dissolved entirely. The standard solutions were diluted as follows : -

48ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
24ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
12ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
6ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
3ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

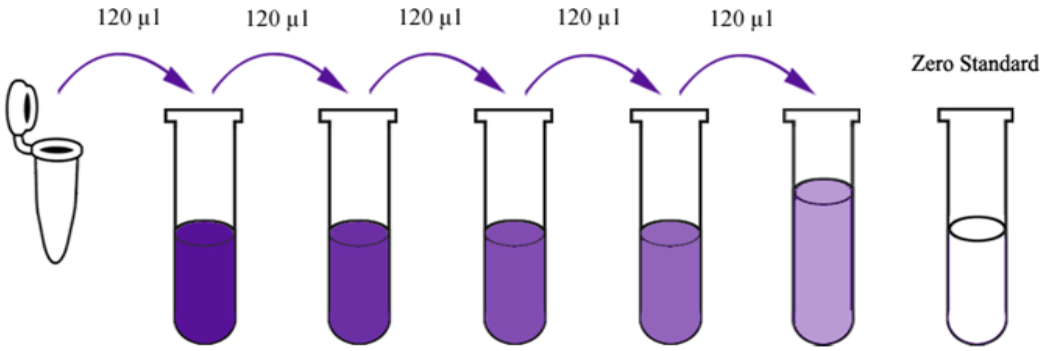

Fig 2.3 : Standard HSP70 dilution.

2.6.4. Calibration Curve of Human HSP70

Table 2.7: Calibration Curve of Human HSP70.

Concentration	Optical density (OD)
0	0.114
3 ng/ml	0.196
6 ng/ml	0.372
12 ng/ml	0.556
24 ng/ml	1.005
48 ng/ml	1.545

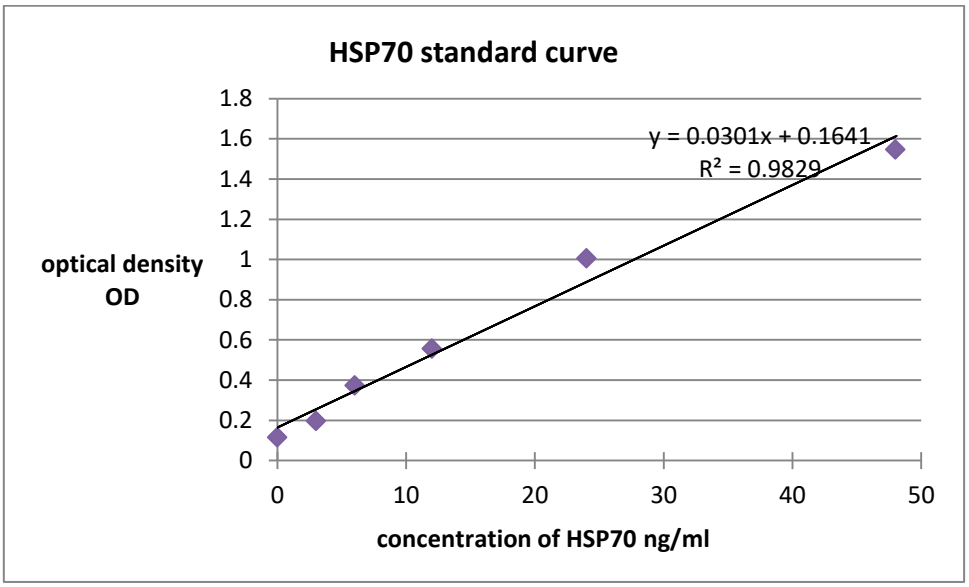

Fig 2.4 : Calibration curve of human HSP70

2.6.5. Assay procedure

1. All reagents, standard solutions, and samples are prepared according to the instructions. Before use, all reagents and samples should be brought to room temperature.
2. The number of strips necessary for the test was determined. For usage, the strips were placed into the frames. The unused strips maintained at 2-8°C.
3. A standard well of 50µl has been added. Because the standard solution contains biotinylated antibody, we did not add antibody to the standard well.
4. 40µl samples were added to samples wells, followed by 10µl anti-HSP-70 antibody, followed by 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix thoroughly. Then used a sealant to cover the plate. Incubated at 37°C for 60 minutes.
5. The sealer has been removed, and the plate has been washed 5 times with wash buffer. Soaked each washed well for 30 seconds to 1 minute in at least 0.35 ml wash buffer. Aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer, for automatic washing. The plate was then blotted using paper towels.
6. 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. Incubated the plate for 10 minutes in the dark at 37°C with a clean sealer.
7. After 50µl of Stop Solution was poured to each well, the blue color obviously turned to yellow. the optical density (OD value) has been Within 10 minutes after applying the stop solution, each well was determined using a micro plate reader set to 450 nm.

2.7. Glycated Hemoglobin HbA1c

Glycosylated protein is produced post-translationally by a complex, nonenzymatic interaction between the glucose and amino groups on proteins. HbA1c is a therapeutically relevant measure of mean glycemia during the previous 120 days, as well as the average erythrocyte life span. Because well controlled studies have shown a tight connection between HbA1c concentrations and mean glycemia, HbA1c is regarded as a more accurate measure in monitoring glycemia. **(Nathan et al., 1984)** .

2.7.1. Principle

A fluorescence immunoassay (FIA) for quantifying HbA1c (Hemoglobin A1c) in human whole blood. It is effective in the long treatment and monitoring of glycemic status in individuals with diabetes mellitus.

The used since a sandwich immunological detection technique in which the detector antibody in buffer binds to the antigen in the sample, making antigen-antibody complexes that migrate on to the nitrocellulose matrix and are collected by the other immobilized antibody on the test strip. The more antigen in the sample, the more antigen-antibody complexes produced, resulting in a brighter fluorescence signal on the detector antibody. The content of glycated hemoglobin in blood is expressed as a percentage of total hemoglobin.

2.7.2. Components

HbA1c kit comprises of 'Cartridges, Hemolysis Buffer Small bottle,' 'Detection Buffer Tubes, and an ID chips.

1- The cartridge contains a test strip, the membrane which has anti human HbA1c at the test line, while rabbit IgG at the control line.
2- The detection buffer contains anti human HbA1c-fluorescence conjugate, anti rabbit IgG-fluorescence conjugate, bovine serum albumin (BSA) as a stabilizer and sodium azide in phosphate buffered saline (PBS) as a preservative.
3- The hemolysis Buffer contains nonionic detergent and sodium azide as preservative in PBS.

HbA1c kit components include:

1) Cartridge Box:
   a) Cartridges 25
   b) ID Chip 1
   c) Instructions for Use
2) Buffer Box for Detection
   a) 25 Detection Buffer Tubes
   b) 1 Hemolysis Buffer Vial (3 ml)

2.7.3. Procedure

1) A total of 100µl of hemolysis buffer has been taken and transferred to a detection buffer tube..

2) 5 µl of whole blood has been drawn from EDTA tube blood and put in to the detection buffer tube.

3) The lid of the detection buffer tube has been Closed and mixed the sample thoroughly by shaking it about 15 times.

4) The cartridge half has been taken out form i-Chamber slot.

5) 75µl of the sample mixture has been pipetted out and loaded it into a sample well in the test cartridge.

6) Then we waited until the sample mixture flowed into the windows (about 10 seconds).

7) The cartridge has been inserted into i-Chamber slot.

8) The cartridge has been leaved Before removing, leave it in the i-Chamber for 12 minutes. When the incubation term is completed, the sample-loaded cartridge is Scanned immediately.

9) The test result has been displayed on the instrument's display panel.

HbA1c concentration inside the test sample either as a percentage (NGSP) or as a mmol/mol (IFCC).) **(Nathan *et al*., 1984)**.

reference range:- NGSP (%): 4.5- 6 %

where National Glycohemoglobin Standardization Program (NGSP)

2.8. Determination of serum LH, FSH and total testosterone concentrations:

2.8.1. Luteinizing Hormone

Human luteinizing hormone (LH, lutropin) is a glycoprotein hormone composed of two different subunits (α and β). The molecular weight of LH is approximately 29 kilo Daltons **(Pierce and Parsons, 1981).** A fluorescence immunoassay (FIA) for quantifying luteinizing hormone (LH) in human serum. It can help with the management and monitoring of fertility abnormalities, the function of reproductive system (ovaries or testicular), or the detecting of ovulation.

LH hormone interpretation the LH / FSH ratio has been used to diagnose the cause of polycystic ovarian disease. Low levels of human LH (LH) and FSH may signal pituitary failure, whereas increased levels of LH and FSH, along with reduced levels of gonadal steroids, may indicate gonadal failure (ovariectomy, menopause, premature ovarian syndrome, Turner Syndrome **(Hall, 1993 ; Rama Raju *et al*., 2013).**

Low gonadotropin concentrations are commonly found in females taking oral steroid-based contraceptives. In males, high LH and FSH with low gonadal steroid concentrations may signal testicular failure or anorchia. Sertoli cell failure may cause LH to be high in Klinefelter's syndrome (**Skakkebaek *et al*., 2017 ; Rama Raju *et al*., 2013).**

2.8.2. Follicle-stimulating hormone

Follicle-stimulating hormone(FSH) is synthesized and secreted by gonadotrophs of the anterior pituitary gland. The alpha subunits of LH, FSH, TSH, and hCG are identical, and contain 92 amino acids. FSH has a beta subunit of 118 amino acids .

The most prevalent cause of elevated serum FSH concentration in a female is menopause, which is currently or has just occurred. High amounts of Follicle-Stimulating Hormone suggest that the gonad's usual limiting feedback is missing, resulting in uncontrolled pituitary FSH secretion. If high FSH levels occur throughout the reproductive age, it is abnormal. Premature menopause, also known as Premature Ovarian Failure, poor ovarian reserve, also known as Premature Ovarian Aging, gonadal digenesis, Turner syndrome, castration, certain forms of Congenital adrenal hyperplasia (CAH), and testicular failure are all conditions associated with high FSH levels (**Randolph *et al*., 2004)**.

2.8.3.Testosterone

Testosterone(17ß-hydroxyandrost-4-en-3-one)is an anabolic steroid synthesized primarily by Leydig cells in the testes of male, the ovary of female, and adrenal glands of both sexes (**Shima *et al*., 2013**).

It is produced from cholesterol, Dehydroepiandrosterone (DHEA), androstened--iol progesterone, and pregnenolone as intermediary substrates. Throughout a man's life, it has a significant and wide-ranging impact on emotional well-being, sexual function, muscular size and strength, activity, cardiovascular health, bone integrity, and cognitive capacity. Only 1 to 15% of testosterone in the blood is in its unbound and physiologically active state. The testosterone that remains is linked to serum proteins **(Carson and Manolagas, 2015).**

2.8.4.Tests Principle for hormones

A sandwich immunological detection approach was studied by multiple researchers, in which dried antibodies in the detector tube connect to antigens in the sample after being diluted with the diluent, producing antigen-antibody complexes. These complexes subsequently migrate through to the nitrocellulose matrix and are collected on the test line by another pair of immobilized antibodies. The greater the number of antigens in the sample, the more antigen-antibody complexes form, resulting in a higher fluorescence signal. The reader then translates this signal to indicate the levels of (LH, FSH, Testosterone) in the sample.

The cartridge contains a test strip, the membrane has (anti human LH ,anti FSH , anti Testosterone)at the test line, with chicken IgY at the control line.

The detection buffer contains anti human(LH,FSH, Testosterone)-fluorescence conjugate, anti chicken IgY--fluorescence conjugate, bovine serum albumin (BSA) as a stabilizer and sodium azide as a preservative in phosphate buffered saline (PBS).

2.8.5. Materials Supplied

Components of(LH,FSH, testosterone) Kit

1Cartridge Package:

a) Cartridges 25.

b) Capillary tube 35 L 25.

c) ID Chip 1.

2) Buffer Box :-

a) Detectors 25.

b) Diluent 1 For AFIAS-50

2.8.6.Tests Procedures

1- A pipette was used to transfer 150µl of diluent to a tube containing detector granular.

2- A total of 35 µl of sample serum were transferred to the detection tube.

3- Close the cover on the detection tube and shake it about 20 times to properly mix the sample.

4- A sample mixed of 75 µl was pipetted out and put in to the cartridge's sample well.

5- The sample-loaded cartridge was kept at room temperature for 12 minutes. The sample-loaded cartridge is scanned immediately after the incubation time has ended.

6- To begin the scanning procedure, push the ‘Select' button on the testing equipment.

7- The test instrument quickly started scanning the sample-loaded cartridge.

8- The test result has been shown on the instrument's display screen..

2.9. Insulin level

2.9.1.Test principle

Sandwich principle. Total duration of assay: 18 minutes.

▪ 1st incubation: A sandwich complex is formed by insulin from a 20µl sample, a biotinylated monoclonal insulin specific antibody, and a monoclonal insulin specific antibody labeled with a ruthenium complex).

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

▪ The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of

a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

▪ Results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve .

2.9.2. Reagents - working solutions

Table 2.8: Reagents solutions of Insulin kit

M	1 container, 6.5ml, streptavidin-coated microparticles (clear cap): streptavidin-coated microparticles 0.72 mg/ml; preservative.
R1	1 bottle, 10 ml Anti-insulin-Abbiotin (gray cap: MESb) buffer 50 mmol/l, pH 6.0; biotinylated monoclonal antiinsulin antibody (mouse) 1 mg/l; preservative.
R2	1 bottle, 10 ml Anti-insulin-AbRu(bpy) (black cap) 1.75 mg/l ruthenium complex labeled monoclonal antiinsulin antibody (mouse); MES buffer 50 mmol/l, pH 6.0; preservative.

2.9.3. Procedure

The procedure was full automated in Cobas e 411.

Reference range

Fasting < 25 mIU/l < 174 pmol/l

2.10. Fasting blood glucose FBG concentration

2.10.1. Principle

The glucose oxidase (GOD) converts glucose to D-gluconate, releasing hydrogen peroxide in the process. A combination of phenol and 4-aminoantipyrine (4-AA) is oxidized by hydrogen peroxide in the presence of peroxidase (POD) to create a red quinoneimine dye proportionate to the quantity of glucose in the sample.

$$\beta\text{-D-Glucose} + H_2O + O_2 \xrightarrow{GOD} \text{D-Gluconate} + H_2O_2$$

$$\text{4-AA} + \text{Phenol} \xrightarrow{H_2O_2\ /POD} \text{Quinoneimine} + H_2O$$

2.10.2. Reagent composition

Table 2.9: Reagent Composition of Blood Glucose kit

1-R1	Monoreagent. Phosphate buffer 100 mmol/l pH 7.5, glucose oxidase > 10 KU/l, peroxidase > 2 KU/l, 4- aminoantipyrine 0.5 mmol/l, phenol 5 mmol/l
2-CAL	Glucose standard. Glucose 100 mg/dl (5.55 mmol/l).

2.10.3. Materials required

− Photometer or colorimeter capable of measuring absorbance at 500 ± 20 nm

− Constant temperature incubator set at 37ºC

− Pipettes to measure reagent and samples

2.10.4. Procedure

1. Reagents were brought and samples to room temperature.

2. The following has been drawn in to labelled tubes:

Tubes	Blank	Sample	Cal.
R1.Monoreagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	10 µl	-
CAL. Standard	-	-	10 µl

3. The tubes has been mixed and let stand 10 minutes at room temperature or 5 minutes at 37ºC.

4. The absorbance (A)has been read of the samples and the standard at 500 nm against the reagent blank.

The color is pink stable for about 2 hours protected from light.

