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Faculty of Medicine
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Estimation of serum gonadotrophins in relation to anti-Müllerian hormone in polycystic ovary syndrome patients attended infertility centers in khartoum state

A thesis submitted in partial fulfillment of the requirement for the degree of master of biochemistry

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بسم الله الرحمن الرحيم

قال تعالى:

(قل إنما أنا بشر مثلكمُ يوحى إليّ أنّمآ إلهكم إلّه واحّد فمَن كَان يرْجَحُوا لقاء رَبِّه فَليَعْمَلُ عَمَلًا صَالِحًا وَلَا يُشَرِّكَ بِعِبَادَة رَبِّه أَحَدًا)

صدق الله العظيم

سورة الكهف الآية (110)
Dedication

To

My beloved parents
For making everything worthwhile

My brothers and sister
For giving me love and inspiration

My uncle Abdalmounaim
For encouragement and pushing me forward

My blue heart my comrade Raghda
For her abundant support and her love

My faithful friends Alaa and Jihad
For their support and encouragement

My friends
For their support

OM ALHASSAN, 2018
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OM ALHASSAN, 2018
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<td>Polycystic ovary syndrome</td>
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<tr>
<td>AMH</td>
<td>Anti-Müllerian Hormone</td>
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<tr>
<td>LH</td>
<td>Serum Luteinizing Hormone</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating Hormone</td>
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<tr>
<td>LH/FSH</td>
<td>Luteinizing Hormone to Follicle stimulating Hormone ratio</td>
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<td>FSH/LH</td>
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<td>MIS</td>
<td>Müllerian Inhibiting substance</td>
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<td>HA</td>
<td>Hyperandrogenic anovulation</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of health</td>
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<td>FNPO</td>
<td>Follicle number per ovary</td>
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<tr>
<td>BMI</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophin releasing hormone</td>
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<tr>
<td>OC</td>
<td>Oral contraceptive</td>
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Abstract

Polycystic ovary syndrome (PCOS), a heterogeneous disorder of unclear etiology, is an important cause of both ovulatory and menstrual irregularity and androgen excess in women. In PCOS, the ovary doesn't make all of the hormones it needs for an egg to fully mature. The follicles may start to grow and build up fluid but ovulation does not occur instead it remain as cysts.

The objective of this study is to assess serum gonadotrophins in relation to anti-Müllerian hormone in polycystic ovary syndrome.

The study was carried out in 2 Fertility Centers at Khartoum State, at the University of Khartoum fertility center (U.K.F.C) (Saad Abu Alela hospital) and Dr. Elsir Abu EL hassen fertility center; during the period from December 2017 to March 2018.

Ninety nine Patients with PCOS were included in the study. Their ages range between less than 22 to above 32 years and thirty matched normal individuals were taken as control group, and were tested for serum Gonadotrophins (Follicle stimulating hormone (FSH), luteinizing hormone (LH)) and anti-Müllerian hormone (AMH) Levels.

The serum values of FSH in normal subject were higher (9.19+_0.66) than in patients with PCOS (6.46+_0.20), while the patients with PCOS were higher than normal subjects in the LH (9.21ng/ml), AMH (8.74ng/ml) levels.

The FSH/LH ratio in normal subjects was higher (1.49 ng/mL) than patients with PCOS (0.98 ng/mL). While the patients with PCOS were higher LH/FSH ratio(1.48 ng/ml) than the normal subjects in (0.88 ng/mL).
FSH concentration correlated significantly (0.316) and positively (P<0.01) with LH of the patients with PCOS and FSH/LH and LH/FSH of the normal subjects. The highest correlation coefficient was found between LH and LH/FSH in patients with PCOS followed by the negative correlation coefficient of FSH/LH and LH/FSH in patients with PCOS. Moreover, the association between AMH and FSH/ LH was significantly (0.246) negatively (P<0.05) correlated in the patients with PCOS.

There was no significant difference in serum hormones concentration between different age groups being studied.

There was no significant difference in the concentration of hormones in the serum of the subjects, taken from different geographical areas of the Sudan.
المستخلص

يعتبر تكييس المبايض مرض متباين يحدث لأسباب غير واضحة وهو أهم الأسباب لعدم انتظام عمله التنبيب والدوادرة الشهرية وزيداء الهرمونات الذكرية عند النساء. تكييس المبايض يؤدي إلى عدم تصنيع كل الهرمونات التي تحتاجها البويضة للنضج الكامل حيث تبدأ الحيوصات في النمو وتغذى بالسوائل لكن عملية التنبيب لا تحدث وتتبي كأكياس.

هدف من هذه الدراسة قياس الهرمونات المحفزة للمبايض ومعرفة مدى علاقةها مع مضادات هرمون ميلر في حالة مرض تكييس المبايض.

هذه الدراسة أجريت في مركزين للخصوبة وال عليك في ولاية الخرطوم مركز جامعه الخرطوم للخصوبة وال عليك (مستشفى سعد أبو العلا) ومراكز السر أبو الحسن للخصوبة وال عليك خلال الفترة من ديسمبر 2017 إلى مارس 2018.

