بســم الله الرحمن الرحيــم

Sudan University of Science and Technology (SUST)

College of Graduate Studies

Estradiol and Cholesterol level among Females with Polycystic Ovarian Syndrome in Khartoum State

مستوى الا ستراديول و الكولسترول فى الإناث المصابات بمتلازمة تكيس المبايض في ولاية الخرطوم

A dissertation submitted in partial fulfillment for the requirement of M.Sc. degree in Medical Laboratory Science - Clinical chemistry

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قال تعالى:

(ويَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ العِلْمِ إِلَّا قَلِيلًا).

صدق الله العظيم

سورة الاسراء (الاية رقم٨٥)

Dedication

To …the masses of the Sudanese people, who are holding the burden of suffering and the cause…

To… the martyrs who sacrificed their souls cheaply to build a democratic homeland for us...!

To all the mothers, the tea and the marginal works that are struggling for a decent living.

To My Father; who taught me Patriotic love & the meaning of Humanity…

To My Mother soul…

To my brothers, sisters and friends …

Sincere gratitude extending any person who assisted me in one way or another.

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Abstract

Polycystic ovary syndrome (PCOS) is a common endocrine disease in women, characterized by heterogeneous presentation of hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology (PCOM).

This study was carried out to assess plasma levels of cholesterol and estradiol among female with polycystic ovarian syndrome (PCOs). Forty clinically diagnosed polycystic ovarian syndrome (PCOs) in the period between February to May 2017,were chosen randomly from Alsir Abu Hassan Center for Fertility in Khartoum State, and forty apparently healthy individuals as control group, to evaluate the plasma levels of cholesterol and estradiol .Plasma estradiol was measured by using ELISA machine and cholesterol measured by BS-380 chemistry analyzer and results were analyzed using Statistical Package for Social Science (SPSS), computer programmed version 11.5.

Anti-Müllerian hormone (AMH) used for the diagnosis of PCOs and when it is levels in the patients compared to the control group. (Mean±SD: 10.6±5.92 versus 1.4±0.22) ng/ml respectively with *P.Value* 0.000. The study showed that, the mean plasma levels of cholesterol was significantly increased in PCOs female patients. (Mean±SD: 207.6±33.4 versus112±33.4 mmoL /L) respectively with P.vale 0.000.

Also the finding of this study showed that, estradiol levels were insignificant difference in PCOs compared with control group. (Mean±SD: 86±39versus 101±38.6 pg/ml) respectively with *P.Value 0.086.*
Person correlation showed that, there was insignificant negative correlation between cholesterol level and the level of estradiol(r= -0.123, *P.Value* = 0.448). It is concluded that: the plasma levels of cholesterol is higher in PCOs Female patients.

مستخلص الدراسة

متلازمة تكيس المبايض (PCOs) هو المرض الاكثر شيوعا في النساء يصيب الغدد الصماء ، وتتميز باظهار اعراض غير متجانسة من فرط الأندروجين،اختلال التبويض واشكال تكيس فى المبيض (PCOM).

وقد أجريت هذه الدراسة لقياس مستويات البلازما من الكوليسترول وهرمون الاستراديول بين النساء المصابات بمتلازمة المبيض عديد التكيس (PCOs).

تم جمع 40 عينة مشخصة بمتلازمة تكيس المبايض خلال الفترة من فبراير إلى مايو 2017م، وتم اختيارهن عشوائيا من مركز السر أبو حسن للخصوبة في ولاية الخرطوم، وأربعين من الأفراد الأصحاء كمجموعة تحكم، لتقييم وتقدير (PCOs) على مستويات البلازما من الكوليسترول واستراديول.

تم تحليل هرمون استراديول باستخدام جهاز إليزا و الكولسيترول باستخدم جهاز التحليل الكيميای bs- 380والنتائج باستخدام حزمة إحصائية للعلوم الاجتماعية (SPSS)، الكمبيوتر المبرمج النسخة 11.

(AMH) استخدمت لتشخيص (PCOs) وعندما قارنت مستوياته في المرضى مع مجموعة التحكم (10.6 ± 5.92 مقابل 1.4 ± 0.22) نج / مل وكان الاحتمال الاحصائى للمقارنة 0.000

وأظهرت الدراسة أن مستويات البلازما من الكوليسترول اظهر زيادة كبيرة في المرضى الذين يعانون من متلازمة تكيس المبايض (207.6 ± 33.4 مقابل 112 ± 33.4مم / ل وكان الاحتمال الاحصائى للمقارنة 0.000).

كما اظهرت نتائج الدراسة ان مستويات استراديول كانت ضعيفة في متلازمة تكيس المبايض مقارنة مع مجموعة التحكم. (86 ± 39 مقابل 101 ± 38.6 بج / مل وكان الاحتمال الاحصائى للمقارنة 0.08 أظهرت علاقة الارتباط وجود علاقة سالبة غير معنوية بين الكوليسترول والاستراديول (معامل بيرسون للارتباط=- .123، مستوى المعنوية=0.448) .

وخلصت الدراسة إلى أن مستويات البلازما من الكولستيرول قد زادت بشكل ملحوظ في المرضى الذين يعانون من متلازمة تكيس المبايض.

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List of abbreviations

ACTH	Adrenocorticotropic hormone
AE	Androgen Excess
AES	Androgen Excess Society
ASRM	American Society for Reproductive Medicine
ASNs	Androgen-secreting neoplasms
AMH	Anti-Müllerian hormone hormones
BMI	Body mass index
CAH	Classic congenital adrenal hyperplasia
CYP	Cytochrome P
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrostenedione sulfate
DHT	Dihydrotestosterone
ESHRE	European Society for Human Reproduction and Embryology
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HDL	High density lipoprotein
LH	Luteinizing hormone
mRNA	Massager Ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCAH	Non-classic congenital adrenal hyperplasia
NICHD	National Institute of Child Health and Human Development
NIH	National Institutes of Health
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovary syndrome
P450	Protein 450
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
STRAW	Stages of Reproductive Aging Workshop
TBG	Thyroxine-binding globulin
TSH	Thyroid-stimulating hormone
T/C	Thymine /Cytosine
UK	United Kingdom
UTR	Untranslated region
HMG	Hydroxyl-3-methylglutaryl
COA	Coenzyme A
CAMP	Cyclic adenosine monophosphate

Chapter one

Introduction, Rationale

Objective

1.1. Introduction:

Polycystic ovarian syndrome (PCOS) is the most common endocrinopathy of women , is combination of chronic an ovulation or oligomenorrhoea and clinical or biochemical hyperandrogenism and ovarian polycystic changes observed by ultrasound (Damonti,2008), affect about 6–8% of the population (Rudnica,2016). In recent years, acceptance of the concept that PCOS is a heterogeneous disorder (that is, capable of having somewhat different manifestations in different people) and the exact cause not known until now, and it is a very common problem among patients attending infertility clinics. The diagnosis depend on above criteria and rule out other causes of hyperandrogenism .Recent study conducted to assess Serum Anti-Müllerian hormone as laboratory predictor in infertile women with PCOS (tayrab.et al,2014).17B-oestradiol is the principle hormone produce by the ovaries also synthesized in the placenta from androgens secreted by the fetal adrenal glands(William *et al.,* 2012).Dyslipidemia is the most common abnormality in PCOS (Lergo et al ,2001).Women with PCOS would be predicted to be at high risk for dyslipidemia because they have elevated androgen levels and are frequently obese. Moreover, since they are also often hyperinsulinemic and insulin resistant, they would also be expected to be at increased risk for the dyslipidemia associated with insulin resistance. (DeFronzo,1997).

1.2-Rationale

Polycystic ovary syndrome (PCOS) is the most common endocrine disease in women worldwide in reproductive age. Dyslipidemia increase the risk of cardiovascular disease .Almost all scientific papers or researches agree there is excess cholesterol in PCOS patients

Ability to measure total cholesterol accurately is essential for establishing the increase risk of cardiovascular disease

The measurement of cholesterol and estradiol may be used as biochemical marker for strength and supporting the diagnosis of polycystic ovary syndrome (PCOS).

There is no research conducted or scientific papers published in the Sudan in this subject exactly and very little at the level of world.

1.3-Objectives

General objective

To evaluate the plasma cholesterol and estradiol levels among female with polycystic ovarian syndrome.

Specific objectives

1. To estimate the plasma level of cholesterol and estradiol among female with polycystic ovarian syndrome compared to apparently healthy female as control group.

2. To correlate between plasma level of cholesterol and estradiol in female with PCOS and other variable.

Chapter two

Literature Review

2. Literature review

2.1-Polycystic ovary syndrome

An international consensus definition of PCOS has defined patients with PCOS at least 2 of the following criteria: Oligomenorrhoea or amenorrhea. Clinical and/or biochemical signs of excessive androgen secretion. Presence of at least 12 follicles measuring 2-9mm in diameter, an ovarian volume >10ml, or both. Only one ovary needs to meet this criterion. Although ultrasound scan is therefore not essential to make the diagnosis. PCOs is very common having prevalence in women of child bearing age 5-10% and may be higher in women of South Asian origin. There is no single diagnostic criterion to confirm the clinical diagnosis. clinical manifestation include infrequent or absent menses, anovulatory infertility, signs of androgen excess (hirsutism ,acne or amenorrhea) although the classical profile of PCOS is that of hyper secretion of LH and androgens with normal concentrations of FSH, a wide spectrum of findings are seen and abnormalities in LH are not always present. In addition to establishing the diagnosis, it is also important to exclude disorders with similar presenting features such as Classic congenital adrenal hyperplasia , Cushing's syndrome and androgen-secreting tumors. many women with PCOS have an increased risk of insulin resistance which, with the prevalence of obesity, is a powerful risk factor for progression to type 2 diabetes .They also have an increased long-term risk of endometrial hyperplasia /cancer (Simon *et al*., 2013).

Polycystic ovary syndrome (PCOS) is a common hormone disorder that affects approximately 5 million women of reproductive age in the United States. Women with PCOS have difficulty becoming pregnant (i.e., are infertile) and may have high levels of androgen hormones from the ovary and adrenal gland. In addition to fertility impairment, a woman with PCOS may have some of the following symptoms and findings:- Irregular or no menstrual periods in women of

reproductive age (ovulatory dysfunction), acne, weight gain, excess hair growth on the face and body, thinning scalp hair, ovarian cysts (polycystic ovarian morphology) and Mental health problems. Women with PCOS are often resistant to the biological effects of insulin and, as a consequence, may have high insulin levels. Women with PCOS are at risk for type 2 diabetes, high cholesterol, and high blood pressure. Obesity also appears to worsen the condition. The degree of obesity may vary by ethnicity. In 1990, the National Institute of Health (NIH) held a conference on PCOS to create both a working definition of the disorder and diagnostic criteria. The outcome of this conference, the NIH Criteria, served as a standard for researchers and clinicians for more than a decade. In 2003, a consensus workshop in Rotterdam in the Netherlands developed new diagnostic criteria, the Rotterdam Criteria. The Androgen Excess (AE) and PCOS Society proposed the AE-PCOS Criteria in 2006 (David *et al.,* 2012).