Calculations:-

$$\frac{\text{Ab Sample}}{\text{Ab Standard}} \text{ x Con Standard = mg/dl glucose in sample}$$

Reference value in adults 70 - 105 mg/dl (3.89 - 5.83 mmol/l)

2.11. Homeostatic model assessment for insulin resistance (HOMA-IR)

HOMA-IR is mean homeostatic model assessment and express on Insulin resistance can be calculate by using the equation that uses fasting glucose and insulin concentration as the following equation **(Matthews *et al.*, 1985 ; Salgado *et al.*, 2010)**

HOMA-IR = glucose (mmol/l) × insulin (mU/ml)/ 22.5

Or HOMA-IR = Glucose (mg / dl) × Insulin (μU / ml) / 405

Using fasting values. The normal HOMA IR value of healthy human ranges from the reference values for HOMA-IR as:-

" HOMA-IR less than 2.60 as the normal range

HOMA-IR from 2.60–3.80 as ‘‘borderline high’’ without labeling these individuals as having insulin resistance.

HOMA-IR more than 3.80 as ‘‘high’’ having clear correlates of insulin resistance **(Qu *et al*., 2011).**

Fasting blood sugar was changed from mg/dl to mmol/L by dividing it by 18 **(Salgado *et al*., 2010).**

2.12. Biostatistics analysis

In this study the data were calculated by Microsoft Excel 10 with the Statistical Package for the Social Sciences (SPSS) version 23. Descriptive statistic were expressed as mean $\pm$ standard deviation (SD) and used T-test for different between two group patient and control while, used one- way ANOVA test for three groups. Pearson's correlation coefficient was used to evaluate the correlations between all variables **(Pearson's Correlation Coefficient, 2019).** Least significant differences (LSD) were used to make comparisons within groups. Significant differences were considered as $p< 0.05$ and more was non significant . ROC curve was also analyzed to find sensitivity and specificity for markers within groups of infertility.

Chapter three

Results and Discussion

Chapter three

3-Results and Discussion

The study included 81 women in reproductive age from (18-45) years old, They were classified in to two groups 50 female with PCOS as a patient group and 31 female apparently healthy as a control group.

The patient group can be investigaed by some criterias including irregular menstrual cycle , acne, hersutism, abnormal hormonal value (ratio LH/FSH) and ultrasound imaging test . In order to prevent PCOS consequences, protein and enzyme markers are frequently used for prognosis and patient monitoring.

3.1. Comparison in biochemical parameters between groups

Independent T-test statistics are used for parameters to compare between studies groups, and the results were expressed by mean± standard deviation (SD) and extraction P-value to show the difference variation ,considered significant if P-value are $P \leq 0.05$, whereas $p > 0.05$ is non-significant while $P < 0.001$ is high significant

Table 3.1 is show all studied parameters studied included LH, FSH, Testo , HbA1c, FBG, fasting Insulin , BMI, HOMA-IR, ZAG level, HSP70 level.

.

Table 3.1: Clinical characteristics of PCOS with control subject.

Parameters	Patient group N=50 Mean± SD	Control group N=31 Mean± SD	P value
Age (years)	29 ± 6	30 ± 7	P> 0.05
BMI (kg/m^2)	30.8 ± 5.0	26. 7 ± 3.6	P<0.05
WC (cm^2)	43 ± 4.5	40.8 ± 1.6	P≤ 0.05
LH (mIU/ml)	10.1 ± 3.7	4.3 ± 1.6	P<0.001
FSH (m.IU/ml)	5.47 ± 1.3	7.8 ± 1.85	P<0.001
Testosterone	0.44 ± 0.25	0.21 ± 0.05	P≤ 0.05
HbA1c %	5.5 ± 0.45	5.2 ± 0.3	P≤0.05
FBG (mg/dl)	95.4 ± 9.2	87 ± 6	P< 0.001
F Ins (mIU/ml)	18.5 ± 2.1	16.3 ± 1.0	P ≤ 0.05
HOMA-IR	4.3 ± 0.6	3.5 ± 0.3	P ≤ 0.05
Ratio LH/FSH	1.8 ± 0.46	0.55 ± 0.1	P<0.001
ZAG (μg/ml)	99.09 ± 27.5	143.3 ± 14.7	P<0.001
HSP70 (ng/ml)	15.8 ± 4.7	9.7 ± 3.7	P<0.001

BMI: Body Mass Index ; FBS: Fasting Blood Sugar ; FSH: Follicle-Stimulating Hormone HbA1c:Hemoglobin A1c ; HOMA-IR : Homeostatic Model Assessment of Insulin Resistan HSP70 : Heat Shock Protein 70 ; LH : Luteinizing Hormone ; SD: Standard Deviation ; WC : Waist Circumference ; ZAG: Zinc Alpha 2 Glycoprotein, NS: t-test p- value ≥ 0.05 ; S: p-value ≤0.05 ; HS: p- value ≤0.01

According to the presented data show the mean of HSP70 levels (15.8± 4.77ng/ml) (9.7 ±3.7ng/ml) for patients and controls group respectively which is significantly increased in patients group of PCOS in ($P<0.001$) and this result agreed with study that Increased blood Hsp70 levels in PCOS patients and linked to a combination of IR, oxidative stress, and limited chronic inflammation in PCOS patients, indicating that Hsp70 may play a role in the pathophysiology of PCOS **(Gao *et al.*, 2013)** while, another study disagreed with our results and it showed that HSP70 was significantly higher in ovarian tissues in PCOS in rats than the controls, but significantly lower in serum **(Wu *et al.*, 2019).** Results of current study was found ZAG levels (99.09±27.5);(143.3±14.7µg/ml) for patients and control groups respectively were significantly decreased in PCOS women than in control group($P<0.001$). this result agrees with study of Zheng et al study **(Zheng *et al.*, 2019)** that found ZAG levels lower in women with PCOS, although they can be raised with the use of metformin.

According to results in table 3.1 There was statistically non-significant changes in age mean values between the normal control group and the patients groups of PCOS.

In the presented data explained by figure 3.1 showed the mean levels of ZAG (µg/ml) while figure 3.2 showed the mean levels of HSP70 (ng /ml) increased in PCOS patient than controls group .

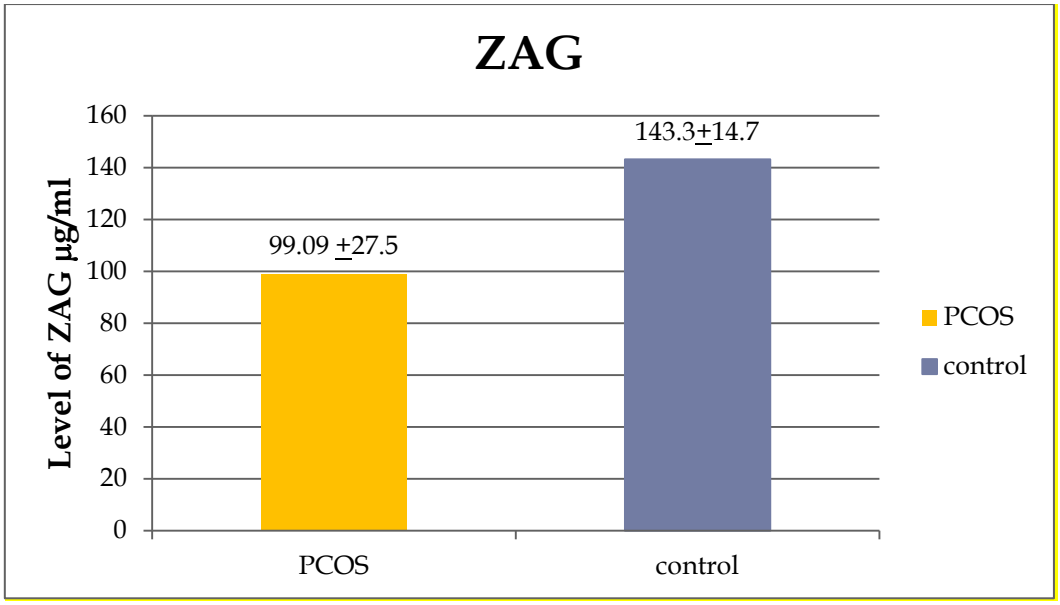

Fig 3.1 : The levels of ZAG in patients group of PCOS versus controls subject

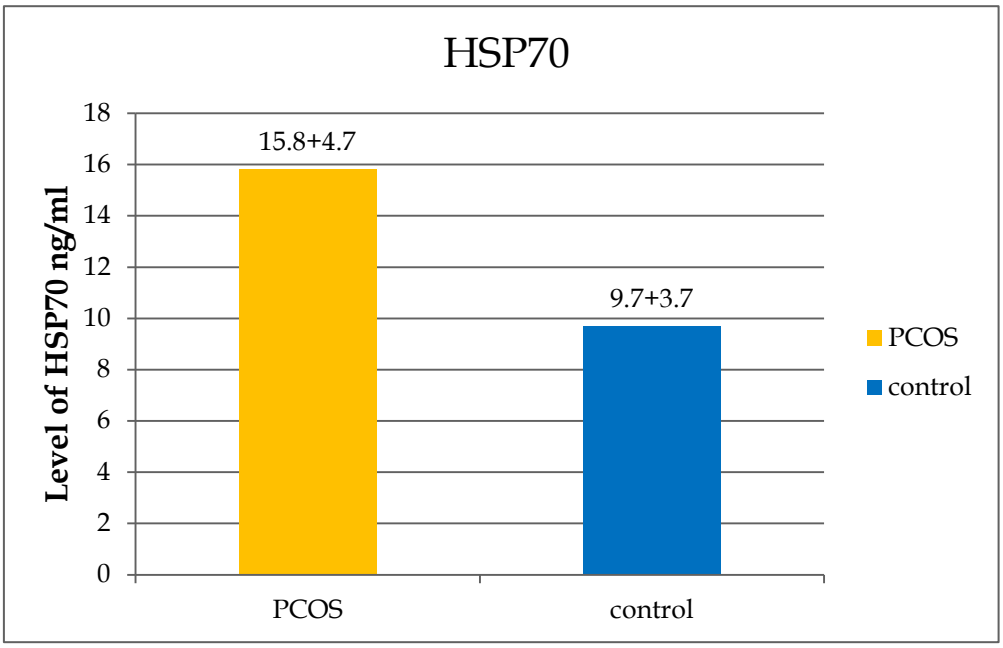

Fig 3.2 :The levels of Heat shock protein70 in patients group compare to control group.

It show in table 3.1 our finding results that elevated high significant in biomarkers measured in hormonal LH, Ratio LH/FSH and testosterone level this agrees with study (**Malini and Roy George, 2018)** that's PCOS and infertility are caused by an unusual increase in LH levels, as well as increased levels of ovarian testosterone and the LH/FSH ratio and highly significant increase of metabolic biomarkers FBG, FIns , BMI, HOMA-IR in patients group compare to healthy group.

These results agreed with study **(DeUgarte, Bartolucci and Azziz, 2005)** that PCOS was defined as the presence of two or more of the following symptoms: oligo- or anovulation, and hyperandrogenemia, and polycystic ovarian cysts on ultrasound, according to the Rotterdam consensus workshop also obesity, insulin resistance, and hyperinsulinemia are all common symptoms of PCOS.

Additionally some causes of the increase of LH level that, insulin was shown to clearly increase androgen production from theca cells resulting in an increase of testosterone levels, an androgenic ovarian environment, and anovulation (resulting in low progesterone levels), Increased leptin levels due to an excess of adipose cells affect Gonadotropin-releasing hormone (GnRH) secretion and these correlated with fertilization rate **(The ObG Project, 2019).**

In the results finding high testosterone levels in patient of PCOS compare the control group in p-value $P \leq 0.05$ can exacerbate abdominal obesity and inflammation **.** Our findings are in agreement research conducted by the study which the Patients with PCOS that have a high BMI are more probable to have androgen overload functional disorders **(Yuan *et al*., 2016).**

Also finding highly significant decrease in FSH levels in patients is a result that agreed with study by **Malini and Roy, 2018** which found that deficient FSH levels participate to impaired follicular development in PCOS. figure 3.3 showed FSH, LH hormonal levels in patients and controls.

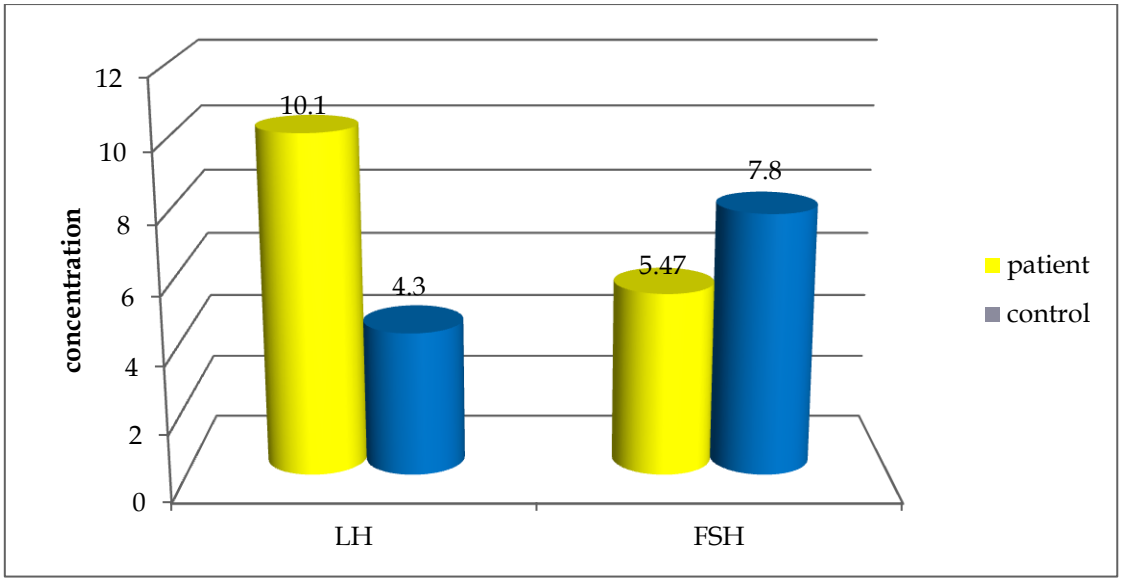

Fig 3.3: Concentration of LH and FSH hormones in patients and controls

3.1.1. Hirsutism

In this study we found the percentage of hirsutism in the group of patients women with PCOS was high, reaching 68%, while the percentage of patients without hirsutism was 32%. From patients in the study. Many studies agreed with research by **(Panse *et al.*, 2019)** that it has been PCOS is the most common reason of hirsutism, which is characterized by excessive hair growth in androgen-dependent areas PCOS is the most prevalent cause of androgen excess. .

Another study agreed with our results that PCOS is the leading cause of hirsutism, with 72 to 82 % of cases **(Bode, Seehusen and Baird 2012)**.

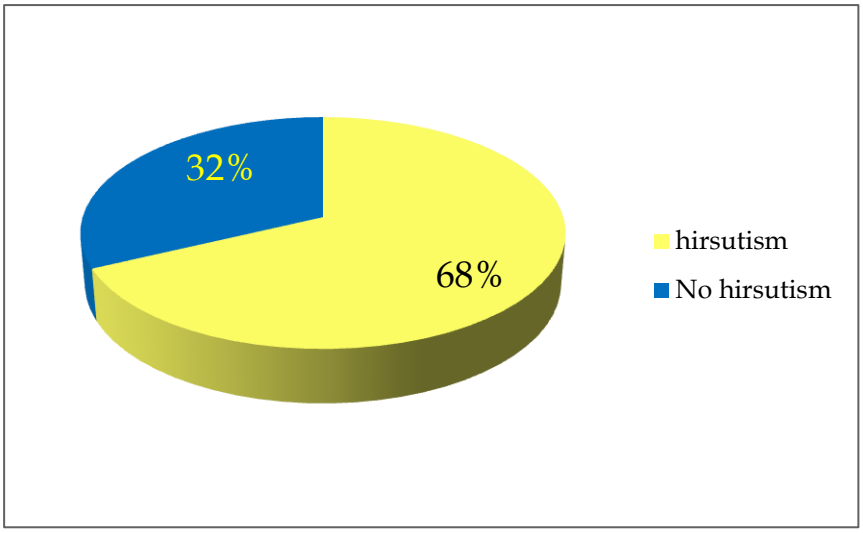

Fig 3.4:The percent of hirsutism in patients group.