تذمتند التكييس تساع وتسعون مريضة بتكييس المبايض وثمانون نساء سليمات كضوابط للدراسة أعمارهم تتراوح بين أقل من 22 سنة إلى أكثر من 32 سنة قبل تكييز الهرمونات المحفزة للمبايض (الهرمون المحفز للحيولة الهرمون الشائع للجسم الأصفر) ومضادات هرمون محلية.

القيم المتصورة للهرمون المحفز للحيولة في النساء السليمات كان أعلى (9.19 ± 0.66) من مريضات التكييس (6.46 ± 0.20). لكن نسبة الهرمون المشف للجسم الأصفر (8.9 نانوجرام/مل) ومضادات هرمون ميلر (7.1 نانوجرام/مل) تركزهما أعلى عند المريضات بالتكيس.

النسبة بين الهرمون المحفز للحيولة والهرمون المشف للجسم الأصفر في الحالات الطبيعية أعلى (0.9 نانوجرام/مل) بينما مريضات التكييز أعلى (1.48 نانوجرام/مل) في النسبة بين الهرمون المشف للجسم الأصفر والهرمون المحفز للحيولة بالنسبة للحالات السليمة (0.88 نانوجرام/مل).

الهرمون المشف للحيولة له علاقة كبيرة (0.31) وموجبة (أقل من 0.01) مع الهرمون المشف للجسم الأصفر للمرئيات وأيضا مع النسبة بين الهرمون المشف للحيولة والهرمون المشف للجسم الأصفر للسليمات. أعلى ارتباط يوجد بين الهرمون المشف للجسم الأصفر والنسبة بين الهرمون المشف للجسم الأصفر والهرمون المشف للحيولة ممترأ بعلاقة سلبية بين نسبة الهرمون المشف للحيولة والهرمون المشف للجسم الأصفر والهرمون المشف للجسم الأصفر والهرمون المشف للحيولة للمرئيات.

علامة على ذلك توجد علاقة سالبة (أقل من 0.05) بشكل كبير (0.24) بين مضادات هرمون ميلر ونسبة بين الهرمون المشف للحيولة والهرمون المشف للجسم الأصفر للمرئيات. ونسبة الهرمون المشف للجسم الأصفر للمرئيات. ولا يوجد اختلاف بين نسبة الهرمونات الثلاث في مختلف الفئات العمرية.

ولا توجد اختلاف بين تراكيز الهرمونات بالنسبة لمختلف المناطق الجغرافية في السودان.
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Chapter one

1-Introduction

1-1 Background

Polycystic ovary syndrome (PCOS), a heterogeneous disorder of unclear etiology, is an important cause of both ovulatory and menstrual irregularity and androgen excess in women [1, 2].

Polycystic ovary syndrome is a serious condition resulting in ovaries which cannot ovulate an oocyte. Polycystic ovaries are the main cause of infertility in women. [3] women with PCOS, have ovaries that don't make all of the hormones it needs for an egg to fully mature. The follicles may start to grow and build up fluid but ovulation does not occur. Instead, some follicles may remain as cysts. For these reasons, ovulation does not occur. [3]

When fully expressed, the manifestations include ovulatory dysfunction, androgen excess and polycystic ovaries. It is recognized as one of the most common endocrine/metabolic disorders of women. This syndrome was first described by Stein and Leventhal in 1935 who described seven women suffering from amenorrhea, hirsutism, and enlarged ovaries with multiple cysts [4], although the presence of sclerocystic ovaries had been recognized for at least 90 years prior to their report [1].

In the 1990s, two new aspects of PCOS became apparent. First, in many instances polycystic ovaries are inherited and this could be through either the mother or father. The polycystic ovary, therefore, can be considered part of an individual's genetic makeup and remains so for life. The symptoms of PCOS however, may change at different times of life [5].

Polycystic ovaries were later found to exist in some women with subtle
endocrine disorders. The wide range and frequency of symptoms made it difficult to establish a consistent clinical picture.[6]

Polycystic ovary syndrome (PCOS) also called hyperandrogenic anovulation (HA) \[11\] or Stein-Leventhal syndrome.[4] Women with PCOS may have enlarged ovaries that contain small collections of fluid – called follicles-located in each ovary as seen during an ultrasound examination. It is thought to be one of the leading causes of female sub-fertility \[7, 8, 9\] and the most frequent endocrine problem in women of reproductive age \[10\].

PCOS is associated with various endocrine abnormalities such as increased serum LH level relative to FSH release. Because of the pulsatile nature of their release; a single test fails to detect an increase ratio of LH: FSH and increase serum testosterone. LH is sufficient to cause anovulation. Estimation of these hormones aids in the diagnosis of PCOS \[12, 13\].

However, there is a considerable inter individual variation in presentation. Major hormonal studies include estimation of AMH, FSH, and LH in serum.

Luteinizing hormone in both sexes stimulates secretion of sex steroids from the gonads. In the tests, it stimulates the synthesis and secretion of testosterone. The ovaries respond to LH stimulation by secretion of testosterone, which is converted into estrogen by adjacent granulosa cells. LH is required for continuous development and function of corpora lutea. The name luteinizing hormone was derived from this effect of inducing luteinization of ovarian follicles.

