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Antimullerian Hormone as a Predictor of Ovarian Reserve and Ovarian Response in *IVF* Candidates

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university of other institute, except where due acknowledgment has been made in the text.

Signature	Name	Date
Μοηαμμεδ	**Mohammed Marwan Atta Laqqan**	**15-06-2010**

Dedication

To my father and my mother who taught me how to give

To my wife who supported me wholeheartedly

To my son Odai

To my brothers and sisters

To all my teachers who supported me

To all my friends who spare no effort to help

To all my students who helped me

To all of them I dedicate this work

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Antimullerian Hormone as a Predictor of Ovarian Reserve and Ovarian Response in *IVF* Candidates

Abstract

Background: Antimullerian hormone (AMH) is expressed only in the gonads. In male, it is secreted by immature sertoli cells and in female by adult granulosa cells of the ovary. AMH is responsible for the regression of mullerian ducts in male fetus. Absence of AMH results in development of female fetus reproductive organs and it is believed to control the formation of primary follicles.

Objective: To assess AMH in early follicular phase as a predictor of ovarian reserve among females undergoing *in vitro* fertilization (IVF) in the Gaza Strip.

Methods: In this case-control prospective study, a meeting interview was used for filling in the questionnaire. AMH was determined by enzyme linked immunosorbent assay in 162 women: 81 women undergoing IVF at Al-Basma Fertility Center in Gaza City (cases) and 81 healthy women, having at least given birth to one healthy child (controls). The number of oocytes and embryos were recorded for each female in the controls group and the occurrence of pregnancy was followed for the three months. Data were computer analyzed using SPSS statistical package version 13.

Results: The AMH mean level in the cases was significantly higher as compared to the controls (3.5±2.3 ng/mL vs. 1.7±0.5 ng/mL; p=0.00). The AMH in cases was significantly decreased with increasing age (3.7±2.0, 3.6±2.4 and 2.1±2.1 ng/mL at ≤25, 26-35 and >35 years, respectively; F=2.327 and p=0.104). The total number of retrieved oocytes was inversely associated with age (12.5±4.5, 11.0±5.4 and 6.9±4.7 at age ≤25, 26-35 and >35 years, F=4.793 and p=0.011). Results showed that the ovarian response to Menotrophin (FSH 75IU, LH 75 IU) stimulation was better with younger age (<4, 4-8, 9-16 and >16 oocytes at mean age of 36.5±5.0, 30.6±5.9, 27.0±4.5 and 26.3±5.2, respectively; F=4.934 and p=0.003). There was a significant positive association between ovarian response in terms of total number of oocytes and AMH levels (<4, 4-8, 9-16 and >16 oocytes at 1.0±0.5, 2.3±1.8,

3.7±1.8 and 5.90±2.9 ng/mL, respectively; F=9.174 and p=0.000), implying that AMH can be used as a good predictor of ovarian reserve and ovarian response. IVF results showed that the chance of pregnancy success increased with decreased age (F=3.077 and p=0.05). Moreover, the maximum level of AMH was observed in females who achieved positive pregnancies (4.5±2.5 ng/mL) followed by negative pregnancies (2.9±1.8 ng/mL) and no cleavage (2.1±1.5 ng/mL) with significant differences (F=6.862 and p=0.002). It is worth-mentioning that AMH levels may be associated with ovarian responsiveness, but not with the probability to achieve a pregnancy. The maximum number of total oocytes was recorded in females who achieved positive pregnancies (13.7±5.1 oocytes). Correlation coefficient revealed that the number of mature oocytes showed strong positive correlation with the AMH levels (r=0.469, p=0.001).

Conclusion: AMH can be used in IVF programs as a good predictor of ovarian reserve and ovarian response.

Keywords: Anti-mullerian hormone (AMH), Ovarian reserve, *In vitro* fertilization (IVF) candidates, Gaza Strip

استخدام هرمون إلانتي مولارين كمؤشر على مخزون المبيض ومدي استجابة مبيض المرشحين لعملية التلقيح الصناعي

ملخص الدراسة

مقدمة: هرمون إلانتي مولارين يتم تكوينه عبر الغدد التناسلية فقط. حيث يفرز في الذكور بواسطة خلايا السورتولي الغير ناضجة بينما في الإناث تنتجه الخلايا الحبيبية الخاصة بالمبيض، وجود هذا الهرمون خلال مرحلة تكوين الجنين الذكري يكون مسئول عن اختفاء قنوات مولر بينما عدم وجوده خلال تكوين الجنين الأنثوي يسمح بتطور الأعضاء التناسلية الأنثوية. ويعتقد انه يتحكم بعملية تكوين الحويصلات الأولية.

الهدف من الدراسة: تحديد مستوى هرمون الانتي مولارين خلال الطور الحويصلي المبكر وإمكانية استخدامه كمؤشر على مخزون المبيض ومدي استجابة مبيض السيدات الراغبات في الخضوع لعملية التلقيح الصناعي في قطاع غزة.

الطريقة: تم إجراء مقابلة شخصية مع 162سيدة تم توزيعهم كالتالي: 81 سيدة يمثلن العينة الضابطة و 81 سيدة يرغبن بإجراء عملية التلقيح الصناعي في مركز البسمة للإخصاب وهم عبارة عن العينة المستهدفة من اجل إكمال البيانات والتحقق من استيفاء الشروط. ومن ثم تم تحديد مستوى هرمون الانتي مولارين بواسطة طريقة الإليزا، وتحديد عدد البويضات والأجنة الناتجة من عملية التلقيح. بالإضافة إلي متابعة سيدات العينة المستهدفة لمدة ثلاثة أشهر من اجل التحقق من حدوث الحمل من عدمه. وكل هذه النتائج تم تحليلها باستخدام "SPSS "13.

النتائج: من خلال هذه الدراسة وجدنا أن مستوي هرمون الانتي مولارين في العينة المستهدفة (أعلى من مستواه في العينة الضابطة وكان كما يلي (3.5±2.3ng/mL، 1.7±0.5ng/mL) على التوالي. كما أن مستوى هرمون الانتي مولارين يقل مع زيادة العمر في العينة المستهدفة كالتالي (3.7±2.0، 3.6±2.4، 2.1±2.1 ng/mL عند أقل من 25، 26-35، أكثر من 35 سنة على التوالي) وعدد البويضات الكلي أيضا يقل مع زيادة العمر كما هو موضح (12.5±4.5، 11.0±5.4، 6.9±4.7) وهذا النقصان ذا دلالة إحصائية واضحة. ووجد أيضا أن استجابة مبيض السيدات لعملية التحفيز تتحسن كلما قل العمر (أقل من 4، 4-8، 9-16 وأكثر من 16 بويضة كان العمر 26.3±5.2، 27.0±4.5، 30.6±5.9 و 36.5±5.0 على التوالي) وهذا التحسن ذا دلالة إحصائية واضحة مع العمر وهناك دلالة إحصائية بين مستوى هرمون الانتي مولارين ومدي الاستجابة المبيض لعملية التلقيح والتي كانت على النحو التالي (أقل من 4، 4-8، 9-16 وأكثر من 16 بويضة كان مستوى الهرمون 1.0±0.5، 2.3±1.8، 3.7±1.8 و 5.90±2.9 ng/mL على التوالي) لذلك يمكن استخدام هرمون الانتي مولارين كمؤشر على مخزون المبيض ومدي استجابة المبيض لتحفيز خلال عملية التلقيح الصناعي. كما وجد أن نتائج التلقيح الصناعي تميل إلي النجاح (حدوث حمل) كلما قل العمر،

وأن أعلى مستوي لهرمون الانتي مولارين يظهر في الحالات التي حدث فيها حمل (ng/mL2.5±4.5) مقارنة بحالات عدم حدوث حمل أو عدم انقسام البويضات الملقحة. وهذا يدل على أن ارتفاع مستوى هرمون الانتي يعمل على تحسين استجابة المبيض أثناء عملية التلقيح الصناعي ، كما وجد أن أعلى عدد بويضات ظهر في الحالات التي ينتج عنها حمل (13.7±5.1 بويضة). كما أظهرت الدراسة أيضاً أن هناك ارتباط ايجابي قوي بين عدد البويضات الناضجة ومستوى هرمون الانتي مولارين.

الاستنتاج: لقد أظهر نتائج هذه الدراسة أن هناك إمكانية لاستخدام هرمون الانتي مولارين كمؤشر على مخزون المبيض ومدي استجابة المبيض للتحفيز خلال عملية التلقيح الصناعي.

الكلمات المفتاحية: هرمون إلانتي مولارين، مخزون المبيض، المرشحين لتلقيح الصناعي، قطاع غزة.

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Abbreviations

AFC	**Antral follicle count**
AMH	**Antimullerian hormone**
AMHR	**Antimullerian hormone receptor**
ART	**Assisted reproductive technology**
COH	**Controlled ovarian hyperstimulation**
E2	**Estradiol hormone**
ELISA	**Enzyme linked immunosorbent assay**
FSH	**Follicle-stimulating hormone**
GIFT	**Gamete intrafallopian tube transfer**
GnRH	**Gonadotropin-releasing hormone**
HCG	**Human chorionic gonadotropin**
IVF	**In vitro fertilization**
ICC	**Intra-class correlation coefficient**
ICSI	**Intracytoplasmic sperm injection**
LH	**Luteinizing hormone**
MIF	**Mullerian-inhibiting factor**
MIS	**Mullerian-inhibiting substance**
OHSS	**Ovarian hyperstimulation syndrome**
PIH	**Prolactin-inhibiting hormone**
PRL	**Prolactin hormone**
RTU	**Conjugate-Ready-to-Use**
T3	**Triiodothyronine**
T4	**Thyroxine**
TGF-β	**Transforming growth factor-β**
TMB	**Tetramethylbenzidine**
TRH	**Thyrotropin-releasing hormone**
TSH	**Thyroid-stimulating hormone**
WHO	**World Health Organization**
ZIFT	**Zygote intrafallopian tube transfer**

Chapter I Introduction

1.1. Overview

Antimullerian hormone (AMH), also known as mullerian-inhibiting substance (MIS) or mullerian-inhibiting factor (MIF) is responsible for the regression of mullerian ducts in the male fetus. The development of the mullerian ducts is controlled by the presence or absence of this hormone. AMH is only expressed in the gonads, and in male it is secreted by immature sertoli cells and in female by adult granulosa cells of the ovary (Josso, 1986; Maheswaran *et al.*, 2001).

AMH is a dimeric glycoprotein and it is structurally related to inhibin and activin. It is a member of the transforming growth factor-β (TGF-β) family (Kobayashi and Behringer, 2003).

Normal human female serum contains measurable amounts of AMH during the reproductive life span. AMH level varies slightly with the menstrual cycle, reaching a maximum in the late follicular phase (Hasegawa *et al*.,1996). The AMH gene is located on chromosome 19, while the gene AMHR2 which codes for its receptor is located on chromosome 12 (Cate *et al.*, 1986).

The hormone and its receptor are expressed in the granulosa cells of preantral and small antral follicles; therefore an important role for AMH in the regulation of follicle selection or maturation has been hypothesized (Hirobe *et al*., 1992).

AMH may be functionally active in the female gonad affecting the transition from resting primordial follicles into growing follicles. Furthermore, AMH may be involved in the recruitment of follicle-stimulating hormone (FSH)- sensitive follicles in the early antral stage (Wunder *et al*., 2008).

AMH can be used for

1. Evaluating fertility potential and ovarian response in *in vitro* fertilization (IVF) – Serum AMH levels correlate with the number of early antral follicles. This makes it useful for prediciting ovarian response in an *in vitro* fertilization cycle. Women with low AMH levels are more likely to be poor ovarian responders (Wunder *et al*., 2008).
2. Measuring ovarian ageing – diminished ovarian reserve, is signaled by reduced baseline serum AMH concentrations. Women with poor ovarian reserve who have entered the menopause have low levels of AMH (Wunder *et al*., 2008).

E2 is produced mainly by the ovary, placenta, and in smaller amounts by the adrenal cortex (Kronenberg *et al.*, 2008). There are two peaks of secretion: one just before ovulation and one during the mid-luteal phase. After menopause, estrogen secretion declines to low levels (Ganong, 2003). The action of E2 is to facilitate the growth of the ovarian follicles and increase the motility of the uterine tubes.

FSH and LH are released from the anterior pituitary gland and transported by the blood stream to their site of action. The action of FSH is to stimulate the growth of ovarian follicles; that is, it initiates egg development in cycles of approximately 28 days. FSH also stimulates secretion of estrogen by the follicle cells (Scanlon and Sanders, 2007). LH, on the other hand, is responsible for ovulation i.e., the release of a mature ovum from an ovarian follicle. LH then stimulates that follicle to develop into the corpus luteum, which secretes progesterone, also under the influence of LH (Scanlon and Sanders, 2007).

1.2. Aim of the study

The overall objective of this study is to assess AMH in early follicular phase as a predictor of ovarian reserve among females undergoing IVF in the Gaza Strip.

The specific objectives are:

1. To measure the level of AMH in women preparing for IVF as compared to healthy women.
2. To determine estradiol (E2), follicle-stimulating hormone (FSH), luteinizing hormone (LH)**,** prolactin hormone (PRL) and thyroid-stimulating hormone (TSH) in both cases and control women.
3. To study the correlation between AMH levels and age of women undergoing IVF.
4. To study the correlation between AMH levels and ovarian response to Menotrophin (FSH 75IU, LH 75 IU) stimulation during IVF program.

1.3. Significance:

This is the first study to assess AMH as a predictor of ovarian reserve and ovarian response in IVF candidates in Gaza Strip.

AMH is hypothesized to play a role in the detection of the ovarian reserve and may be used as a marker for ovarian aging.

Chapter II literature Review

2.1. Ovaries

The female reproductive system includes the ovaries that are responsible for producing eggs and certain sex hormones. Once women reach sexual maturity, they experience a monthly fertility cycle that is regulated by four hormones (estrogens, progesterone, follicle-stimulating hormone and luteinizing hormone). These hormones control the maturation and release of a few eggs every month, and affect the lining of the womb making it ready to receive a fertilized egg (Snell, 2007). Mature eggs are released from the ovary and move along the fallopian tube to the uterus. If an egg is fertilized, it tries to embed itself in the uterus wall, ready to develop into a fetus. If no egg becomes implanted, the uterus lining is shed, and this results in a flow of menstrual blood, know as the period (Marieb, 2006).

2.1.1. Structure and location of the ovary

The mature ovary is roughly a bean-shaped structure. It consists of an outer cortex and a central medulla with the addition of an inner hilus that serves as the point of entry and exit of blood vessels and nerves (Snell, 2007). The ovaries are located in the pelvis, attached to a structure called the broad ligament by the mesovarium, that part of the broad ligament extending between the attachment of the mesovarium and the lateral wall of the pelvis is called the suspensory ligament of the ovary (Kronenberg *et al*., 2008). The round ligament of the ovary, which represents the remains of the upper part of the gubernaculum, connects the lateral margin of the uterus to the ovary (Scanlon and Sanders, 2007). The ovary usually lies against the lateral wall of the pelvis in a depression called the ovarian fossa, bounded by the external iliac vessels above and by the internal iliac vessels behind (Frederic *et al*., 2008). The position of the ovary is, however, extremely variable, and it is often found hanging down in the rectouterine pouch. During pregnancy, the enlarging uterus pulls the ovary up into the abdominal cavity. After childbirth, when the broad ligament is lax, the ovary takes up a variable position in the pelvis (Snell, 2007).