3.1.2. Body mass index

We used Pie charts to describe the proportion of patients for each body mass index groups as normal weight 18-24.9 kg/m^2 has lowest percentage obtain 14% of patient groups , over weight (pre obesity) 25-29.9 kg/m^2 was 36% of patients and obesity over 30 kg/m^2 obtain higher percentage 50% of patient groups . numerous studies researches agreed with findings research such as (**Diamanti- Kandarakis, 2007 ; Al-Tu'ma *et al*.,2015)** That a common problem feature on PCOS women, which a high proportion of female with PCOS are obese and obesity and abdominal fat accumulation worsen the physiological, hormonal, and metabolic symptoms of polycystic ovary syndrome PCOS .

Another study that PCOS women with high levels of total testosterone and LH had a metabolic defect, as obese and overweight PCOS women. PCOS women had a higher prevalence of overweight/obesity **(Liu *et al*., 2019).**

One of the reasons why women with PCOS gain weight or have a hard time reducing weight is the inability of insulin to work correctly.

Obesity has the potential to cause serious health problems dyslipidemia, irregular ovarian cycles, and anovulation are all symptoms of Increased PCOS. BMI may be a significant factor in endocrine and metabolic disorders. Obesity can have a significant effect on the syndrome's development and progression, often linked to an increase in metabolic abnormalities and cardiovascular risk factors **(Diamanti-Kandarakis, 2007)**.

we found most of patient PCOS have high BMI as show in figure 3.5

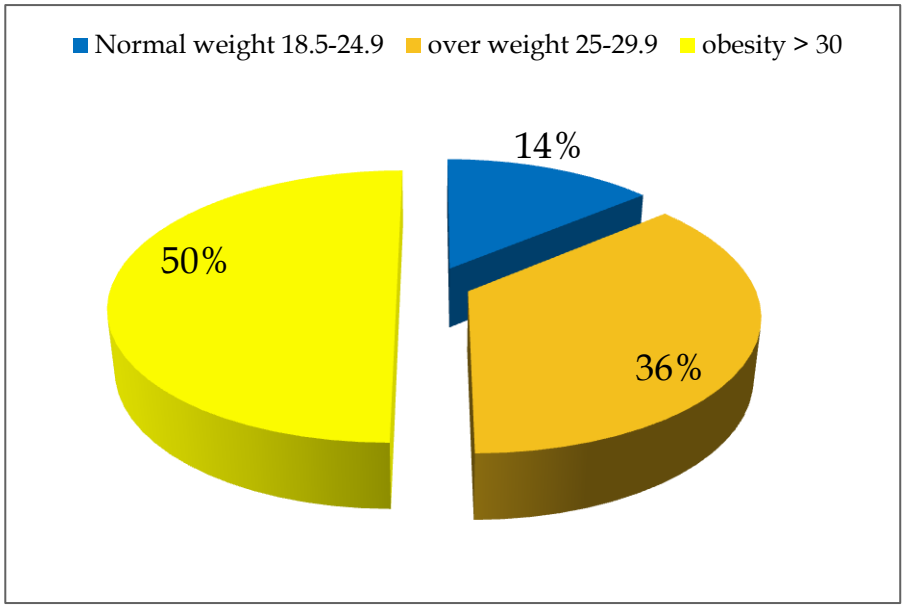

Fig 3.5 : BMI proportion in patients groups.

3.1.3. Metabolic parameters

In this study fasting blood glucose ,HbA1c , fasting insulin level and HOMA-IR were measured to know different levels of metabolic rate between groups patient and control, FBG results (95.4 ± 9.2)(87 ± 6) (mg/dl), HbA1c (5.5 ± 0.45)(5.2 ± 0.3)%, Fasting insulin (18.5 ± 2.1)(16.3 ± 1.0) (m.IU/ml) and HOMA-IR (4.3 ±0.6) (3.5±0.3) for patient and control respectively as shown in table (3.1) were appeared increase in patient group with (primary infertility and secondary infertility) than control group or fertility group.

The result of FBG level indicated that women in POCS groups had significantly higher levels than women in the control group. Women with PCOS have significantly decreased insulin sensitivity lead to Insulin resistance causes elevated blood glucose levels, which are then transported to the liver, where the glucose is converted to fat and stored in the body, resulting in weight gain and, eventually, obesity resulting agreed with study of **(Dahan and Reaven, 2019).**

When noticing the result high fasting insulin level, IR and Increased insulin levels induce the ovaries to release testosterone, causing testosterone levels to increase. Insulin has an inhibition effect on hepatic sex hormone binding globulin SHBG synthesis, resulting in an increase in free testosterone, as well as a stimulatory effect on ovarian androgen production, promoting hyperandrogen--ism **(Suresh and Vijayakumar, 2014).**

3.2. Comparison of groups by fertility

The comparing is performed between three groups were divided according to the degree of fertility by using one-way ANOVA test analysis namely primary infertility, secondary infertility for patients groups while fertility for controls groups. These groups include a group of primary infertility PCOS (n=32), secondary infertility PCOS (n=18) for patients groups and fertility (n=31) for

contols groups. Table 3.2 shown the testing parameters according to the fertility groups

Table 3.2 :- Comparison of groups by fertility types for parameter and markers

parameters	PCOS		control	Sig
	Primary infertility N=32 G1	**secondary infertility N=18 G2**	**fertility N=31 G3**	
Age (years)	29.3 ± 5	32.0 ± 6.4	30.9 ± 7.1	NS
BMI (k/m^2)	31.8 ± 4.3 ^{b*}	30.1 ± 5.4 ^{b*}	26.7 ± 3.6	S
LH (mIU/ml)	11.2 ± 3.3 ^{b**}	10.0 ± 4.3 ^{b**}	4.3 ± 1.6	HS
FSH (mIU/ml)	5.4 ± 1.4 ^{b**}	5.5 ± 1.1 ^{b**}	7.8 ± 1.8	HS
Testosterone	0.47 ± 0.27 ^{a*,b**}	0.39 ± 0.20 ^{b*}	0.21 ± 0.05	S HS
FBG	95.7 ± 10.1 ^{b**}	94.8 ± 7.4 ^{b**}	87.7 ± 6.0	HS
HbA1c %	5.47 ± 0.45 ^{b*}	5.40 ± 0.41	5.2 ± 0.37	S NS
Fins (mIU/ml)	18.6 ± 2.3 ^{b*}	18.4 ± 1.9 ^{b*}	16.3 ± 1.6	S
HOMA-IR	4.25 ± 0.6 ^{b*}	4.0 ± 0.5 ^{b*}	3.5 ± 0.3	S
LH/FSH ratio	1.88 ± 0.44 ^{b**}	1.80 ± 0.5 ^{b**}	0.55 ± 0.12	HS
ZAG µg/ml	94.0 ± 27.0 ^{a*,b**}	108.0 ± 26.7 ^{b*}	143.3 ± 14.7	S HS
HSP70 (ng/ml)	16.3 + 4.9 ^{b**}	15.1 + 4.45 ^{b**}	9.7 + 3.75	HS

NS: t-test p- value ≥ 0.05 ; S: p-value ≤0.05 ; HS: p- value ≤0.01

a; Comparison between G1&G2 ; b: Comparison between G1&G3 or G2&G3

****--significant correlation p≤0.05 ; **-- highly significant p<0.001***

PCOS is the main cause of infertility due to anovulation. Therefore, the study groups were divided according to fertility, to observe the effect of the severity of the syndrome on the degree of fertility **(Bellver *et al*., 2017)**.

Another research found PCOS accounts for ~80% of cases of anovulatory infertility in women **(Balen *et al.*, 2016)**. The results we reached in this study after dividing the groups according fertility degree (primary infertility, secondary infertility , fertility) that the hormonal disorder in primary infertility was higher than secondary infertility in patient groups and this agreed with study done by Al- Balen et al that increased luteinizing hormone levels (LH) in the blood have a negative effect on fertility **(Balen *et al.*, 2016)**. Also showed that however women with PCOS was take longer time to become pregnant their long-term fertility is not impaired **(Balen *et al.*, 2016)**.

As show in figure 3.8 that explains mean of hormones (LH, FSH, Testosterone within the fertility groups.

The study found that classifying the groups depended on fertility the highest BMI was in primary infertility group (31.8 ± 4.3) secondary infertility (30.1± 5.4) compare to fertility group(26.7 ± 3.6) k/m^2 and that agreed with study that a higher body mass index (BMI) is associated to A more complex treatment plan for fertility compared to normal-weight women with the syndrome, is one of the leading causes of infertility **(Cena, Chiovato and Nappi, 2020)** this result due to high level of fasting insulin , IR, FBG and androgen hormones levels.

For express on mean of ZAG levels within groups in figure 3.6 as shown

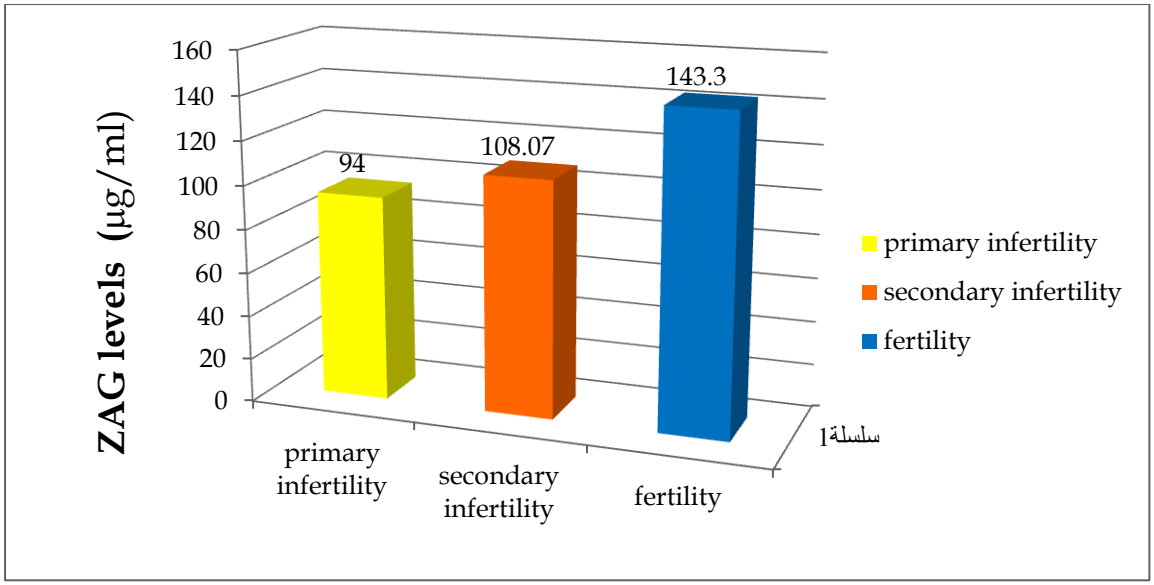

Fig 3.6 : The levels of ZAG within groups of fertile

The mean of ZAG level in groups were in primary infertility G1(94±27µg/ml) and secondary infertility group G2 (108.07 ± 26.7 µg/ml) as patients groups, while fertility group G3 (143.3 ± 14.7 µg/ml) which were diagnosed decreased levels significantly ($p< 0.001$) when compared infertilities groups with the fertility and showed decrease significant between groups G1,G2 in $P\leq0.05$ that's may be mean in lower ZAG level lead to increase infertility degree showed in study of **(Lai *et al.*, 2016)** that indicate circulating ZAG levels were significantly decreased in subjects of PCOS in general ,while notably, no study has quantified circulating ZAG levels in infertility women within PCOS groups to date, Except study in fertilization ZAG is presence in human seminal fluid is 6 times more than it's amount in the human serum, which indicating it's role in fertilization **(Hassan et al., 2008 ; Ding et al., 2007)**.

The mean HSP70 levels were increased significantly in PCOS women groups of primary infertility and secondary infertility groups than control group(fertility group) in $p< 0.001$ value these results agreed with study performed by **(Sisti *et al.*,2015)**Induction of heat shock protein production and activation when cells

under a variety of stresses-elevated temperature, hypoxia, inflammation, infection, oxidative stress, and nutrient deprivation.

Also Induction of Hsp70 in the ovary might explain why women with polycystic ovarian syndrome have lower fertility **(Sisti *et al*., 2015)**.

Whereas, the mean of HSP70 level in the primary infertility group (16.3 + 4.9 ng/ml)was higher than the secondary infertility (15.1 + 4.45 ng/ml) and it was farther than secondary group from the control group (9.7 + 3.75 ng/ml) as shown in the figure 3.7.

In other words the secondary infertility group was closer to the control groups than in primary infertility but did not reach significance in p-value.

Figure 3.7 was explain HSP70 level in fertility groups

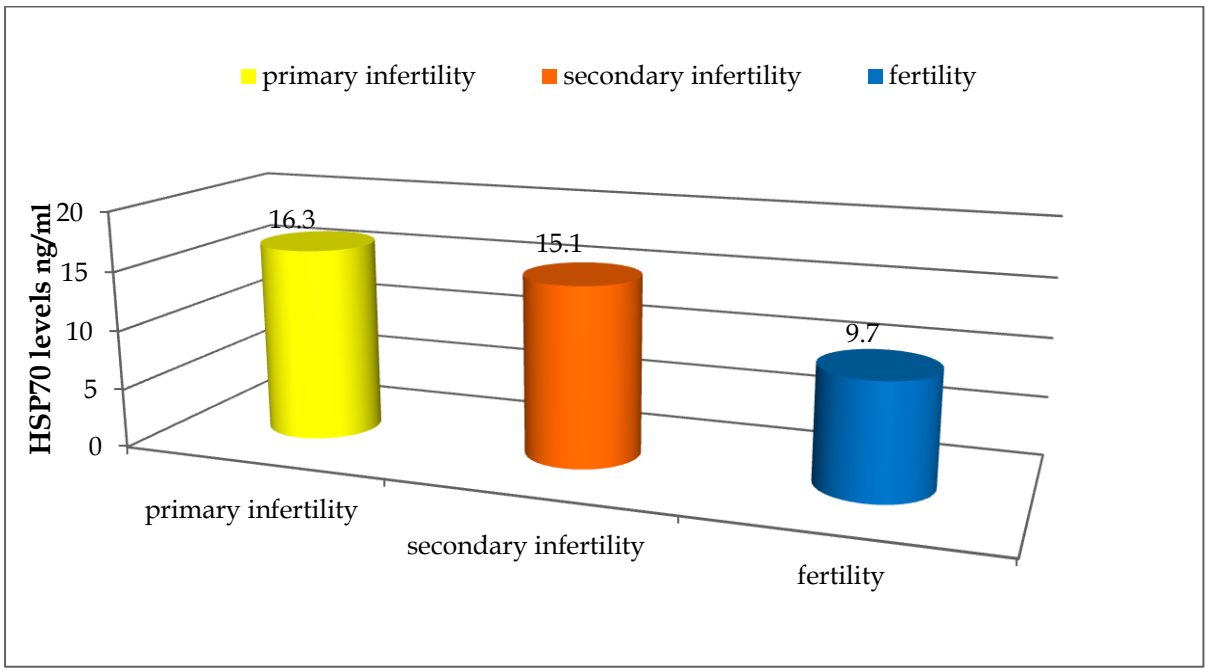

Fig 3.7 : The levels of HSP70 within groups of fertile.