Follicle-stimulating hormone (FSH) stimulates the maturation of ovarian follicles and critical for sperm production. It supports the function of Sertoli cells, which in turn support many aspects of sperm cell maturation. Traditionally the level of LH and FSH, as well as the LH: FSH ratio, can offer significant insight into the PCOS patients.
AMH also known as Müllerian Inhibiting Substance (MIS) is a new diagnostic marker of ovarian function. The level of AMH increased in PCO patients, and may become part of its diagnostic criteria.\textsuperscript{[14,15]} AMH levels correlate well with the ovarian antral follicle count and were the only levels that decreased longitudinally over time compared with FSH, estradiol, and inhibin-B levels. AMH levels do not vary significantly during the menstrual cycle and can therefore, be drawn on any day of the cycle.

1.2. Justification:

Polycystic ovarian syndrome (PCOS) is very common among women of reproductive age. The lack of well-defined diagnostic criteria makes identification of this common disease confusing to many clinicians. Also, the varied manifestations of the disorder, the estimation of serum gonadotrophins and anti-Müllerian hormone in the PCOS patients often will vary over time as the patient enters different stages of life with different goals.

According to our knowledge there is scarcity informations about PCOS in Sudan. Thus our study is an attempt to provide the possible explanation for this problem.
1.3. Objectives:

1.3.1 General objectives:

- To assess serum gonadotrophins in relation to anti-Müllerian hormone in polycystic ovary syndrome patients attended University of Khartoum fertility center (U.K.F.C) (Saad Abu Alela Hospital ) and Dr. Alsir Abu elhassan fertility center.

1.3.2 Specific objectives:

- To estimate serum luteinizing hormone (LH) concentration (ng/ml).
- To estimate serum follicle stimulating hormone (FSH) concentration (ng/ml).
- To compare serum luteinizing hormone to follicle stimulating hormone Ratio (ng/ml).
- To estimate serum anti-Müllerian hormone (AMH) concentration (ng/ml).
Chapter Two

2. Literature review:

2.1 Pathophysiology of Ovaries:

The ovaries are female gonads. Paired oval, each of them about 2-3 cm, the size and appearance of the ovaries depend on both age and the stage of the menstrual cycle. In the young adult they are almond shaped, solid, grayish pink and approximately 3 cm long, 1.5 cm wide and 1 cm thick. In the child, the ovaries are small structures, approximately 1.5 cm long. They have a smooth surface and at birth contain between 1 and 2 million primordial follicles, some of which will ripen into mature follicles in the reproductive years. The ovaries increase to adult size in the months preceding puberty. This considerable increase is brought about by proliferation of the stromal cells and by the commencing maturation of the ovarian follicles. After the menopause, no active follicles are present and the ovary becomes a small, shrunken structure with a wrinkled surface. The ovary is the only intra-abdominal structure not to be covered by peritoneum. Each ovary is attached to the cornu of the uterus by the ovarian ligament and at the hilum to the broad ligament by the mesovarium, which contains its supply of vessels and nerves. Laterally, each ovary is attached to the suspensory ligament of the ovary with folds of peritoneum which becomes continuous with that overlying the psoas major. Anterior to the ovary lie the Fallopian tubes, the superior portion of the bladder and the uterovesical pouch. The ovary is bound behind by the ureter where it runs downwards and forwards in front of the internal iliac artery. [46]
The surface of the ovaries is covered by a single layer of cuboidal cells, the germinal epithelium. Beneath this is an ill-defined layer of condensed connective tissue, the tunica albuginea, which increases in density with age. At birth, numerous primordial follicles are found, mostly in the cortex, but some are found in the medulla. With puberty, some form each month into Graafian follicles, which, at later stages of their development, form corpora lutea and ultimately atretic follicles, the corpora albicans. \[46\]

Polycystic ovary syndrome (PCOS) is one of the outstanding matters of endocrinological and gynecological investigation due to its complex pathogenesis and its multiple clinical expressions. PCOS are the most common endocrine disorder in reproductive age women and affect approximately 7-12% of this population worldwide \[47\]. Most recent research supports the opinion that androgen excess is a prerequisite diagnostic criterion for PCOS \[48\].

2.2 Pathogenesis:

Polycystic ovaries develop when the ovaries are stimulated to produce excessive amounts of male hormones (androgens), particularly testosterone by either release of excessive luteinizing hormone (LH) by the anterior pituitary gland or through high levels of insulin in the blood (hyperinsulinaemia) in women whose ovaries are sensitive to this stimulus. \[16, 17\] Along with that reduced levels of sex-hormone binding globulin can result in increased free androgens.

Pre- antral and small antral follicles produce AMH six times the density of pre-antral follicles compared with the normal ovary in PCOS.\[18\] High AMH levels in PCOS also due to increased production by individual follicles.\[19\]
Possible role of AMH in the pathophysiology of PCOS is through its counteraction on FSH in promoting follicular growth. The size of the 2-5 mm follicle pool is an independent and important contributor to the follicular arrest of PCOS.