The ovaries are surrounded by a thin fibrous capsule, the tunica albuginea. This capsule is covered externally by a modified area of peritoneum called the germinal epithelium. The term germinal epithelium is a misnomer because the layer does not give rise to ova (Scanlon and Sanders, 2007). Oogonia develop before birth from primordial germ cells. Before puberty, the ovary is smooth, but after puberty, the ovary becomes progressively scarred as successive corpora lutea degenerate. After menopause, the ovary becomes shrunken and its surface is pitted with scars (Kronenberg et al., 2008).

2.1.2. Development of the ovary

The female sex chromosome causes the genital ridge on the posterior abdominal wall to secrete estrogens. The presence of estrogen and the absence of testosterone induce the development of the ovary and the other female genital organs. The sex cords contained within the genital ridges contain groups of primordial germ cells. These become broken up into irregular cell clusters by the proliferating mesenchyme (Krohmer, 2004). The germ cells differentiate into oogonia, and by the third month, they start to undergo a number of mitotic divisions within the cortex of the ovary to form primary oocytes. These primary oocytes become surrounded by a single layer of cells derived from the sex cords, called the granulosa cells. Thus, primordial follicles have been formed, but later, many degenerate (Krohmer, 2004; Snell, 2007).

2.1.3. Ovarian hormones

The ovaries are the organs responsible for the production of the female germ cells, the ova, and the female sex hormones (Frederic *et al.*, 2008). The ovaries secrete large amounts of estrogens and small amounts of androgens. Androgens are also secreted from the adrenal cortex in both sexes. The ovaries also secrete progesterone, a steroid that has special functions in preparing the uterus for pregnancy (Scanlon and Sanders, 2007).

Particularly during pregnancy, the ovaries secrete the polypeptide hormone relaxin, which loosens the ligaments of the pubic symphysis and softens the cervix, facilitating delivery of the fetus. In both sexes, the gonads secrete other polypeptides, including AMH and inhibin B (Ganong, 2003). AMH is responsible for the regression of mullerian ducts in the male fetus. These ducts give rise to the female reproductive structures, oviducts, uterus, and cervix (Maheswaran *et al*., 2001). As Shown in Figure 2.1.

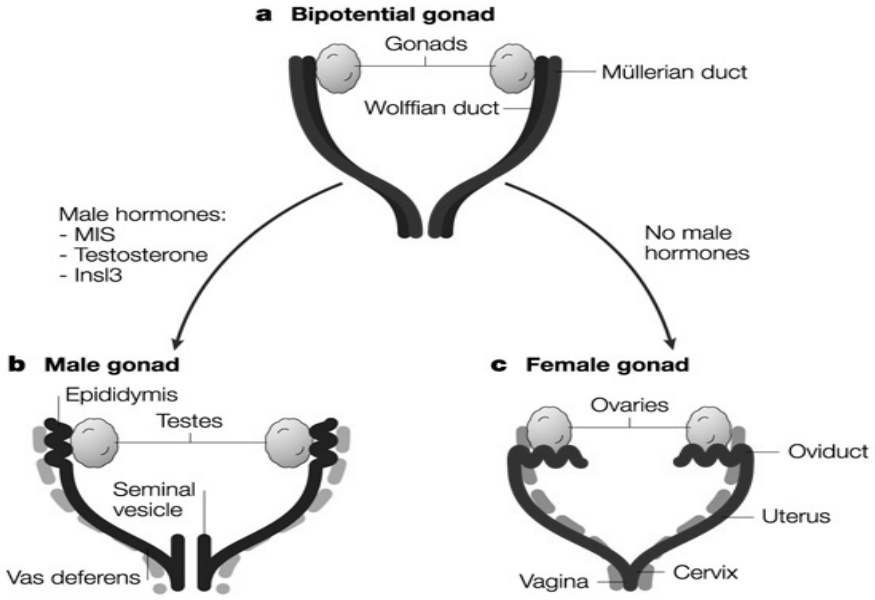

Figure 2.1. Action of AMH on mullerian ducts (Adopted from Kobayashi and Behringer, 2003).

MIS: Mullerian inhibiting substance
Insl3: insulin-like growth factor

Inhibin B inhibits FSH secretion (Josso and Picard *et al*., 1986). The secretory and gametogenic functions of the gonads are both dependent upon the secretion of the anterior pituitary gonadotropins, FSH and LH. The sex hormones and inhibin B feed back to inhibit gonadotropin secretion. In postpubertal females an orderly, sequential secretion of gonadotrophins is necessary for the occurrence of menstruation, pregnancy, and lactation (Ganong, 2003).

2.1.3.1. Estradiol

Estradiol (E2) is a C18 steroid characterized by the presence of an aromatic A ring (Gruber *et al.*, 2002), a phenolic hydroxyl group at C-3, and either a hydroxyl group (estradiol) or a ketone group (estrone) at C-17, synthesized from cholesterol (Kronenberg *et al*., 2008).

Secretion and target site

It is the most potent natural estrogen, produced mainly by the ovary, placenta, and in smaller amounts by the adrenal cortex (Kronenberg *et al.*, 2008). There are two peaks of secretion: one just before ovulation and one during the mid-luteal phase. After menopause, estrogen secretion declines to low levels (Ganong, 2003). In blood stream, 98% of estrogen circulates bound to sex hormone binding globulin. To a lesser extent it is bound to other serum protein such as albumin. Estrogen activity is affected via estradiol-receptor complexes which trigger the appropriate response at the nuclear level in the target site. These sites include the follicles, uterus, breast, hypothalamus and pituitary gland (Siiteri *et al*., 1982; Ganong, 2003).

Actions

Steroid hormones are soluble in the lipids of the cell membrane and diffuse easily into a target cell. Once inside the cell, the steroid hormone combines with a protein receptor in the cytoplasm, and this steroid protein complex enters the nucleus of the cell. Within the nucleus, the steroid-protein complex activates specific genes, which begin the process of protein synthesis. The enzymes produced bring about the cell's characteristic response to the hormone (Kronenberg *et al*., 2008). Estrogens facilitate the growth of the ovarian follicles and increase the motility of the uterine tubes. They increase uterine blood flow and have important effects on the smooth muscle of the uterus (Gruber *et al*., 2002). Estrogens decrease FSH secretion. Under some circumstances, they inhibit LH secretion (negative feedback); in other circumstances, they increase LH secretion (positive feedback). Women are sometimes given large doses of estrogens for 4-6 days to prevent conception after coitus during the fertile period (Ganong, 2003).

Regulation

The rising E2 concentration is understood to exert a positive feedback influence at the level of the pituitary where it influences the secretion of the gonadotropins, FSH and LH which are essential for follicular maturation and ovulation, respectively (Scanlon and Sanders, 2007).

2.2. Pituitary gonadotropins

2.2.1. Follicle stimulating hormone

Follicle stimulating hormone (FSH) is a glycoprotein consisting of α and β subunits with an approximate molecular mass of 35.5 KDa. The α-subunit is similar to that of other pituitary hormones (e.g., TSH and CGH), while the β-subunit is unique (Kosasa, 1981). The sugar part of the hormone is composed of fucose, galactose, mannose, galactosamine, glucosamine, and sialic acid. The half-life of human FSH is about 170 minutes (Ganong, 2003).

Secretion and target site

The hypothalamic gonadotropin-releasing hormone (GnRH) causes release of FSH from the anterior pituitary gland and the hormone is transport by the blood stream to its site of action, i.e., the ovary (Scanlon and Sanders, 2007).

Actions

FSH binds to a specific FSH glycoprotein transmembrane receptor, which is located exclusively on granulosa cells in ovarian follicles. Subsequent activation of the G-protein-coupled adenylate cyclase system results in cell proliferation and differentiated function (Ganong, 2003). FSH is named for one of its functions in women. Within the ovaries are ovarian follicles that contain potential ova (egg cells). FSH stimulates the growth of ovarian follicles; that is, it initiates egg development in cycles of approximately 28 days. FSH also stimulates secretion of estrogen by the follicle cells (Scanlon and Sanders , 2007).

2.2.2 Luteinizing hormone

Luteinizing hormone (LH) is a glycoprotein consisting of α and β subunits with an approximate molecular mass of 30 KDa. The α-subunit is similar to that of other pituitary hormones (e.g., TSH and CGH), while the β-subunit is unique (Kosasa, 1981). The carbohydrate content of LH is between 15% and 30% (Ganong, 2003).

Secretion and target site

The hypothalamic gonadotropin-releasing hormone (GnRH) causes release of LH from the anterior pituitary gland and the hormone is transported by the blood stream to their site of action, i.e., the ovary (Scanlon and Sanders, 2007).

Actions

The receptors for LH are serpentine receptors coupled to G_{s-} protein and adenylyl cyclase signal translocation system (Ganong, 2003). In women, LH is responsible for ovulation, the release of a mature ovum from an ovarian follicle. LH then stimulates that follicle to develop into the corpus luteum, which secretes progesterone, also under the influence of LH (Scanlon and Sanders, 2007).

2.2.3. Regulation of pituitary gonadotropin hormones

The principal regulator of LH and FSH secretion is gonadotropin-releasing hormone (GnRH). GnRH is a ten amino acid peptide that is synthesized and secreted from hypothalamic neurons and binds to receptors on gonadotrophs (Purves *et al*., 2008). GnRH stimultes secretion of LH, which in turn stimulates gonadal secretion of the sex steroids testosterone, estrogen and progesterone. In a classical negative feedback loop, sex steroids inhibit secretion of GnRH and also appear to have direct negative effects on gonadotrophs (Purves *et al*., 2008).

This regulatory loop leads to pulsatile secretion of LH and, to a much lesser extent, FSH. The number of pulses of GnRH and LH varies from a few per day to one or more per hour. In females, pulse frequency is clearly related to stage of the cycle. Numerous hormones influence GnRH secretion, and positive and negative control over GnRH and gonadotrophin secretion is actually considerably more complex than depicted in the Figure 2.2. For example, the gonads secrete at least two additional hormones - inhibin and activin - which selectively inhibit and activate FSH secretion from the pituitary (Purves *et al*., 2008).

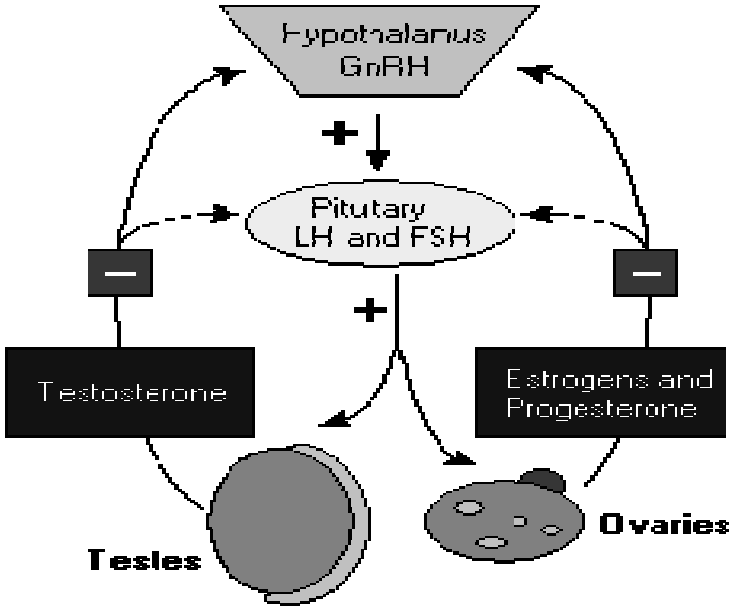

Figure 2.2. Regulation of Pituitary gonadotropin hormones (Adopted from Purves *et al*., 2008)

2.2.4. Prolactin hormone

Prolactin hormone (PRL) consists of a single polypeptide chain containing approximately 200 amino acid residues and three disulfide bridges and has a considerable structural similarity to human growth hormone. The half-life of PRL, like that of growth hormone, is about 20 minutes. (Ganong, 2003).

Secretion and target site

PRL is secreted from the lactotrophs of the anterior pituitary gland and acts on mammary glands (Scanlon and Sanders, 2007).

Actions

The primary physiological action of the hormone is on the mammary glands where it is involved in the growth of the glands and in the induction and maintenance of milk production (Tietz, 1995). There is evidence to suggest that PRL may be involved in steroidogenesis in the gonad, acting synergistically with LH. The action of the hormone is not exerted on the cell nucleus. PRL receptor resembles the growth hormone receptor and is one of the superfamily of receptors that includes the growth hormone receptor and receptors for many cytokines and hematopoietic growth factors (Ganong, 2003).

Regulation

PRL secretion is topically inhibited by the hypothalamus and section of the pituitary stalk leads to an increase in circulating PRL. Thus, the effect of the hypothalamic prolactin-inhibiting hormone (PIH) dopamine is normally greater than the effects of the various hypothalamic peptides with prolactin- releasing activity (Scanlon and Sanders, 2007). In humans, PRL secretion is increased by exercise, surgical and psychologic stresses, and stimulation of the nipple. Secretion is increased during pregnancy, reaching a peak at the time of parturition (Tietz, 1995). L-Dopa decreases prolactin secretion by increasing the formation of dopamine. Dopamine agonists also inhibit secretion PRL because they stimulate dopamine receptors. Estrogens produce a slowly developing increase in PRL secretion as a result of a direct action on the lactotrophs (Ganong, 2003).

2.2.5. Thyroid stimulating hormone

Thyroid stimulating hormone (TSH) is a 28 to 30 KDa glycoprotein (like LH, FSH and HCG); it is a heterodimeric cystine-knot glycoprotein consisting of a common α-subunit and a unique β-subunit (Szkudlinski *et al*., 2002; Ganong, 2003).

Secretion and target site

TSH is synthesized and secreted from thyrotrophs of the anterior pituitary gland and its target organ is the thyroid gland (Scanlon and Sanders, 2007). The TSH receptor is found mainly on thyroid follicular cells. Stimulation of the receptor increases triiodothyronine (T3) and thyroxine (T4) production and secretion (Parmentier *et al*., 1989).

Actions

This hormone receptor, like other glycoprotein hormones are serpentine receptors coupled to G_s and adenylyl cyclase (Ganong, 2003). Secretion of T4 and T3 is stimulated by thyroid-stimulating hormone from the anterior pituitary gland. When the metabolic rate (energy production) decreases, this change is detected by the hypothalamus, which secretes TRH. TRH in turn stimulates the anterior pituitary to secrete TSH, which stimulates the thyroid to release T4 and T3, which raise the metabolic rate by increasing energy production (Scanlon and Sanders, 2007).

Regulation

The most important controller of TSH secretion is thyroid-releasing hormone (TRH). Thyroid-releasing hormone is secreted by hypothalamic neurons into hypothalamic-hypophyseal portal blood, finds its receptors on thyrotrophs in the anterior pituitary and stimulates secretion of TSH. One interesting aspect of TRH is that it is only three amino acids long. Its basic sequence is glutamic acid-histidine-proline (Szkudlinski *et al*., 2002). Secretion of thyroid-releasing hormone, and hence, TSH, is inhibited by high blood levels of thyroid hormones in a classical negative feedback loop.