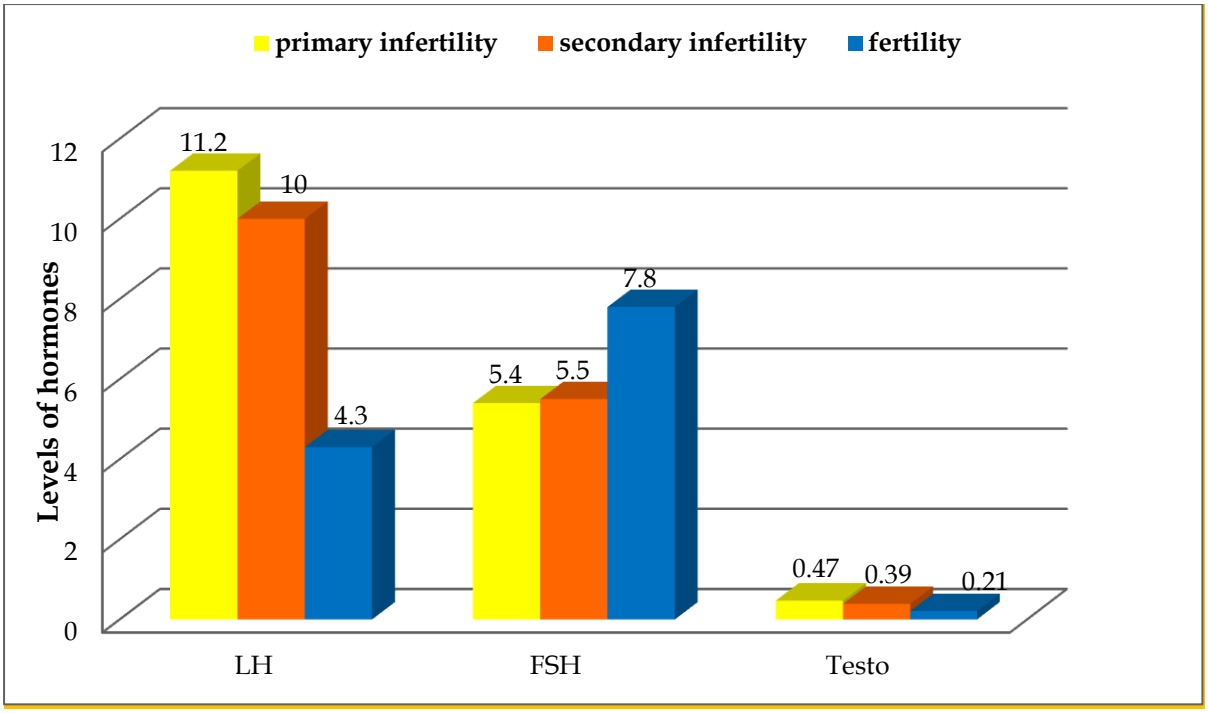

Fig 3.8 :The levels of LH,FSH and Testosterone hormones within groups of fertile.

As for the results of the groups, we found that the secondary infertility group had lower values showed statistically significant decreases in PCOS Signs , but did not normalize value.

3.3. Correlation Study

Correlations study between markers ZAG & HSP70 with all of the studied variables were estimated, by using Pearson's correlation coefficient (r) for the evaluation of data, the strength of Pearson correlation describe as

0.2-0.39 ------ low
0.4-0.69 ------- moderate
0.7-0.89 ---------high

Shown in (table 3.3) If the value of the correlation coefficient is negative sign, then this indicates the existence of an inversed relationship between the variables (negative correlation) while, the positive sign indicates direct proportion (positive correlation) **(Pearson's Correlation Coefficient, 2019).**

We found during this study that the correlation between ZAG and body mass index, insulin resistance and LH was highly significant negative correlation an inverse relationship in $p<0.001$ and significant negative correlation with insulin level in $p\leq0.05$. while HSP70 levels significant positive correlation with IR, insulin levels as shown below in table 3.3.

Table 3.3: The correlation between HSP70, ZAG and Parameters of groups.

Parameters	HSP 70		ZAG	
	P-Value	r	P-Value	r
BMI (K/m^2)	0.881	0.022	0.001	-0.439**
LH (mIU/ml)	0.771	0.042	0.001	-0.447**
FSH (mIU/ml)	0.66	-0.062	0.36	0.132
Testosterone	0.37	-0.128	0.14	-0.21
HbA1c %	0.85	0.03	0.30	-0.148
FBG (mg/dl)	0.354	0.134	0.2	-0.154
Fasting Ins (mIU/ml)	0.003	0.414**	0.014	-0.345*
IR	0.021	0.326*	0.005	-0.388**

NS: t-test p- value ≥ 0.05 ; S: p-value ≤0.05 ; HS: p- value ≤0.01
****--significant correlation p≤0.05 ; **-- highly significant p<0.01***
+r: Pearson's correlation coefficient

3.3.1.Correlation of ZAG level and parameters

The results concerning ZAG showed a negative correlation with BMI this result agreed with study of **(Lei *et al.*, 2017)** which appear ZAG had positive correlation with age but a negative correlation with Fat %, BMI, blood pressure, triglycerides, FBG, fasting insulin, HbA1c and HOMA-IR **(Lei *et al.*, 2017)**.

This result related to three mechanism for ZAG act as a lipid-mobilizing factor and increases lipolysis by activating hormone-sensitive lipase through increased stimulation intracellular adenylyl cyclase cAMP levels and in adipose tissue, it lowers the levels of lipogenic enzymes including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and diglycerid-glycerol acyltransferase (DGAT)., and act as increased insulin sensitivity also increase the expression of adiponectin therefor ZAG plays an essential role in regulating whole-body **(Severo *et al.*, 2019) .**

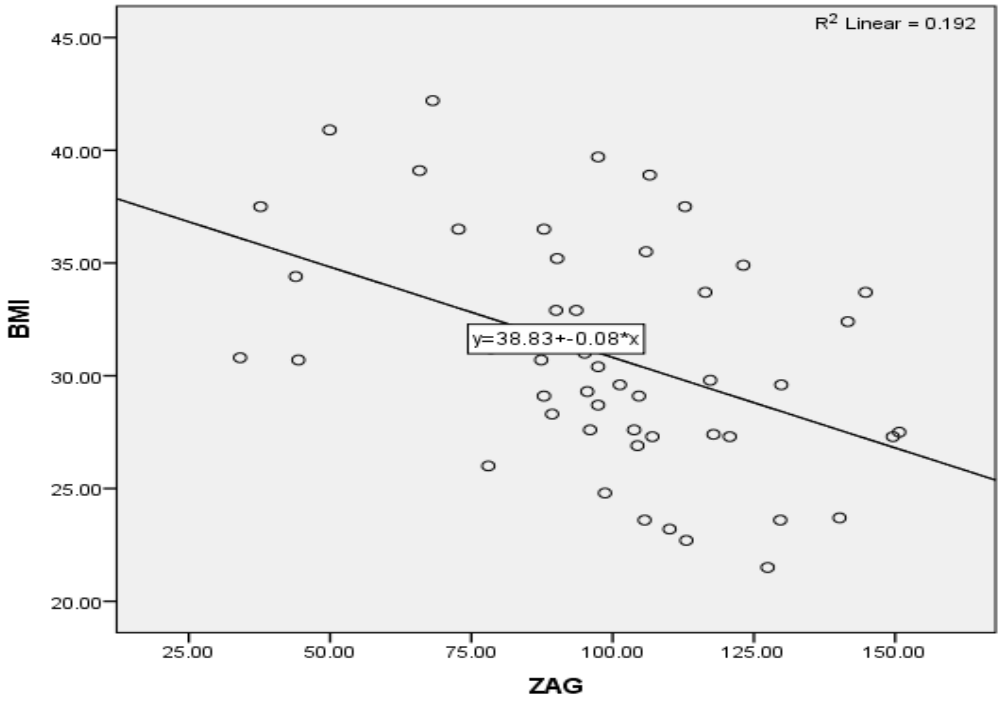

Fig 3.9: The correlation of serum levels of ZAG and body mass index in patients group .

Another study obtained that Adiponectin levels decrease by obesity and insulin resistance develop, has been shown to protect against development of insulin resistance and inflammation **(Khan and Joseph, 2014).** These mechanisms agrees with the results of the research, where the results appeared negative correlation between the ZAG and BMI and IR that shown in figure 3.9 and figure 3.11.

When, we divided the study groups according to weight (normal weight, over weight and obesity) the ZAG level in women with PCOS of normal weight, overweight and obesity groups were (117.8+14.8 μg/ml) (108.8+20 μg/ml) (87.6+29.5 μg/ml) respectively, results were decreased significantly ($p<0.05$) when compared with control groups normal weight (148.9 +9.9 μg/ml), over weight (147.9+11.0 μg/ml) and obesity (128.4+16.0 μg/ml). These findings were agreed with research Lai which circulating ZAG levels had significantly lower in PCOS women, overweight/obese subjects ($BMI \geq 25\ kg/m^2$) than individuals with a BMI of less than 25 kg/m^2 **(Lai *et al*., 2016).**

Table 3. 4: The level of ZAG in controls and patients with POCS (dividing according to weight)

		mean	SD deviation	number	p-value
patient	**normal weight**	117.8	14.8	7	0.001
	over weight	108.8	20.1	18	0.001
	obesity	87.6	29.5	25	0.001
Control	**normal weight**	148.9	9.9	10	--
	over weight	147.9	11.0	13	--
	obesity	128.4	16.0	8	--

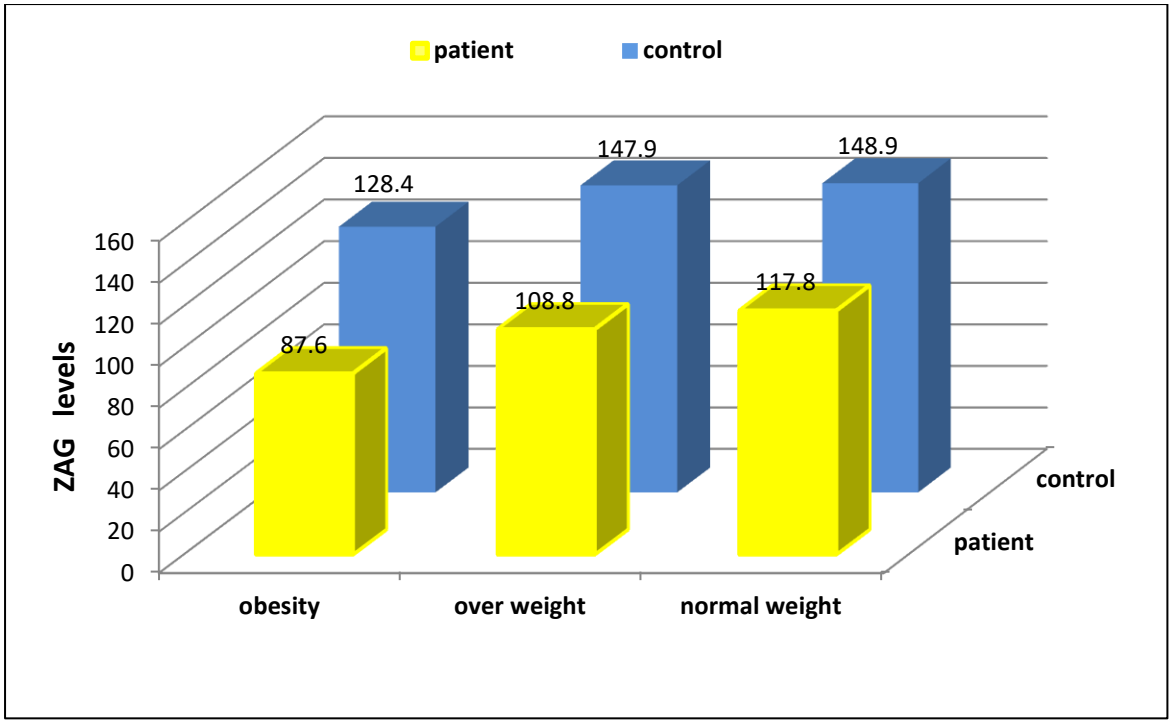

Fig 3.10: The concentration of ZAG in controls and PCOS patients (dividing according to weight).

According to another identified study that showed the obese people have low zinc and ZAG levels in their blood, while also a decrease of expression of genes encoding this protein **(Severo *et al.*, 2019).**

Another researches proved the importance of zinc when using zinc supplement in women with PCOS reduced hirsutism **(Jamilian *et al.*, 2016).**

In Cervantes study which agreed with decrease zinc level on acne and zinc was an effective treatment for acne vulgaris, proliferation of acnes is directly inhibited by regulation of protein, lipid and suppressed sebaceous gland activity **(Cervantes *et al.*, 2017).**

On the other hand obesity activates theca cells to LH stimulation and enlarge functional ovarian hyperandrogenis by up regulating ovarian androgen production **(Glueck and Goldenberg, 2019).**

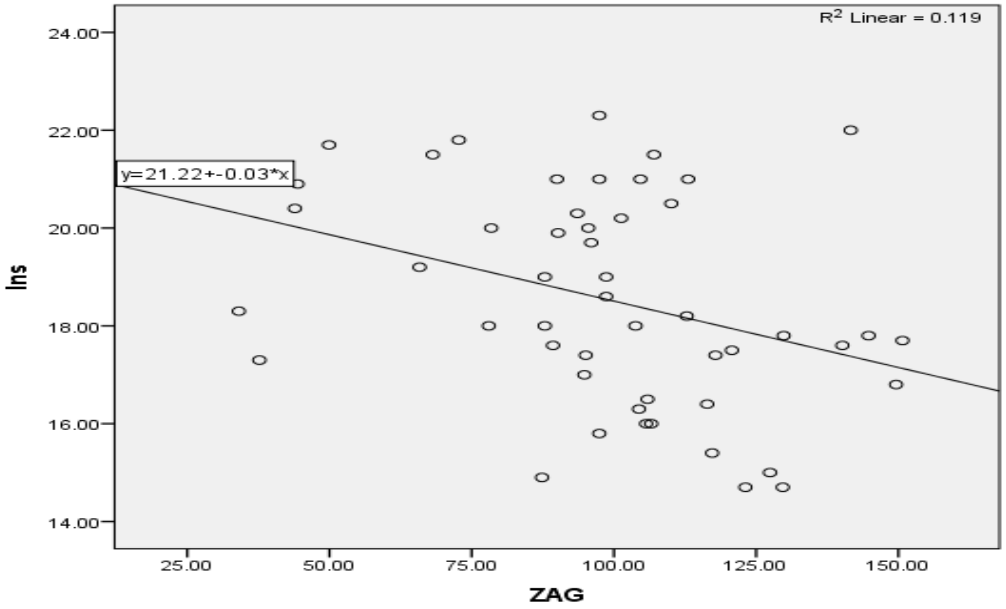

Fig 3.11 : The correlation of serum levels of ZAG and insulin levels in patients group .