Hyperandrogenism is very frequent in adolescent girls and is a source of concern for the girl herself, her family, and the clinician. Androgen excess during puberty produces a variety of clinical signs and symptoms that must be appropriately recognized, evaluated, and treated. Unfortunately for the pediatric endocrinologist, the criteria are so broad that many adolescents are presenting with transitory functional hyperandrogenism and menstrual disorders during puberty risk being misdiagnosed. The well known long-term sequelae of PCOS now present a challenge for pediatric endocrinologists to make an early diagnosis (in the pubertal period) and to treat these teenagers both symptomatically and prophylactically. The striking trend toward adolescent obesity should reinforce our responsibilities. We therefore propose to screen for PCOS all adolescents presenting oligo-amenorrhea within two years after menarche, particularly if hyperandrogenism is associated with low birth weight, family history of PCOS, abdominal obesity, and/or insulin resistance (Charles *et al.,* 2006).

This is a condition showing features of hyperandrogenism with anovulation and abnormal ovarian morphology and is the most common cause of anovulatory infertility. Presenting clinical symptoms may also include hirsutism, menstrual disturbances, enlarged polycystic ovaries and infertility. Plasma testosterone and androstenedione concentrations are often increased. The plasma LH may be elevated with normal FSH. Because plasma SHBG concentrations are reduced in obese individuals, the plasma concentration of free testosterone is often increased. The plasma prolactin concentrations may also be high. Multiple small sub capsular ovarian cysts may be demonstrated on ultrasound scanning of the ovaries. Polycystic ovary syndrome is also associated with insulin resistance, obesity and elevated plasma insulin concentrations, which may stimulate androgen production from the ovarian theca interna cells. Individuals may also have hyperlipidaemia, glucose intolerance and hypertension (Martin, 2012).

2.1.1-Androgens excess

 A patient with androgen excess has variable degrees of excess hair on the face, chest, abdomen and thighs, acne, and obesity. PCOS is clinically defined by hyperandrogenism with chronic an ovulation without underlying disease of the adrenal or pituitary glands. This syndrome characterized by infertility, hirsutism and obesity (in approximately half of those affected), and various menstrual disturbances from amenorrhea to irregular vaginal bleeding. Relatively low FSH concentrations and disproportionately high LH concentrations are common in PCOS. Serum androstenedione and testosterone (total and free concentrations) are elevated with mean concentration 50%to150% higher than normal. PCOS patient have substantial estrogen production because of the peripheral conversion of androgens to estrogen. The anovulation is caused by continuous estrogen stimulation of the endometrium (Carl *et al*., 2006).

2.1.1.1-Hirsutism

Hirsutism is defined as the excessive growth of terminal hair in women and child in distribution similar to that occurring in post pubertal men (Carl *et al.,* 2006). Clinical features of hyperandrogenism frequently seen in PCOS include hirsutism, acne, and androgenic alopecia. Here, we review the prevalence of these features in this disorder hirsutism is the presence of terminal hairs on the face and/or body in a female in a male-type pattern. The most common method of determining the presence of hirsutism uses a visual score. (Azziz *et al*., 2009). Various methods have been proposed. The most commonly used method is a modification of a method originally reported by Ferriman and Gallwey. Nine body areas, including the upper lip, chin, chest, upper back, lower back, upper and lower abdomen, upper arm, and thigh, are assigned a score of 0–4 based on the density of terminal hairs. A score of 0 represented the absence of terminal hairs, a score of 1 minimally evident terminal hair growth, and a score of 4 extensive terminal hair growths. The cutoff value should be established after the study of a large population of unselected women. Using this approach, cutoff values for defining hirsutism have been variously reported to be a score of 6 or greater, 7 or more, and 8 or more.(Azziz *etal*.,2009). However, we should note that the prevalence of hirsutism in PCOS will vary according to the race and ethnicity of the population being studied. These data suggest that the degree of body and terminal hair growth and the prevalence of hirsutism are not significantly different between unselected White and Black women. Consequently, it is likely that there will be little difference in the prevalence of hirsutism between Black and White PCOS women, although this remains to be confirmed. Consistent with the lower population prevalence of hirsutism observed in East Asian women, a comparative study of patients with PCOS from the United States (primarily Mexican Americans), Italy, and Japan noted that Japanese women had a significantly lower mean hirsutism score than their non-

Asian counterparts. However, the lesser prevalence of hirsutism among East Asian PCOS patients may not extend to all groups in the region. For example, wijeyaratne and colleagues observed that hirsutism was more prevalent and more severe among PCOS patients of Southern Asian extraction (Pakistani, Bengali, Gujarati, or Dravidian Indian) than Whites. Likewise, among women of Indian descent in New Zealand, about two thirds of women with PCOS presented with clinical evidence of hirsutism, similar to the prevalence found in women of European, Maori, and Pacific Island descent. Although it is clear that there is racial variation in hair growth patterns, race-specific normative ranges have not been well established, which is required to determine whether a particular woman has excessive amounts of body of facial hair. Overall, hirsutism is an important feature of PCOS, affecting approximately 65% to 75% of patients with PCOS, including women of White, Black, and Southeast Asian race. The prevalence of hirsutism in PCOS is likely to be less among women of East Asian extraction (Azziz *et al*., 2009).

2.1.1.2-Acne

Acne affects approximately 12% to 14% of white PCOS patients although the prevalence of this dermatologic abnormality varies with ethnicity: it is reportedly higher in Asian Indians and lower in Pacific Islanders. In a study of 248 women with PCOS in Italy, acne alone in the absence of other pilosebaceous features was present in 23.4%. Among 716 patients with PCOS, 14.5% presented with acne, either alone or in combination with hirsutism. In a prospective study of women presenting for blood donation, Asuncion and colleagues noted that of the 10 women diagnosed with PCOS, four (40%) had acne, three without associated hirsutism. However, various surveys have noted a relatively high prevalence of acne in the general population, particularly among younger women. Approximately 20% of individuals in their midteens and 15% of those in their early 20s complain of acne; even 10% of women in their 30s and 5% of women

40 to 60 years old will complaint of, albeit mild, acne. Consequently, the degree to which PCOS increases the risk of acne above the general population prevalence is unclear. The variability in the prevalence of acne is compounded by the fact that there is no single scoring system used. Overall, although acne affects 15% to 25% of PCOS patients, it is unclear whether the prevalence of acne is significantly increased in these patients over that observed in the general population (Azziz *et al.,* 2009).

2.1.1.3-Androgenic alopecia

Scalp hair loss in women is a distressing complaint with significant psychologic morbidity. It usually represents the pilosebaceous unit response to endogenous androgens and may be associated with acne and hirsutism. Androgen sensitivity of the pilosebaceous unit varies, and there is poor correlation between clinical features and evidence of biochemical hyperandrogenism. The presence of DHT, formed from the 5a-reduction of tin the dermal papilla, is associated with a higher 5a-reductase activity in the hairs plucked from a scalp presenting with androgenic alopecia. In addition to androgen excess, other potential etiologies of alopecia or diffuse scalp hair loss in any woman may be genetic (i.e., familial premature scalp follicular loss), environmental (e.g., damage following the use or abuse of hair cosmetics), and nutritional (e.g., poor protein intake, zinc deficiency, iron-deficient anemia). Androgenic alopecia is a recognized sign of PCOS. However, the prevalence of this abnormality in PCOS is unclear. Although we previously noted that PCOS patients may account for _10% to 40% of all women with alopecia, literature defining the incidence of alopecia in either normal women or women with PCOS is sparse. The pattern of hair loss in PCOS generally involves thinning of the crown with preservation of the anterior hairline. Androgenic related alopecia in women with PCOS tends to be seen in the anterior midvertex area extending to the crown. The anterior hairline remains intact in women with PCOS and significant a bit emporal scalp hair recession is unusual except in

virilizing syndromes. Unfortunately, a loss of at least 25% of scalp hair is needed before a woman becomes aware of thinning of her scalp hair. The sole presence of alopecia or diffuse scalp hair loss in women may be the sole dermatologic sign of PCOS (Azziz *et al.,* 2009).

2.1.1.4-Amenorrhoea

Amenorrhoea can be primary (menstruation has never occurred) or secondary. Oligomenorrhoea is sparse or infrequent menstruation; it can be due to less severe forms of some of the causes of amenorrhoea. Primary amenorrhoea can occur as part of the syndrome of female hypogonadism, but can also be present in normally feminized women. The commonest cause of amenorrhoea in women of child-bearing age is pregnancy, and this possibility, however unlikely, must always be excluded. The finding of an apparently high plasma LH concentration may suggest pregnancy before a pregnancy test is performed: chorionic gonadotrophins cross-reacts in some assays for LH. Pregnancy apart, amenorrhoea in normally feminized women is most frequently due to a hormonal disturbance that results in a failure of ovulation. Causes include: disordered hypothalamo-pituitary function, related to weight loss (30–35% of cases in most series) or hyperprolactinemia (10–12%), but idiopathic in some 10% of cases, ovarian dysfunction (e.g. autoimmune disease leading to premature menopause) (10–12%), increased androgen production (particularly polycystic ovary syndrome (PCOS) and late-onset congenital adrenal hyperplasia) (30–35%). Weight loss can lead to a decrease in the frequency of the pulsatility of GnRH secretion and thus decreased secretion of LH and FSH. Menstruation almost always ceases if weight falls below 75% of the ideal, and may do so with smaller losses. Regular menstruation returns if weight is regained. Severe stress and intensive exercise regimens, such as are adopted by elite long-distance runners, ballet dancers and gymnasts, can also lead to amenorrhoea, probably for complex neuroendocrinological reasons in addition to any effect of decreased body weight.

Amenorrhoea due to excessive androgen secretion is often associated with hirsutism or even virilism. Uterine dysfunction is an uncommon cause of amenorrhoea. It can be excluded, if necessary, by the progestogen challenge test. If medroxyprogesterone acetate is given orally (10 mg daily for 5 days), the occurrence of vaginal bleeding 5–7 days later signifies that the uterus was adequately oestrogenized. If bleeding does not occur, the test is repeated, giving oestrogen (ethinyloestradiol, 50 mg daily for 21 days, with progestogen on the last 5 days). Absence of bleeding indicates uterine disease. If bleeding occurs, oestrogen deficiency is present. The diagnosis of hormonal causes of amenorrhoea requires basal measurements of plasma FSH, LH and prolactin concentrations. A high FSH concentration is indicative of ovarian failure (and is more sensitive in this respect than LH). If LH, but not FSH, is elevated, and the patient is not pregnant, the most likely diagnosis is PCOS, and pelvic ultrasonography should be performed. If LH and FSH concentrations are normal or low, a pituitary or hypothalamic disorder should be sought, by anatomical studies and dynamic testing of the hypothalamo-pituitary axis in a manner similar to that described for male hypogonadism. As in males, however, the results of such tests do not always distinguish between pituitary and hypothalamic disorders. The management of amenorrhoea depends on the cause, and whether fertility is required. In hyperprolactinemia, the treatment is directed to the underlying cause wherever possible (e.g. withdrawal of drugs, treatment of hypothyroidism). In ovarian, pituitary or hypothalamic disease, when fertility is not required, cyclical oestrogen and (if the patient has a uterus) progestogen replacement is given. In established ovarian failure, pregnancy is only possible using donated ova. If fertility is required in pituitary failure, treatment is with human FSH and LH; HCG may be required to mimic the mid-cycle LH peak and stimulate ovulation. Careful monitoring of plasma oestradiol concentrations is necessary to detect hyper stimulation, which carries a risk of multiple pregnancies and the production of ovarian cysts. Patients with hypothalamic disease may

respond to clomiphene. This substance blocks oestradiol receptors in the hypothalamus and may stimulate GnRH (and thus LH and FSH) secretion. Nonresponders are treated with pulsatile GnRH. Clomiphene is also useful in inducing ovulation in patients with PCOS. When it has not been possible to distinguish between hypothalamic and pituitary disease, a failure to respond to pulsatile GnRH sug gests that amenorrhoea is due to pituitary dysfunction (William *et al*., 2012).