Free or unbound T3 and T4 exert a negative feedback mechanism on the synthesis and release of TSH and TRH (Figure 2.3) to maintain circulating thyroid hormone levels within the required range. (Szkudlinski *et al*., 2002; Ganong, 2003).

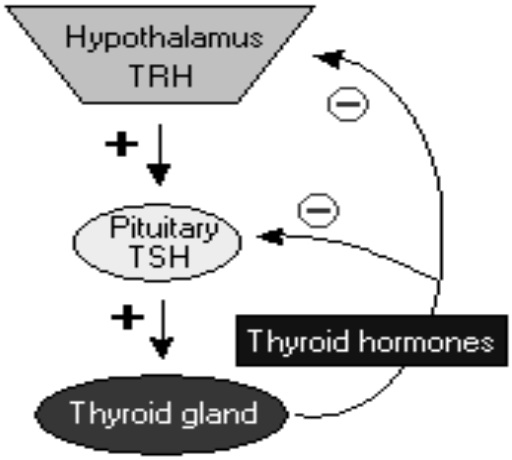

Figure 2.3. Regulation of TSH release (Adopted from Purves *et al*., 2008).

2.3. Menstrual cycle

The reproductive system of women, unlike that of men, shows regular cyclic changes that teleologically may be regarded as periodic preparations for fertilization and pregnancy. In humans, the cycle is a menstrual cycle, and its most conspicuous feature is the periodic vaginal bleeding (menstruation) that occurs with the shedding of the uterine mucosa (Jones, 1997). The length of the cycle is notoriously variable in women, but an average is 28 days from the start of one menstrual period to the start of the next. By common usage, the days of the cycle are identified by number, starting with the first day of menstruation (Ganong, 2003). Since the cyclic production of estrogens and progesterone by ovaries is, in turn, regulated by the anterior pituitary gonadotrophic hormones, FSH and LH, it is important to understand how these "hormonal pieces" fit together (Jones, 1997; Marieb, 2006).

2.3.1. Phases of the menstrual cycle

Days 1-5: Menstrual phase

During this interval, the superficial functional layer of the thick endometrial lining of the uterus is sloughing off, or becoming detached, from the uterine wall. This is accompanied by bleeding for three to five days (Jones, 1997). The detached tissues and blood pass through the vagina as the menstrual flow by day 5; growing ovarian follicles are beginning to produce more estrogen (Ganong, 2003; Scanlon and Sanders *et al*., 2007).

Days 6-14: Proliferative phase or follicular phase

This phase is stimulated by rising estrogen levels produced by growing follicles of the ovaries, the basal layer of the endometrium regenerates the functional layer, glands are formed in it, and the endometrium blood supply is increased. Ovulation occurs in the ovary at the end of this stage, in response to the sudden surge of LH in the blood (Ganong, 2003; Scanlon and Sanders *et al*., 2007).

Days 15-28: Secretory phase or luteal phase

Rising levels of progesterone production by the corpus luteum of the ovary act on the estrogen-primed endometrium and increase its blood supply even more (Jones, 1997). Progesterone also causes the endometrial gland to increase in size and to begin secreting nutrients into the uterine cavity. These nutrients will sustain a developing embryo (if one is present) until it becomes implanted (Ganong, 2003; Scanlon and Sanders *et al*., 2007).

2.4. Ovarian cycle

The ovarian cycle (Figure 2.4) starts on the first day of the menstrual cycle. The levels of all the hormones - estrogen, progesterone, FSH and LH - which are primarily responsible for maintaining menstruation are at the baseline levels. The low level of estrogen in the blood at this time stimulates the pituitary gland to start producing FSH (Ganong, 2003). The level of FSH rises and stimulates the Graafian follicles in the ovaries to develop. This indicates the start of the ovarian cycle in menstruation. The ovarian cycle is subdivided into two phases (Krohmer, 2004).

2.4.1. Follicular phase

The follicular phase starts with the development of about 20 Graafian follicles in the ovary under the stimulation of the hormone FSH released from the pituitary gland. These follicles grow steadily, releasing the hormone estrogen into the blood stream. At around the 10^{th} day of the follicular phase, one of the follicles becomes distinctly larger than the others. It continues to grow, becoming larger and larger (dominant follicle) to become mature, while the growth of the others is arrested. These eventually die out (Ganong, 2003). The estrogen released by the follicles, acts on the endometrium of the uterus and stimulates it to proliferate, indicating the start of the uterine cycle. The proliferative phase is thus also called the estrogenic phase. The dominant follicle usually ruptures when it is about 18 – 20 mm in size and releases an ovum at about the 14-16^{th} day of the menstrual cycle. This process is called ‘ovulation’. The follicular phase ends at this stage and the luteal phase begins (Krohmer, 2004).

2.4.2. Luteal phase

As soon as the graafian follicle ruptures and releases the ovum (ovulation) the cells of the follicle itself undergoes certain changes. Fat globules get deposited in them, they grow larger, and they assume a yellowish color. These cells are called luteal cells and the follicle now forms the 'corpus luteum' (Ganong, 2003). The cells of the corpus luteum are capable of producing the hormone ‘progesterone’ which, like estrogen, acts on the uterine endometrium. Hence this phase is also called the progestogenic phase. The level of progesterone reaches a peak at 22 – 26^{th} day of the cycle (Krohmer, 2004).

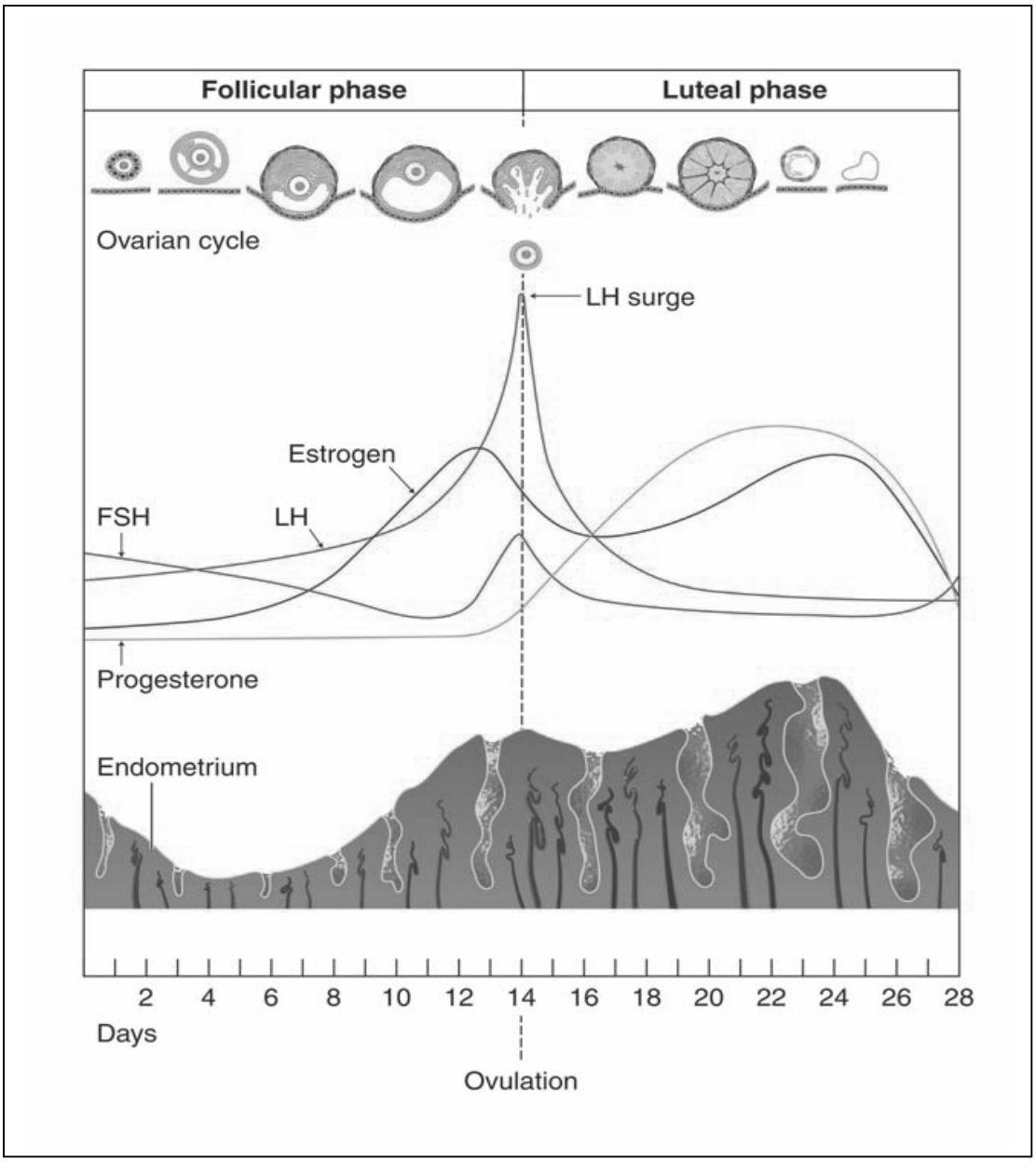

Figure 2.4. The ovarian cycle: The development of the egg and uterine lining as they are associated with pituitary and ovarian hormones. As the egg is preparing to leave the follicle, estrogen and LH levels rise while FSH remains constant. Just before the egg is released, LH levels surge. At this point, the uterine lining is very thick, ready to provide nutrients for a fertilized egg. If the egg is not fertilized, progesterone levels rise, the egg is expelled from the body, and the uterine lining is released through the process of menstruation (Adopted from Krohmer, 2004)**.**

2.5. Anti-Mullerian hormone

Antimullerian hormone (AMH) is a glycoprotein dimer composed of two 72 KDa monomers linked by disulfide bridges (Cate *et al*., 1986). AMH belongs to the transforming growth factor-β (TGF-β) superfamily and binds to AMH receptor 2 (AMHR2). The superfamily includes TGF-B and the various inhibin and activin glycoproteins. All members of this superfamily are dimeric glycoproteins (Maheswaran *et al.*, 2001; La Marca *et al.*, 2006)

Secretion and target sites

AMH is produced by ovarian granulosa cells in female. After puberty, when menstrual cycling begins, circulating AMH slowly decrease throughout life and becomes undetectable at menopauses (Maheswaran *et al*., 2001; Durlinger *et al*., 2002). AMH continues to be expressed in the growing follicles in the ovary until they have reached the size and differentiation state at which they are to be selected for dominance. In the human, this occurs at the antral stage when the follicle size is 4–6 mm (Weenen *et al.*, 2004).

Action

AMH receptors, like other glycoprotein hormone, are serpentine receptors coupled to G_s and adenylyl cyclase signal transduction system (Jorgensen *et al*., 1999; Weenen *et al*., 2004). AMH seems to act only in the reproductive organs (Lee and Donahoe, 1993). The most striking effect of AMH is its capacity to induce regression of the mullerian ducts, the anlage of the female internal reproductive organs. In the absence of AMH, mullerian ducts of both sexes develop into uterus, fallopian tubes and the upper part of the vagina (Munsterberg and Lovell, 1991; Lee and Donahoe, 1993).

The AMH has two sites of action.

1. It inhibits initial follicle recruitment and,
2. Inhibits FSH-dependent growth and selection of preantral and small antral follicles (Visser *et al.*, 2002), as illustrated in Figure 2.5.

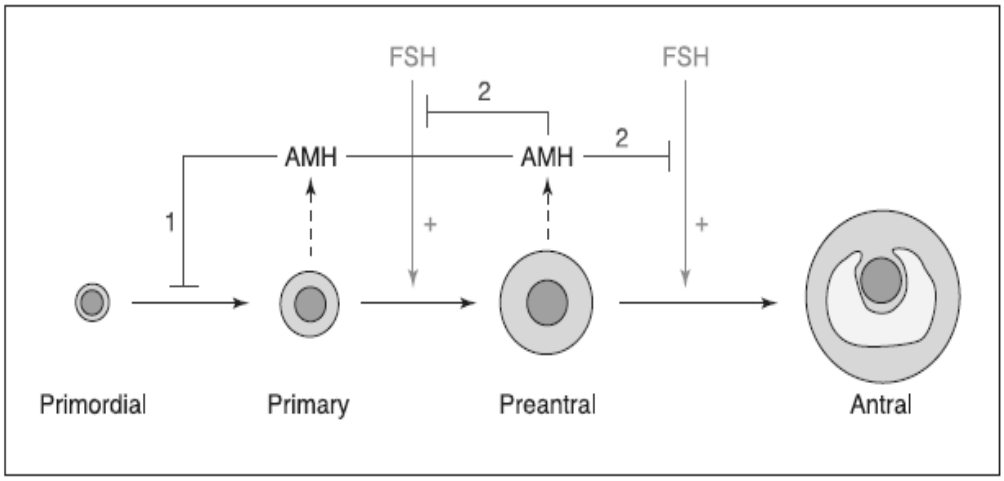

Figure 2.5. A model for the action of AMH in the ovary (Adopted from Visser *et al*., 2002).

2.5.1. Antimullerian hormone as a predictor of ovarian reserve

Fanchin *et al*. (2003), compared the relationship between serum AMH levels and other markers of ovarian function with early antral follicle count on day 3. A total of 75 infertile women were studied prospectively. On cycle day 3, serum levels of AMH, inhibin B, estradiol, FSH and LH levels were measured, and the number of early antral follicles (2-10 mm in diameter) were estimated by ultrasound scanning to compare the strengths of hormonal-follicular correlations. Median serum levels of AMH, inhibin B, E2, FSH and LH were 1.39 ng/ml (0.24-6.40), 90 (16-182) pg/ml, 31 (15-111) pg/ml, 7.0 (2.9-19.3) mIU/ml and 4.7 (1.2-11.7) mIU/ml, respectively, and the mean follicular count was 12 (1-35). Serum AMH levels were more strongly correlated ($p<0.001$) with follicular count ($r=0.74$, $p<0.0001$) than were serum levels of inhibin B ($r=0.29$, $p<0.001$), E2 ($r=-0.08$, p=NS), FSH ($r=-0.29$, $p<0.001$) or LH ($r=0.05$, p=NS).

Muttukrishna *et al*. (2004), in their prospective study have investigated whether FSH, AMH and inhibin B could be useful in predicting the ovarian response to gonadotrophin stimulation in assisted reproduction patients who are considered to be poor responders. Blood samples from 69 patients were collected on day five or six in the early follicular phase of an untreated menstrual cycle. Among the 69 patients, 52 patients completed an IVF cycle and 17 patients had to cancel the cycle because of poor ovarian response to gonadotrophin stimulation. Mean FSH levels were significantly higher ($p<0.05$) in the cancelled group (10.69±2.27 mIU/mL) as compared to the cycle-completed group (7.89±0.78 mIU/mL). Mean AMH levels were significantly lower ($p<0.01$) in the cancelled group (0.175±0.04ng/mL) as compared to the cycle-completed group (1.13±0.2 ng/mL). Mean inhibin B levels were significantly lower ($p<0.001$) in the cancelled group (70±12.79 pg/mL) as compared to the cycle-completed group (126.9±8.8 pg/mL). Predictive statistics showed that AMH is the best single marker and that the combination of FSH, AMH and inhibin B is modestly better than AMH alone.