In primary human adipocytes, suppressing ZAG decreased the expression of adiponectin (ADI),insulin receptor substrate-1(IRS-1),and glucose transporters4 (GLUT4) genes, showing that ZAG plays an essential role in adipose tissue insulin sensitivity and BMI **(Balaz *et al*., 2014 ; Wang *et al*., 2020)**

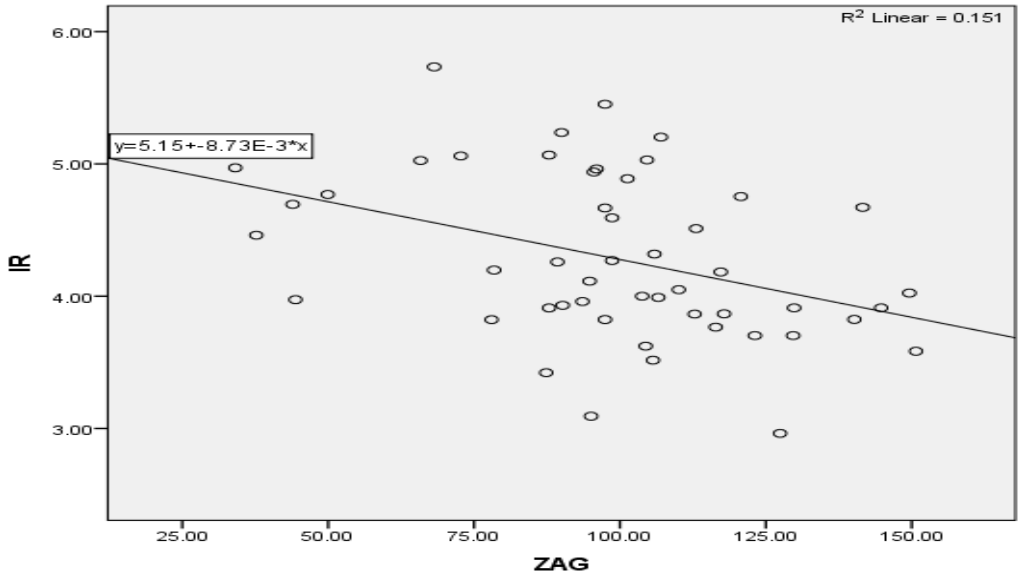

Fig 3.12: The correlation of serum levels of ZAG and IR in patients group

Circulating ZAG levels were significantly lower in topics with metabolic syndrome (MetS) than in those without MetS or in subjects with a greater number of MetS components such as IR and central adiposity **(Lei *et al*., 2017)**

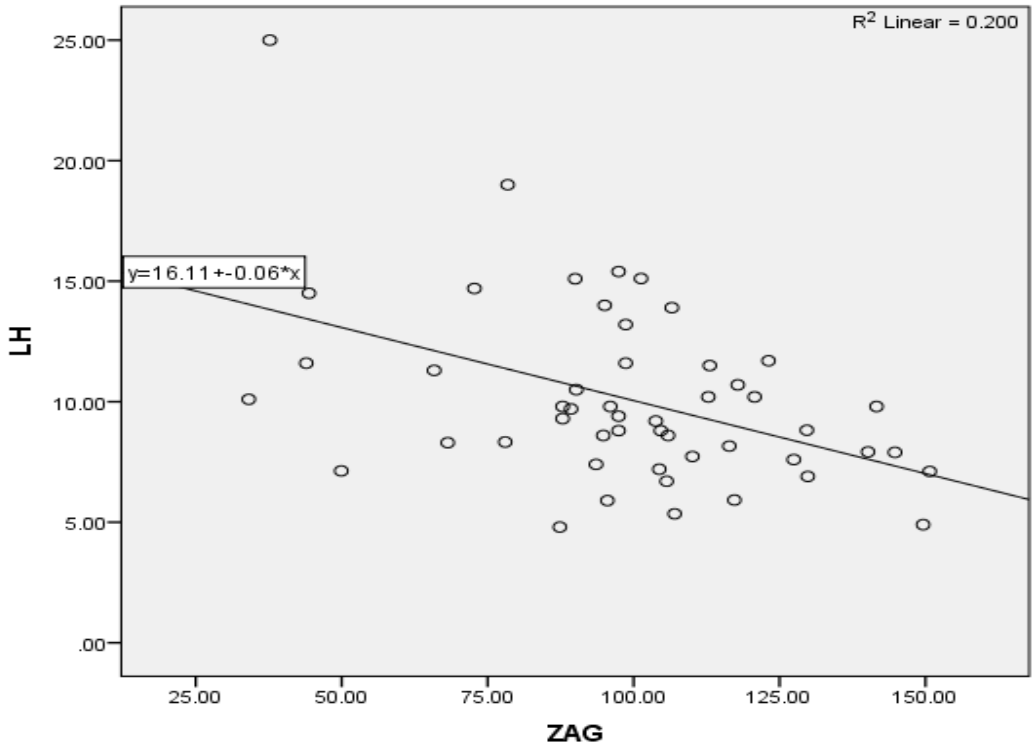

Fig 3.13 : The correlation of serum levels of ZAG and LH levels in patients group .

We show in result of marker ZAG levels found negative correlation with other parameters but not in significant p-value .

3.3.2. Correlation of HSP70 with parameters

The results of correlation in table (3.3) found that the concentration of HSP70 and FSH hormone was negatively in Pearson's correlation coefficient (r) was (-0.062) but not in significant p-value that agrees with study of that FSH prevented the stimulatory effect of serum deprivation on the transcription of HSP70.2 , HSP72 . FSH can prevent stress-related changes in HSPs. The use of HSPs as stress and hormone indicators and mediators on ovarian function (**Sirotkin and Bauer, 2010).**

Long-term stress has a role in the development of insulin resistance and might be linked to a number of other PCOS pathogenic variables than insulin resistance **(Ersin , 2021**). also in other study that was increased serum HSP70 levels with increase the duration of diabetes **(Nakhjavani *et al*., 2010)**.

The result in the study was positive correlation with IR and fasting insulin that's agreed with study which increased blood Hsp70 levels are linked to a combination of IR, oxidative stress, and limited chronic inflammation in PCOS patients **(Gao *et al.*, 2013).**

There is a positive correlation between leptin and HSP70 in chronic inflammation, such as type 2 diabetes. It's possible that the link between serum HSP70 and leptin indicates a greater level of chronic inflammation **(Nakhjavani *et al.*, 2013).** There was a positive correlation between serum leptin, BMI, and insulin in PCOS. The mean BMI, LH, and LH: FSH ratios were all higher in PCOS women. PCOS women had significantly higher levels of androgen as well as fasting insulin. LH hormone synthesis is stimulated by leptin **(Chakrabarti,2013).**

Another study showed that Infection-induced ovarian inflammation could also cause HSP70 induction, resulting in decreased oocyte viability, Induction of Hsp70 in the ovary may explain why women with polycystic ovarian have reduced fertility (**Sisti *et al.*, 2015)**.

Positive correlation between HSP70 levels with increased levels of other parameters but of no significant value. Excepting with Insulin and IR as shown in figure 3.14

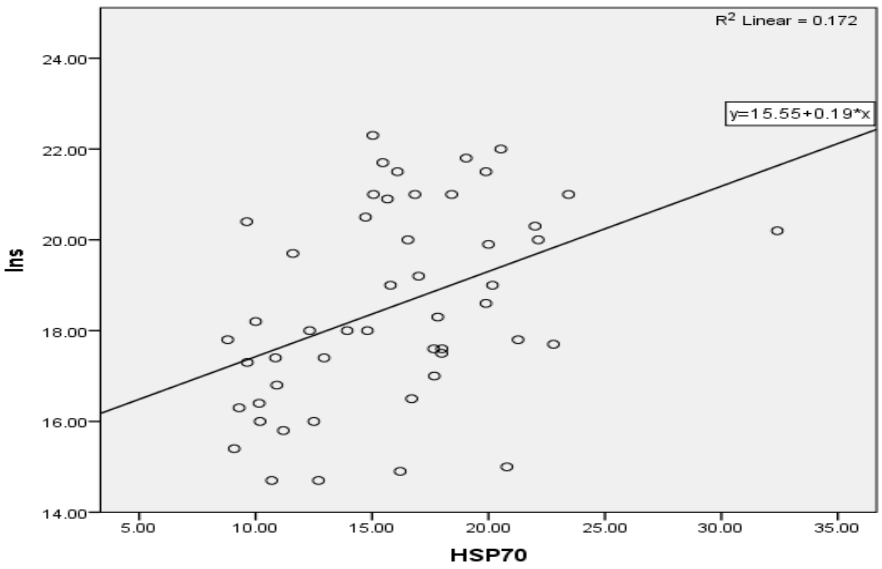

Fig 3.14: The correlation between serum HSP70 & Insulin level in patients

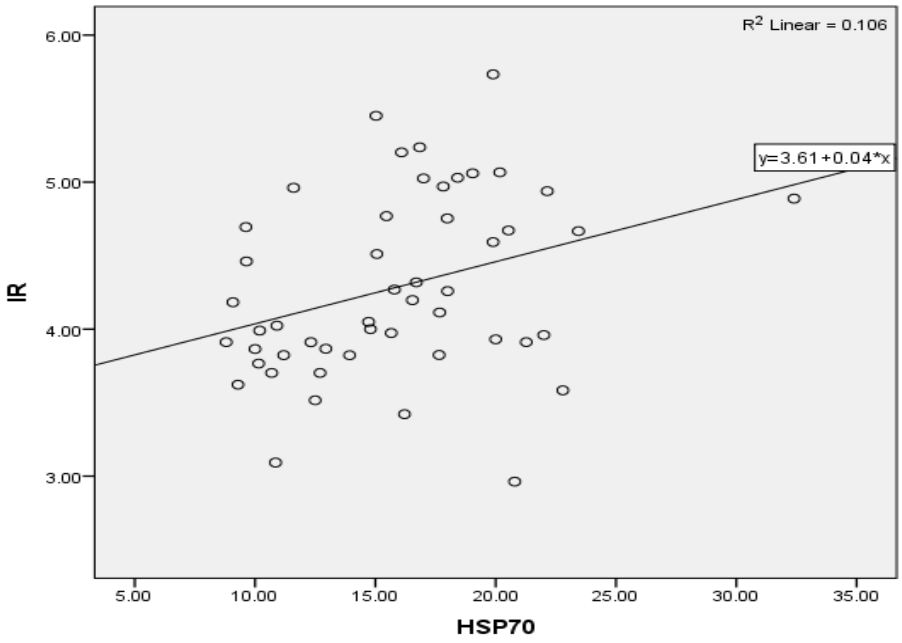

Fig 3.15: The correlation between HSP70 level & IR in patients group

Finding also in the study that no correlation between HSP70 and obesity and this disagree with the study that shows HSP70 concentration inversely correlated with BMI, percentage body fat, waist circumference **(Islam *et al.*, 2014).** while, the result agreed with other study that shown both BMI and WC were not correlated with HSP70 levels in serum, and HSP70 levels remained unchanged while BMI or WC increased in T2D patients and healthy controls **(Alemi *et al*., 2019).**

Folate supplementation reduced plasma homocysteine while increasing glutathione levels. These changes indicate a reduction in oxidative stress, which may decrease in serum Hsp70 in patient with type 2 DM **(Hunter-Lavin *et al*., 2004)**, the same mechanism is found in patient with PCOS use folate to decrease homocysteine level in blood **(Gholinezhad-Chari, Esmaeilzadeh andGhadimi,2017).**

3.4. Receiver Operator Characteristics Curve

3.4.1. ROC analysis for serum ZAG

The receiver operator characteristic (ROC) curve shows a large variance power for ZAG as shown in figure 3.16

The cut-off value of ZAG concentration in PCOS with primary infertility was 120μg/ml with a sensitivity of 94% and a specificity of 84% and the area under the curve (AUC) was 0.944, while the cut-off value of ZAG concentration in secondary infertility was 132μg/ml with a sensitivity of 77.8% and specificity 78% and the area under the curve 0.859 as shown in the table 3.5.

Table 3.5: Sensitivity and specificity of ZAG μg/ml in women within groups of infertility PCOS compared to control subject

Infertility	Sensitivity	Specificity	Cutoff value	AUC
Primary infertility	94%	84%	120	0.944
Secondary infertility	77.8%	78%	132	0.859

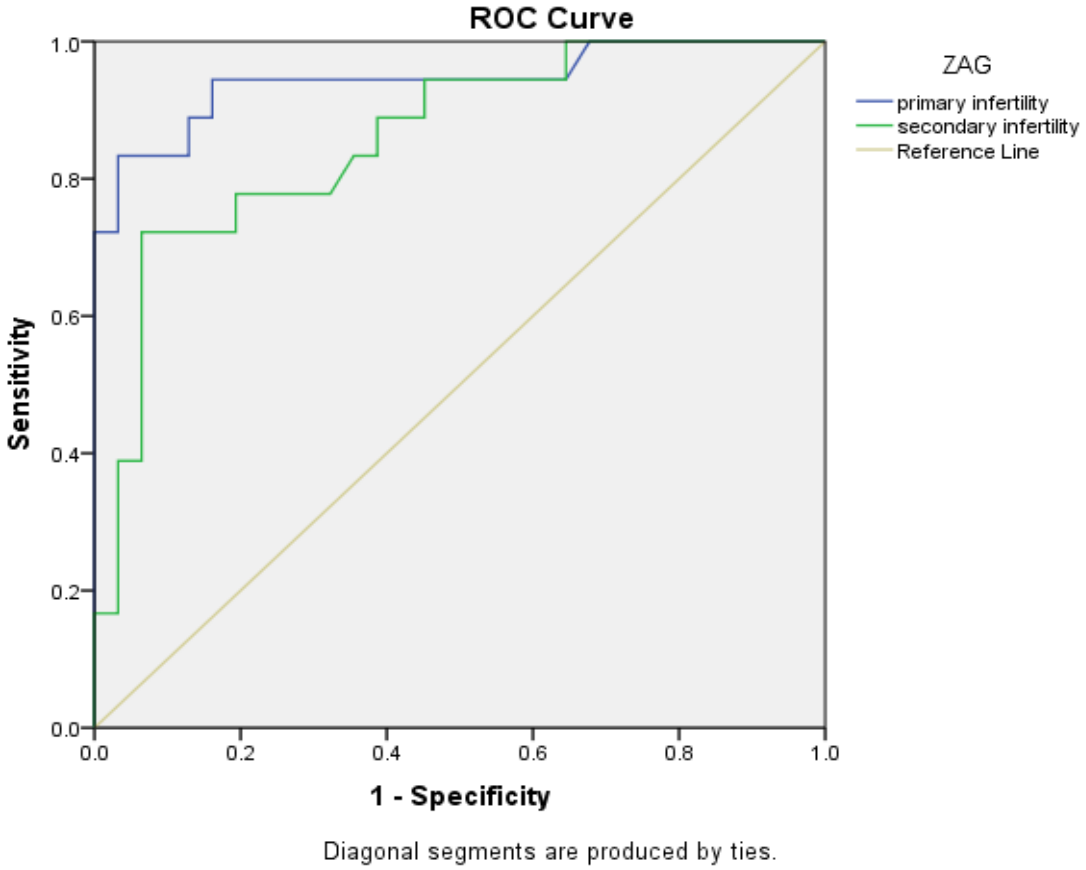

Figure 3.16:-ROC curve of ZAG μg/ml in women within groups in infertility PCOS compared to control subject.

According to the ROC curve analysis, the ZAG may be a useful marker for the diagnosis of PCOS, and determined the optimum ZAG cutoff value for

assessing the degree of infertility this result was agreed with study research **(Lai *et al.*, 2016).**

3.4.2. ROC analysis of serum HSP70

The ROC analysis for serum HSP70 show can detected the syndrome as in figure 3.17.The cut-off value of HSP70 concentration in PCOS with primary infertility was 12 ng/ml with a sensitivity of 83.3% and a specificity of 80% and the area under the curve (AUC) was 0.876, while the cut-off value of HSP70 concentration in secondary infertility was 10.1 ng/ml with a sensitivity of 77.8% and specificity 64% and the area under the curve 0.817 as shown in the table 3.6 and shows in figure 3.17 .