The majority of patients with PCOS has insulin resistance and/or are obese. Their elevated insulin levels contribute to cause the abnormalities seen in the hypothalamic-pituitary-ovarian axis that lead to PCOS. Hyperinsulinemia increase GnRH pulse frequency, LH over FSH dominance, increased ovarian androgen production, decrease follicular maturation, and insulin resistance is a common finding among patients of normal weight as well as overweight patients.

Many cases of PCOS are characterised by a complex positive feedback loop of insulin resistance and Hyperandrogenism. In most cases it cannot be determined which of those two should be regarded causative. Experimental treatment with either antiandrogens or insulin sensitizing agents improves both hyperandrogenism and insulin resistance.

2.3 PCOS and associated complications:

Having polycystic ovary syndrome may make a series of conditions more likely, like type 2 diabetes, high blood pressure, cholesterol and lipid abnormalities, metabolic syndrome, non-alcoholic steatohepatitis, infertility, sleep apnea, depression and anxiety, abnormal uterine bleeding, cancer of uterine lining, gestational diabetes or pregnancy- induced high blood pressure especially if obesity also is a factor.

Untreated polycystic ovary syndrome may be regarded as a disorder that progresses until the time of menopause. On-going studies lend support to the hypothesis that women with the syndrome are at increased risk for the development of
cardiovascular disease. Because the syndrome is also associated with lipid abnormalities, affected women could benefit from measures to prevent cardiovascular disease and the other sequel of longstanding hypertension and diabetes mellitus that are associated with the syndrome.

More important, the long-term effects of unopposed estrogen place women with the syndrome at considerable risk for endometrial cancer, endometrial hyperplasia and, perhaps, breast cancer. The risk of endometrial cancer is three times higher in women with polycystic ovary syndrome than in normal women. In addition, small observational studies have suggested that chronic anovulation during the reproductive years is associated with a three to four times increased risk of breast cancer in the postmenopausal years.

2.4 Role of gonadotrophins in PCOS:

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotropins, hormones made in and released from pituitary gland that control the function of gonads, testes and ovaries, The absolute level of each, as well as the LH: FSH ratio, can offer significant insight into the PCOS patient. The ratio of LH to FSH (follicle stimulating hormone), when measured in international units, is greater than 1:1, as tested on day 3 of the menstrual cycle. A higher level of LH than FSH in the early part of the menstrual cycle is a hallmark of PCOS. Clearly increased LH is related to, if not diagnostic of, PCOS. Elevation in LH is very useful in diagnosis of PCOS. The measurement of FSH will also permit the diagnosis of an occult ovarian failure where the FSH levels are particularly elevated.

Women with PCOS have higher GnRH, which in turn results in an increased in LH/FSH ratio because of the pulsatile nature of their release, a single test fails to
detect an increase ratio of LH: FSH and increase serum testosterone. LH is sufficient to cause anovulation. \[^{30}\]

2.5 Role of antimüllerian hormone (AMH) in PCOS:

AMH is a glycoprotein growth factor and a member of the transforming growth factor super family (TGF-B) with a molecular weight of 140kDa. AMH also known as Müllerian Inhibiting Substance (MIS) is a new diagnostic marker of ovarian function. This hormone is made in the testes of men. However, now we know that in female neonates, AMH is virtually undetectable but increases gradually until puberty and remains relatively stable thereafter, and throughout the reproductive period. \[^{31}\]

AMH has an integral role in the intrauterine development and sex differentiation of the male fetus. It is secreted from the Sertoli cells of the developing testes inhibiting ipsilateral mullerian duct development and thereby allowing the Wolffian duct system to prevail \[^{32}\]. In female, Anti-Müllerian hormone (AMH) is produced by the granulosa cells of the recruited follicles until they become sensitive to FSH. AMH has been identified as a regulator of the recruitment, preventing the depletion of all primordial follicle pool at once. It is primarily produced by the pool of early-growing follicles, which are believed to serve as a proxy for the number of primordial follicles in the ovary. It is widely accepted that the reduction of AMH levels in serum is the first indication of a decline in the follicular reserve of the ovaries. AMH concentration remains stable throughout the menstrual cycle. \[^{34}\]

However, the role of AMH across the female reproductive life-span has only more recently come to light \[^{33}\]. In the ovary, AMH has an inhibitory effect on primordial follicle recruitment as well as on the responsiveness of growing follicles to FSH and leads to anovulation when secreted in excess amounts in PCOS. It has been proved,
however, that follicle number only added 5.3% to variance in the concentration of AMH, and raised production of hormone is an intrinsic property of granulosa cells in PCOS \cite{35, 36, 37}. The ovary-specific expression pattern in granulosa cells of growing non selected follicles makes AMH an ideal marker for the size of the ovarian follicle pool and also a prognostic factor for fertility potential \cite{32}.