Linear regression analysis in the cycle-completed patients showed that although FSH ($r=0.25$, $p<0.05$) and inhibin B ($r=0.35$, $p<0.05$) have a significant linear association with the number of eggs collected, AMH has the greatest association ($r=0.69$, $p<0.001$) with the number of eggs collected among the parameters measured.

Fanchin *et al*. (2005a) studied the dynamics of serum AMH levels during the luteal phase of controlled ovarian hyperstimulation (COH) and its possible association with follicle development. Their study included 34 women undergoing COH with GnRH agonist and FSH. On the day of hCG (dhCG), serum AMH, E2, progesterone and hCG levels were measured, and ovarian follicles were sorted into three size classes: <12, 12–15 and 16–22 mm. Hormonal measurements were repeated 4 days (hCG 1 4) and 7 days (hCG 1 7) after hCG. From dhCG to hCG + 4, a decline in serum AMH levels was observed (-64±3%; $p<0.0001$), which paralleled that of E2 levels. From hCG+4 to hCG+7, an increase in AMH levels occurred (82±28%; $p<0.02$), whose magnitude was correlated with the number of <12 mm follicles ($r=0.68$; $p<0.0001$) but not with other follicle size classes nor with the remaining hormone levels.

In another study, Fanchin *et al*. (2005b) compare the intercycle reproducibility of serum AMH measurements with that of other markers of ovarian follicular status. Forty-seven normoovulatory, infertile women underwent serum AMH, inhibin B, E2 and FSH measurements and early antral follicle (2–12mm in diameter) counts by transvaginal ultrasound on cycle day 3 during three consecutive menstrual cycles. Reproducibility of measurements was estimated using intra-class correlation coefficient (ICC) calculation. The number of replicate measurements theoretically needed to reach satisfactory reliability of results was also assessed in the study. Serum AMH showed significantly higher reproducibility (ICC, 0.89; 95% confidence interval, 0.83–0.94) than inhibin B (0.76; 0.66–0.86; $p<0.03$), estradiol (0.22; 0.03–0.41; $p<0.0001$) and FSH levels (0.55; 0.39–0.71; $p< 0.01$), and early antral follicle counts (0.73; 0.62–0.84; $p<0.001$), and reached satisfactory reliability with a single measurement.

Ficiciog *et al*. (2006) have conducted a prospective study to determine the predictive value of AMH as a marker for ovarian reserve and to compare its value with the markers currently being used. Fifty women undergoing assisted reproduction cycles we recruited in the study. Comparison of day-3 serum AMH levels among women with less than five retrieved oocytes and five or more oocytes were done. Antral follicle count, mature oocyte count, age, basal FSH, E2, maximum serum E2 levels, and pregnancy success were also compared. The mean serum AMH levels of patients with more than five retrieved oocytes were found to be higher (0.67±0.41 vs. 0.15±0.11 pg/mL). Mature oocyte counts, antral follicle counts, and maximum E2 levels were found to be significantly different in the two groups despite similar ages and levels of basal FSH and E2. Although the receiver operator characteristics analysis revealed that the most sensitive and specific indicator of ovarian reserve is the level of AMH, it does not indicate pregnancy success as well.

In another study, Silberstein *et al*. (2006) showed that the pre-antral and early antral follicles secrete mullerian inhibiting substance (MIS), suggesting that MIS may directly reflect ovarian reserve. Since little is known about how ovarian reserve affects oocyte quality, they attempted to assess the predictive value of MIS on embryo morphology and IVF outcome. They measured MIS at the time of HCG administration 36 h prior to oocyte retrieval. A total of 257 patients undergoing IVF were prospectively recruited. The authors measured MIS levels by enzyme-linked immunosorbent assay at the time of HCG administration, and compared the AMH values to day 3 FSH levels in the prediction of embryo morphology and IVF outcome. The distribution of AMH levels was skewed, with a median of 2.7 ng/ml (range 0-28.5 ng/ml). AMH values at the time of HCG administration inversely correlated with basal FSH levels (p=0.002), and both correlated significantly with patient age, number of mature follicles, number of oocytes retrieved and serum estradiol levels.

AMH levels correlated significantly with a greater number of 6-cell embryos and better embryo morphology score, while basal FSH levels did not correlate with these outcome variables. AMH levels ≥2.7 ng/ml portended improved oocyte quality as reflected in a higher implantation rate (p=0.001) and a trend toward a better clinical pregnancy rate (p=0.084).

La Marca *et al*. (2006) have evaluated AMH as a marker of ovarian response. Serum AMH levels were measured in women undergoing assisted reproductive technology (ART). At frequent time-points during the menstrual cycle, suggesting the complete absence of fluctuation. Forty-eight women attending the IVF/ICSI program were investigated. Blood withdrawal for AMH measurement was performed in all the patients independently of the day of the menstrual cycle. Women in the lowest AMH quartile (<0.4 ng/ml) were older and required a higher dose of recombinant FSH than women in the highest quartile (>7 ng/ml). All the cancelled cycles due to absent response were in the group of the lowest AMH quartile, whereas the cancelled cycles due to risk of ovarian hyperstimulation syndrome (OHSS) were in the group of the highest AMH quartile. This study demonstrated a strong correlation between serum AMH levels and ovarian response to gonadotrophin stimulation.

Firouzabadi *et al*. (2008), investigated whether AMH and antral follicle count can be useful in predicting the ovarian reserve and pregnancy outcome. In their prospective study they have included a total of 60 patients attending an ART program. Patients with an oocyte count of ≥4 were considered good responders (group A); those with <4 oocytes were considered as poor responders (group B). On day three of the menstrual cycle, blood sample was taken from each woman for the measurement of serum levels of FSH, LH, E2, and AMH. Thereafter, ovarian ultrasound scanning was performed to evaluate the number and size of antral follicles. Results showed that serum FSH, LH, and E2 levels were not statistically different between the two groups.

Meanwhile, the difference between serum AMH levels, AFC, and retrieved oocyte counts were statistically significant in the two groups. The mean±SD serum AMH level was 34.22±13.95 and 12.53±9.4 pM/mL in groups A and B, respectively (p=0.002). The number of chemical pregnancies was seven versus three in groups A and B, respectively (p=0.014), whereas the number of clinical pregnancies was six versus two in groups A and B, respectively (p=0.52).

Freiesleben *et al*. (2010) have investigated serum AMH through correlations to other basal parameters on 123 patients and according to ovarian response to 75IU recombinant follicle-stimulating hormone (rFSH)/day on 62 patients in ovulatory patients' first rFSH treatment cycle before intrauterine insemination. Mean age of the patients was 33 years. Serum AMH significantly correlated to age (r=-0.38), antral follicle count (AFC) (r=0.68), ovarian volume (r=0.40), FSH (r=-0.31), (p<0.001) and cycle length (r=0.26, p=0.004). Serum AMH median (interquartile range; IQR) was 8.5 pmol/l (1.9-15.1) in hyporesponders (one mature follicle) versus 10.7 (7.3-17.3) in normal responders (2-3 follicles, with a maximum of two follicles 18 mm and no need for dose reduction) and 13.4 (4.4-24.2) in hyperresponders (>2-3 mature follicles or dose reduction).

The study La Marca *et al*. (2010) has shown that the AMH levels in women, may represent the ovarian follicular pool and could be a useful marker of ovarian reserve. The clinical application of AMH measurement has been proposed in the prediction of quantitative and qualitative aspects in ART. AMH seems to be a better marker in predicting ovarian response to controlled ovarian stimulation than age of the patient, FSH, E2 and inhibin B. A similar performance for AMH and antral follicular count has been reported. In clinical practice, AMH measurement may be useful in the prediction of poor response and cycle cancellation and also of hyper-response and ovarian hyperstimulation syndrome.

2.5.2. Antimullerian Hormone as a predictor of ovarian ageing

Van Rooij *et al*. (2002) pointed out that AMH is produced by the granulosa cells of preantral and small antral follicles and its levels can be assessed in serum. Since the number of ovarian follicles declines with increasing age, AMH levels might be used as a marker for ovarian ageing. In their study they investigated the relationship between AMH levels and ovarian response during ovarian stimulation for IVF. A total of 130 patients undergoing their first IVF treatment cycle using a long protocol with GnRH agonist were prospectively included. Blood withdrawal was performed and the number of antral follicles was assessed by ultrasound on day 3 of a spontaneous cycle. Poor response and the number of oocytes were used as primary outcome measures. In a random subset of 23 patients a GnRH agonist stimulation test was performed to investigate whether a rise in FSH and LH would affect AMH levels. The data of 119 patients were analyzed. Serum AMH levels were highly correlated with the number of antral follicles (r =0.77; p<0.01) and the number of oocytes retrieved (r=0.57, p<0.01). A negative association was found between AMH levels and poor ovarian response (fewer than 4 oocytes or cycle cancellation; OR 0.82, 95% CI 0.75–0.90, p<0.01).

Laven *et al*. (2004) concluded that the concentrations of AMH correlate with the number of antral follicles as well as age and constitute an endocrine marker for ovarian aging. In normogonadotropic anovulatory infertile women, the number of early antral follicles is usually increased. To investigate whether AMH concentrations are increased, serum levels in 128 WHO class 2 women were compared with those in 41 normoovulatory premenopausal women of similar age. Serum AMH concentrations were significantly (p<0.001) elevated in WHO class 2 patients [median, 7.6 µg/liter (range, 0.1–40.0)], compared with controls [median, 2.1 µg/liter (0.1–7.4)]. AMH levels correlated well with age in WHO class 2 patients (r=-0.248; p=0.002) as well as in controls (r=-0.465; p=0.005).

However, the relative decline in AMH with age was less pronounced in WHO class 2 patients. In a subset of patients no significant correlation was found between AMH serum concentrations or the FSH response dose, the duration of stimulation, and the total number of ampoules of FSH used.

Van Rooij *et al*. (2005) designed a prospective longitudinal study to assess which of the basal ovarian reserve markers provides the best reflection of the changes occurring in ovarian function over time (i.e. reproductive aging). Eighty-one women with normal reproductive performance during the course of their lives were longitudinally assessed. In this selected group of women, becoming chronologically older was considered as a proxy variable for becoming older from a reproductive point of view. The women were assessed twice, with on average 4-year interval (T1 and T2). The number of antral follicles on ultrasound (AFC) and blood levels of AMH, FSH, inhibin B, and E2 were assessed. The mean ages at T1 and T2 were 39.6 and 43.6 years, respectively. Although AFC was strongly associated with age in a cross-sectional fashion, it did not change over time. The AMH, FSH, and inhibin B levels showed a significant change over time, in contrast to E2 levels, the AMH and AFC were highly correlated with age both at T1 and T2, whereas FSH and inhibin B predominantly changed in women more than 40 years of age. AMH concentrations showed the best consistency, with AFC as second best. The FSH and inhibin B showed only modest consistency, whereas E2 showed no consistency at all.

Disseldorp *et al*. (2008) related AMH levels to the age distribution of reproductive events like onset of menopause to test AMH as a predictor of reproductive status. AMH levels were measured in 144 fertile normal volunteers and used to determine an estimate of mean AMH as a function of age. Estimation of an AMH threshold to predict menopause was done by maximum likelihood using the observed distribution of age at menopause and the predictive distribution from this AMH threshold.

Predictions of age at menopause follow from an individual woman's AMH relative to percentiles of the distribution of AMH for a given age, and the corresponding percentiles of the predictive distribution of age at menopause. There was good conformity between the observed distribution of age at menopause and that predicted from declining AMH levels.

2.6. *In vitro* fertilization

2.6.1. Definition

In vitro fertilization (IVF) is a procedure in which eggs (ova) from a woman's ovary are collected. They are fertilized with sperm in the laboratory, and then the fertilized egg (embryo) is returned to the woman's uterus (Carlson *et al*., 1996).

2.6.2. Preparation

Once a woman is determined to be a good candidate for IVF, she will generally be given "fertility drugs" to stimulate ovulation and the development of multiple eggs. These drugs may include gonadotropin releasing hormone agonists (GnRHa) and human chorionic gonadotropin (HCG). The maturation of the eggs is then monitored with ultrasound tests and frequent blood tests. If enough eggs mature, the physician will perform the procedure to collect them. The woman may be given a sedative prior to the procedure. A local anesthetic may also be used to reduce discomfort during the procedure (Carlson *et al*., 1996; Krohme, 2004).

2.6.3. Description

In vitro fertilization is a procedure in which the joining of egg and sperm takes place outside the woman's body. A woman may be given fertility drugs before this procedure so that several eggs mature in the ovaries at the same time. Eggs (ova) are removed from a woman's ovaries using a long, thin needle. The physician gets access to the ovaries using one of two possible procedures. One procedure involves inserting the needle through the vagina (transvaginally).

The physician guides the needle to the location of the ovaries with the help of an ultrasound machine. In the other procedure, called laparoscopy, a small thin tube with a viewing lens is inserted through an incision in the navel. This allows the physician to see inside the patient and locate the ovaries on a video monitor. Once the eggs are retrieved, they are mixed with sperm in a laboratory dish or test tube (this is the origin of the term "*test tube baby"*). The eggs are monitored for several days. Once there is evidence that fertilization has occurred and the cells begin to divide, they are then returned to the woman's uterus. In the procedure to collecting eggs, enough may be gathered to be frozen and saved (either fertilized or unfertilized) for additional IVF attempts (Carlson *et al* 1996; Krohme, 2004). IVF has been used successfully since 1978, when the first child to be conceived by this method was born in England (Carlson *et al*.,1996). Other types of assisted reproductive technologies might be used to achieve pregnancy. A procedure called intracytoplasmic sperm injection (ICSI) uses a manipulation technique that must be performed using a microscope to inject a single sperm into each egg. The fertilized eggs can then be returned to the uterus as in IVF. In gamete intrafallopian tube transfer (GIFT) the eggs and sperm are mixed in a narrow tube and then deposited in the fallopian tube, where fertilization normally takes place. Another variation on IVF is zygote intrafallopian tube transfer (ZIFT). As in IVF, the fertilization of the eggs occurs in a laboratory dish. And, similar to GIFT, the embryos are placed in the fallopian tube (rather than the uterus as with IVF) (Carlson *et al*., 1996; Krohme, 2004).

CHAPTER III Materials and Methods

3.1. Study design

The present study is a case-control prospective study.

3.2. Study population

The study population consisted of women undergoing *in vitro* fertilization and aged between 20-40 years, who reside in the Gaza Strip. The controls were healthy women having at least one healthy child.

3.3. Sampling and sample size

Women 20 to 40 years old without history of other diseases at their first examination were referred to Al-basma fertility Center in Gaza City between October 2009 to January 2010. Control women were selected from general population with healthy history and have at least one baby. Case and control women were matched for age. The sample size calculations were based on the formula for case-control studies. EPI-INFO statistical package version 3.5.1 was used with 95% CI, 80% power and 50% proportion as conservative and OR > 2. The sample size in case of 1:1 ratio of case control was found to be 73:73. For a no-response expectation, the sample size was increased to 81 women and were recruited from a private fertility clinic (Al-basma fertility Center). The controls also consisted of 81 healthy women.