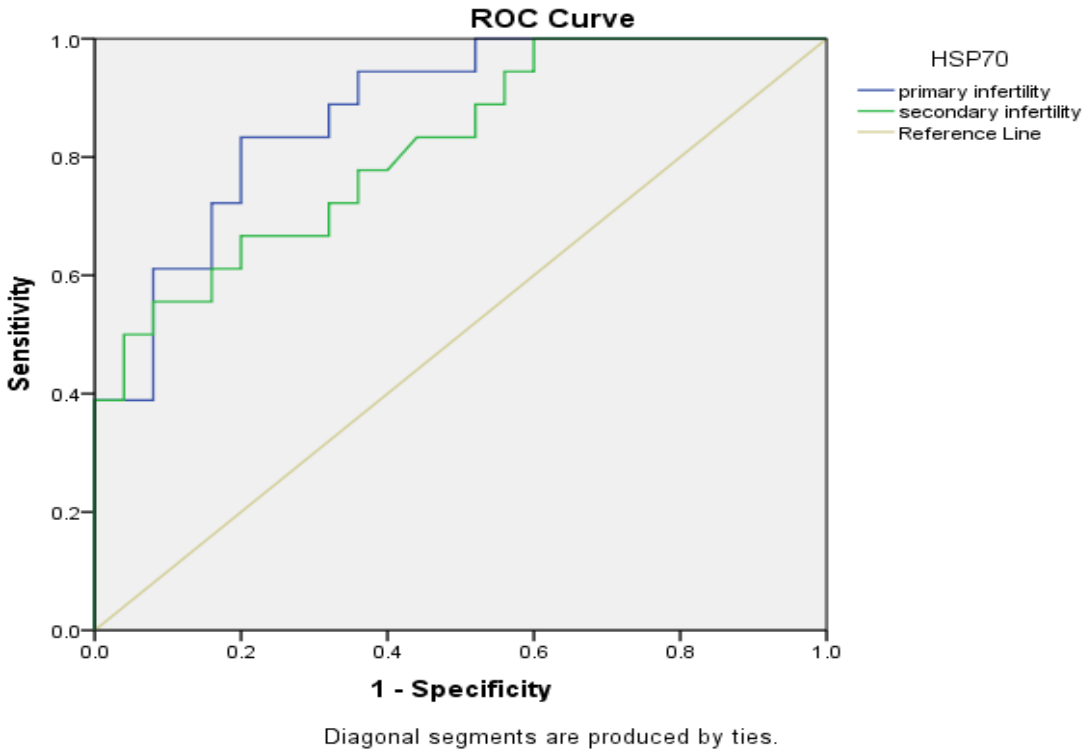

Fig 3.17: ROC curve of HSP70 ng/ml in women within groups in infertility PCOS compared to control subject

Table 3.6: Sensitivity and specificity of HSP70 ng/ml in women within groups of infertility PCOS compared to control subject

Infertility	Sensitivity	Specificity	Cutoff value	AUC
Primary infertility	83.3%	80%	12,0	0.876
Secondary infertility	77.8%	64%	10.1	0.817

These result was agreed with study of **(Gao *et al.*, 2013)** that explained the predictive value indicated by ROC curves research on Hsp70 level shows that it should be an important risk factor and may have clinical significance for PCOS.

Chapter four

Conclusion & Recommendation and Future work

4.1. Conclusion

In conclusion, this study confirmed that:

1- Circulating ZAG decreased in PCOS, especially in obese women and those with high blood glucose.

2- Associations between ZAG expression and obesity, insulin resistance, glucose and lipid metabolism, and hyperandrogenism in patients might therefore be involved in the development of PCOS

3- ZAG level is associated with PCOS and its effects on the fertility in women when ZAG level within normal value, while when it was decrease it will represent as a risk factor and leads to higher degree of infertility.

4- Increased serum Hsp70 levels are associated with the combination of hyper glycaemia, IR, hyper androgenic status in PCOS individuals, which provides supportive evidence that Hsp70 plays a key role in the pathogenesis of PCOS.

5- Increased HSP70 levels it had a significant risk factor and may have clinical relevance for PCOS.

4.2. Recommendation and Future work

1- Zinc appears to be an important regulator of the homeostasis of ZAG in the body; therefore, further studies are needed to clarify the relationship between zinc and ZAG metabolism and its repercussions in obesity or PCOS.

2- Genetic study to investigate the ZAG and HSP70 gene regulation and receptors in PCOS with or without type 2 diabetes mellitus.

3- Study ZAG levels in serum and seminal fluid and it's correlations with male infertility.

4- Study the serum levels of ZAG in PCOS women postprandial and its relation with lipid profile.

5- Suggest study serum HSP70 relationship with cardiovascular disease in postmenopausal women as development of PCOS.

6- Serum HSP70 may as novel marker in pancreatic cancer due to its relationship with insulin level .

7- Suggested that ZAG may be useful for the treatment of obesity and determine the relation of ZAG with gynecologic cancer.

8- At last, More consequent studies were warranted to confirm the clinical utility of circulating of ZAG and Hsp70, especially in diagnosis and prognosis of PCOS and its long-term health cost.

Chapter Five

References

5.1.References

- Albakova, Z., Armeev, G.A., Kanevskiy, L.M., Kovalenko, E.I. and Sapozhnikov, A.M. (2020). HSP70 Multi-Functionality in Cancer. *Cells*,[online]9(3).

- Alemi, H., Khaloo, P., Rabizadeh, S., Mansournia, M.A., Mirmiranpour, H., Salehi, S.S., Esteghamati, A. and Nakhjavani, M. (2019). Association of extracellular heat shock protein 70 and insulin resistance in type 2 diabetes; independent of obesity and C-reactive protein. *Cell Stress & Chaperones*, [online] 24(1), pp.69–75 .

- Al-Tu'ma, F., HadiFarhan, N. and Al-Safi, W.G., 2015. Association between fat mass and obesity Geners9939609 polymorphism with PCOS women in Iraqi population. *Ijppr. Human*, 5(1), pp.62-72

- Araki, T., Gejyo, F., Takagaki, K., Haupt, H., Schwick, H.G., Burgi, W., Marti, T., Schaller, J., Rickli, E. and Brossmer, R. (1988). Complete amino acid sequence of human plasma Zn-alpha 2-glycoprotein and its homology to histocompatibility antigens. *Proceedings of the National Academy of Sciences*, 85(3), pp.679–683.

- Archer, J.S. and Chang, R.Jeffrey. (2004). Hirsutism and acne in polycystic ovary syndrome. *Best Practice & Research Clinical Obstetrics & Gynaecology*, 18(5), pp.737–754 .

- Atalay, M., Oksala, N., Lappalainen, J., Laaksonen, D.E., Sen, C.K. and Roy, S. (2009). Heat shock proteins in diabetes and wound healing. *Current protein & peptide science*, [online] 10(1), pp.85–95.

- Bagherani, N. (2012). The Newest Hypothesis about Vitiligo: Most of the Suggested Pathogeneses of Vitiligo Can Be Attributed to Lack of One Factor, Zinc-α2-Glycoprotein. *ISRN Dermatology*, 2012, pp.1–8.

- Balaz, M., Vician, M., Janakova, Z., Kurdiova, T., Surova, M., Imrich, R., Majercikova, Z., Penesova, A., Vlcek, M., Kiss, A., Belan, V., Klimes, I., Olejnik, J., Gasperikova, D., Wolfrum, C., Ukropcova, B. and Ukropec, J. (2014). Subcutaneous adipose tissue zinc-α2-glycoprotein is associated with adipose tissue and whole-body insulin sensitivity. *Obesity*,22(8),pp.1821–1829 .

- Balen, A.H., Morley, L.C., Misso, M., Franks, S., Legro, R.S., Wijeyaratne, C.N., Stener-Victorin, E., Fauser, B.C.J.M., Norman, R.J. and Teede, H. (2016). The management of anovulatory infertility in women with polycystic ovary syndrome: an analysis of the evidence to support the development of global WHO guidance. *Human reproduction update*, [online] 22(6), pp.687–708.

- Beere, H.M. (2004). `The stress of dying': the role of heat shock proteins in the regulation of apoptosis. *Journal of Cell Science*, [online] 117(13), pp.2641–2651.

- Beere, H.M., Wolf, B.B., Cain, K., Mosser, D.D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R.I., Cohen, G.M. and Green, D.R. (2000). Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nature CellBiology*,[online]2(8),pp.469–475.

- Bellver, J., Rodríguez-Tabernero, L., Robles, A., Muñoz, E., Martínez, F., Landeras, J., García-Velasco, J., Fontes, J., Álvarez, M., Álvarez, C. and Acevedo, B. (2017). Polycystic ovary syndrome throughout a woman's life. *Journal of Assisted Reproduction and Genetics*, 35(1), pp.25–39.

- Bing, C., Bao, Y., Jenkins, J., Sanders, P., Manieri, M., Cinti, S., Tisdale, M.J. and Trayhurn, P. (2004). Zinc- 2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia. *Proceedings of the National Academy of Sciences*, 101(8), pp.2500–2505.

- Blagojevic, I.P., Eror, T., Pelivanovic, J., Jelic, S., Kotur-Stevuljevic, J. and Ignjatovic, S. (2017). Women with Polycystic Ovary Syndrome and Risk of Cardiovascular Disease. *Journal of Medical Biochemistry*, 36(3), pp.259–269.

- Bode, D.V., Seehusen, D.A. and Baird, D.C., 2012. Hirsutism in women. *American family physician*, 85(4), pp.373-380

- Body mass index - BMI. (2019). *WHO (World Health Organization) .int*. [online] Available at: http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi

- Borges, J. and Ramos, C. (2005). Protein Folding Assisted by Chaperones. *Protein & Peptide Letters*, 12(3), pp.257–261.

- Borthakur, A., D Prabhu, Y. and Valsala Gopalakrishnan, A. (2020). Role of IL-6 signalling in Polycystic Ovarian Syndrome associated inflammation. *Journal of Reproductive Immunology*, 141, p.103155.

- Breuninger, S. and Erl, J. (2014). Quantitative Analysis of Liposomal Heat Shock Protein 70 (Hsp70) in the Blood of Tumor Patients Using a Novel LipHsp70 ELISA. *Journal of Clinical & Cellular Immunology*, .*5*(5), p.264

- BT LAB, (2020) Human Heat Shock protein 70 Eliza kit manual ,*Bioassay Technology Laboratory*, http://www.bt-laboratory.com/

- BT LAB, (2020) Human Zinc Alpha 2glycoprotien Eliza kit manual ,*Bioassay Technology Laboratory*, http://www.bt-laboratory.com/

- Buttacavoli, M., Di Cara, G., D'Amico, C., Geraci, F., Pucci-Minafra, I., Feo, S. and Cancemi, P. (2021). Prognostic and Functional Significant of Heat Shock Proteins (HSPs) in Breast Cancer Unveiled by Multi-Omics Approache.*Biology*,10(3),p.247.

- Cao, Y., Ohwatari, N., Matsumoto, T., Kosaka, M., Ohtsuru, A. and Yamashita, S. (1999). TGF-β1 mediates 70-kDa heat shock protein induction due to ultraviolet irradiation in human skin fibroblasts. *Pfl*[?]*gers Archiv European Journal of Physiology*, 438(3), pp.239–244

- Carson, J.A. and Manolagas, S.C. (2015). Effects of sex steroids on bones and muscles: Similarities, parallels, and putative interactions in health and disease. *Bone*,[online]80,pp.67–78.

- Caza, F., Betoulle, S., Auffret, M., Brousseau, P., Fournier, M. and St-Pierre, Y. (2016). Comparative sequence analysis of Hsp70 gene from Mytilus edulis desolationis and Aulacomya ater of the Kerguelen Islands. *Journal of Xenobiotics*, 6(2).p. 41-44.

- Cena, H., Chiovato, L. and Nappi, R.E. (2020). Obesity, Polycystic Ovary Syndrome, and Infertility: A New Avenue for GLP-1 Receptor Agonists. *The Journal of Clinical Endocrinology & Metabolism*, [online] 105(8), pp.e2695–e2709.

- Cervantes, J., Eber, A.E., Perper, M., Nascimento, V.M., Nouri, K. and Keri, J.E. (2017). The role of zinc in the treatment of acne: A review of the literature. *Dermatologic Therapy*,31(1), p.e12576.

- Chakrabarti, J. (2013). Serum leptin level in women with polycystic ovary syndrome: Correlation with adiposity, insulin, and circulating testosterone. *Annals of Medical and Health Sciences Research*, 3(2), p.191.

- Chen, H.-H., Chen, C.-C., Hsieh, M.-C., Ho, C.-W., Hsu, S.-P., Yip, H.-T. and Kao, C.-H. (2020). Graves' disease could increase polycystic ovary syndrome and comorbidities in Taiwan. *Current Medical Research and Opinion*, 36(6), pp.1063–1067.

- Ciechanover, A. and Kwon, Y.T. (2017). Protein Quality Control by Molecular Chaperones in Neurodegeneration. *Frontiers in Neuroscience*, *11*,p 185

- Clerc, F., Reiding, K.R., Jansen, B.C., Kammeijer, G.S.M., Bondt, A. and Wuhrer, M. (2015). Human plasma protein N-glycosylation . *Glycoconjugate Journal*, 33(3), pp.309–343.

- Condorelli, R.A., Calogero, A.E., Di Mauro, M., Mongioi', L.M., Cannarella, R., Rosta, G. and La Vignera, S. (2017). Androgen excess and metabolic disorders in women with PCOS: beyond the body mass index. *Journal of Endocrinological Investigation*, 41(4), pp.383–388.

- Costa, E. C., DE Sá, J., Stepto, N. K., Costa, I., Farias-Junior, L. F., Moreira, S., Soares, E., Lemos, T., Browne, R., & Azevedo, G. D. (2018). Aerobic Training Improves Quality of Life in Women with Polycystic Ovary Syndrome. *Medicine and science in sports and exercise*,50(7),pp.1357–1366.

- Costa-Beber, L.C., Hirsch, G.E., Heck, T.G. and Ludwig, M.S. (2020). Chaperone duality: the role of extracellular and intracellular HSP70 as a biomarker of endothelial dysfunction in the development of atherosclerosis. *Archives of Physiology and Biochemistry*, pp.1–8.

- Craig, E.A. (2018). Hsp70 at the membrane: driving protein translocation. *BMC Biology*, 16(1).p.11

- Crook, M. (2013). Lipoprotein X: clinical implications. Annals of Clinical Biochemistry: An *international journal of biochemistry and laboratory medicine*, 50(2), pp.93–94

- Dahan, M.H. and Reaven, G. (2019). Relationship among obesity, insulin resistance, and hyperinsulinemia in the polycystic ovary syndrome. *Endocrine*, 64(3), pp.685–689.

- Datta, K., Rahalkar, K. and Dubey, D.K. (2017). Heat Shock Proteins (Hsp): Classifications and Its Involvement in Health and Disease. *Research in Pharmacy and Health Sciences*, 3(3), pp.334–337.

- De Jong, J.M.A., Wouters, R.T.F., Boulet, N., Cannon, B., Nedergaard, J. and Petrovic, N. (2017). The β3-adrenergic receptor is dipensable for browning of adipose tissues. *American Journal of Physiology-Endocrinology and Metabolism*, 312(6), pp.E508–E518

- De Medeiros, S.F., Rodgers, R.J. and Norman, R.J. (2021). Adipocyte and steroidogenic cell cross-talk in polycystic ovary syndrome. *Human Reproduction Update*. 27(4). pp. 771–796

- DeUgarte, C.M., Bartolucci, A.A. and Azziz, R. (2005). Prevalence of insulin resistance in the polycystic ovary syndrome using the homeostasis model assessment. *Fertility and sterility*, [online] 83(5), pp.1454–60.

- Diagnostic Criteria in Polycystic Ovary Syndrome. (2003). *Seminars in Reproductive Medicine*, 21(3), pp.267–276

- Diamanti-Kandarakis, E. (2007). Role of obesity and adiposity in polycystic ovary syndrome. *International Journal of Obesity*, 31(S2), pp.S8–S13

- Ding, Z., Qu, F., Guo, W., Ying, X., Wu, M. and Zhang, Y. (2007). Identification of sperm forward motility-related proteins in human seminal plasma. *Molecular Reproduction and Development*, 74(9), pp.1124–1131.

- Duică, F., Dănilă, C.A., Boboc, A.E., Antoniadis, P., Condrat, C.E., Onciul, S., Suciu, N., Creţoiu, S.M., Varlas, V.N. and Creţoiu, D. (2021). Impact of Increased Oxidative Stress on Cardiovascular Diseases in Women With Polycystic Ovary Syndrome. *Frontiers in Endocrinology*, 12.

- Duleba, A.J. and Dokras, A. (2012). Is PCOS an inflammatory process? *Fertility and Sterility*,[online] 97(1),pp.7–12.

- Dumesic, D.A., Phan, J.D., Leung, K.L., Grogan, T.R., Ding, X., Li, X., Hoyos, L.R., Abbott, D.H. and Chazenbalk, G.D. (2019). Adipose Insulin Resistance in Normal-Weight Women With Polycystic Ovary Syndrome. *The Journal of Clinical Endocrinology & Metabolism*,104(6),pp.2171–2183.