Women with PCOS have an increased number of small follicles in the pre-antral and antral stage, and therefore, it is observed that their AMH serum concentrations are higher than their counterpart \cite{33}. first reported this in 1997, there have been several clinical studies that have confirmed the increased levels of serum AMH levels (two to three times) higher in PCOS compared with the levels in women with normal ovaries. Another study reported significantly elevated levels of AMH in normogonadotrophic, normoestrogenic, an-ovulatory patients compared with controls \cite{32}.

2.6 Previous studies:

Early studies have shown that in women with Polycystic Ovary Syndrome (PCOS) both serum and follicular fluid AMH levels are significantly higher compared to controls \cite{38}. Recent studies have confirmed the latter results \cite{39} suggesting that the increased in AMH levels in women with PCOS is related to the large number of small diameter (2–5 mm) follicles \cite{40}. The ovarian morphology of women with PCOS is characterized by the presence of a two- to six-fold increase in the ovarian follicular number (preantral and small antral follicles) \cite{40, 41}, most probably due to hyperandrogenemia \cite{42}. In anovulatory women with PCOS, a suspension of follicular development occurs when the diameter of the follicles ranges from 6–9 mm, just before the selection of the dominant follicle (follicular arrest) \cite{43}. Therefore, high
serum AMH levels in women with PCOS are related, most probably, to the follicular arrest that suspends the selection of the dominant follicle.

In young women with PCOS and in young normal ovulatory women, who served as controls, serum AMH levels did not correlate with serum FSH levels. On the contrary, serum AMH levels were positively correlated with LH levels in women with PCOS ($r = 0.414. p < 0.001$). The increased serum LH levels were the most significant conjunction between ovulation disorders that characterize PCOS, and high serum AMH levels $^{[39]}$.

In addition, women with classic PCOS (1990 NIH-diagnostic criteria) $^{[44]}$ presented significantly higher LH and LH/FSH ratio.

In conditions of increased LH and normal to low FSH, such as in PCOS, AMH serum levels are increased and tend to be associated to serum LH, while in conditions of increased FSH such as premature ovarian failure, AMH serum levels are decreased and tend to be associated to serum FSH in young PCOS women with hyperandrogenemia. Serum AMH levels are linked to serum LH levels, reflecting follicle overstimulation. The evidence that supports the theory of a link between AMH and LH in PCOS comes from both in vitro and in vivo experiments. Serum AMH levels have been directly linked to serum LH levels in the most severe forms of PCOS. LH has also been shown in vitro to directly increase serum AMH levels in PCOS derived granulosa cells. Finally, hyperandrogenism, obesity, insulin resistance and Oral contraceptive (OCs) administration, indirectly affect serum AMH levels, by modulating serum LH. Concerning PCOS, AMH correlation to LH can be used in the future for the assessment of the severity of PCOS, of the amelioration of PCOS under Oral contraceptive (OCs) treatment, as well as of the efficacy of infertility treatment in clomiphene resistant PCOS women $^{[49]}$. Apart from PCOS, the
implications of the theoretical approach that serum AMH levels are modulated by both gonadotropins might become important in clinical practise in a variety of medical conditions. For instance, serum AMH levels might be used in the future as a marker of cysts formation in the ovaries\textsuperscript{[45]} as well as of ovarian endometriosis, or as a marker of ovarian response to treatment of ovarian cysts or ovarian endometriosis by oral contraceptives or surgery. Additionally, new insights concerning the impact of AMH in normal pubertal development\textsuperscript{[46]} with clinical implications in the treatment of infertile women with hypothalamic amenorrhea where serum AMH levels might be used for the assessment of ovarian recovery.
Chapter three
3. Material and methods

3.1 Material

3.1.1 Study design:
Cross-sectional study was conducted in Sudanese women with polycystic ovary syndrome (PCOS).

3.1.2 Study area:
This study was carried out in 2 Fertility Centers at Khartoum State, at the University of Khartoum fertility center (U.K.F.C) (Saad Abu Alela hospital) and Dr. Elsir Abu EL hassan fertility center; during the period from December 2017 to March 2018.

3.1.3 Study subjects:
Patients who have PCOS ninety nine patients and thirty normal females as control measurement of were tested for serum gonadotrophins FSH, LH and anti-Müllerian hormone (AMH) Levels. The inclusion criteria included Sudanese women patients with polycystic ovary syndrome PCOS of all ages. And exclusion criteria rejected patients with heavy polycystic ovary syndrome PCOSH.

3.2 Data collection and clinical assessment:

3.2.1 Interview and questionnaire:
Interview with the patients was carried out to obtain the clinical data. A questionnaire was specifically designed to obtain information which helped in either including or excluding certain individuals in or out from the study.

3.2.2 Sample size:
Ninety nine patients with PCOS (test group) and 30 normal females (control group) were selected randomly.
3.2.3 Blood samples collection:
5ml of venous blood were collected after disinfection with 70% alcohol using plane vacutainers often having their consent.

3.3 Methods:

3.3.1 Biochemical methods:
Blood samples were obtained by venipuncture on day 2-3 of the menstrual period. The serum FSH and LH levels were measured by Automated Enzyme Immunoassay System TOSOH (model AIA 360).
AMH was measured by HumaReader HS using the AMH Gen II, Bekman Coulter enzyme linked immunosorbent assay (ELISA) kits.