Amenorrhea due to androgen excess can be due to adult onset CAH, corticotropin-dependent Cushing syndrome, or polycystic ovary syndrome (PCOS). Some individuals with 21-hydroxylase deficiency do not manifest any developmental abnormalities or salt wasting, but they present with signs of androgen excess. This clinical syndrome, referred to as nonclassic, adult-onset, or late-onset CAH, may be clinically indistinguishable from PCOS. Serum androstenedione and testosterone concentrations (total and free concentrations) are elevated, with mean concentrations 50 to 150% higher than normal. Abnormal bleeding patterns seen in PCOS are due to chronic anovulation and lack of progesterone stimulation and withdrawal. Chronic estrogen exposure without progesterone may predispose patients to endometrial cancer. Some attempt has been made to link PCOS to leptin, a hormone that is secreted by adipocytes and is thought to play a role in regulating food intake and metabolism. Animals that lack leptin are infertile; leptin injection increases gonadotropin secretion and restores fertility. For women with PCOS who wish to conceive, treatment is aimed at ovulation induction. Weight reduction should be attempted first in those women who are overweight, as it often helps to promote ovulation. If ovulation does not occur, then medications such as clomiphene citrate, metformin, and aromatase inhibitors may be useful. Ovarian hyperthecosis, a non-neoplastic lesion of the ovary characterized by the presence of islands of luteinized thecal cells in the ovarian stroma, is sometimes confused with PCOS (Carl *et al.,* 2006).

2.1.1.5-Infertility

Infertility is a common clinical problem, leading approximately one in six couples in the UK to seek professional advice. Investigation is usually considered appropriate when a couple has been unable to conceive after 12 months of trying, assuming regular, unprotected intercourse. It can be primary (conception has never occurred) or secondary, and due to problems affecting either the male or the female. Ovulatory failure, due most frequently to hyperprolactinemia or hypothalamic–pituitary dysfunction, is responsible in approximately 20% of cases, and defective sperm production in about one-quarter. Endocrine causes of infertility are rare in males. A couple in their late 20s was infertile in spite of regular intercourse over a two-year period. Each partner had a child by a previous marriage. The woman's periods had recently become irregular. A semen sample contained a normal count of motile sperm. Physical examination revealed no abnormality. The woman was on steroid replacement treatment for adrenal failure, which had been diagnosed in her late teens (William *et al.*, 2012).

Infertility can result from ovulatory or uterine problems; mechanical problems, including obstruction of the fallopian tubes; male fertility factors; or multiple factors in either sex or combined female and male factors. Ovulatory problems are the most common cause of female infertility. Polycystic ovarian syndrome (PCOS) affects up to 5% of reproductive-age women. It is the most common cause of ovulatory infertility. PCOS is a condition characterized by multiple ovarian cysts, often found in a row, resembling a "string of pearls." Ovarian cysts are fluid-filled sacs arising from follicles swollen with fluid that are prevented from producing mature oocytes. Patients with PCOS also have hormonal imbalances, including decreased levels of LH, FSH, and progesterone and increased androgen production, including excess testosterone and DHEAS causing hirsutism or male facial patterns of hair growth. Insulin resistance is a common associated condition. PCOS is generally diagnosed when two of the following three criteria are present and other possible causes can be ruled out:

clinical or laboratory results showing excess androgen secretion, decreased or absence of ovulation, and ovaries found by imaging techniques such as ultrasound to contain many cysts. Although the exact etiology of the problem is still unknown, genetic factors may be involved. Around the time of menopause, impairment of ovulation may cause infertility with adverse effect on follicle size and oocytes quality despite regular ovulation and normal gonadotropin levels. These factors are considered when treating older women with infertility. Serum levels of LH, FSH, and inhibin A and B may be helpful in assessing infertility and treatment options. Infertility diagnostic testing is as varied as treatment options. The patient workup for infertility includes a careful, detailed history, which can help to limit the number of laboratory tests required. Availability of tests varies from center to center, so availability is one of the considerations for infertility testing. Typical laboratory tests ordered are FSH on day three of the ovulatory cycle, LH, estradiol, prolactin, and TSH levels. Measurement of ovarian and adrenal androgens such as testosterone and DHEAS should be decided on the basis of ovulatory status of the patient and the clinical picture. (Wendy, 2007).

Infertility is defined as the inability to conceive after 1 year of unprotected intercourse. It has been estimated that 93% of healthy couples practicing unprotected intercourse should expect to conceive within 1 year, and 100% will be successful within 2 years. a specific cause of infertility is identified in ≈80% of couples: one third are due to female factors alone, one third to male factors alone, and one third to a combination of problems. Primary infertility refers to couples or patients who have had no previous successful pregnancies. Secondary infertility encompasses patients who have previously conceived, but are currently unable to conceive. These types of infertility generally share common causes. Infertility problems often arise as a result of hormonal dysfunction of the hypothalamic-pituitary-gonadal axis. Measurements of peptide and steroid hormones in the serum are therefore essential aspects of the evaluation of

infertility. This section focuses on hormonal and biochemical aspects of evaluating infertility (Carl *et al*., 2006).

2.1.2-Genetic of Polycystic Ovarian Syndrome

The mode of inheritance of PCOS remains unknown, and recent studies indicate that this disorder could be a complex trait. This means that several genes are interacting with environmental factors to provoke the phenotype. In contrast, biochemical parameters, including fasting insulin levels or hyperandrogenemia, seem to be highly heritable parameters, suggesting that some clinical signs, symptoms, or biochemical parameters of PCOS could be transmitted as Mendelian autosomal dominant or X-linked traits, but the genetic studies have not as yet concluded the pattern of heredity. While studies, so far, are unable to exclude an autosomal or X-linked dominant mode of inheritance, the heritability of PCOS is probably more complex, similar to that of type 2 diabetes mellitus or cardiovascular disease. However, a positive family history appears to be the most informative risk factor for the development of PCOS. Furthermore, environmental factors alter the clinical and biochemical presentation in those with genetic predisposition to PCOS. A relation between PCOS with the X chromosome aneuploidies and polyploidies in addition to other cytogenetic abnormalities has been confirmed. Some of the cases of PCOS may represent an intermediate condition in a spectrum that extends from the streak gonad of Turners syndrome to the normal ovary. The concept is that at least some cases of PCOS may be due to X chromosomal factors causing an abnormal follicular apparatus. In addition, large deletion of the long arm of chromosome 11 was seen in some of the PCOS cases. However, there is no large cytogenetic study to identify karyotype abnormalities. There are different candidate genes as a cause of PCOS; such as genes involved in steroid hormone synthesis and action, genes involved in carbohydrate metabolism and fuel homeostasis, genes involved in gonadotropin action and regulation; and genes in the major histocompatability region, which could account for certain PCOS features. Increased androgen secretion and

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insulin resistance persist in cultured theca cells and skin fibroblasts, respectively, from women with PCOS, which suggest that these are intrinsic, presumably genetic, defects. Different studies have indicated a genetic susceptibility to PCOS. It was shown that polycystic ovaries and hyperandrogenemia are present in 50% of sisters of affected women. Therefore genetic analyze of candidate genes have been performed. Both linkage and association studies have suggested that PCOS can be explained by the interaction of a small number of key genes with environmental, particularly nutritional factors. Hyperandrogenemia is genetically determined and the result of familial studies indicating that hyperandrogenism clusters as a dominant genetic trait. The steroid synthesis gene CYP11a, coding for P450 cholesterol side chain cleavage and the insulin gene regulatory region may be involved. However, it is unlikely that the hyperandrogenemia of PCOS is principally determined by polymorphisms or mutations in the genes encoding a single steroidogenic enzyme activity, such as CYP17 or CYP11a. In addition, an increase of mRNA abundance in PCOS has been found in corresponding to the genes of aldehyde dehydrogenase-6 and retinol dehydrogenase-2, which both increases the expression of 17a-hydroxylase. Recent studies have found a significant prevalence of CYP21 mutation, gene encode the 21-hydroxylase enzyme mimic the PCOS phenotype, in the supposed PCOS population (Sheikhha *et al*., 2007).

The first step in steroidogenesis is the conversion of cholesterol into progesterone, catalyzed by the P450 Cytochrome side chain cleavage enzyme encoded by CYP11a gene located at 15q. Investigation of CYP11A gene showed a significant association between serum testosterone levels and the alleles of the CYP11a with a 5' untranslated region (UTR) consisting of repeats of a (tttta) n pent nucleotide, a variable number tandem repeat (VNTR) polymorphis. Further investigation is required due to these controversial results in order to confirm a role in the a etiology of PCOS of this gene. Another part in steroidogenesis is the conversion of 17-hydroxyprogesterone into 11-deoxycortisol which is catalyzed

by the 21-hydroxylase enzyme encoded by CYP21. The deficiency of this enzyme is responsible for most cases of congenital adrenal hyperplasia and increased serum 17-hydroxyprogesterone levels are correlated with its deficiency. It is a common finding among women with functional hyperandrogenism or PCOS an increased serum 17-hydroxyprogesterone response to ACTH stimulation. Furthermore, patients having both heterozygote CYP21 mutations and clinical symptoms exhibit a PCOS-like phenotype. Accordingly, mutations of CYP21 have been investigated as a candidate gene in patients with PCOS. Two studies showed that children with premature pubarche and adolescent girls with hyperandrogenism were heterozygous for mutations in CYP21. On the other hand, there are other researchers that found no clear concordance between the CYP21 genotype and the functional origin of androgen excess. Overall, CYP21 and associated mutations do not seem to play a key role in the development of PCOS. The conversion of pregnenolone and progesterone into 17-hydroxypregnenolone and 17-hydroxyprogesterone, respectively and of these steroids into dehydroepiandrosterone (DHEA) and Δ4-Androstendione (Δ4A) is catalyzed by the P450c17α enzyme. This enzyme has both 17α-hydroxylase and 17, 20-lyase activities and is encoded by CYP17 located at 10q. It was reported increased P450c17α expression and enzymatic activity in ovarian theca cells from women with PCOS as well as increased transactivation of the CYP17 promoter. Moreover, it was showed that CYP17 expression is dysregulated at the level of mRNA stability in PCOS theca cells. Another study identified a rare T/C single nucleotide polymorphism (SNP) in the promoter region of CYP17 increasing the susceptibility to develop PCOS. Subsequently, more comprehensive studies have failed to detect a significant linkage between CYP17 and PCOS. Although CYP17 gene does not seem to be a candidate gene in the pathophysiology of PCOS, it should be noted that post-translational regulation of this gene product might play a role in the pathophysiology of PCOS. The enzyme complex aromatase converts androgens to estrogens. This enzyme complex is composed of

the Cytochrome P450 aromatase and the NADPH Cytochrome P450 reductase, and P450arom is encoded by CYP19 located at 15p. Aromatase deficiency has been reported in a number of hyper androgenic patients. It has been demonstrated that granulosa cells obtained from medium-sized follicles of women with PCOS have little aromatase activity. Similarly, it has been showed that when compared to the control follicles, all PCOS follicle contained low levels of P450arom mRNA, estradiol, and lower aromatase stimulating bioactivity. These findings indicate that the aromatase activity might be decreased in PCOS follicles, and that the possible androgen excess resulting might contribute to abnormal follicle development. Association studies utilizing SNPs and haplotypes showed association with PCOS symptoms and serum testosterone levels (Prapas *et al.,* 2009).