3.4. The inclusion criteria

1. Regular menstrual cycle (cycle length, 25–35 days, duration of menstruation, three to eight days).
2. Not on hormone therapy for three months.
3. Have not been subject to surgical operation in the reproductive system.

3.5. Questionnaire

A meeting interview was used for filling in the questionnaire for both cases and controls (Annex 1). All interviews were conducted face to face by the researcher himself. During the survey the interviewer explained to women any of the questions that were not clear to them. Most questions were of one of two types: the yes/no question, which offer a dichotomous choice; and the multiple choice questions, which offer several fixed alternatives. The questions were direct and brief and the validity of the questionnaire was tested by three specialists. The questionnaire included personal information such as age and address, medical information such as regularity of the menstrual cycle and cause of infertility. Pilot study was done prior to beginning real data collection to know the length and clarity of questionnaire and to evaluate the outcome. Ten women were interviewed. At the end of the pilot study, a comprehensive revision to questionnaire was made and modified as necessary. The pilot subjects were not included in the study.

3.6. Ethical considerations

The researcher obtained the necessary approval to conduct the study from Helsinki committee in Gaza Strip (Annex 2). Helsinki committee is an authorized professional body for giving permission to researchers to conduct their studies with ethical concern in the area. An official letter (Annex 3) of request was sent to Al-basma fertility Center to obtain approval to make some hormonal assays. Women were given an explanation about the purpose of the study and assurance about the confidentiality of the information and that the participation was optional.

3.7. Hormonal assays

Blood samples were collected from the median cubital vein by a well trained nurse. About 5 ml blood were drawn from each woman on day three of the cycle by plastic pyrogen-free disposable syringe (Medi-Hut International-Korea) into a plastic tube and left for a short time to allow blood to clot. Clear serum samples was then obtained by centrifugation at 3500 rpm for 10 minutes and then the serum was separated and stored at a -20°C until use. AMH, E2, FSH, LH, PRL and TSH analyses were carried out at the Medical Relief Laboratory.

Equipment and Disposables

- Stat fax 303 plus ELISA reader
- Alcohol swab
- Syringes and needles
- Plasters
- Plastic tubes
- Distilled water
- Micropipettes 10µl -1000µl
- Yellow and blue tips
- Eppendrof tubes
- Timer
- Centrifuge
- Ice box for keeping the sample during transportation.
- Deep freezer (-20°C) for specimen storage

3.7.1. Antimullerian hormone assay

AMH level was determined according to Durlinger *et al*. (1999) method using Diagnostic Systems Laboratories Inc ELISA kit for AMH.

A. Principle of the assay

The active MIS/AMH ELISA is an enzymatically amplified two-site immunoassay. In the assay, standards, controls, and samples are incubated in microtitration wells which have been coated with anti-MIS/AMH antibody. After incubation and washing, anti-MIS/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 well. Lastly, an acidic stopping solution is added. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measured at 450 nm and between 600 and 630 nm. The absorbance measured is directly proportional to the concentration of MIS/AMH in the sample.

B. Kit components

One stripholder containing 96 microtitration wells coated with anti-MIS/AMH IgG, six MIS/AMH reference standard labeled from B-G with concentrations of approximately (0.05, 0.10, 0.25, 1.8, 7.5 and 15 ng/mL). MIS/AMH controls, MIS/AMH Antibody-Biotin conjugate—ready-to-use (RTU). streptavidin-enzyme conjugate-RTU. MIS/AMH assay buffer, wash buffer, diluent solution and stopping solution. TMB chromogen solution,.

C. Procedure

All samples and reagents were allowed to reach room temperature (~25°C). Reagents were mixed by gentle inversion before use. Standards, controls and samples were assayed in duplicates.

1. Microtitration strip was marked for use.
2. Twenty µL of the standards, controls and samples were added into appropriate wells.

3. One hundred µL of the MIS/AMH assay buffer were added into each well using a precision pipette.
4. The wells were incubated and shaken at 500-700 rpm on an orbital microplate shaker, for one hour at room temperature (~25°C).
5. Each well was aspirated and washed 5 times for 30 seconds with wash solution using an automatic microplate washer or manually using a precision pipette. Then the plate was inverted on an absorbent material to dry it.
6. One hundred µL of the antibody-biotin conjugate solution were added into each well using a precision pipette.
7. The wells were incubated and shaken at 500-700 rpm on an orbital microplate shaker, for one hour at room temperature (~25°C).
8. Each well was aspirated and washed 5 times for 30 seconds with wash solution using an automatic microplate washer or manually using a precision pipette. Then the plate was inverted on an absorbent material to dry it.
9. One hundred µl of the streptavidin-enzyme conjugate--RTU were added into each well using a precision pipette.
10. The wells were incubated and shaken at 500-700 rpm on an orbital microplate shaker, for 30 minutes at room temperature (~25°C).
11. Each well were aspirated and washed 5 times for 30 seconds with wash solution using an automatic microplate washer or manually using a precision pipette. Then the plate was inverted on an absorbent material to dry it.
12. One hundred µL of the TMB chromogen solution were added into each well using a precision pipette.
13. The wells were incubated and shaken at 500-700 rpm on an orbital microplate shaker, for 10-15 minutes at room temperature (~25°C).
14. One hundred µL of the stopping solution were added into each well using a precision pipette.
15. The absorbance of the solution in each well was read within 30 minutes, at 450 nm (450-620nm).

D. Calculation of results

The absorbance for each standard, control, or sample was obtained , and then a standard curve was prepared by plotting the absorbance readings for each of the standards along the Y-axis versus MIS/AMH concentrations in ng/mL along the X-axis, using a linear curve-fit. The MIS/AMH concentrations of the samples were determined from the standard curve (Figure 3.1) by matching their mean absorbance readings with the corresponding MIS/AMH concentrations.

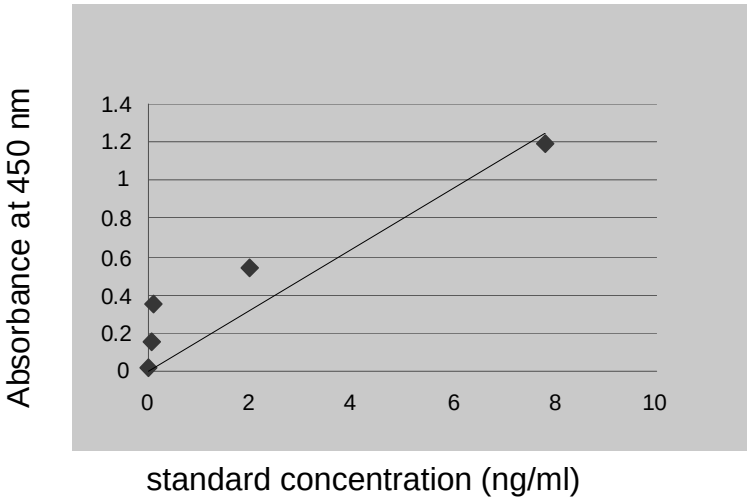

Figure 3.1: Antimullerian hormone standard curve

E. Reference values of AMH in adult females

Interpretation	AMH Blood Level (ng/ml)
High fertility	>3.0
Normal fertility	>1.0
Low fertility	0.3 - 0.9
Very Low fertility	<0.3

3.7.2. Estradiol assay

E2 level was determined according to Tietz (1995) method using TECO Diagnostics ELISA kit for E2.

A. Principle of the assay

The E2 ELISA is based on the principle of competitive binding between E2 in the test specimen and E2-HPR conjugate for a constant amount of rabbit antiestradiol. In the incubation, goat anti-rabbit IgG-coated wells are incubated with 25µl E2 standards, controls, or samples, 100µl estradiol-HPR conjugate reagent and 50µl rabbit antiestradiol reagent at room temperature (18-25 °C) for 90 minutes. During the incubation, a fixed amount of HRP-labeled E2 competes with the endogenous E2 in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. Thus, the amount of E2 peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increase. Unbound E2 peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB reagent is added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCL, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled E2 in the sample.

B. Kit components

One stripholder containing 96 microtitration wells coated with anti-rabbit IgG, six estradiol reference standards with concentrations of approximately (0, 10, 30, 100, 300 and 1000 pg/ml). Rabbit anti-estradiol reagent, estradiol-HRP conjugate reagent, estradiol reference controls, TMB chromogen solution and stop solution.

C. Procedure

All samples and reagents were brought to room temperature (~25°C). Reagents were mixed by gentle inversion before use. Standards, controls and samples were assayed in duplicates**.**

1. Microtitration strip was marked for use.
2. Twenty-five µL of the standards, controls and samples were added into appropriate well.
3. One hundred µL of estradiol-HRP conjugate reagent were added into each well using a precision pipette.
4. Fifty µL of rabbit anti- estradiol reagent were added into each well using a precision pipette.
5. The wells were mixed for 30 seconds.
6. The wells were incubated for 90 minute at room temperature (~25°C).
7. Each well was aspirated and washed 5 times with distilled water.
8. One hundred µL of TMB reagent were added into each well using a precision pipette and gently mixed for 10 seconds.
9. The wells were incubated for 20 minutes at room temperature (~25°C).
10. One hundred µL of stop solution were added into each well using a precision pipette and mixed for 30 seconds.
11. The absorbances of the solution in each well were read within 15 minutes, at 450 nm.

D. Calculation of results

The absorbance for each standard, control, or sample was obtained, and then a standard curve was prepared by plotting the absorbance readings for each of the standards along the Y-axis versus standard concentrations in pg/ml along the X-axis, The absorbance values for each sample was used to determine the corresponding concentration of estradiol in pg/ml from the standard curve (Figure 3.2).

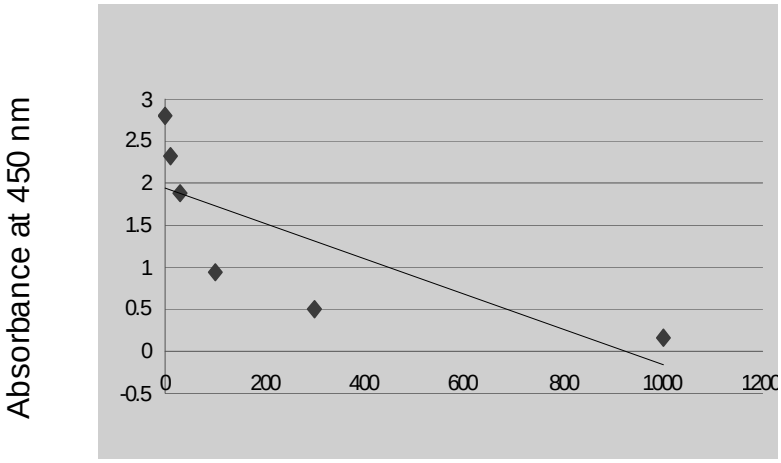

Figure 3.2: Estradiol hormone standard curve

E. Reference values of E2 in adult females

The normal range of this hormones in adult females during early follicular phase is between 25-100 pg/ml

3.7.3. Follicle Stimulating hormone assay

FSH level was determined according to Vitt *et al*. (1998) method using TECO Diagnostics ELISA kit for FSH.

A. Principle of the assay

The essential reagents required for an immunoenzymometric assay include excess amount of antibodies (both enzyme conjugated and immobilized) with high affinity, high specificity and contain different epitopes with distinct recognition and native antigen. In this assay procedure, the immobilization takes place at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-FSH antibody. Upon mixing, a reaction results between the native antigen contained in serum, the monoclonal biotinylated antibody and the enzyme-labeled antibody, without competition or steric hindrance, to from a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen value, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

B. Kit components

The kit consist of one stripholder containing 96 microtitration wells coated with streptavidin, six FSH reference standards with concentrations of approximately (0, 5.0, 10, 25, 50 and 100 mIU/mL), enzyme conjugate, TMB chromogen solution, stop solution and wash solution concentrate.

C. Procedure

All samples and reagents were allowed brought to room temperature (~25°C). Reagents were mixed by gentle inversion before use. Standards, controls and samples were assayed in duplicates.

1. Microtitration strip was marked for use.
2. Fifty µL of the standards, controls and samples were added into each appropriate well.
3. One hundred µL of conjugate reagent were added into each well using a precision pipette and then mixed for 30 seconds.
4. The wells were incubated for 60 minutes at room temperature (~25°C).
5. Each well was aspirated and washed 3 times by added 300 µL of working wash solution.
6. One hundred µL of TMB reagent were adding into each well and gently mixed for 10 second.
7. The wells were incubated in the dark for 15 minutes at room temperature (~25°C) without shaking.
8. Fifty µL of stop solution were added into each well and gently mixed for 10-20 second.
9. The absorbance for each well was read at 450 nm.

D. Calculation of results

The absorbance for each standard, control, or sample was obtained, and then a standard curve was prepared by plotting the absorbance reading for each of the standards along the Y-axis versus standard concentration in mIU/mL along the X-axis, The mean absorbance value for each sample was used to determine the corresponding concentration of FSH in mIU/mL from the standard curve (Figure 3.3)

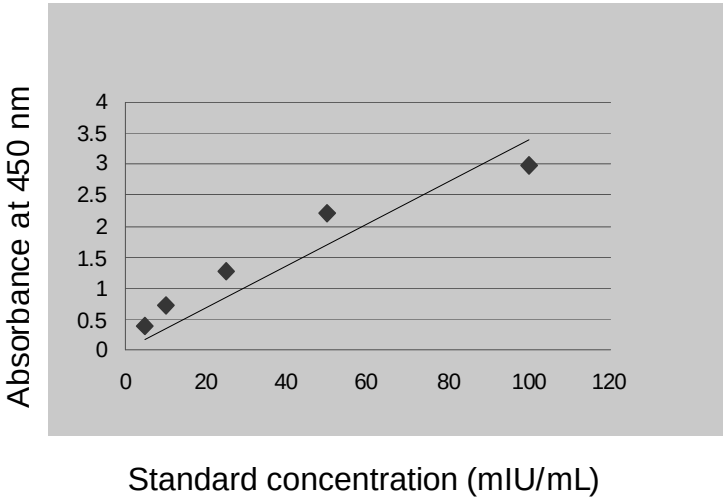

Figure 3.3: Follicle stimulating hormone standard curve.

E. Reference values of FSH in adult females

The normal range of this hormones in adult females during early follicular phase is between 3.0-12.0 mIU/mL

3.7.4. Luteinizing hormone assay

LH level was determined according to Lenton *et al*. (1982) method using TECO Diagnostics ELISA kit for LH.

A. Principle of the assay

The essential reagents required for an immunoenzymometric assay include excess amount of antibodies (both enzyme conjugated and immobilized) with high affinity, high specificity and contain different epitopes with distinct recognition and native antigen. In this assay procedure, the immobilization takes place at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated

monoclonal anti-LH antibody. Upon mixing, a reaction results between the native antigen contained in serum, the monoclonal biotinylated antibody and the enzyme-labeled antibody, without competition or steric hindrance, to from a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen value, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

B. Kit components

The kit consists of one stripholder containing 96 microtitration wells coated with streptavidin, six LH reference standards with concentrations of approximately (0, 5.0, 25, 50, 100 and 200 mIU/mL), enzyme conjugate, TMB chromogen solution, stop solution and wash solution concentrate.