- Ersin, B.T. (2021). Stress- induced Hormonal Levels in Blood and Symptomatic Changes in PCOS Patients. *dspace.rsu.lv*. Riga Stradins University .Berit.Tülin.Ersin.26410.

- Freije, J.P., Fueyo, A., Uría, J. and López-Otin, C. (1991). Human Zn-α2 -glycoprotein cDNA cloning and expression analysis in benign and malignant breast tissues. *FEBS Letters*, 290(1-2), pp.247–249

- Gao, H., Meng, J., Xu, M., Zhang, S., Ghose, B., Liu, J., Yao, P., Yan, H., Wang, D. and Liu, L. (2013). Serum Heat Shock Protein 70 Concentration in Relation to Polycystic Ovary Syndrome in a Non-Obese Chinese Population. *PLoS ONE*, 8(6), p.e67727.

- Gao, S.-X., Guo, J., Fan, G.-Q., Qiao, Y., Zhao, R.-Q. and Yang, X.-J. (2018). ZAG alleviates HFD-induced insulin resistance accompanied with

decreased lipid depot in skeletal muscle in mice. *Journal of Lipid Research*, 59(12), pp.2277–2286

- Gao, T. and Newton, A.C. (2002). The Turn Motif Is a Phosphorylation Switch That Regulates the Binding of Hsp70 to Protein Kinase C. *Journal of Biological Chemistry*, 277(35), pp.31585–31592

- Gholinezhad-Chari, M., Esmaeilzadeh, S. and Ghadimi, R. (2017). The effect of metformin treatment on the serum levels of homocysteine, folic acid, and vitamin B12 in patients with polycystic ovary syndrome. *Journal of Human Reproductive Sciences*, 10(2), p.95.

- Glueck, C.J. and Goldenberg, N. (2019). Characteristics of obesity in polycystic ovary syndrome: Etiology, treatment, and genetics. *Metabolism*,92,pp.108–120.

- Halimova, Z.Yu., Narimova, G.J., Jabborova, G.M. and Mirsaidova, U.A. (2019). Case of Pituitary corticotropinoma - from PCOS to obvious Cushing's. *Endocrine Abstracts* . (Vol. 63). P688 .

- Hall, J.E. (1993). Polycystic Ovarian Disease as a Neuroendocrine Disorder of the Female Reproductive Axis. *Endocrinology and Metabolism Clinics of North America*, 22(1), pp.75–92

- Hartl, F.U., Bracher, A. and Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature*, 475(7356), pp.324–332.

- Haslbeck, M. and Vierling, E. (2015). A First Line of Stress Defense: Small Heat Shock Proteins and Their Function in Protein Homeostasis. *Journal of Molecular Biology*, [online] 427(7), pp.1537–1548.

- Hassan, Md.I., Waheed, A., Yadav, S., Singh, T.P. and Ahmad, F. (2008). Zinc 2-Glycoprotein: A Multidisciplinary Protein. *Molecular Cancer Research*, 6(6), pp.892–906.

- Ho, C.-W., Chen, H.-H., Hsieh, M.-C., Chen, C.-C., Hsu, S.-P., Yip, H.-T. and Kao, C.-H. (2020). Increased Risk of Polycystic Ovary Syndrome and It's Comorbidities in Women with Autoimmune Thyroid Disease. *International Journal of Environmental Research and Public Health*, 17(7), p.2422.

- Hoter, A. and Naim, H.Y. (2019). Heat Shock Proteins and Ovarian Cancer: Important Roles and Therapeutic Opportunities. *Cancers*, 11(9), p.1389.

- Hoter, Rizk and Naim (2019). The Multiple Roles and Therapeutic Potential of Molecular Chaperones in Prostate Cancer. *Cancers*, [online] 11(8), p.1194.

- Hunter-Lavin, C., Hudson, P.R., Mukherjee, S., Davies, G.K., Williams, C.P., Harvey, J.N., Child, D.F. and Williams, J.H.H. (2004). Folate supplementation reduces serum Hsp70 levels in patients with type 2 diabetes. *Cell Stress & Chaperones*, 9(4), p.344.

- Ikwegbue, P., Masamba, P., Oyinloye, B. and Kappo, A. (2017). Roles of Heat Shock Proteins in Apoptosis, Oxidative Stress, Human Inflammatory Diseases, and Cancer. *Pharmaceuticals*, 11(1), p.2.

- Islam, A., Hait, S., Andrews-Shigaki, B., Carus, S. and Deuster, P. (2014). Plasma HSP70 Levels Correlate with Health Risk Factors and Insulin Resistance in African American Subjects. *Experimental and Clinical Endocrinology&Diabetes*,122(08), pp.496 .

- Jamilian, M., Foroozanfard, F., Bahmani, F., Talaee, R., Monavari, M. and Asemi, Z. (2016). Effects of Zinc Supplementation on Endocrine Outcomes

in Women with Polycystic Ovary Syndrome: a Randomized, Double-Blind, Placebo-Controlled Trial. *Biological trace element research*, 170(2), pp. 271-278.

- Kedar, K.V., Rewatkar, M.M. and Akare, M.D. (2019). Thyroid dysfunction in women with polycystic ovarian syndrome: a comparative study. *International Journal of Reproduction, Contraception, Obstetrics and Gynecology*, 8(5), p.1943

- Khan, M. and Joseph, F. (2014). Adipose Tissue and Adipokines: The Association with and Application of Adipokines in Obesity .*Scientifica*,2014,pp.1-7.

- Krause, M., Heck, T.G., Bittencourt, A., Scomazzon, S.P., Newsholme, P., Curi, R. and Homem de Bittencourt, P.I. (2015). The Chaperone Balance Hypothesis: The Importance of the Extracellular to Intracellular HSP70 Ratio to Inflammation-Driven Type 2 Diabetes, the Effect of Exercise, and the Implications for Clinical Management. *Mediators of Inflammation*, 2015,pp.1–12.

- Krause, M., Keane, K., Rodrigues-Krause, J., Crognale, D., Egan, B., De Vito, G., Murphy, C. and Newsholme, P. (2014). Elevated levels of extracellular heat-shock protein 72 (eHSP72) are positively correlated with insulin resistance in vivo and cause pancreatic β-cell dysfunction and death in vitro. *Clinical Science*, 126(10), pp.739–752.

- Kyrou, I., Karteris, E., Robbins, T., Chatha, K., Drenos, F. and Randeva, H.S. (2020). Polycystic ovary syndrome (PCOS) and COVID-19: an overlooked female patient population at potentially higher risk during the COVID-19 pandemic. *BMC Medicine*, 18(1), p.1-10

- Lai, Y., Chen, J., Li, L., Yin, J., He, J., Yang, M., Jia, Y., Liu, D., Liu, H., Liao, Y. and Yang, G. (2016). Circulating Zinc-α2-glycoprotein levels and Insulin Resistance in Polycystic Ovary Syndrome . *Scientific Reports*, 6(1). p1-10.

- Lau, A.M., Zahid, H., Gor, J., Perkins, S.J., Coker, A.R. and McDermott, L.C. (2019). Crystal structure of zinc-α2-glycoprotein in complex with a fatty acid reveals multiple different modes of protein-lipid binding. *Biochemical Journal*, 476(19), pp.2815–2834.

- Legro, R. (2008). New Directions in Polycystic Ovary Syndrome. *Seminars in Reproductive Medicine*, 26(1), pp.003–004.

- Lei, L., Li, K., Li, L., Fang, X., Zhou, T., Zhang, C., Luo, Y., Liu, H., Li, X., Zheng, H., Zhang, L., Yang, G. and Gao, L. (2017). Circulating zinc-α2-glycoprotein levels are low in newly diagnosed patients with metabolic syndrome and correlate with adiponectin .*Nutrition&Metabolism*,14(1), 1-7.

- Li, Z. and Srivastava, P. (2004). Heat-shock proteins. *Current protocols in immunology*, [online] Appendix 1, p.Appendix1T. *58*(1), A-1T .

- Liu, J., Han, H., Fan, Z., El Beaino, M., Fang, Z., Li, S. and Ji, J. (2018). AZGP1 inhibits soft tissue sarcoma cells invasion and migration. *BMC Cancer*, 18(1), 1-8.

- Liu, Q., Xie, Y., Qu, L., Zhang, M. and Mo, Z. (2019). Dyslipidemia involvement in the development of polycystic ovary syndrome. *Taiwanese Journal of Obstetrics and Gynecology*, 58(4), pp.447–453

- Malini, N.A. and Roy George, K. (2018). Evaluation of different ranges of LH:FSH ratios in polycystic ovarian syndrome (PCOS) – Clinical based case control study . *General and Comparative Endocrinology* ,260,pp.51–57.

- Marshall, J.C. and Dunaif, A. (2012). Should all women with PCOS be treated for insulin resistance? *Fertility and Sterility*, [online]97(1),pp.18–22.

- Martin KA, Chang RJ, Ehrmann DA, et al. (2008). Evaluation and treatment of hirsutism in premenopausal women: an Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism*, 93(4), 1105-1120.

- Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F. and Turner, R.C. (1985). Homeostasis model assessment: insulin resistance and ?-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*,[online]28(7),pp.412–419.

- Matz, J.M., Blake, M.J., Tatelman, H.M., Lavoi, K.P. and Holbrook, N.J. (1995). Characterization and regulation of cold-induced heat shock protein expression in mouse brown adipose tissue. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 269(1), pp.R38–R47 .

- Mayer, M.P. (2010). Gymnastics of Molecular Chaperones. *Molecular Cell*, 39(3), pp.321–331.

- Mayer, M.P., Brehmer, D., Gässler, C.S. and Bukau, B. (2001). Hsp70 chaperone machines. *Advances in protein chemistry*, 59, pp.1–44.

- Merkin, S.S., Phy, J.L., Sites, C.K. and Yang, D. (2016). Environmental determinants of polycystic ovary syndrome. *Fertility and Sterility*, [online] 106(1), pp.16–24.

- Molina, M.N., Ferder, L. and Manucha, W. (2015). Emerging Role of Nitric Oxide and Heat Shock Proteins in Insulin Resistance. *Current Hypertension Reports*, 18(1), 1-13

- Morin-Papunen, L.C., Duleba, A.J., Bloigu, A., Järvelin, M.-R., Saikku, P. and Pouta, A. (2010). Chlamydia antibodies and self-reported symptoms of oligo-amenorrhea and hirsutism: A new etiologic factor in polycystic ovary syndrome? *Fertility and Sterility*, 94(5),pp.1799–1804.

- Murphy, M.E. (2013). The HSP70 family and cancer. *Carcinogenesis*, 34(6), pp.1181–1188.

- Nadir, A., Temizkan, S., Ozderya, A., Temizkan, O., Orbay, E. and Aydin, K. (2018). Dehydroepiandrosterone sulfate/free androgen index ratio predicts a favorable metabolic profile in patients with polycystic ovary syndrome. *Gynecological Endocrinology*, 35(2), pp.128–132

- Nakhjavani, M., Morteza, A., Khajeali, L., Esteghamati, A., Khalilzadeh, O., Asgarani, F. and Outeiro, T.F. (2010). Increased serum HSP70 levels are associated with the duration of diabetes. *Cell Stress and Chaperones*, 15(6), pp.959–964.

- Nakhjavani, M., Morteza, A., Nargesi, A.A., Mostafavi, E. and Esteghamati, A. (2013). Appearance of leptin–HSP70 correlation, in type 2diabetes.*MetaGene*,1,pp.1–7.

- Narayansingh, R. M., Senchyna, M., Vijayan, M. M., & Carlson, J. C. (2004). Expression of prostaglandin G/H synthase (PGHS) and heat shock protein-70 (HSP-70) in the corpus luteum (CL) of prostaglandin F2α-treated immature superovulated rats.*Canadian Journal of Physiology and Pharmacology*, 82(6), 363–371.

- Nathan, D.M., Singer, D.E., Hurxthal, K. and Goodson, J.D. (1984). The Clinical Information Value of the Glycosylated Hemoglobin Assay. *New England Journal of Medicine*, 310(6), pp.341–346.

- Pallavi R. Panse.., M., P. and M., B. (2019). Hirsutism Beyond PCOS. *Journal of Evidence Based Medicine and Healthcare*, 6(43), pp.2833–2835.

- Pagnon, J., Matzaris, M., Stark, R., Meex, R.C.R., Macaulay, S.L., Brown, W., O'Brien, P.E., Tiganis, T. and Watt, M.J. (2012). Identification and Functional Characterization of Protein Kinase A Phosphorylation Sites in the Major Lipolytic Protein, Adipose Triglyceride Lipase. *Endocrinology*, 153(9), pp.4278–4289

- Palomba, S., de Wilde, M.A., Falbo, A., Koster, M.P.H., La Sala, G.B. and Fauser, B.C.J.M. (2015). Pregnancy complications in women with polycystic ovary syndrome. *Human reproduction update*, 21(5),pp.575–592.

- Pearson's Correlation Coefficient. (2019). *Encyclopedia of Public Health*, Springer,Dordrecht. eBook Packages Medicine Reference Module Medicine pp.1090–1091.

- Pierce, J.G. and Parsons, T.F. (1981). Glycoprotein Hormones: Structure and Function. *Annual Review of Biochemistry*,50(1),pp.465–495.

- Pirkkala, L., Nykänen, P., & Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB journal* : official publication of the Federation of American Societies for Experimental Biology, 15(7),pp.1118–1131.

- Pockley, A.G., Shepherd, J. and Corton, J.M. (1998). Detection of heat shock protein 70 (HSP70) and anti-HSP70 antibodies in the serum of normal individuals. *Immunological Investigations*,27(6),pp.367–377.

- Qu, H.-Q., Li, Q., Rentfro, A.R., Fisher-Hoch, S.P. and McCormick, J.B. (2011). The Definition of Insulin Resistance Using HOMA-IR for Americans of Mexican Descent Using Machine Learning. *PLoS ONE*, 6(6), p.e21041.

- Radons, J. (2016). The human HSP70 family of chaperones: where do we stand? *Cell Stress and Chaperones*, 21(3), pp.379–404.

- Rajkhowa, M., Brett, S., Cuthbertson, D.J., Lipina, C., Ruiz-Alcaraz, A.J., Thomas, G.E., Logie, L., Petrie, J.R. and Sutherland, C. (2009). Insulin resistance in polycystic ovary syndrome is associated with defective regulation of ERK1/2 by insulin in skeletal muscle in vivo. *Biochemical Journal*, 418(3), pp.665–671.

- Rama Raju, G., Chavan, R., Deenadayal, M., Govindarajan, M., Gunasheela, D., Gutgutia, R., Haripriya, G., Patel, N. and Patki, A. (2013). Luteinizing hormone and follicle stimulating hormone synergy: A review of role in controlled ovarian hyper-stimulation. *Journal of Human ReproductiveSciences*, [online] 6(4),p.227.

- Randolph, J.F., Sowers, M., Bondarenko, I.V., HarlowS.D., Luborsky, J.L. and Little, R.J. (2004). Change in Estradiol and Follicle-Stimulating Hormone across the Early Menopausal Transition: Effects of Ethnicity and Age. *The Journal of Clinical Endocrinology & Metabolism*, 89(4), pp.1555–1561.

- Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). (2004). *Human Reproduction*, 19(1), pp.41–47

- Romauch, M. (2020). Zinc-α2-glycoprotein as an inhibitor of amine oxidase copper-containing3. *OpenBiology*,10(4),p.190035 .

- Rosenzweig, R., Nillegoda, N.B., Mayer, M.P. and Bukau, B. (2019). The Hsp70 chaperone network. *Nature Reviews. Mol ecular Cell Biology*, [online] 20(11), pp.665–680.