2.1.3-Obesity and Complication in Polycystic Ovarian Syndrome

Obesity is a feature of PCOS and about half of the patients presenting in secondary care have a body mass index of greater than 30. A further 25% are 'overweight', with a body mass index of greater than 25. Ideal body mass index is considered to be between 20 and 25. Body mass index (BMI) is calculated as the weight in kg divided by the height in meters squared (kg/m2). All features of PCOS are made worse by increased weight and are ameliorated by weight loss. Insulin resistance is considered to be present when the body requires more insulin than normal to regulate glucose homeostasis. In the general population, a relationship between obesity and insulin resistance has been recognized for many years. The more obese a patient is the more insulin resistant they are likely to be. Not all women with PCOS can be shown to be insulin resistant. However, insulin resistance, when present in PCOS is greater than can be accounted for by obesity alone. Insulin resistance proceeds, and is a strong predictor for, type 2 diabetes, which is more common in young women with PCOS. In PCOS the incidence of type 2 diabetes and hypertension also increases with age. Insulin resistance is also

associated with specific atherogenic abnormalities of lipoprotein metabolism which are raised triacylglycerols and low HDL cholesterol. Altered endothelial function is also recognized. These abnormalities are important features of the metabolic syndrome, which predicts higher risk of later diabetes and cardiovascular disease in middle age. In spite of many studies showing increased cardiovascular risk markers in PCOS, strong evidence for increased cardiovascular mortality, a hard end point, is lacking. Pregnancy outcomes in polycystic ovary syndrome Patients with PCOS have an increased risk of gestational diabetes. There is also an increased risk of pregnancy associated hypertension and pre-eclampsia. Babies tend to be delivered early and are more likely to be admitted to a neonatal intensive care unit. Perinatal mortality is higher. Whether these abnormalities are specifically associated with PCOS remains to be established as similar problems are found in obese women without PCOS. Insulin and androgen action Insulin, in addition to its essential role in regulating glucose homeostasis, is a hormone of many actions. Some of these are particularly relevant to the abnormalities found in PCOS. Insulin, together with LH, acts on the ovary to increase the ovarian secretion of androstenedione and testosterone. Insulin directly increases androstenedione secretion by ovarian theca cells acting via its own receptor. Measures which decrease insulin secretion by reducing insulin resistance, such as dietary manipulation, and treatment with insulin sensitizing agents such as metformin and thiazolidenedione drugs, which reduce circulating insulin, also reduce circulating androgens (Nessar, 2011).

 Sex hormone binding globulin, which is lower in patients with PCOS, is secreted by the liver, a major target of insulin action. Insulin down-regulates the hepatic production of SHBG, independent of its actions on glucose regulation. Therefore SHBG is a marker of insulin action on the liver and low SHBG is a marker for insulin resistance. In the circulation, SHBG has a direct role in controlling the concentrations of circulating non-protein bound or free testosterone and DHT. These are regarded as the biologically active fraction. As testosterone and DHT

are not involved in a feedback control with the hypothalamus and pituitary in women, rising SHBG levels reduce their biological availability and vice versa. Thus SHBG is an important controlling factor in the expression of androgen action, especially in women. Therefore insulin has two main effects on androgen action. First, it increases ovarian androgen secretion and second, it enhances androgen bioavailability. Disordered follicular maturation in PCOS The principal cause of infertility due to PCOS is abnormal development of ovarian follicles with the accumulation of small follicles 'the cysts (Nessar, 2011).

Women with PCOS present an adverse reproductive profile, including a high risk of pregnancy-induced hypertension, preeclampsia, and gestational diabetes mellitus. Patients with PCOS present not only a higher prevalence of classic cardiovascular risk factors, such as hypertension, dyslipidemia, and type-2 diabetes mellitus, but also of nonclassic cardiovascular risk factors, including mood disorders, such as depression and anxiety. Moreover, at the moment, clinical data on cardiovascular morbidity and mortality in women with PCOS are controversial. Women with PCOS show an increased risk of endometrial cancer compared to non-PCOS healthy women, particularly during premenopausal period. Currently, we are unable to clarify if the increased PCOS early- and long-term risks are totally due to PCOS or mostly due to obesity, in particular visceral obesity that characterized the majority of PCOS patients. In any case, the main endocrine and gynecological scientific societies agree to consider women with PCOS at increased risk of obstetric, cardio metabolic, oncology, and psychological complications throughout life, and it is recommended that these women be accurately assessed with periodic follow-up(Palomba *et al*., 2015).

2.1.4-Diagnositic Criteria of Polycystic Ovarian Syndrome

In 1990, the first formal attempt to consolidate a clinical definition of PCOS by the National Institute of Child Health and Human Development resulted in PCOS being defined as the combined presence of Clinical and/or biochemical signs of hyperandrogenism and Oligo- or chronic anovulation in the absence of all other reasons for anovulatory infertility. The NICHD criteria were deliberately listed in order of perceived importance. The use of these criteria defined PCOS as a syndrome whose primary determinant was a derangement in androgen homeostasis with consequent effects on menstrual cyclicity. Ultrasonographic evidence of polycystic ovaries was concluded to be "suggestive" of PCOS but not necessarily diagnostic. This prevailing opinion reflected the paucity of British and European attendees at the meeting to define the NICHD criteria, because Ultrasonographic evidence of polycystic ovaries had long been considered definitive evidence of PCOS in the UK and most of Europe. The NICHD criteria represented a very important first step towards establishing a universally accepted clinical definition for PCOS. However, it is important to recognize that the criteria were based on majority opinion and not clinical trial evidence. In the years that followed, it became apparent that the clinical presentation of PCOS was much more variable than that described by the NICHD criteria, and that polycystic morphology of the ovaries was a consistent finding in women demonstrating biochemical and clinical evidence of the syndrome (Marla *et al.*, 2008).

In 2003, the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine amended the consensus criteria to include polycystic ovaries as a third diagnostic marker and to allow for a diagnosis of PCOS if two of three criteria were met Oligo- or chronic anovulation, Clinical and/or biochemical signs of hyperandrogenism and Polycystic ovaries, in exclusion of other etiologies of androgen excess and

anovulatory infertility is necessary. These "Rotterdam criteria" were intended to broaden the phenotypic expression of the syndrome and to redefine PCOS as primarily a syndrome of ovarian dysfunction. The Rotterdam criteria are controversial. Fulfilling two of three diagnostic criteria implies that PCOS can be diagnosed in the absence of androgen excess or menstrual irregularity the very factors that were once considered absolute requisites for the syndrome. While most agree that PCOS exists as a spectrum, it has been difficult to reconcile the absence of androgen excess in the diagnosis (Marla *et al*., 2008).

In 2006, the Androgen Excess Society formed a task force to review existing data on the phenotypic expression of PCOS and Patient demonstrates both: Hirsutism and/or hyperandrogenemia and Oligo-anovulation and/or polycystic ovaries and exclusion of other etiologies of androgen excess and anovulatory infertility are necessary. The AES concluded that although there was good evidence for features of PCOS (e.g., mild insulin resistance and mild ovarian dysfunction) in women with polycystic ovaries, androgen excess, and regular menstrual cycles, there was conflicting evidence supporting the presence of such features of PCOS in women with polycystic ovaries and ovulatory dysfunction but without clinical or biochemical signs of hyperandrogenism. The AES has proposed a new set of diagnostic criteria that acknowledge the wide prevalence of morphologic polycystic ovaries and the wide heterogeneity of PCOS. They do not, however, recognize a mild variant of the syndrome in which little is known about metabolic status or long-term health risks (Marla *et al*., 2008).

All current definitions of PCOS require that other disorders of androgen excess or ovulatory function be excluded. Principally, the former includes 21-hydroxylase-deficient non-classic congenital adrenal hyperplasia (NCAH), Cushing's syndrome, androgen-secreting neoplasms (ASNs), and drug-induced or iatrogenic hyperandrogenism (IH): the latter includes thyroid dysfunction and hyperprolactinemia. NCAH is excluded by the measurement of a follicular phase (pre-ovulatory) basal 17ahydroxyprogesterone (17-HP) level, which if >2–4ng/ml

mandates an acute adrenocorticotropic hormone (ACTH) stimulation test. Alternatively, evaluation for Cushing's syndrome, ASNs, or IH should be instituted if the history and physical exam suggests their possibility .Thyroid dysfunction and hyperprolactinemia can be excluded by the routine measurement of thyroid-stimulating hormone (TSH) and prolactin levels, although the prevalence of these disorders in women with overt hyperandrogenism is relatively low. Overall, patients being evaluated for PCOS should, at a minimum, have NCAH excluded by a basal 17-HP level, and possibly thyroid dysfunction and hyperprolactinemia, by TSH and prolactin levels (Bradley *et al*., 2007)

2.2-Estradiol

 The principal ovarian hormone is 17β-oestradiol.Oestrogens are also secreted by the corpus luteum and the placenta. Oestrogens are responsible for the development of many female secondary sexual characteristics. They also stimulate the growth of ovarian follicles and the proliferation of uterine endometrium during the first part of the menstrual cycle. They have important effects on cervical mucus and vaginal epithelium, and on other functions associated with reproduction. Plasma concentrations of oestrogens are low before puberty. During puberty, oestrogen synthesis increases and cyclical changes in concentration occur thereafter until the menopause, unless pregnancy occurs. After the menopause, the sole source of oestrogens is from the metabolism of adrenal androgens; plasma concentrations fall to very low values. In the plasma, oestrogens are transported bound to protein, 60% to albumin and the remainder to SHBG. Only 2–3% remains unbound. Oestrogens stimulate the synthesis of SHBG and also that of other transport proteins, notably thyroxine-binding globulin (TBG) and transcortin, and thus increase total thyroxine and total cortisol concentrations in the plasma. Slowly rising or sustained high concentrations of oestrogens, together with progesterone, inhibit pituitary gonadotrophin secretion by negative feedback, but the rapid rise in oestrogen

concentration that occurs prior to ovulation stimulates LH secretion (positive feedback). Oestradiol is present in low concentrations in the plasma of normal men. Approximately one-third is secreted by the testes, the remainder being derived from the metabolism of testosterone in the liver and in adipose tissue. The stimulated corpus luteum secretes large amounts of oestrogens and progesterone, but after six weeks the placenta becomes the major source of these hormones. There is a massive increase in the production of oestriol during pregnancy, but production of oestrone and oestradiol increases also. Oestradiol is synthesized in the placenta from androgens secreted by the fetal adrenal glands. Its measurement in maternal plasma or urine was formerly used to assess feto-placental function, but now has been superseded by ultrasonography, which can be used to provide direct measurements of fetal growth and placental blood flow. The same applies to measurements of other placental products, for example human placental lactogen and placental alkaline phosphatase (a heat-stable isoenzyme), which have been used in the past as indicators of placental function (William *et al.,* 2012).