3.3.2 Background to Automated Enzyme Immunoassay System TOSOH (model AIA 360):

3.3.2.1 Instrumentation:
Automated Enzyme Immunoassay System TOSOH (model ALA 360) instrument is basically a spectero photometer. It consists of a display and an Operation Panel, Carousel unit for reagent cup holder and the sample holder, it rotates the specimen and reagent cups. Substrate Compartment, this is used for the enzyme substrate bottle and the disinfect ethanol solution bottle. In the outside Printer Unit and Bottle Tray where the diluents, wash solution, waist fluid and disinfecting ethanol bottles are stored.

3.3.2.2 The principle:
Automated Enzyme Immunoassay System (TOSOH) technique for gonadotrophins measurement is defined as a method for determining the concentration of the FSH
and LH in samples by Fluorometric enzyme immunoassay (FEIA) using A ready-to-use “all-in-one” test cup which consist of aluminum top seal, lyophilized conjugate and magnetic beads coated with immobilized antibodies/antigens. The substrate and fluorescence kinetic detection to give the concentration of gonadotrophins in sample.

3.3.3 Background to HumaReader HS:

3.3.3.1 Instrumentation:

The HumaReader HS is a microprocessor-controlled, general-purpose photometer system designed to read and calculate the results of ELISA assays in micro titer plate. All human methods are preprogrammed and multiple calculations are available. They consist of power pilot lamp, touch panel to Display program and plate carrier and amicro plate.

3.3.3.2 Principle:

The HumaReader HS technique for AMH measurement using the AMH Gen II ELISA kits which is an enzymatically amplified two-site immunoassay. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm and between 600 and 630nm. The absorbance measured is directly proportional to the concentration of AMH in the samples. A set of AMH calibrators is used to plot a calibration curve of absorbance versus AMH concentration. The AMH concentration in the samples can then be calculated from this calibration curve.
3.3.3.3 Material and reagents:

All materials and reagents used in this study were of analytical grade:

1-Ab PLATE consists of Anti-AMH Gen II Antibody Coated Microtitration strips, the microtitration wells with mouse monoclonal anti-AMH IgG Immobilized to the inside wall of each well. AMH GenII Sample DILUENT: One bottles ,13 ml ,containing buffer with bovine serum albumin (BSA)<0.5 %PRO*300and sodium azide.

2-BIO_ CON_J RTU: AMH Gen II antibody-Biotin Conjugate: One bottle,13 ml containing biotinylated anti-AMH antibody in buffer with protein (bovine ,mouse)<0.3% Pro Clin 300 and sodium azide.

3-STERP-CONJ-RTU: Sterptavidin-Enzyme Conjugate: One bottle,13 ml, containing streptavidin –HRP in buffer with protein (mouse,fish) and<10% methanol.

4-ASSAY-BUFFER: AMH GenII Assay Buffer: Two bottle, 13ml, containing buffer with BSA ,protein ,<0.3% proClin 300 and sodium azide.

5- TMB SOLN: TMB Chromogen Solution :One bottle,11 ml containing a solution of TMB in citrate buffer with hydrogen peroxide.

6- WASHCON I: Wash Concentrate I: 100 ml , containing buffered saline with a nonionic detergent.

7-STOP-SOLN-A: Stopping Solution A: 0.2 M sulfuric acid.

3.3.3.4 Preparation of Reagents:

1-Wash Solution: Dilute 1 part Wash Concentrate 1 with 9 parts deionized water.

2-Microtitration Wells: Select the number of coated wells required for the assay.

3.3.3.5 Sample preparation:

For the determination of serum AMH the calibrators , controls and samples are incubated in microtitration wells which have been coated with anti-AMH antibody.
After incubation and washing, anti-AMH detection antibody labeled with biotin is added to each well. After a second incubation and washing step, streptavidin-horseradish peroxidase (HRP) is added to the wells. After a third incubation and washing step, the substrate tetramethylbenzidine (TMB) is added to the wells. Lastly an acidic stopping solution is added. The AMH concentration in the samples can be calculated from the calibration curve of absorbance versus AMH concentration.

3.4 Ethical consideration:

- Written consent was obtained from Ethical Committee – International University of Africa.
- The objectives of the study were explained to all individuals participating in this study.

3.5 Data analysis:

Data was managed and analyzed using statistical package for the social sciences (SPSS) program.
Chapter four

4. Results

This study aimed to investigate the serum gonadotrophins and anti-Müllerian hormone levels in patients with polycystic ovary syndrome, so as to find a correlation to either reinforce or refute any claims of relationship between them as a diagnostic or screening tool.

The study targeted 99 patients with PCOS and 30 healthy persons.