C. Procedure

All samples and reagents were brought to room temperature (~25°C). Reagents were mixed by gentle inversion before use. Standards, controls and samples were assayed in duplicates.

1. Microtitration strip was marked for use.
2. Fifty µL of the standards, controls and samples were added into each appropriate well.
3. One hundred µL of conjugate reagent were added into each well using a precision pipette and then mixed for 30 seconds.
4. The wells were incubated for 60 minutes at room temperature (~25°C).
5. Each well was aspirated and washed 3 times by adding 300 µL of working wash solution.
6. One hundred µL of TMB reagent were added into each well and gently mixed for 10 second.
7. The wells were incubated in the dark for 15 minutes at room temperature (~25°C) without shaking.

8. Fifty µL of stop solution were added into each well and gently mixed for 10-20 second.
9. The absorbance for each well was read at 450 nm.

D. Calculation of results

The absorbance for each standard, control, or sample was obtained, and then a standard curve was prepared by plotting the absorbance reading for each of the standards along the Y-axis versus standard concentration in mIU/mL along the X-axis, The mean absorbance value for each sample was used to determine the corresponding concentration of LH in mIU/mL from the standard curve (Figure 3.4).

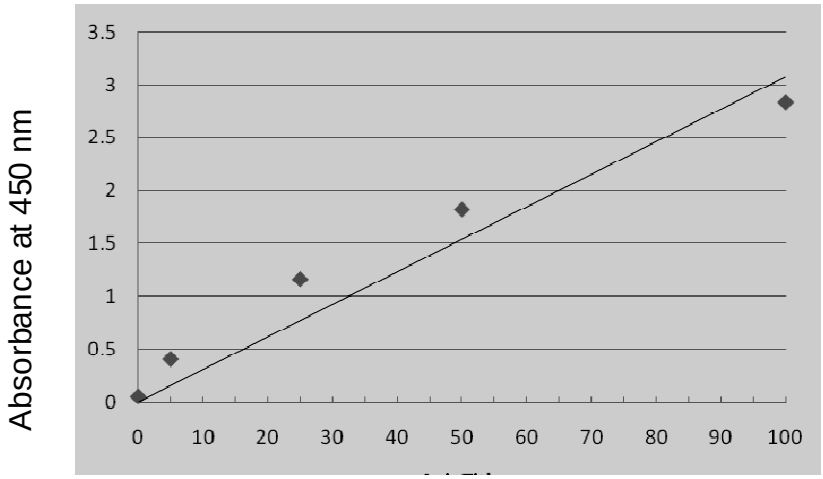

Figure 3.4: Luteinizing hormone standard curve.

E. Reference values of LH in adult females

The normal range of this hormones in adult females during early follicular phase is between 0.5-10.5 mIU/mL**.**

3.7.5. Prolactin hormone assay

PRL level was determined according to Tietz *et al*. (1992) method using TECO Diagnostics ELISA kit for PRL.

A. Principle of the assay

The essential reagents required for sandwich enzyme-linked immunoassay include excess amount of antibodies (both enzyme conjugated and immobilized) with high affinity, high specificity and contain different epitopes with distinct recognition and native antigen. In this assay, a certain amount of prolactin hormone calibrator, patient specimen or control is first added to a microplate well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of PRL) are added and the reactants mixed. Immobilization of the tagged PRL occurs through the interaction of streptavidin coated on the well and the added biotinylated monoclonal anti-PRL antibody. Upon mixing a reaction results between the native antigen contained in serum, the monoclonal biotinylated antibody and the enzyme-labeled antibody, without competition or steric hindrance, to from a soluble sandwich complex bound to the surface of microplate through the streptavidin-biotin system. After equilibrium is attained the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By several different serum references of known antigen value, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

B. Kit components

The kit consists of one stripholder containing 96 microtitration wells coated with streptavidin, six PRL reference standards with concentrations of approximately (0, 5.0, 10, 25, 50 and 100 ng/mL), enzyme conjugate, TMB chromogen solution, stop solution and wash solution concentrate.

C. Procedure

All samples and reagents were brought to room temperature (~25°C). Reagents were mixed by gentle inversion before use. Standards, controls and samples were assayed in duplicates.

1. Microtitration strip was marked for use.
2. Twenty-five µL of the standards, controls and samples were added into each appropriate well.
3. One hundred µL of conjugate reagent were added into each well using a precision pipette.
4. The wells were mixed for 30 seconds.
5. The wells were incubated for 60 minute at room temperature (~25°C).
6. Each well was aspirated and washed 3 times by adding 300 µL of working wash solution.
7. One hundred µL of TMB reagent were added into each well using a precision pipette and gently mixed for 10 seconds.
8. The wells were incubated in the dark for 15 minute at room temperature (~25°C).
9. Fifty µL of stop solution were added into each well using a precision pipette and mixed for 10-20 seconds.
10. The absorbance of the solution in each well was read at 450 nm.

D. Calculation of results

The absorbance for each standard, control, or sample was obtained, and then a standard curve was prepared by plotting the absorbance reading for each of the standards along the Y-axis versus standard concentration in ng/mL along the X-axis, The mean absorbance value for each sample was used to determine the corresponding concentration of PRL in ng/mL from the standard curve (Figure 3.5).

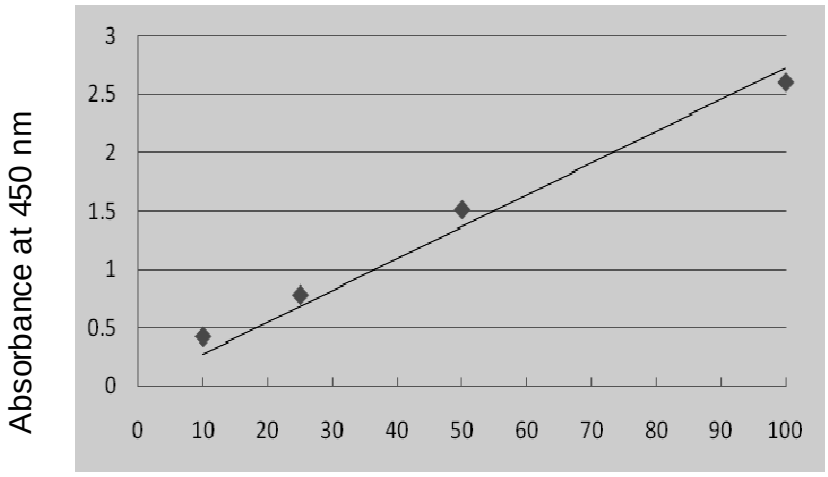

Figure 3.5: Prolactin hormone standard curve.

E. Reference values of PRL in adult females

The normal range for this hormone in adult females is between 1.2-19.5 ng/mL.

3.7.6 Thyroid stimulating hormone assay

TSH level was determined according to Boelaert *et al*. (2005) method using TECO Diagnostics ELISA kit for TSH.

A. Principle of the assay

The essential reagents required for immunoenzymetric assay include excess amount of antibodies (both enzyme conjugated and immobilized) with high affinity, high specificity and contain different epitopes with distinct recognition and native antigen. In this assay procedure, the immobilization takes place at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-TSH antibody. Upon mixing, a reaction results between the native antigen contained in serum, the monoclonal biotinylated antibody and the enzyme-labeled antibody, without competition or steric hindrance, to from a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. After equilibrium is attained the antibody-bound fraction is separated from

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

B. Kit components

The kit consist of one stripholder containing 96 microtitration wells coated with streptavidin, seven TSH reference standards with concentrations of approximately (0, 0.5, 2.5, 5.0, 10, 20 and 40 µIU/mL). enzyme conjugate, TMB chromogen solution, stop solution and wash solution.

C. Procedure

All samples and reagents were allowed to reach room temperature (~25°C). Reagents were mixed by gentle inversion before use. Standards, controls and samples were assayed in duplicate.

1. Microtitration strip was marked for use.
2. Fifty µL of the standards, controls and samples were added into each appropriate well.
3. One hundred µL of conjugate reagent were added into each well using a precision pipette and then mixed for 30 seconds.
4. The wells were incubated for 60 minutes at room temperature (~25°C).
5. Each well was aspirated and washed 3 times by adding 300 µL of working solution.
6. One hundred µL of TMB reagent were added into each well and gently mixed for 10 second.
7. The wells were incubated in the dark for 15 minutes at room temperature (~25°C) without shaking.
8. Fifty µL of stop solution were added into each well and gently mixed for 10-20 second.
9. The absorbance for each well was read at 450 nm.

D. Calculation of results

The absorbance for each standard, control, or sample was obtained, and then a standard curve was prepared by plotting the absorbance readings for each of the standards along the Y-axis versus standard concentrations in µIU/mL along the X-axis, The absorbance value for each sample was used to determine the corresponding concentration of TSH in µIU/mL from the standard curve (Figure 3.6).

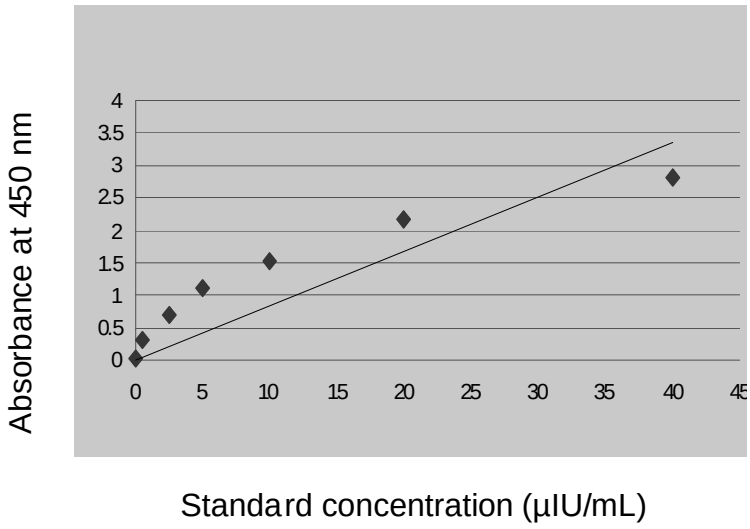

Figure 3.6: Thyroid stimulating hormone standard curve.

E. Reference values of TSH in adult females

The normal range is between 0.4 to 6.0 µIU/mL.

3.8. Data analysis

Data were computer analyzed using SPSS/ PC (Statistical Package for the Social Science Inc. Chicago, Illinois USA, version 13.0) statistical package. Simple distribution of the study variables and cross tabulation were applied. The independent-sample t-test procedure was used to compare means of quantitative variables by dividing cases into two qualitative groups such as the relationship between patient and controls hormones. The one-way ANOVA test was used for analysis of variance for average hormone level as quantitative variable by qualitative variable such as the relationship between hormone level by the result of IVF classes. Correlation coefficient (r) between the number of mature oocytes and the different parameters investigated was used. The results in all the above mentioned procedures were accepted as statistically significant when the p-value was less than 5% ($p<0.05$).

Chapter IV Results

4.1. Characteristics of the study population

The study population comprised 162 females; 81 have a problem in the process of pregnancy and were seeking *in vitro* fertilization (cases) at Al-basma fertility Center, Gaza Strip. The remaining 81 females served as the control group. The mean age of cases was 28.7±5.4 years old whereas that of the controls was 29.1±6.4 years.

4.1.1. Some clinical aspects of *In vitro* fertilization cases (n=81)

Table 4.1. summarizes the clinical characteristics of cases based on the cause of infertility, repetition of *in vitro* fertilization (IVF) and the result of IVF. Medical records of women undergoing IVF program showed that males were the main cause of infertility representing 58 (71.6%). Forty six (56.8%) cases had repeat IVF whereas 35 (43.2%) have not been subjected to IVF before. The result of IVF was positive in 33 (40.2%) and negative in 39 (48.1%) of the cases. However, IVF showed no cleavage (no embryo) in 9 (11.1%) of the cases.

Table 4.1. Clinical characteristics of *in vitro* fertilization cases (n=81).

Clinical characteristic	**n**	**%**
Cause of infertility		
Male	58	71.6
Female	6	7.4
Idiopathic	17	21.0
Repeated *in vitro* fertilization		
Yes	46	56.8
No	35	43.2
IVF result		
Positive*	33	40.7
Negative**	39	48.1
No cleavage	9	11.1

* Positive: Pregnancy occurred.
** Negative: No pregnancy.

4.2. Hormonal levels in cases as compared to controls

The mean levels of AMH, E2, FSH, LH, PRL and TSH in cases as compared to controls are presented in Table 4.2. The AMH mean level in cases (3.5±2.3 ng/mL) was found to be significantly increased as compared to that of controls (1.7±0.5 ng/mL) showing % difference of 105.9% (t=7.06 and p=0.000). Similarly, E2 showed a significant increase in cases as compared to controls (35.5±15.1 vs. 27.6±8.3 pg/ml, % difference=28.6, t=4.11 and p=0.000). On the other hand, no significant change was observed in the mean levels of FSH, LH, PRL and THS between cases and controls (p=0.506, 0.330, 0.662 and 0.372, respectively).

Table 4.2. The mean levels of AMH, E2, FSH, LH, PRL and TSH in cases as compared to controls.

Hormones	Control (n= 81)	Case (n= 81)	% difference	t	p-value
AMH (ng/mL)	1.7±0.5	3.5±2.3	105.9	7.061	0.000
E2 (pg/ml)	27.6±8.3	35.5±15.1	28.6	4.111	0.000
FSH (mIU/mL)	7.5±3.2	7.2±2.2	-4.0	-0.667	0.506
LH (mIU/mL)	5.3±1.2	5.6 ±2.6	5.7	0.977	0.330
PRL (ng/mL)	14.7±6.2	14.3±5.1	-2.7	-0.438	0.662
TSH (µIU/mL)	2.4±2.2	2.1±1.6	-12.5	-0.895	0.372

All values are expressed as mean ±SD.

p> 0.05: not significant, p<0.05: significant.

4.3. The relationship between age and the parameters investigated in the study population

4.3.1. The relationship between age and hormonal levels

The mean levels of AMH, E2, FSH, LH, PRL and TSH in relation to the age of the study population are illustrated in Table 4.3. According to their age, the study population was divided into three groups: ≤25, 26-35 and >35 years. The mean levels of AMH in controls was significantly decreased with increasing age scoring 2.2±0.6 ng/mL, 1.8±0.3 ng/mL and 1.3±0.3 ng/mL at ≤25, 26-35 and >35 years, respectively (F=38.80 and p=0.001). Similar trend was found for cases but the change was not significant (3.7±2.0 ng/mL, 3.6±2.4 ng/mL and 2.1±2.1 ng/mL at ≤25, 26-35 and >35 years, respectively; F=2.327 and p=0.104). The mean levels of E2 fluctuated among different age groups showing the maximum levels of 30.6±10.3 pg/ml and 37.0±16.93 pg/ml at age 26-35 years for both controls and cases, respectively (F=5.286 and P=0.007, F=0.528 and p=0.592). FSH and LH also showed fluctuation in the different age groups in both controls and cases. However, only the difference in the mean levels, of FSH in controls was significant (F=4.401 and p=0.015). PRL and TSH levels of controls and cases were not significantly varied among different age groups, (PRL: F=0.210, p=0.811 and F=2.544, p=0.085; TSH: F=2.295, p=0.108 and F=1.092, p=0.341 in controls and cases, respectively).