Estrogens are secreted by the ovarian follicles and by the placenta in pregnancy (and to a much lesser extent by the adrenal glands and testes). Estrogen promotes development and maintains the female reproductive system, including the uterus, fallopian tubes, and vagina. It is responsible for development and maintenance of secondary female sex characteristics. Estrogen peaks at midcycle, causing a decrease in FSH but promoting the LH surge at midcycle. There are three primary estrogens: estradiol-17, estrone, and estradiol. Estradiol is the principal estrogen synthesized by the ovaries. Hyperprogesteronemia: Prevents menstrual cycle from occurring and Causes infertility, abortion of fetus (Anna *et al*., 2010).

Dynamic changes in circulating estradiol level, including increase at menarche and decrease at menopause, occur in a woman's lifetime. Circulating estradiol levels decrease drastically during the menopausal transition, though the levels differ among races. It has been reported that estradiol levels in both Japanese and

Chinese women were lower than those in Caucasians, Hispanic and African-Americans. This dynamic decrease in estradiol level induces menopausal symptoms, such as hot flashes and night sweat, urogenital symptoms, osteoporosis, coronary heart disease, stroke and possibly early onset of Alzheimer's disease in postmenopausal women. However, not only estrogen but also other endocrinological hormones may be involved in the occurrence of these diseases. Little attention has been paid to roles of endogenous androgens in women despite the results of studies suggesting that androgens may play important roles. Androgens are known to be important for normal physiology in women and to play key roles in the physical, sexual and emotional well-being of women. Therefore, it is necessary to take account of androgens as well as estrogen when considering women's health (Yasui *et al.,* 2012).

2.3- Cholesterol:

Cholesterol is an unsaturated steroid alcohol containing four rings (A, B, C, and D), and it has a single C-H side chain tail similar to a fatty acid in its physical properties .It is present in dietary fat , and can be synthesize in the liver by mechanism that is under close metabolic regulation . It is fairly water insoluble, it does however contain a polar hydroxyl (OH) group on its A- ring (burtis et al.,2008).

 Cholesterol is, therefore, also an amphepatic lipid and is found on the surface of lipid layers along with phospholipids. It is oriented in lipid layers so that the four rings and the side chain tail are buried in the membrane in a parallel orientation to the fatty acid acyl chains on adjacent phospholipid   molecules. The polar hydroxyl group on the cholesterol A-ring faces outward, away from the lipid layer, allowing it to interact with water by non covalent hydrogen bonding (nowak ,2009)

It can also exist in an esterified form called cholesteryl ester, with the hydroxyl group conjugated by an ester bond to a fatty acid, in the same way as in triglycerides .In contrast to free cholesterol, there are no polar groups on

cholesteryl esters, making them very hydrophobic. Because it is not charged ,cholesteryl esters are classified as a neutral lipid and are not found on the surface of lipid layers but instead are located in the center of lipid drops and lipoproteins, along with triglycerides (Nowak ,2009).

2.3.1-Cholesterol functions:

It is play an important role in: (a) cell membrane structure, (b) Precursor of steroid hormones , bile acids and vitamin D (burtis et al.,2008).

2.3.2 Cholesterol absorption:

All cholesterol in the intestine is present in the un esterified form(free).Esterified cholesterol which contain fatty acid attached to hydroxyl group on the A- ring , is rapidly hydrolyzed in the intestine to free cholesterol and fatty acid by cholesterol esterase secrete from pancreas and small intestine . Before being absorbed , cholesterol is first solubilized through a process called emulsification which occurs by the formation of mixed micelles that contain:

 (a) Un esterified cholesterol

(b) fatty acid

(c)mono glyceride

 (d)phospholipid

(e)conjugated bile acids

Most cholesterol absorption occur in the middle jejunum and terminal ilium mediated by enterocyte surface proteins ,Once cholesterol enter the intestinal mucosal cell , it packaged with TG and phospholipid and large protein called apo lipo protein(apo) B-48 into large lipoprotein particles called chylomicron ,that enter blood stream by lymphatic system (burtis .et al,2008).

2.3.3 Cholesterol Biosynthesis:

About 20–25% of total daily cholesterol production occurs in the liver; other sites of higher synthesis rates include the intestines, adrenal glands, and reproductive organs . Synthesis within the body starts with one molecule of acetyl CoA and one molecule of acetoacetyl-CoA, which are hydrated to form 3-hydroxy-3-

methylglutaryl CoA (HMG-CoA) .This molecule is then reduced to mevalonate by the enzyme HMG-CoA reductase. This is the regulated, rate-limiting and irreversible step in cholesterol synthesis (Zoller ,2003).

Mevalonate is then converted to 3-isopentenyl pyrophosphate in three reactions that require ATP. Mevalonate is decarboxylated to isopentenyl pyrophosphate, which is a key metabolite for various biological reactions. Three molecules of isopentenyl pyrophosphate condense to form farnesyl pyrophosphate through the action of geranyl transferase .Two molecules of farnesyl pyrophosphate then condense to form squalene by the action of squalene synthase in the endoplasmic reticulum, ido-squalene cyclase then cyclizes squalene to form lanosterol. Finally, lanosterol is converted to cholesterol through a 19-step process (Mehta ,2013).

2.3.4 Regulation of Cholesterol Biosynthesis:

Cholesterol synthesis is controlled by certain hormones like glucagon and insulin but the main step that regulates cholesterol synthesis is the conversion of HMG-CoA to mevalonate in presence of HMG-CoA reductase. This enzyme HMG-CoA reductase is thus the rate limiting enzyme and controls excessive cholesterol formation by feedback mechanism.When the concentration of intracellular cholesterol increases, levels of HMG-CoA reductase are reduced by decreasing the gene transcription of this enzyme (Methta ,2013).

As mentioned above the enzyme HMG-CoA reductase is also regulated by hormones to regulate cholesterol synthesis. Covalent modification of the enzyme itself causes activation and deactivation of the enzyme and increase or decrease in levels of hormonal release .The phosphorylated form of HMG-CoA reductase is the inactivated state, whereas the dephosphorylated form is in the active state. Dephosphorylation is promoted by insulin thereby activating the enzyme and in turn increasing cholesterol synthesis whereas phosphorylation is triggered by glucagon thereby deactivating the enzyme and in turn reducing cholesterol synthesis. These hormones exert their effects through a series of reactions via (cyclic Adenosine Mono Phosphate) cAMP (Jeremy ,2011).

Also when the concentrations of cholesterol is high in the cells, an enzyme called acyl CoA-cholesterol acyl transferase is released which elevates esterification of cholesterol for storage. Moreover high cholesterol level sends a signal to the gene encoding Low Density Lipoprotein (LDL) receptors to decreases transcription and in turn reduce production of receptors. These causes a fall in level of uptake of cholesterol from the blood (Meha ,2013).

2.3.5 Cholesterol catabolism:

Cholesterol is susceptible to oxidation and easily forms oxygenated derivatives known as oxysterols , three different mechanisms can form these; autoxidation, secondary oxidation to lipid peroxidation, and cholesterol-metabolizing enzyme oxidation. A great interest in oxysterols arose when they were shown to exert inhibitory actions on cholesterol biosynthesis .This finding became known as the ―oxysterol hypothesis‖ . Additional roles for oxysterols in human physiology include their participation in bile acid biosynthesis, function as transport forms of cholesterol, and regulation of gene transcription (Russell,2000).

Cholesterol is oxidized by the liver into a variety of bile acids . These, in turn, are conjugated with glycine, taurine, glucuronic acid, or sulfate. A mixture of conjugated and non conjugated bile acids, along with cholesterol itself, is excreted from the liver into the bile. Approximately 95% of the bile acids are reabsorbed from the intestines, and the remainder are lost in the feces . The excretion and reabsorption of bile acids forms the basis of the enterohepatic circulation, which is essential for the digestion and absorption of dietary fats. Every day, up to 1 g of cholesterol enters the colon. This cholesterol originates from the diet, bile, and desquamated intestinal cells, and can be metabolized by the colonic bacteria. Cholesterol is converted mainly into coprostanol, a non absorbable sterol that is excreted in the feces. A cholesterol-reducing bacterium origin has been isolated from human feces (Wolkoff .Cohen ,2009).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1-Study approach

A quantitative method was used to evaluate the plasma cholestral and estradiol levels among female with polycystic ovarian syndrome during the period from February to May 2017.

3.2-Study design and Study area

This cross sectional (case control) study was conducted in Khartoum state, the capital of Sudan.

3.3- Study population and Sample size

Sudanese infertile women which came to fertility center , which will diagnosis as PCOS (diagnosed according to hormonal profile and ultrasound and confirm the diagnosis by Anti-mullrien Hormone above 4ng/ml consider PCOS were be recruited as case and other infertile Sudanese women without PCOS which AMH below 4ng/ml and other normal fertile as control group was enrolled.

3.4-Inclusion criteria

Sudanese female with polycystic ovarian syndrome, and healthy female were included in this study.

3.5-Exclusion criteria

The criteria of exclusion based on excluding any menopausal and women receiving contraceptives.

3.6-Ethical consedration

The study was revised and ethically approved by the ethical committee of the Faculty of Medical Laboratory sciences, Sudan Unversity for science and technology .Consent was taken regarding acceptance to participate in the study and re-assurance of confidentialty. Before the specimen was collected, the donors knew that this specimen was collected for research purpose.

3.7-Data collection

Data were collected using a structural interviewing questionnaire, which was designed to collect and maintain all valuable information concering each case examined.

3.8-Sample collection and processing

About 2.5 ml of venous blood were collected from each participant (both case and control). The sample collected under aseptic conditions and placed in sterile lithium heparin containers and centrifuged for 5 minutes at 3000 RPM to obtain plasma then sample were kept in plain containers at $-70C^0$ until the time of analysis.

3.9-Estimation of estradiol by using ELISA mehod:

3.9-1-Principle of method:

In which one of the reaction components is bound to a solid-phase surface. In this Technique, an aliquot of sample is allowed to interact with the solid-phase antibody. After washing, a second antibody labeled with enzyme is added to form an Ab–Ag–Ab–enzyme complex. Excess free enzyme–labeled antibody then is washed away, and the substrate is added; the conversion of substrate is proportional to the quantity of antigen (Carl *et al*., 2006).