Table 1. Serum hormones concentration in normal and patients with PCOS

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=30)</th>
<th>Patients (n=99)</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (ng/mL)</td>
<td>9.19±0.66</td>
<td>6.46±0.20</td>
<td>0.000</td>
</tr>
<tr>
<td>LH (ng/mL)</td>
<td>6.80±0.44</td>
<td>9.21±0.64</td>
<td>0.002</td>
</tr>
<tr>
<td>AMH (ng/mL)</td>
<td>2.71±0.14</td>
<td>8.74±0.40</td>
<td>0.000</td>
</tr>
<tr>
<td>FSH/LH</td>
<td>1.49±0.13</td>
<td>0.98±0.06</td>
<td>0.000</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>0.88±0.11</td>
<td>1.48±0.10</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Mean ± Standard error of mean

Table (1) showed the hormones concentration in the serum of normal and patients with PCOS, the results revealed highly significant (P<0.01) differences between patients with PCOS and control in all studied hormones. FSH in normal subjects was higher (9.19 ng/mL) than in patients with PCOS (6.46 ng/mL). While the patients with PCOS were higher than normal subjects in LH (9.21ng/ml) and AMH (8.74ng/ml).

The FSH/LH ratio in normal subjects was higher (1.49 ng/mL) than patients with PCOS (0.98 ng/mL). While the patients with PCOS were higher LH/FSH ratio (1.48 ng/ml) than the normal subjects in (0.88 ng/mL).
Table 2. Association between studied hormones with each other in normal and patients with PCOS

<table>
<thead>
<tr>
<th></th>
<th>FSH</th>
<th>LH</th>
<th>AMH</th>
<th>FSH/LH</th>
<th>LH/FSH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FSH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.181**</td>
<td>1</td>
<td></td>
<td>0.316**</td>
<td>1</td>
</tr>
<tr>
<td>Patients</td>
<td>0.095</td>
<td>1</td>
<td></td>
<td>0.095</td>
<td>1</td>
</tr>
<tr>
<td><strong>AMH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>-0.066</td>
<td>0.191</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>-0.132</td>
<td>0.095</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FSH/LH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.654**</td>
<td>-0.571**</td>
<td>-0.178</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>0.057</td>
<td>-0.681**</td>
<td>-0.246</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>LH/FSH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.632**</td>
<td>0.305</td>
<td>0.209</td>
<td>-0.780**</td>
<td>1</td>
</tr>
<tr>
<td>Patients</td>
<td>-0.182</td>
<td>0.815**</td>
<td>0.142</td>
<td>-0.785**</td>
<td>1</td>
</tr>
</tbody>
</table>

**= significant differences at P<0.01
*= significant differences at P<0.05

Table (2) FSH concentration correlated significantly (0.316) and positively (P<0.01) with LH of the patients with PCOS and FSH/LH and LH/FSH of the normal subjects. The highest correlation coefficient was found between LH and LH/FSH in patients with PCOS followed by the negative correlation coefficient of FSH/LH and LH/FSH in patients with PCOS. Moreover, the association between AMH and FSH/ LH was significantly (0.246) negatively (P<0.05) correlated in the patients with PCOS.

Table 3. Serum hormones concentration in different age groups

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Less than 22 (n=11)</td>
</tr>
<tr>
<td>FSH</td>
<td>7.42±0.60</td>
</tr>
<tr>
<td>LH</td>
<td>8.64±1.49</td>
</tr>
<tr>
<td>AMH</td>
<td>8.65±1.09</td>
</tr>
<tr>
<td>FSH/LH</td>
<td>1.05±0.13</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.20±0.22</td>
</tr>
</tbody>
</table>

Means ± Standard error of mean
The results presented in table (3) indicated that, there was no significant difference in serum hormones concentration between different age groups being studied otherwise there were higher number of PCOS cases found in the age group 22-32 (n=90) that means it is the main age range at which the women have high capacity in their reproductive system and the PCOS clear in them.

Table 4. Serum hormones concentration in different geographical areas of the Sudan

<table>
<thead>
<tr>
<th>Area of Sudan</th>
<th>Center (n=63)</th>
<th>West (n=5)</th>
<th>East (n=8)</th>
<th>North (n=13)</th>
<th>South (n=10)</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>6.92±0.25</td>
<td>6.44±1.01</td>
<td>7.35±0.61</td>
<td>7.05±1.12</td>
<td>8.93±1.22</td>
<td>0.257</td>
</tr>
<tr>
<td>LH</td>
<td>8.68±0.65</td>
<td>6.50±1.18</td>
<td>9.24±1.03</td>
<td>8.81±1.08</td>
<td>8.73±1.95</td>
<td>0.943</td>
</tr>
<tr>
<td>AMH</td>
<td>7.40±0.43</td>
<td>9.98±2.67</td>
<td>4.56±0.93</td>
<td>8.15±1.67</td>
<td>6.54±1.07</td>
<td>0.200</td>
</tr>
<tr>
<td>FSH/LH</td>
<td>1.10±0.07</td>
<td>1.11±0.25</td>
<td>0.92±0.17</td>
<td>0.94±0.15</td>
<td>1.49±0.33</td>
<td>0.313</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.34±0.10</td>
<td>1.12±0.27</td>
<td>1.35±0.22</td>
<td>1.55±0.31</td>
<td>1.18±0.31</td>
<td>0.868</td>
</tr>
</tbody>
</table>

Mean ± Standard error of mean

The results presented in table (4) clearly showed that there was no significant difference in the concentration of hormones in the serum of patients with PCOS, concerning the geographical areas of the Sudan. High numbers of PCOS cases were found in the central part of Sudan, followed by north, south and the low number of patients with PCOS cases were from western and eastern part of Sudan.
Chapter five

5.1. Discussion:

This prospective study was conducted to evaluate the relevance of routine gonadotrophins (fsh, lh) and AMH measurement in infertile females with PCOS.