Table 4.3. The mean levels of AMH, E2, FSH, LH, PRL and TSH in relation to age of the study population.

Hormone	Age (year)	Control	F	P-value	Case	F	p-value
AMH (ng/mL)	**≤25** **26-35** **>35**	2.2±0.6 1.8±0.3 1.3±0.3	38.80	0.001	3.7±2.0 3.6±2.4 2.1±2.1	2.327	0.104
E2 (pg/ml)	**≤25** **26-35** **>35**	23.1±5.6 30.6±10.3 27.9±6.7	5.286	0.007	34.6±12.7 37.0±16.9 32.0±14.7	0.528	0.592
FSH (mIU/mL)	**≤25** **26-35** **>35**	6.7±1.7 6.5±3.7 8.6±3.2	4.401	0.015	7.0±2.0 7.4±2.3 6.5±2.2	0.765	0.469
LH (mIU/mL)	**≤25** **26-35** **>35**	5.5±1.3 5.0±0.5 5.3±1.4	0.870	0.423	5.8±2.9 5.5±2.4 5.0±2.7	0.308	0.736
PRL (ng/mL)	**≤25** **26-35** **>35**	13.9±3.6 14.7±8.0 15.1±5.9	0.210	0.811	13.4±5.2 15.5±5.0 12.0±4.2	2.544	0.085
TSH (µIU/mL)	**≤25** **26-35** **>35**	3.3±4.1 2.1±0.7 2.1±1.1	2.295	0.108	2.4±2.1 1.9±1.0 2.3±1.6	1.092	0.341

All values are expressed as mean ±SD.

$p > 0.05$: not significant, $p < 0.05$: significant.

4.3.2. The relationship between age and various types of oocytes and embryos

Table 4.4. illustrates the mean number of various types of oocytes and embryos of cases in relation to different age groups. The number of mature oocytes was significantly decreased with increasing age with means of 9.4±3.3, 8.4±4.2 and 4.7±4.9, at age groups ≤25, 26-35 and >35 years, respectively (F=5.225 and p=0.007). This inverse relationship was exhibited in immature oocytes, but the change in their mean number among the different age groups was not significant (3.0±2.9, 2.9±2.5 and 2.2±1.4, F=0.370 and p=0.692). When the total number of oocytes was counted, their mean number was also decreased with increasing age. This change was significant (12.5±4.5, 11.0±5.4 and 6.9±4.7, F=4.793 and p=0.011). The change in the number of oocytes with age coincided with the AMH change describe earlier i.e., decreased AMH levels with increased age. The mean number of embryos formed from the collected mature oocytes also decreased with increasing age, but no significant change was detected among the different age groups (4.0±1.6, 3.3±1.9 and 2.6±1.8, F=2.656 and p=0.077).

Table 4.4. The mean number of various types of oocytes and embryos of cases in relation to the different age groups.

Oocytes	**Age (≤25 years) (n= 31)**	**Age (26 to 35 years) (n=40)**	**Age (> 35 years) (n= 10)**	**F**	**p - value**
Number of mature oocytes	9.4±3.3	8.4±4.2	4.7±4.9	5.225	0.007
Number of immature oocytes	3.0±2.9	2.9±2.5	2.2±1.4	0.370	0.692
Total number of oocytes	12.5±4.5	11.0±5.4	6.9±4.7	4.793	0.011
Number of embryo	4.0±1.6	3.3±1.9	2.6±1.8	2.656	0.077

All values are expressed as mean ±SD.

p> 0.05: not significant, p<0.05: significant.

4.4. Ovarian response of women attending IVF program in relation to the parameters investigated

The cases were divided into four groups based on their response prior to IVF:
Poor responders: <4 oocytes were collected.
Normal responders: 4-8 oocytes were collected.
Good responders: 9-16 oocytes were collected
High responders: >16 oocytes were collected.

4.4.1. Ovarian response in relation to age

Table 4.5. demonstrates women response attending IVF program in relation to age. Out of the 81 woman, 2 (2.5%), 26 (32.1%), 43 (53.1%) and 10 (12.3%) gave <4, 4-8, 9-16 and >16 oocytes during ovarian stimulation of IVF program. When related to age, the total number of oocytes increased with decreased age, showing <4, 4-8, 9-16 and >16 oocytes at mean age of 36.5±5.0, 30.6±5.9, 27.0±4.5 and 26.3±5.2, respectively. This inverse relationship between the total number of oocytes and age was found to be significant F=4.934 and p=0.003). This indicates that younger women yield more oocytes.

Table 4.5. Ovarian response of women attending IVF program in relation to age

Number of oocytes	**%**	**Age (year)**
Poor responders (<4 oocytes) (n= 2)	2.5	36.5±5.0
Normal responders (4-8 oocytes) (n= 26)	32.1	30.6±5.9
Good responders (9-16 oocytes) (n= 43)	53.1	27.0±4.5
High responders (> 16 oocytes) (n= 10)	12.3	26.3±5.2
F	4.934	
p-value	0.003	

Age is expressed as mean±SD.
p<0.05: significant.

4.4.2. Ovarian response in relation to hormonal levels

Table 4.6. gives the mean levels of AMH, E2, FSH, LH, PRL and TSH in relation to the total number of oocytes retrieved upon ovarian stimulation of IVF program. The increase in the mean levels of AMH paralleled the increase in the total number of oocytes, showing 1.0±0.5 ng/mL, 2.3±1.8 ng/mL, 3.7±1.8 ng/mL and 5.90±2.9 ng/mL in poor, normal, good and high responders, respectively. This successive change was found to be significant (F=9.174 and p=0.000). In contrast, E2 level was generally decreased with the increase in the total number of collected oocytes showing mean values of 63.7± 4.9 pg/ml, 38.1±18.3 pg/ml, 32.9±12.0 pg/ml and 34.0±13.4 pg/ml. This inverse relationship was significant (F=3.313 and p=0.024). However, the level of the FSH, LH, PRL and TSH varied among the different responders. Such variation was not significant (F=0.542, p=0.655; F=1.394, p=0.251; F=0.279, p=0.840 and F=1.554, p=0.207, respectively).

Table 4.6. The mean levels of AMH, E2, FSH, LH, PRL and TSH in relation to the number of oocytes retrieved upon ovarian stimulation of IVF program.

Hormones	**(<4 oocytes) (n= 2)**	**(4-8 oocytes) (n= 26)**	**(9-16 oocytes) (n= 43)**	**(> 16 oocytes) (n= 10)**	**F**	**p-value**
AMH (ng/mL)	1.0±0.5	2.3±1.8	3.7±1.8	5.90±2.9	9.174	0.000
E2 (pg/ml)	63.7± 4.9	38.1±18.3	32.9±12.0	34.0±13.4	3.313	0.024
FSH (mIU/mL)	5.6±3.3	7.5±2.2	7.1±2.3	6.9±1.8	0.542	0.655
LH (mIU/mL)	8.0±3.8	5.2±2.6	5.9±2.6	4.7±2.0	1.394	0.251
PRL (ng/mL)	13.7±2.7	14.4±4.6	14.6±5.3	12.9 ± 6.2	0.279	0.840
TSH (µIU/mL)	3.2±1.9	1.6±1.1	2.3±1.8	2.5±1.3	1.554	0.207

All values are expressed as mean ±SD.

p> 0.05: not significant, p<0.05: significant.

4.4.3. Ovarian response in relation to number of mature and immature oocytes and number of embryos

The mean number of mature and immature oocytes and number of embryos in relation to poor, normal, good and high responders to ovarian stimulation is shown in Table 4.7. The mean number of mature oocytes increased in the different responders to ovarian stimulation during IVF program as: 2.0±0.0, 4.2±2.4, 9.7±2.1 and 14.1±3.7 in poor, normal, good and high responders, respectively. This increase was found to be significant (F=53.738 and p=0.000). Similar trend was observed for the mean number of immature oocytes and embryos with F=10.453, p=0.000 and F=13.694, p=0.000, respectively.

Table 4.7. The mean number of mature and immature oocytes and number of embryos in relation to poor, normal, good and high responders to ovarian stimulation during IVF programs.

Oocytes	**(<4 oocytes) (n= 2)**	**(4-8 oocytes) (n= 26)**	**(9-16 oocytes) (n= 43)**	**(> 16 oocytes) (n= 10)**	**F**	**p-value**
Number of mature oocytes	2.0± 0.0	4.2±2.4	9.7±2.1	14.1±3.7	53.738	0.000
Number of immature oocytes	0.5±0.7	2.0±1.3	2.7±2.2	6.3±3.8	10.453	0.000
Number of embryo	0.0±0.0	2.3±1.7	4.1±1.4	4.5±0.7	13.694	0.000

All values are expressed as mean±SD

p> 0.05: not significant, p<0.05: significant.

4.5. The relationship between the outcome of IVF and the parameters investigated

4.5.1. The relationship between the outcome of IVF and age

The result of IVF (positive pregnancy, negative pregnancy and no cleavage) in relation to age of cases is provided in Table 4.8. Out of the 81 women enrolled in the IVF programs, 33 (40.7%) had pregnancy, 39 (48.2%) had no pregnancy and in 9 (11.1%) no cleavage occurred (no embryo). IVF results showed that positive pregnancy occurred in women aged 27.4±4.8 years where as negative pregnancy and no cleavage occurred at ages 28.1±5.7, 32.3±5.5, respectively. When related to age, IVF result showed that the chance of IVF success increased with decreased age (F=3.077 and p= 0.05). This implies that younger women had more chance to have pregnancy through IVF.

Table 4.8. The outcome of IVF in relation to age of cases.

IVF outcome	%	Age (mean ± SD)
*Positive (n= 33)	40.7	27.4±4.8
**Negative (n= 39)	48.2	28.1±5.7
No cleavage (n= 9)	11.1	32.3±5.5
F	3.077	
p-value	0.050	

* Positive: pregnancy occurred.
** Negative: no pregnancy.
p<0.05: signifiant**.**

4.5.2. The relationship between the outcome of IVF and hormone level

Table 4.9. summarizes the outcome of IVF (positive pregnancy, negative pregnancy and no cleavage) in relation to the mean levels of AMH, E2, FSH, LH, PRL and TSH. The maximum mean level of AMH was observed in positive pregnancy (4.5±2.5), followed by negative pregnancy (2.9±1.8) and no cleavage (2.1±1.5). The difference in AMH levels among these classes was significant (F=6.862 and p=0.002). The mean levels of the other hormones were slightly varied in different classes of IVF outcome but with no significant difference (p>0.05).

Table 4.9. IVF outcome in relation to the levels of AMH, E2, FSH, LH, PRL and TSH.

Hormones	* Positive (n= 33)	** Negative (n= 39)	No cleavage (n= 9)	F	p-value
AMH (ng/mL)	4.5±2.5	2.9±1.8	2.1±1.5	6.862	0.002
E2 (pg/ml)	32.8±10.1	35.6±17.4	44.5±17.2	2.771	0.121
FSH (mIU/mL)	7.4±2.1	7.0±2.1	7.2± 3.1	0.335	0.717
LH (mIU/mL)	5.5±2.5	5.7±2.6	5.4±3.1	0.075	0.928
PRL (ng/mL)	14.3±5.5	14.5±5.0	13.6±4.1	0.112	0.894
TSH (µIU/mL)	2.1±1.1	2.2±1.9	2.0±1.1	0.090	0.914

All values are expressed as mean ±SD.

* Positive: pregnancy occurred.

** Negative: no pregnancy.

p> 0.05: not significant, p<0.05: significant.

4.5.3. The relationship between the outcome of IVF and the number of the various types of oocytes and embryos

Table 4.10 shows the outcome of IVF (positive pregnancy, negative pregnancy and no cleavage) in relation to the mean number of the different types of oocytes and embryo. The mean number of mature oocytes decreased among different classes of IVF outcome showing the mean values: 10.5±3.9 in positive pregnancy, followed by 7.3±3.5 in negative pregnancy and by 4.8 ± 4.1 in no cleavage. The difference between these three classes of IVF outcome was found to be significant (F=11.262 and p=0.000). This trend was also evident for immature oocytes, but the change in their mean number among the classes of IVF outcome was not significant (3.1±2.4, 2.8±2.9 and 1.9±1.6, F=0.817 and p= 0.446). When the total number of oocytes was counted, their mean number was also decreased with different classes of IVF outcome. This change was statistically significant (13.7±5.1, 9.8±4.3 and 6.7±4.1, F=10.820 and p=0.000). The mean number of embryos produced from the collected mature oocytes also significantly decreased among the three IVF classes (4.5±0.8, 3.4±1.5 and 0.0±0.0, F=50.561 and p=0.000).

Table 4.10. The outcome of IVF in relation to the mean number of the different types of oocytes and embryo.

Variable	Positive (n= 33)	Negative (n=39)	No cleavage (n= 9)	F	p-value
Number of mature oocytes	10.5±3.9	7.3 ± 3.5	4.8±4.1	11.262	0.000
Number of immature oocytes	3.1±2.4	2.8 ± 2.9	1.9±1.6	0.817	0.446
Total number of oocytes	13.7±5.1	9.8 ± 4.3	6.7±4.1	10.820	0.000
Number of embryo	4.5±0.8	3.4 ± 1.5	0.0±0.0	50.561	0.000

All values are expressed as mean ±SD.

* Positive: pregnancy occurred.

** Negative: no pregnancy.

p> 0.05: not significant, p<0.05: significant.

4.6. Correlation between the number of mature oocytes and the different parameters investigated

Table 4.11 summarizes the correlation between the number of mature oocytes collected and the different parameters investigated. AMH and number of embryos showed positive strong correlation with the number of mature oocytes (r=0.469, p=0.001 and r=0.512, p=0.001, respectively). Strong but negative correlation was observed between age and estradiol with mature oocytes (r=-0.355, p=0.001 and r=-0.248, p=0.025, respectively). However, weak correlation was observed between FSH, LH, PRL and TSH with number of mature oocytes (r=-0.001, p=0.992; r=-0.025, p=0.825; r=0.013, p=0.906 and r=0.120, p=0.287, respectively).

Table 4.11. Correlation coefficients between the number of mature oocytes collected and the different parameters investigated.

Variable	r	P-value
Age	-0.355	0.001
AMH (ng/mL)	0.469	0.001
E2 (pg/ml)	-0.248	0.025
FSH (mIU/mL)	-0.001	0.992
LH (mIU/mL)	-0.025	0.825
PRL (ng/mL)	0.013	0.906
TSH (µIU/mL)	0.120	0.287
Number of embryo	0.512	0.001

r: Correlation coefficients.

p> 0.05: not significant, p<0.05: significant.