3.9-2-Procedure of estradiol measurement (Appendix II)

3.10-Total cholesterol measurement:

Cholestraeol was measured by using test kits reagent of Bio system (spain) provided by soliana co.ltd.

The laboratory analysis of samples was performed at Omdurman teaching hospital central laboratory by using BS-380 chemical analyzer (BS-380 is full automated chemical analyzer produce by mindray which provide 300 test per hour).

The method that used in this study is the enzymatic (cholesterol oxidase) method.

3.10.1.Principle:

Use cholesterol oxidase reactions along with cholesterol esterase and peroxidase reaction for the color or final determination.

Reaction:

Cholesterol+H2O $_{cholesteryl\ esterase}$ Cholesterol +Fatty acid

Cholesterol +O2 $_{cholesterol\ oxidase}$ cholestenone +H2O2

2H2O2 + 4-aminoantipyrine + phenol $_{peroxidase}$ pink color (*Wendy Arneson ,Jean Brickell ,2007*)**.**

3-10-2-Procedure of cholesterol measurement (appendiex III)

3.11-Quality control

The precision and accuracy of all methods used in this study were checked by commercially prepared control sample before it is application for the measurement of test and control samples.

3.12-Statistical analysis

Data was analyzed to obtain means standard deviation and correlation of the sampling using statistical package for social science (SPSS) computer Programmed version 11.5, t test and person correlation were used for comparison correlation between variable.

Chapter Four

Results

4. Results

The biochemical results of serum total cholesterol and estradiol in patients with polycystic ovarian syndrome are given in tables and figures:

Table (4-1): Illustrate the mean concentration of AMH levels,cholestereol and estradiol of patients with PCOs compared with control group. AMH were significantly increased in PCOs compared with control groups. Minimum (6.2 versus 0.01) ng/ml and maximum (28.6 versus 5.0) ng/ml. (Mean±SD: 10.6±5.92 versus 1.4±0.22) ng/ml respectively with *P.Value* 0.000.

cholesterol were significantly increase in pcos compare with control group (Mean±SD: 207.6±33.4 versus112±33.4 mmoL /L) respectively with P.vale 0.000. The estradiol levels were insignificant different in PCOs compared with control group. (Mean±SD: 86±39versus 101±38.6 pg/ml) with *P.Value 0.086).*

Table (4-2): Illustrate the correlation (P.vale , r) of age with (estradiol , cholesterol and AMH) in patient with polycystic ovary syndrome.

There was no correlations between age and AMH , Estradiol and cholesterol (r,value=0.189,P.vaule=0.242),(r.value=0.140,P.vaule=0.388),(r,value =-0.22,P.value=0.171) respectively .

Figure (4-1): Show correlation between cholesterol concentration and estradiol , there was insignificants week negative correlation (r,value= -0.123, *P.Value* = 0.448).

Figuer (4-2):show correlation between cholesterol and AMH, there was no correlation (r ,value= 0.065,P.value=0.690)

Figure(4-3): show correlation between estradiol and AMH , there was no correlation (r,value =0.121,P.value= 0.458).

Table (4-1): The mean concentration of cholesterol and estradiol in polycystic ovarian syndrome patients and control group.

Variable	PCOS N=40 Mean±SD	Control N=40 Mean±SD	*P.Value*
Cholesterol mmol/l	207.6±33.4	112±33.4	0.0000
Estradiol pg/ml	86±39	101±38.6	0.086
AMH	10.6±5.92	1.4±0.22	0.000

*Result given in mean ± SD, *P-Value* ≤ 0.05 Consider significant.

* Independent sample T test was used for comparison.

Table (4-2): The person correlation (P.vale , r.value) of age with (estradiol , cholesterol and AMH) in patient with polycystic ovary syndrome.

Correlation	r	P.vale
Age with AMH	0.189	0.242
Age with estradiol	0.140	0.388
Age with cholesterol	0.22	0.171

*result are given as P.vale and r small

* *P-Value* ≤ 0.05 Consider significant.
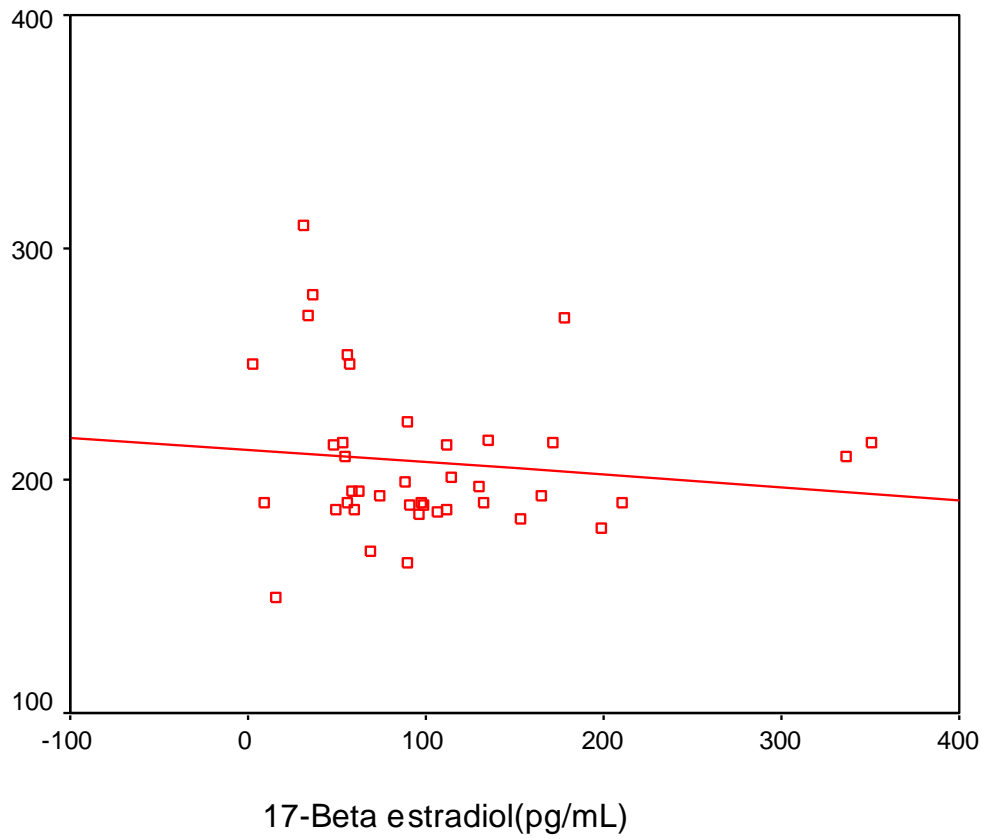
Figure (4-1): Show correlation between cholesterol concentration and estradiol (r,value = -0.123, *P.Value* = 0.448).
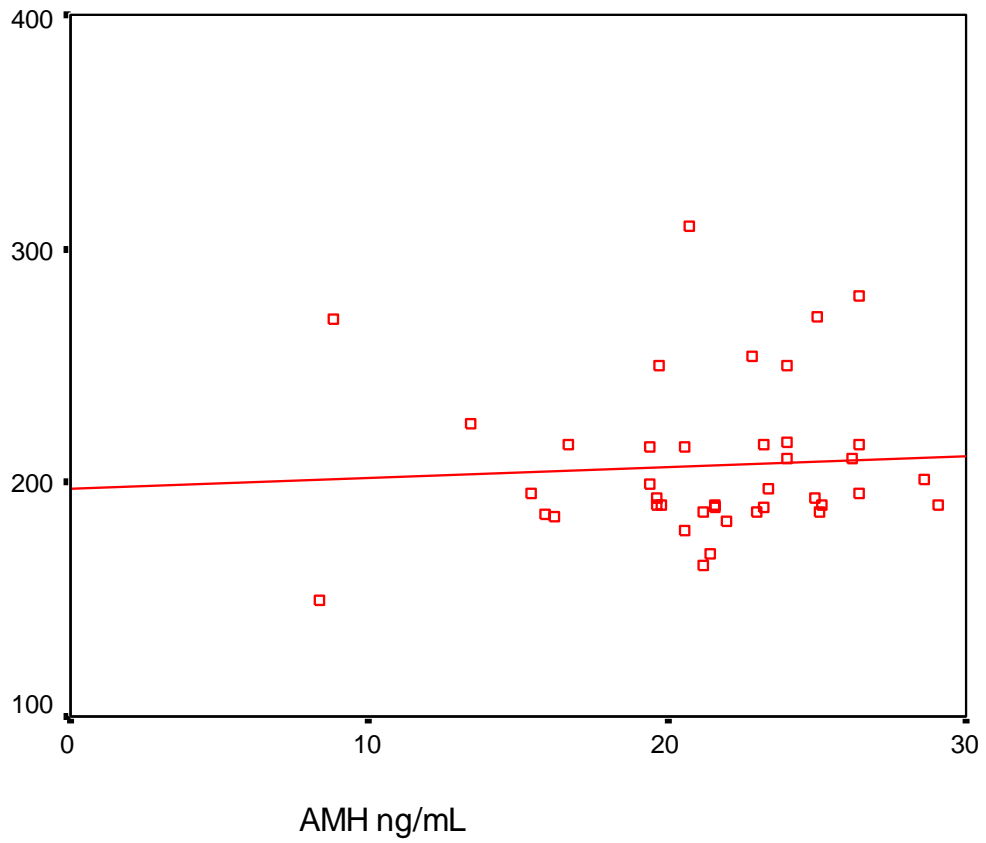
Figuer(4-2):show correlation between cholesterol and AMH(r,value = 0.065,P.value=0.690)
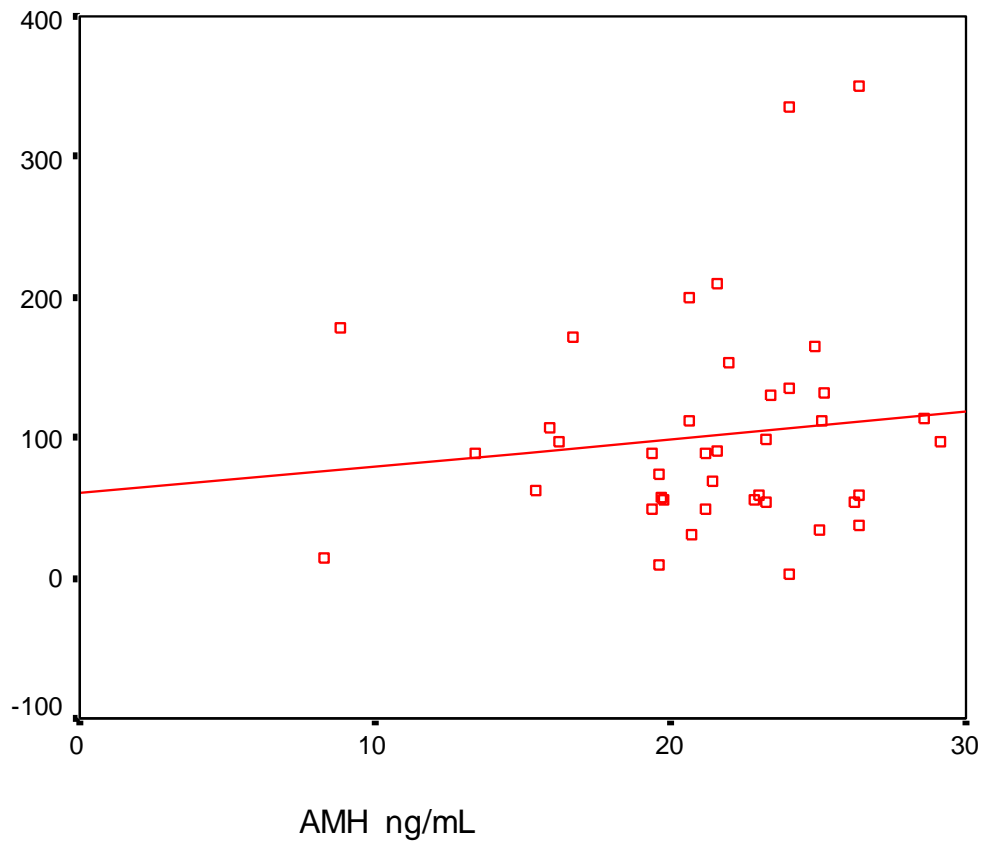
Figure(4-3): show correlation between estradiol and AMH (r,value =0.121,P.value= 0.458).