- Russell, S.T. and Tisdale, M.J. (2009). Antidiabetic Properties of Zinc-α2-Glycoprotein in ob/ob Mice. *Endocrinology*, 151(3), pp.948–957

- Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. *Nature Reviews Molecular Cell Biology*,14(10),pp.630–642 .

- Sajjadi, A.Y., Mitra, K. and Grace, M. (2013). Expression of heat shock proteins 70 and 47 in tissues following short-pulse laser irradiation: Assessment of thermal damage and healing. *Medical Engineering & Physics*, 35(10), pp.1406–1414.

- Salgado, A.L.F. de A., Carvalho, L. de, Oliveira, A.C., Santos, V.N. dos, Vieira, J.G. and Parise, E.R. (2010). Insulin resistance index (HOMA-IR) in the differentiation of patients with non-alcoholic fatty liver disease and healthy individuals. *Arquivos de gastroenterologia*, 47(2), pp.165-169.

- Sanchez, L.M., Lopez-Otin, C. and Bjorkman, P.J. (1997). Biochemical characterization and crystalization of human Zn- 2-glycoprotein, a soluble class I major histocompatibility complex homolog. *Proceedings of the National Academy of Sciences*, 94(9), pp.4626–4630

- Schlesinger, M.J. (1990). Heat shock proteins. *Journal of Biological Chemistry*, 265(21), pp.12111–12114.

- Severo, J.S., Morais, J.B.S., Beserra, J.B., dos Santos, L.R., de Sousa Melo, S.R., de Sousa, G.S., de Matos Neto, E.M., Henriques, G.S. and do Nascimento Marreiro, D. (2019). Role of Zinc in Zinc-α2-Glycoprotein Metabolism in Obesity: a Review of Literature. *Biological Trace Element Research*, 193(1), pp.81–88

- Shahin, L., Hyassat, D., Batieha, A., Khader, Y., El-Khateeb, M. and Ajlouni, K. (2019). Insulin Sensitivity Indices in Patients with Polycystic

Ovary Syndrome with Different Body Mass Index Categories. *Current Diabetes Reviews*, 16(5), 483-489.

- Shaikh, N., Dadachanji, R. and Mukherjee, S. (2014). Genetic Markers of Polycystic Ovary Syndrome: Emphasis on Insulin Resistance. *International Journal of Medical Genetics*, 2014, pp.1–10.

- Sharif, E.(2019). New markers for the detection of polycystic ovary syndrome.*Obstetrics & Gynecology International Journal*,10(4).pp.257-268.

- Shima, Y., Miyabayashi, K., Haraguchi, S., Arakawa, T., Otake, H., Baba, T., Matsuzaki, S., Shishido, Y., Akiyama, H., Tachibana, T., Tsutsui, K. and Morohashi, K. (2013). Contribution of Leydig and Sertoli Cells to Testosterone Production in Mouse Fetal Testes. *Molecular Endocrinology*, 27(1), pp.63–73.
- Singla, R., Gupta, Y., Khemani, M. and Aggarwal, S. (2015). Thyroid disorders and polycystic ovary syndrome: An emerging relationship. *Indian Journal of Endocrinology and Metabolism*, 19(1),p.25.

- Sirotkin, A.V. and Bauer, M. (2010). Heat shock proteins in porcine ovary: synthesis, accumulation and regulation by stress and hormones. *Cell Stress and Chaperones*, 16(4), pp.379–387.

- Sisti, G., Kanninen, T.T., Ramer, I. and Witkin, S.S. (2015). Interaction between the inducible 70-kDa heat shock protein and autophagy: effects on fertility and pregnancy. *Cell Stress and Chaperones*, 20(5), pp.753–758.

- Skakkebaek, A., Moore, P.J., Pedersen, A.D., Bojesen, A., Kristensen, M.K., Fedder, J., Laurberg, P., Hertz, J.M., Østergaard, J.R., Wallentin, M. and Gravholt, C.H. (2017). The role of genes, intelligence, personality, and social engagement in cognitive performance in Klinefelter syndrome. *Brain and Behavior*, 7(3), p.e00645.

- Sumathi, M., Chitra, P., Sakthi Prabha, R. and Srilatha, K. (2021). Study and detection of PCOS related diseases using CNN. *IOP Conference Series: Materials Science and Engineering*, 1070, p.012062.

- Suresh, S. and Vijayakumar, T. (2014). Correlations of Insulin Resistance and Serum Testosterone Levels with LH:FSH Ratio and Oxidative Stress in Women with Functional Ovarian Hyperandrogenism. *Indian Journal of Clinical Biochemistry*, 30(3), pp.345–350.

- Szydlarska, D., Machaj, M. and Jakimiuk, A. (2017). History of discovery of polycystic ovary syndrome. *Advances in Clinical and Experimental Medicine*, [online] 26(3), pp.555–558.

- The ObG Project. (2019). PCOS Part 1: Pathophysiology of PCOS book in OBG library Availableat: https://www.obgproject.com/2019/06/12/pcos-part-1-sensitive-care-of-the-pcos-patient

- Ullah, Z., Iqbal, A., Baloch, M.K., Nishan, U. and Shah, M. (2020). New insights into the zinc-α2-glycoprotein (ZAG) scaffold and its metal ions binding abilities using spectroscopic techniques. *LifeSciences*,249,p.117462.

- Vostakolaei, M.A., Hatami-Baroogh, L., Babaei, G., Molavi, O., Kordi, S. and Abdolalizadeh, J. (2020). Hsp70 in cancer: A double agent in the battle between survival and death. *Journal of Cellular Physiology*. 236(5), 3420-3444.

- Wang, L., Liu, M., Ning, D., Zhu, H., Shan, G., Wang, D., Ping, B., Yu, Y., Yang, H., Yan, K., Pan, H. and Gong, F. (2020). Low Serum ZAG Levels Correlate With Determinants of the Metabolic Syndrome in Chinese Subjects. *Frontiers in Endocrinology*, 11. 154.

- Waseem, N., Waseem, H., Abbas, B., Ali, R. and Aftab, K. (2021). Pregnancy in a Secondary Infertile Woman with Polycystic Ovarian

Syndrome (PCOS) –A Case Report. *Scholars Journal of Medical Case Reports*, 9(1), pp.70–73.

- WHO. Waist Circumference and Waist–Hip Ratio: Report of a WHO Expert Consultation. Geneva. World health organization; 2008.[cited2019,Jun5th].Availableat https://apps.who.int/iris/bitstream/handle/10665/44583/9789241501491_eng.pdf;jsessionid=483DD9BF86B110BAD5996EA4EF69CBB8?sequence=1

- Wu, G., Hu, X., Ding, J. and Yang, J. (2019). Abnormal expression of HSP70 may contribute to PCOS pathology. *Journal of Ovarian Research*, 12(1)**,** 74 .

- Xiao, X.-H., Qi, X.-Y., Wang, Y.-D., Ran, L., Yang, J., Zhang, H.-L., Xu, C.-X., Wen, G.-B. and Liu, J.-H. (2018). Zinc alpha2 glycoprotein promotes browning in adipocytes. *Biochemical and Biophysical Research Communications*, 496(2), pp.287–293.

- Yildiz, B.O. and Azziz, R. (2007). The adrenal and polycystic ovary syndrome. *Reviews in Endocrine and Metabolic Disorders*, 8(4), pp.331–342.

- Yuan, c., Liu, x., Mao, Y., Diao, F., Cui, Y. & Liu, J. 2016. Polycystic ovary syndrome patients with high BMI tend to have functional disorders of androgen excess: A prospective study. *Journal of biomedical research*, 30(3):197-202.

- Zhang, X., Xu, Z., Zhou, L., Chen, Y., He, M., Cheng, L., Hu, F.B., Tanguay, R.M. and Wu, T. (2010). Plasma levels of Hsp70 and anti-Hsp70 antibody predict risk of acute coronary syndrome. *Cell Stress and Chaperones*, 15(5), pp.675–686.

- Zheng, S., Liu, E., Zhang, Y., Long, T., Liu, X., Gong, Y., Mai, T., Shen, H., Chen, H., Lin, R., Zheng, Y., Xie, Y. and Wang, F. (2019). Circulating zinc-α2-glycoprotein is reduced in women with polycystic ovary syndrome, but can be increased by exenatide or metformin treatment. *Endocrine Journal*, 66(6), pp.555–562.

الخلاصة

متلازمة تكيس المبايض (PCOS) هي اختلال هرموني نتيجة لارتفاع مستوى الأندروجين (هرمون الذكورة) في الإناث ، وعلامات عدم نضج البيوض وتكيسات المبيض وعدم انتظام الدورة الشهرية ، والعقم ، ونمو الشعر الزائد على الوجه. يحفز الأنسولين المفرط في الجسم المبيضين على إنتاج الأندروجين الزائد ويزيد من خطر الإصابة بمرض السكري من النوع ٢.

زنك ألفا ٢ جليكوبروتين (ZAG) هو ببتيد متعدد وحيد السلسلة بوزن جزيئي ٤٠-٤٣ كيلو دالتون موجود في غالبية سوائل الجسم. يؤدي انخفاض مستويات ZAG إلى انخفاض معدلات تحلل الدهون المرتبط بتراكم الدهون.

بروتينات الصدمة الحرارية (HSP) هي مرافقات جزيئية تلعب دورًا رئيسيًا في الحفاظ على توازن البروتين ونقله. تشارك بروتينات الصدمة الحرارية التي يبلغ وزنها الجزيئي حوالي ٧٠ كيلو دالتون (Hsp70) في المساعدة في طي البروتين ، ومنع تراكم البروتين ونقل البروتينات عبر الأغشية. ارتبط ارتفاع المصل Hsp70 ارتباطًا إيجابيًا بمقاومة الانسولين والإجهاد التأكسدي والالتهاب في متلازمة تكيس المبايض: يتسبب الالتهاب في إطلاق Hsp70 في الدم ، وقد توفر مستويات Hsp70 في المصل علامة على العمليات الالتهابية.

الاهداف

الهدف من الدراسة هو تقييم مستويات ZAG في مصل مرضى متلازمة تكيس المبايض وارتباطها بالعقم مقارنةً بالمجموعة الضابطة ، ودراسة الارتباط بين مستوى ZAG مع المؤشرات الحيوية الهرمونية ومقاومة الأنسولين ، وتقييم مستويات HSP70 في مصل مرضى PCOS و مقارنتها مع تلك الموجودة في المجموعة الضابطة ، والتحقيق في علاقتها مع المؤشرات الحيوية الهرمونية ومقاومة الأنسولين.

الموضوع والطرق

اشتملت دراسة الحالات والشواهد على ٨١ أنثى ، في سن الإنجاب [١٨-٤٥ سنة] تم تشخيص ٥٠ منهن بمتلازمة تكيس المبايض ، في حين أن الـ ٣١ الأخرى كانت ضوابط صحية تم تقسيمها إلى ثلاث مجموعات اعتمادا على الخصوبة وهي العقم الأولي ، العقم الثانوي والخصوبة تم جمع العينات من نوفمبر ٢٠٢٠ حتى مايو ٢٠٢١.

تم قياس مستويات ZAG ومستويات HSP70 باستخدام تقنية ELISA من شركة Bioassay Lab - الصين والمتغيرات الكيميائية الحيوية التي تم حساب الوزن المحسوب لمؤشر كتلة الجسم مقسومًا على الارتفاع المربع بالمتر (كجم / م ٢) الهرموني LH و FSH و Testosterone وسكرالتراكمي HbA1c عن طريق المقايسة المناعية الفلورية او التألق المناعي (Boditech –Korea)) ، تم قياس مستوى الجلوكوز في الدم أثناء الصيام (FBG) بواسطة مقياس الطيف الضوئي ((Liner-SPAIN، HOMA-IR ، الأنسولين الصائم بواسطة (Cobas e411 (Roche-Germany).

النتائج

أظهرت نتائج هذه الدراسة أن متوسط مستويات ZAG (ميكروغرام / مل) وجد انخفاضًا كبيرًا في النساء المصابات بمتلازمة تكيس المبايض مقارنة بمجموعة التحكم (P <0.001) ، ووجدت أن الارتفاع الكبير في المؤشرات الحيوية الهرمونية التي تقيس هرمون التستوستيرون ، LH ، نسبة LH / FSH والعلامات الحيوية الأيضية FBGمستوى سكر الصائم و F Insulin مستوى الانسولين ,HOMA-IR مقاومة الانسولين ومستوى السكر التراكمي HbA1c ومؤشر كتلة الجسم BMI في مجموعة المرضى مقارنة بالمجموعات الصحية بينما انخفاض بشكل ملحوظ في هرمون FSH في مجموعات المرضى مقارنة بمجموعات التحكم في P≤0.05. كما توجد علاقة ارتباط معنوية سالبة بين مستوى ZAG ومؤشر كتلة الجسم ومقاومة الأنسولين وهرمون LH 0.001> p.

أظهرت النتائج التي توصلنا إليها في هذه الدراسة بعد تقسيم المجموعات حسب درجة الخصوبة (عقم أولي ، عقم ثانوي ، خصوبة) أن الاضطراب الهرموني ومؤشر كتلة الجسم في المرحلة الأولية كان أعلى من العقم الثانوي في مجموعات المرضى. وجد أيضًا انخفاضًا كبيرًا في قيمة ZAG بين مجموعات العقم الأولي والعقم الثانوي في P≤0.05 والذي قد يعني الانخفاض في مستوى ZAG يؤدي إلى زيادة درجة العقم.

كان متوسط مستويات HSP70 (نانوغرام / مل) في مجموعة المرضى أعلى بكثير من ذلك في المجموعة الضابطة في (P <0.001) وجد أيضًا ارتباطًا إيجابيًا معنويًا بين HSP70 و مقاومة الانسولينIR ، والأنسولين الصائم في P≤0.05، P <0.001 على التوالي

الاستنتاج

ينخفض مستوى ZAG في مرضى متلازمة تكيس المبايض وكان له علاقة سلبي مع مؤشر كتلة الجسم ، ومقاومة الانسولين ، والهرمون اللوتيني . وينخفض مع مستويات غير طبيعية من الهرمونات فيؤثر على الخصوبة ، فعندما ينخفض يؤدي ذلك إلى ارتفاع درجة العقم. هذا يعني أن مستويات ZAG في العقم الأولي أقل من مستوياتها في العقم الثانوي.

يزداد مستوى HSP70 في مريض متلازمة تكيس المبايض وكان له ارتباط إيجابي بمستوى أنسولين الصائم ومقاومة الانسولين IR. وتم العثور على مستويات عالية معنوية لجميع الهرمونات والمعلمات بما في ذلك LH وهرمون التستوستيرون الكلي ومستوى سكر الدم FBG و الأنسولين الصائم والسكر التراكمي HbA1c في النساء المصابات بمتلازمة تكيس المبايض مقارنة مع مجموعة التحكم ، باستثناء انخفاض قيم FSH.يؤدي انخفاض مستويات ZAG في مصل النساء المصابات بالـ PCOS إلى زيادة خطر الإصابة باضطراب التمثيل الغذائي ، ويرتبط ذلك بزيادة خطر الإصابة بالعقم.وترتبط الزيادة في مستويات HSP70 في مصل النساء المصابات بالـ PCOS بزيادة خطر الإصابة باضطراب التمثيل الغذائي ومضاعفاتها في زيادة العقم.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء/كلية الطب
قسم الكيمياء والكيمياء الحياتية

ارتباط الزنك ألفا ٢ كلايكوبروتين وبروتين الصدمة الحرارية ٧٠ مع متلازمة تكيس المبايض لدى النساء العراقيات المصابات بالعقم

رسالة

مقدمة الى مجلس كلية الطب/ جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الكيمياء الحياتية السريرية

من قبل

لهيب شاكر محمود المياحي

بكالوريوس علوم كيمياء / جامعة بغداد/١٩٩٧

إشراف

ا.م.د. شيماء زهراو ندى
فرع الكيمياء والكيمياء الحياتية
كلية الطب – جامعة كربلاء

١٤٤٣ هـ

2021 م