Table 1 & 2 in the current study we demonstrated the concentration of Serum hormones and Associated between them in normal and patients with PCOS indicated that there was highly significant differences (P<0.01) in all studied hormones. FSH in normal subjects was higher (9.19 ng/mL) than in patients with PCOS (6.46 ng/mL), while the patients with PCOS were higher than normal subjects in LH (9.21ng/ml) and AMH (8.74ng/ml) confirm those of previous studies \[38, 39, 40,41,42,43,46\] showing that AMH levels are 2- to 3-fold higher in women with PCOS compared to healthy women. The FSH/LH ratio in normal subjects was higher (1.49 ng/mL) than patients with PCOS (0.98 ng/mL). While the patients with PCOS have higher in LH/FSH ratio (1.48 ng/ml) than the normal subjects, which are comparable with the results of previous studies \[50, 51\].

FSH concentration correlated significantly (0.316) and positively (P<0.01) with LH of the patients with PCOS and FSH/LH and LH/FSH of the normal subjects. The highest correlation coefficient was found between LH and LH/FSH in patients with PCOS followed by the negative correlation coefficient of FSH/LH and LH/FSH in patients with PCOS. Moreover, the association between AMH and FSH/ LH was significantly (0.246) negatively (P<0.05) correlated in the patients with PCOS. Pigny et al \[49\] found no relationship between AMH, LH and LH\FSH in PCOS and controls While Begway et al. \[50\] found positive correlation between AMH and LH, LH\FSH in both groups.
There was no significant difference in serum hormones concentration between different age groups being studied and. Pigny et al. [49] found no relationship between AMH and age, LH and LH\FSH in PCOS and controls and there was no significant difference in the concentration of hormones in the serum of the subjects, taken from different geographical areas of the Sudan.
5.2 Conclusions:

The prevalence of infertility is significantly high worldwide. Amongst the female infertility PCOS is an important cause and which must be correctly diagnosed for the effective treatment. Oocyte number and quality decline with age; however, fertility varies significantly even among women of the same age. Serum antiMüllerian hormone (AMH), is one of the hormone biomarker of follicle number has become known in recent years.\textsuperscript{[52]}

In conditions of increased LH and normal to low FSH, such as PCOS, AMH serum levels are increased and tend to be associated to serum LH while in young PCOS women with hyperandrogenemia serum AMH levels are linked to serum LH levels, reflecting follicle overstimulation. The evidence that supports the theory of a link between AMH and LH in PCOS comes from both in vitro and in vivo experiments. Serum AMH levels have been directly linked to serum LH levels in the most severe forms of PCOS. LH has also been shown in vitro to directly increase serum AMH levels in PCOS derived granulosa cells. Finally, hyperandrogenism, obesity, insulin resistance and OCs administration, indirectly affect serum AMH levels, by modulating serum LH.
5.3 Recommendations:

- AMH correlation to LH could be used in the future for the assessment of the severity of PCOS, of the amelioration of PCOS under oral contraceptive (OCs) treatment, as well as of the efficacy of infertility treatment in clomiphene resistant women with PCOS.

- Since serum AMH being more stable during the entire menstrual periods could be used as a better marker over FSH and LH for diagnosis of polycystic ovary syndrome especially where the ultra sonographic examination of the ovaries is not feasible.

- Studies are needed to determine the cut off of AMH for diagnosis of PCOS.
REFERENCES


7. Goldenberg N, Glueck C. Medical therapy in women with polycystic ovary syndrome before and during pregnancy and lactation. Minerva Ginecol, 2008; 60(1): 63-75. PMID 18277353.


39. Piouka A, Farmakiotis D, Macut D, Gerou S, Katsikis I, Panidis D. Anti-Mullerian hormone levels are increased in women with classical PCOS and are negatively influenced by obesity. Am J Endocrinol Metab 2009;296:E238–43.


45. Hagen CP, Aksglaede L, Sørensen K, Main KM, Boas M, Cleemann L. Serum levels of anti-Müllerian hormone as a marker of ovarian function in 926 healthy


47. Panidis D, Farmakiotis D, Rousso D, Katsikis I, Kourtis A, Diamanti-Kandarakis E. Serum luteinizing hormone levels are markedly increased and significantly correlated with D4-androstenedione levels in lean women with polycystic ovary syndrome. Fertil Steril 2005; 84: 538-540.


4.5 Appendices:

Questionnaire:

NO…………………………………………………………

Patient name…………………………………………

Age………………………………………………

Geographical Area……………………………………

LH Concentration……………………………………

FSH Concentration……………………………………

AMH Concentration…………………………………