Chapter Five

Discussion, Conclusion

Recommendations

5. Discussion

Polycystic ovary syndrome (PCOS) is a common endocrine disease in women, ch aracterized by heterogeneous presentation of hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology (PCOM) (Palomba *et al*., 2015).

In this study, the average age of woman was from (17 to 30 years).This finding is consistent with studies by (Rousseau et al.,2009) and (Johnstone et al,.2010) , who had reported that the proportion of women with PCO decreased with age . this can be caused by a decrease in the number of antral follicles throughout the reproductive years that occurs in normal women, a phenomenon that also applies to patients with PCOS (Murphy et al.,2006).

We use AMH as diagnostic tool for polycystic ovary syndrome there are many studies reported that there is increase in AMH concentration with PCOS woman when compare with normal woman (wiewko et al.,2014). this increase is due to increased synthesis and secretion of AMH by polycystic ovarie. (Mulders et al.,2004, Pellat et al.,2004). Reported that AMH production increases approximately 75 times higher in each polycystic ovarian granulose cell (pellae et al.,2010) This finding is supported by Catteau-Jonard et al., who found increased mRNA expression of AMH in ovarian granulosa cells(Amner et al.,2009). Elevated serum AMH levels in PCOS patients may also be caused by disturbances in folliculogenesis, resulting in the accumulation of excessive pre-antral and small antral follicles (Wang et al.,2007). Cessation of antral follicle development toward the dominant follicle is due to suppression of aromatase activity by AMH and by lower follicle sensitivity to FSH (Singer et al.,2009,Biouca et al.,2009).

Obesity and insulin resistance occur frequently in association with this syndrome. Cardiovascular risk factors seem to cluster in women with PCOS compared with general population as result of increase concentration of cholesterol (walid et al.,2002).

Dyslipidemia is one of the important risk factor associated with PCOS. Abnormal lipid metabolism is one of the main metabolic characteristics of PCOS patients. The result of this study show that PCOS patients had higher total cholesterol concentration when compared with control group which are similer to the results observed in PCOS patients in another study (Valkenburg,2008) also in agreement with some studies that suggested that PCOS patients are hyperlipidemic with higher total cholesterol compare to control group (Madhu et al .,2012),(Shoeib et al,2015) and (Cristian.,2012).

While estradiol showed insignificant difference with *p-value* 0.086. This finding agreed with studies done by (Shang-Gwo *et al.,* 2008) and (Amato *et al.,* 2010).

Also the result showed that, there was no correlations were found between AMH and plasma levels of cholesterol and estradiol .This finding was agreed with estradiol in studies done by (A.F. Begawy *et al.*, 2010).

 Also the result showed that, there was no correlations were found between age and plasma levels of cholesterol an, estradiol and AMH.

5.2- Conclusion

From the results and finding of this study, it is concluded that: the plasma levels of total cholesterol is increase in PCOs female patients , estradiol was affected, no correlations were found between cholesterol and plasma levels of estradiol.

5.3-Recommendations

1.using of cholesterol as indicator of increase the risk of cardiovascular disease in patient with polycystic ovary syndrome

2. More studies should be carried out to investigation like measurement of HDL , LDL cholesterol and

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Appendix

Appendix I

Questionnaire

Sudan University of science and Technology

College of graduate studies

Estradiol and Cholesterol level among Female with Polycystic Ovarian Syndrome

Patient name or code:-………………………………………………………………

Age:……………….Years

Methods of diagnosis:-

………………………………………………………………………………………

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Duration of disease: Months (   )   Years (   )

Laboratory investigations:-

Test name	Concentration
Cholesterol	**Mmol/l**
Estradiol	**pg/ml**

Appendix II

| BXE0860A |
| 96 Tests |
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| FOR IN-VITRO DIAGNOSTIC USE ONLY |

ESTRADIOL

ELISA

Summary:

Measurement of estradiol in serum or plasma is considered to be the most reliable way to assess its rate of production.

Estradiol (17β-estradiol) is a steroid hormone (molecular weight of 272.3 daltons), which circulates predominantly protein-bound. In addition to estradiol, other natural steroidal estrogens include estrone, estriol and their metabolites. Natural estrogens are hormones secreted principally by the ovarian follicles and also by the adrenals, corpus luteum and placenta. In males, by the testes. Exogenous estrogens (natural or synthetic) elicit, to varying degrees, all the pharmacologic responses usually produced by endogenous estrogens.

Estrogenic hormones are secreted at varying rates during the menstrual cycle throughout the period of ovarian activity. During pregnancy, the placenta becomes the main source of estrogens. At menopause, ovarian secretion of estrogens declines at varying rates. The gonadotropins of the anterior pituitary regulate secretion of the ovarian hormones, estradiol and progesterone; hypothalamic control of pituitary gonadotropin production is in turn regulated by plasma concentrations of the estrogens and progesterone. This complex feedback system results in the cyclic phenomenon of ovulation and menstruation.

Estradiol determinations have proved of value in a variety of contexts, including the investigation of precocious puberty in girls and gynecomastia in men. Its principal uses have been in the differential diagnosis of amenorrhea and in the monitoring of ovulation induction.

The kit uses a specific anti-estradiol antibody and does not require prior sample extraction of serum or plasma. Crossreactivity to other naturally occurring and structurally related steroids is low.

Principle:
**Delayed Competitive Enzyme Immunoassay (TYPE 9):**

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated antibody with a serum containing the antigen, a reaction results between the antigen and the antibody. The interaction is illustrated by the following equation:

$$Ag + Ab_{Btn} \rightleftharpoons AgAb_{Btn}$$

Ab_{Btn} = Biotinylated Antibody
Ag = Antigen (Variable Quantity) $AgAb_{Btn}$ = Immune Complex

After a short incubation, the enzyme conjugate is added. (This delayed addition permits an increase in sensitivity for low concentration samples). Upon the addition of the enzyme conjugate, competition reaction results between the enzyme analog and the antigen in the sample for a limited number of antibody binding sites (not consumed in the first incubation).

$$^{Enz}Ag + Ag + rAb_{Btn} \underset{k_a}{\overset{k_d}{\rightleftharpoons}} AgAb_{Btn} + ^{Enz}AgAb_{Btn}$$
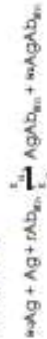
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$^{Enz}AgAb_{Btn}$ = Enzyme-antigen Conjugate-Antibody Complex
rAb_{Btn} = Biotinylated Antibody not reacted in first incubation
k_a = Rate Constant of Association
k_d = Rate Constant of Dissociation
$k = k_a / k_d$ = Equilibrium Constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.

$$AgAb_{Btn} + ^{Enz}AgAb_{Btn} + Streptavidin_{WP} \Rightarrow Immobilized complex$$

$Streptavidin_{WP}$ = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface

The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Reagent Materials Provided:
A. Estradiol Calibrators - 0.5ml/vial

Seven (7) vials of serum reference for estradiol at concentrations of 0 (A), 20 (B), 100 (C), 250 (D), 500(E), 1500 (F) and 3000 (G) in pg/mL. Store at 2-8° C. A preservative has been added. The calibrators can be expressed in molar concentrations (nM/L) by multiplying by 2.72.

For example: 1pg/ml x 3.67 = 3.67 pM/L

B. Estradiol Enzyme Reagent - 6.0ml/vial

One (1) vial of Estradiol (Analog) - horseradish peroxidase (HRP) conjugate in a protein-stabilizing matrix red with dye. Store at 2-8°C.

C. Estradiol Biotin Reagent - 6.0ml

One (1) bottle of reagent contains anti-estradiol biotinylated purified rabbit IgG conjugate in buffer, green dye and preservative. Store at 2-8°C.

D. Streptavidin Coated Plate - 96 wells

One 96-well microplate coated with 1.0μg/ml streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Concentrate Solution - 10ml

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

F. Substrate Reagent - 12ml/vial

One (1) bottle containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G. Stop Solution - 8ml/vial

One (1) vial containing a strong acid (H_2SO_4). Store at 2-30°C.

H. Production Instructions

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

1. Pipette capable of delivering 25μl and 50μl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
3. Adjustable volume (200-1000μl) dispenser(s) for conjugate
4. Microplate washer or a squeeze bottle (optional)
5. Microplate Reader with 450nm and 620nm wavelength absorbance capability
6. Absorbent paper for blotting the microplate wells
7. Plastic wrap or microplate cover for incubation steps
8. Vacuum aspirator (optional) for wash steps
9. Timer
10. Quality control materials

Precautions:
For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

Reagent Preparation:
1. Wash Buffer
Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at room temperature (20-27°C) for up to 60 days.

Note: Do not use the working substrate if it looks blue.

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Test Procedure:

Before proceeding with assay, bring all reagents, serum references and controls to room temperature (20-27°C).

1. Format the micro plates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused micro well strips back into the aluminium bag, seal and store at 2-8°C.

2. Pipette 0.025ml (25µL) of the appropriate serum reference, control or specimen into the assigned well.

3. Add 0.050ml (50µl) of the Estradiol Biotin Reagent to all wells.

4. Swirl the microplate gently for 20-30 seconds to mix.

5. Cover and incubate for 30 minutes at room temperature.

6. Add 0.050ml (50µl) of Estradiol Enzyme Reagent to all wells.

Add directly on top the reagents dispensed in the wells.

7. Swirl the microplate gently for 20-30 minutes to mix.

8. Cover and incubate for 90 minutes at room temperature.

9. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

10. Add 350µl of wash buffer [see Reagent Preparation Section], decant [tap and blot] or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container [avoiding air bubbles] to dispense the wash. Decant the wash and repeat two (2) additional times.

11. Add 0.100ml (100µl) of working substrate solution to all wells [see Reagent Preparation Section]. Always add reagents in the same order to minimize reaction time differences between wells. DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION.

12. Incubate at room temperature for twenty (20) minutes.

13. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

14. Read the absorbance in each well at 450nm [using a reference wavelength of 620-630nm to minimize well imperfections] in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Note: Dilute the samples suspected of concentrations higher than 3000pg/ml 1:5 and 1:10 with estradiol "0" pg/ml calibrator or mate patient serum pools with a known low value for estradiol.

Quality Control:

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should

set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

Calculation of Results:

A dose response curve is used to ascertain the concentration of estradiol in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

2. Plot the absorbance for each duplicate serum reference versus the corresponding estradiol concentration in pg/ml on linear graph paper [do not average the duplicates of the serum references before plotting].

3. Connect the points with a best-fit curve.

4. To determine the concentration of estradiol for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, read the concentration [in pg/ml] from the horizontal axis of the graph [the duplicates of the unknown may be averaged as indicated]. In the following example, the average absorbance (1.202) intersects the dose response curve at (160pg/ml) estradiol concentration [see Figure 1].

Figure 1

Estradiol Value / 1000 in pg/ml

Note: Multiply the horizontal values by 1000 to convert into pg/ml

QC Parameters:

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0pg/ml should be ≥1.3.

2. Four out of six quality control pools should be within the established ranges.

A. Assay Performance

1. It is important that the time of reaction in each well is held constant for reproducible result.

2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.

3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.

4. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time-deviation during reaction.

5. Plate readers measure vertically. Do not touch the bottom of the wells.

6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious result.

7. Use components from the same lot. No intermixing of reagents from different batches.

8. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from this may yield inaccurate results.

9. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used for using this device.

Example 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (pg/ml)
Cal A	A1	2.268	2.256	0
	B1	2.244		
Cal B	C1	1.839	1.849	20
	D1	1.860		
Cal C	E1	1.409	1.426	100
	F1	1.443		
Cal D	G1	1.017	1.003	250
	H1	0.989		
Cal E	A2	0.698	0.723	500
	B2	0.748		
Cal F	C2	0.480	0.487	1500
	D2	0.493		
Cal G	E2	0.390	0.388	3000
	F2	0.385		
Patient	G2	1.202	1.202	160
	H2	1.203		

The above data and table is for example only. Do not use it for calculating your results.

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B. Interpretation

1. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

Expected Ranges of Values:

In agreement with established reference intervals for a "normal" adult population and females during gestation the expected ranges for the Estradiol ELISA Test Kit are detailed in table 1.

Table 1: Expected Values for the Estradiol Test Kit

	Median	Range
Females		
Follicular Phase	48	9-175
Luteal Phase	103	44-196
Periovulatory	209	107-281
Treated Menopausal	122	42-289
Untreated Menopausal	7.3	ND-20
Oral Contraceptives	13	ND-103
Males	19	4-94

During pregnancy the estradiol serum levels rise rapidly till the end of the third trimester.

It is important to keep in mind that establishment of a range if values which can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors; the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analyst using the method with a population indigenous to the area in which the laboratory is located.

Performance Characteristics:

Precision

The within and between assay precision of the Estradiol ELISA Test Kit were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

Table 2
Within Assay Precision (Values in pg/ml)

Sample	N	x	σ	C.V.
Low	20	85.9	7.6	8.8%
Normal	20	260.5	20.3	7.8%
High	20	495.3	33.7	6.8%

Table 3
Between Assay Precision (Values in pg/ml)

Sample	N	x	σ	C.V.
Low	10	89.3	8.2	9.2%
Normal	10	245.5	23.7	9.7%
High	10	467.2	38.3	8.2%

*As measured in ten experiments in duplicate over a ten day period

Accuracy

The Estradiol ELISA Test Kit was compared with a chemiluminescence immunoassay method. Biological specimens from low, normal and relatively high estradiol level populations were used (The values ranged from 10pg/ml-4300pg/ml). The total number of such specimens was 63. The least square regression equation and the correlation coefficient were computed for this estradiol ELISA in comparison with the reference method. The data obtained is displayed in table 4.

Table 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (Y)	139	Y = 10+0.985*(X)	0.979
Reference (X)	148		

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

Sensitivity:

The Estradiol ELISA Test Kit has a sensitivity of 6.5pg/ml. The sensitivity was ascertained by determining the variability of the 0pg/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

Specificity:

The % cross-reactivity of the estradiol antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of estradiol needed to displace the same amount of labeled analog.

Substance	Cross Reactivity	Substance	Cross Reactivity
Androstanedione	0.0003	Estriol	<0.0001
Dihydrotestosterone	0.0008	DHEA sulfate	<0.0001
Cortisone	<0.0001	Estradiol	<0.0001
Corticosterone	<0.0001	Estrone	<0.0001
Cortisol	0.0004	Testosterone	<0.0001

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IVD For in Vitro Diagnostic Use Only
LOT Lot Number
REF Catalogue Number
Storage temperature
Expiry Date (Year / Month)
Warning, Read Enclosed Documents
Instructions For Use
Manufactured By

REVISED NOV /09

Appendix III

COD 11805 1 x 50 mL	COD 11535 1 x 200 mL	COD 11506 1 x 500 mL	COD 11539 1 x 1 L
STORE AT 2-8ºC				
Reagents for measurement of cholesterol concentration Only for in vitro use in the clinical laboratory				

CHOLESTEROL
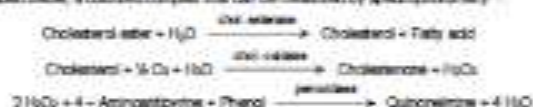
CE

CHOLESTEROL
CHOLESTEROL OXIDASE/PEROXIDASE

PRINCIPLE OF THE METHOD

Free and esterified cholesterol in the sample originates, by means of the coupled reactions described below, a coloured complex that can be measured by spectrophotometry.

$$Cholesterol\ ester + H_2O \xrightarrow{\ cholesterol\ esterase\ } Cholesterol + Fatty\ acid$$

$$Cholesterol + ½O_2 + H_2O \xrightarrow{\ cholesterol\ oxidase\ } Cholestenone + H_2O_2$$

$$2\ H_2O_2 + 4 - Aminoantipyrine + Phenol \xrightarrow{\ peroxidase\ } Quinoneimine + 4\ H_2O$$

CONTENTS

	COD 11805	COD 11535	COD 11506	COD 11539
A. Reagent	1 x 50 mL	1 x 200 mL	1 x 500 mL	1 x 1 L
B. Standard	1 x 5 mL	1 x 5 mL	1 x 5 mL	1 x 5 mL

COMPOSITION

A. Reagent: Pipes 35 mmol/L, sodium cholate 0.5 mmol/L, phenol 28 mmol/L, cholesterol esterase > 0.2 U/mL, cholesterol oxidase > 0.1 U/mL, peroxidase > 0.8 U/mL, 4-aminoantipyrine 0.5 mmol/L, pH 7.0.

B. Cholesterol Standard: Cholesterol 200 mg/dL (5.18 mmol/L). Aqueous primary standard.

STORAGE

Store at 2-8ºC

Reagent and Standard are stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use.
Indications of deterioration:
- Reagent: Presence of particulate material, turbidity, absorbance of the blank over 0.200 at 500 nm (1 cm cuvette).
- Standard: Presence of particulate material, turbidity.

REAGENT PREPARATION

Reagent and Standard are provided ready to use.

ADDITIONAL EQUIPMENT

- Thermostatic water bath at 37ºC
- Analyzer, spectrophotometer or photometer able to read at 500 ± 20 nm

SAMPLES

Serum or plasma collected by standard procedures.
Cholesterol is stable for 7 days at 2-8ºC. Heparin, EDTA, oxalate and fluoride may be used as anticoagulants.

PROCEDURE

1. Bring the Reagent to room temperature.
2. Pipette into labelled test tubes: (Note 1)

	Blank	Standard	Sample
Cholesterol Standard (B)	—	10 µL	—
Sample	—	—	10 µL
Reagent (A)	1.0 mL	1.0 mL	1.0 mL

3. Mix thoroughly and incubate the tubes for 10 minutes at room temperature (16-25ºC) or for 5 minutes at 37ºC.
4. Measure the absorbance (A) of the Standard and Sample at 500 nm against the Blank. The colour is stable for at least 2 hours.

CALCULATIONS

The cholesterol concentration in the sample is calculated using the following general formula:

$$\frac{A_{Sample}}{A_{Standard}} \times C_{Standard} = C_{Sample}$$

If the Cholesterol Standard provided has been used to calibrate (Note 2):

$\dfrac{A_{Sample}}{A_{Standard}}$	× 200 = mg/dL cholesterol
	× 5.18 = mmol/L cholesterol

REFERENCE VALUES

The following uniform cut-off points have been established by the US National Cholesterol Education Program and have also been adopted in many other countries for the evaluation of coronary artery disease risk.

Up to 200 mg/dL = 5.2 mmol/L	Desirable
200-239 mg/dL = 5.2-6.21 mmol/L	Borderline-High
> 240 mg/dL = > 6.24 mmol/L	High

QUALITY CONTROL

It is recommended to use the Biochemistry Control Serum level I (cod. 18005, 18009 and 18042) and II (cod. 18007, 18010 and 18043) to verify the performance of the measurement procedure.
Each laboratory should establish its own internal Quality Control scheme and procedures for corrective action if controls do not recover within the acceptable tolerances.

METROLOGICAL CHARACTERISTICS

- Detection limit: 0.3 mg/dL = 0.008 mmol/L.
- Linearity limit: 1000 mg/dL = 26 mmol/L. For higher values dilute sample 1/2 with distilled water and repeat measurement.
- Repeatability (within run):

Mean Concentration	CV	n
121 mg/dL = 3.13 mmol/L	1.1 %	20
257 mg/dL = 6.66 mmol/L	0.8 %	20

- Reproducibility (run to run):

Mean Concentration	CV	n
121 mg/dL = 3.13 mmol/L	1.9 %	25
257 mg/dL = 6.66 mmol/L	1.9 %	25

- Trueness: Results obtained with this reagent did not show systematic differences when compared with reference reagents (Note 2). Details of the comparison experiments are available on request.
- Interferences: Lipemia (triglycerides 10 g/L) does not interfere. Bilirubin (>10 mg/dL) and hemoglobin (>5 g/L) may affect the results. Other drugs and substances may interfere.

These metrological characteristics have been obtained using an analyzer. Results may vary if a different instrument or a manual procedure are used.

DIAGNOSTIC CHARACTERISTICS

Cholesterol is a sterol of high molecular weight and possesses the cyclopentanophenanthrene skeleton. Dietary cholesterol is partially absorbed and it is also synthesized by the liver and other tissues. Cholesterol is transported in plasma by lipoproteins. It is excreted unchanged into bile or after transformation to bile acids.
Increased total cholesterol values are associated with a progressively escalating risk of atherosclerosis and coronary artery disease.
Clinical diagnosis should not be made on the findings of a single test result, but should integrate both clinical and laboratory data.

NOTES

1. This reagent may be used in several automatic analyzers. Instructions for many of them are available on request.
2. Calibration with the provided aqueous standard may cause a matrix related bias, specially in some analyzers. In these cases, it is recommended to calibrate using a serum based standard (Biochemistry Calibrator, cod. 18011 and 18044).

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Quality System certified according to
EN ISO 13485 and EN ISO 9001 standards

Appendix IV