Chapter V Discussion

In vitro fertilization (IVF) entails egg fertilization with sperm *in vitro*, and then returning the fertilized egg (zygote) to the woman's uterus. This process is employed worldwide particularly in developing countries. In the Gaza Strip, women seek IVF program mostly when the man had fertility problems and the process of pregnancy is delayed. However, there is no published data on IVF in Gaza Strip. The present study is the first one to identify some clinical aspects of IVF as will as to asses AMH as a predictor of ovarian reserve and ovarian response to gonadotrophin stimulation in IVF candidates in the Gaza Strip.

5.1. Characteristics of the study population

Data presented in this study dealt with 81 women enrolled in IVF programs (cases) and 81 controls. The mean age of the cases in the present study (28.7 years) was close to that reported in Egyptian (29 years) and Iranian (29.1 years) studies (Shahin, 2007; Dehghani-Fieouzabadi *et al*., 2008), but lower than that reported from the Netherlands (33.8 years), (van Rooij *et al*., 2002). The younger age of women seeking IVF in developing countries, including Gaza Strip, could be explained in the context of social habits where most families have the desire to have children immediately after marriage.

5.1.1. Some clinical aspects of *In vitro* fertilization in cases

Medical records of women undergoing IVF showed that males were the main cause of infertility, representing (71.6%). This finding supports the previous idea that most men had the desire to have children immediately after marriage and that they encourage women to undergo IVF. In this context more than half of the women had repeated the process of IVF. The outcome of IVF was positive in 40.2% of the cases. In agreement with this result, a study from the Netherlands showed that out of 112 women undergoing IVF, 40 (36%) of them achieved viable pregnancy (Smeenk *et al*., 2007).

However Shahin (2007) reported that the pregnancy rate among patients who had an IVF trial in different settings was 23.1%. Nevertheless, IVF treatment outcome is highly variable and difficult to predict.

5.2. Hormonal levels in cases as compared to controls

The mean levels of AMH, E2, FSH, LH, PRL and TSH recorded in the present study for cases were more or less close to those reported by Fanchin *et al*., (2003) and Ficicioglu *et al*., (2006). When compared to controls AMH and E2 where significantly higher than those encountered in the controls (3.5±2.3 vs. 1.7±0.5 ng/mL and 35.5±15.1 vs. 27.6±8.3 pg/ml, respectively). On the other hand no significant changes were observed in the mean levels of FSH, LH, PRL and TSH between cases and controls. It has been hypothesized that the high level of AMH (>3 ng/ml) indicates higher fertility rate in females (Durlinger *et al*., 1999). Consequently, the high level of AMH observed in most women undergoing IVF means that they have normal ovarian reserve with a good opportunity to get pregnant.

It was concluded that poor response to stimulation in IVF, indicative of a diminished ovarian reserve, is associated with reduced baseline serum AMH concentrations (e.g., van Rooij *et al.*, 2002). Therefore, when women have normal ovarian reserve and good response, failure of IVF should be due to other infertility cases, e.g., male specific factor (like Y chromosome microdeletion). This conclusion is supported by the medical records of women undergoing IVF which showed that males were the main cause of infertility. Although E2 levels in the cases were higher than those of controls, they are still within the normal range of 25-100 pg/ml (Tietz, 1995), indicating that E2 alone is not sufficient to predict the female reproductive potential.

5.3. The relationship between age and the parameters investigated in the study population

5.3.1. The relationship between age and hormonal levels

Data presented in this study showed that the mean levels of AMH in controls and cases decreased with increasing age. This inverse relationship is in agreement with that found by Van Rooij *et al*. (2005) who reported that serum

AMH levels decline with age in normal women with proven fertility. They added that serum AMH represents the best endocrine marker to assess the age-related decline of reproductive capacity. On the other hand, E2, FSH, LH, PRL and TSH, generally showed fluctuations in different age groups in both controls and cases.

5.3.2. The relationship between age and various types of oocytes and embryos

As shown in the present results, the mean number of various types of oocytes and embryos of cases decreased with increasing age, with significant changes detected in the number of mature oocytes and in the total number of retrieved oocytes. Similar result was reported by La Marca *et al*. (2007) who found an inverse relationship between age and the total number of oocytes in Italian women attending IVF programs i.e., the number of total oocytes decreased with increasing age. This coincides with the prevailing concept of female reproductive aging suggesting that the decline of the oocytes pool determines the age-dependent loss of female fertility (Te Velde *et al*., 2002). Because chronological age at the final stage of reproductive aging (i.e., the occurrence of menopause) shows a considerable individual variation, it is most likely that women differ with regard to the status of the oocytes pool at a given age. The change in the number of oocytes correlates with AMH change where AMH levels decrease with increased age.

5.4. Ovarian response of women attending IVF program in relation to the parameters investigated

5.4.1. Ovarian response of women attending IVF program in relation to age

Women undergoing IVF showed that ovarian response is better with decreasing age, showing poor response (<4 oocytes) at the oldest age (36.5 years) and high response (>16 oocytes) at the youngest age (26.3 years). This means that younger women have a better chance of a successful IVF. In their studies on ovarian reserve and ovarian response in IVF women, Van Rooj *et al*., (2002) and La Marca *et al*. (2007). found that the poor responses

were detected at ages (36.3 and 32.5 years). This inverse relationship between women response and age was also documented in other studies (e.g., Ficicioglu *et al.*, 2006; Dehghani-Firouzabadi *et al.*, 2008).

5.4.2. Ovarian response in IVF candidates in relation to hormonal levels

The present study illustrated that the mean levels of AMH showed a progressive increase parallel to the total number of oocytes in poor, normal, good and high responders. La Marca *et al*. (2006) reported similar successive increase in the total number of oocytes as a result of increasing AMH levels in responder women attending IVF. In their report the total mean number of oocytes was significantly lower and higher in poor and high responders, respectively, than in other groups. Significant decrease in AMH levels was found in poor responders as compared to good responders. In agreement with this positive trend, Seifer *et al*. (2002) found that the mean serum AMH concentration was more than two and a half-fold in the group with ≥11 collected oocytes as compared to the group with ≤6 oocytes. In addition, Dehghani-Firouzabadi *et al*. (2008) reported significant increase in good responder women undergoing IVF (≥4 oocytes) as compared to poor responders (<4 oocytes). Accordingly, serum AMH levels can predict ovarian response during IVF treatment cycles. In contrast to AMH, the total number of oocytes in different response classes increased with significant decreased in the levels of E2. This inverse relation was also reported by Van Rooij *et al*. (2002), Fanchin *et al* (2003) and Ficicioglu *et al.*, (2006), but with no significant change indicating that E2 is a poor marker of ovarian response. Finally, the differences between the mean levels of the remaining hormones including FSH, LH, PRL and TSH and total number of oocytes count were not statistically significant among the different classes of responders.

5.4.3. Ovarian response in women attending IVF in relation to number of mature and immature oocytes and number of embryos

Results presented here revealed that the mean number of mature and immature oocytes and number of embryos were significant in different classes of responders to ovarian stimulation from poor to high responders. This finding is in agreement with that found by La Marca *et al.* (2006 and 2007)

and Dehghani-Firouzabadi *et al*. (2008) who reported that the mean number of oocytes was significantly lower in poor respondent women than in normal, good and high respondent women attending IVF programs. This leads to the conclusion that the ovarian response can be regarded as a reflection of the ovarian reserve.

5.5. The relationship between the outcome of IVF and the parameters investigated

5.5.1. The relationship between the outcome of IVF and age

In vitro fertilization results presented in this study showed that the chance of IVF success increased with decreased age; 33 (40.7%) of cases ending up with pregnancy were 27.4 years old. This implies that younger women had a better chance to have a successful pregnancy. This is supported by the aforementioned result that ovarian reserve and response increased with decreasing age making more chance for pregnancy to occur. In addition, our result is congruent with that of Gnoth *et al*. (2008) who found that pregnancy outcome of IVF program was significantly higher in younger women than that in the older ones. Similar result was also reported by Smeenk *et al*. (2007), but the difference in the pregnancy outcome of IVF program was not significant

5.5.2. The relationship between the outcome of IVF and hormone level

The results of IVF classes showed that the maximum level of AMH was observed in the cases which yielded positive pregnancy, followed by negative pregnancy and no cleavage, with significant difference in AMH levels among these three classes. The observed high mean levels of AMH can be linked to the increased mean number of collected oocytes and consequently to increase the chance to obtain higher mean numbers of embryos. The final outcome is to improve the chance of pregnancy. This implies that AMH levels may be an indicator for ovarian responsiveness but not with embryo quality or the probability to achieve a pregnancy, Smeenk *et al*. (2007) recorded a higher mean level in AMH in pregnant women undergoing IVF as compared to those who failed and came to the similar conclusion that AMH predicts

ovarian responsiveness. The mean levels of E2, FSH, LH, PRL and TSH were slightly varied in different classes of IVF outcome with no significant differences between the classes. Similar results were exhibited by Smeenk *et al*. (2007). It is worth mentioning here that although AMH level seems to be a good marker for ovarian response to stimulation drug IVF it can't however, predict the outcome of the IVF process since 40.7% of the cases with elevated AMH did not end up a viable pregnancy.

5.5.3. The relationship between the outcome of IVF and the number of the various types of oocytes and embryos

The mean number of mature, immature and total number of oocytes, and the mean number of embryos correlated with the success of IVF. Moreover, the increase in the mean number of total oocytes in positive pregnancy coincides with increased mean levels of AMH, giving a better chance for higher mean number of mature of oocytes and embryos, and consequently higher probability for pregnancy to occur. Similar result was obtained by Smeenk *et al*. (2007) who found that the mean number of total oocytes was higher in women with successful pregnancy.

5.6. Correlation between the number of mature oocytes and the different parameters investigated

As depicted from correlation coefficient results, AMH and number of embryos showed positive strong correlation with the number of mature oocytes. This reconfirms the previous result that elevated levels of AMH increased the mature number of oocytes offering more chance to higher number of embryos. This could lead us to the hypothesis that AMH has an indirect effect on the number of embryos. Ficicioglu *et al*. (2006) found strong positive correlation between AMH and the number of mature oocytes in women attending IVF. On the other hand, strong negative correlation was observed between age and E2 with mature oocytes. Such negative correlation between age and mature number of oocytes was also reported by Ficicioglu *et al*. (2006). This further supports the previous inverse relationship between age and the number of oocytes.

Chapter 6 Conclusions & Recommendations

6.1 Conclusions

The present study investigated 162 females; 81 with inability to conceive naturally and seeking *in vitro* fertilization (cases) at Al-basma fertility Center, Gaza Strip. The remaining 81 females served as the control group. The aim of this study was to assess AMH in early follicular phase as a predictor of ovarian reserve and ovarian response for stimulation among females attending IVF programs. The mean age of cases was 28.7±5.4 years whereas that of the controls was 29.1±6.4 years. The conclusions that can be drawn from this study include:

1. The mean levels of AMH and E2 in cases were significantly higher as compared to controls. However, no significant change was observed in the mean levels of FSH, LH, PRL and TSH between the two groups.
2. The mean levels of AMH were significantly increased in respondent women from poor (<4 oocytes) to high (>16 oocytes) making it a good predictor of ovarian reserve and ovarian response in IVF candidates.
3. In contrast, E2 levels were generally decreased from poor to high respondents. However, the levels of the FSH, LH, PRL and TSH were variable among different responses to ovarian stimulation.
4. There is an inverse relationship between AMH and age in both controls and cases with significant difference in controls. E2, FSH, LH, PRL and TSH, hormones, showed fluctuation in different age groups.
5. The ovarian response of the cases was inversely related to the age; i.e., the poor response (<4 oocytes) and the high response (>16 oocytes) were observed in oldest and youngest ages, respectively.
6. The mean number of total and mature oocytes in cases was significantly decreased with increasing age.
7. The mean number of mature and immature oocytes, and embryos was significantly increased in different response groups of ovarian stimulation from poor to high responses.

8. The IVF results showed that the chance of IVF success increased with decreased age i.e., younger women have a better chance of having pregnancy.
9. The maximum level of AMH was observed in positive pregnancy followed by negative pregnancy and then by no cleavage. The difference in AMH levels among these classes of IVF result was significant. It is worth mentioning that AMH levels may be associated with ovarian responsiveness (increase the total number of oocytes), but not with the probability to achieve a pregnancy. The mean levels of E2, FSH, LH, PRL and TSH were slightly varied with no significant change.
10. The mean number of total and mature oocytes, and embryos was significantly increased in positive pregnancy.
11. The AMH levels and the number of embryos showed positive strong correlation with the number of mature oocytes. Strong but negative correlation was observed between age and estradiol with mature oocytes. However, weak correlation was observed between FSH, LH, PRL and TSH and the number of mature oocytes.
12. Medical records of women attending IVF program showed that males are the main cause of infertility.

Recommendations

1. AMH can be used in IVF programs as a good predictor of ovarian reserve and ovarian response.
2. AMH can be used as a marker for ovarian aging.
3. Younger age (<30 years) is preferable for women intending to undergo IVF trials, as the total number of oocytes is higher and there is a higher opportunity to achieve pregnancy.
4. Further research is recommended on a) other hormones like inhibin B and its relation to AMH and on b) the relationship between AMH and ovarian hyperstimulation.

References

Boelaert K. and Franklyn J.A. (2005). *Thyroid hormone in health and disease.* Endocrinology, 187-201.

Carlson J.K., Eisenstat A.S., Terra Diane Ziporyn D.T. (1996). *The Harvard Guide to Women's Health,* 2nd Ed. Cambridge, Harvard University Press, USA.

Cate R.L., Mattaliano R.J., Hession C., Tizard R., Farber N.M., Cheung A.,Nin fa E.G., Frey A.Z., G ash D.J., Chow E.P. (1986). *Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells*. Cell, 45 (5):685-698.

Dehghani-Firouzabadi R., Tayebi N., Asgharnia M. (2008). *Serum Level of Anti-Mullerian Hormone in Early Follicular Phase as a Predictor of Ovarian Reserve and Pregnancy Outcome in Assisted Reproductive Technology* Cycles. Archives of Iranian Medicine, 11, 4.

Disseldorp J., Faddy M., Themmen A., De Jong F., Peeters P., Schouw Y., Broekmans F. (2008). *Relationship of Serum Antimullerian Hormone Concentration to Age at Menopause*. Clinical Endocrinology and Metabolism, 293(6):2129–2134.

Durlinger A.L., Kramer P., Karels P., Hoogerbrugges J.W., van Leeuwen E.C. (1999). *Endocrinology*, 140, 5789-5796.

Fanchin R., Schonauer L., Righini C., Guibourdenche J., Frydman R., Taieb J. (2003). *Serum anti-Mullerian hormone is more strongly related to ovarian follicular status than serum inhibin B, FSH, and LH on day 3*. Human Reproduction, 18(2):323 – 327.

Fanchin R., Mendez Lozano D., Louafi N., Achour-Frydman N., Frydman R., Taieb J. (2005a). *Dynamics of serum anti-Mullerian hormone levels during the luteal phase of controlled ovarian hyperstimulation*. Human Reproduction, 20, 747-751.

Fanchin R., Taieb J., Lozano D., Ducot B., Frydman R., Bouyer J. (2005b). *High reproducibility of serum anti-Mullerian hormone measurements suggests a multi-staged follicular secretion and strengthens its role in the assessment of ovarian follicular status.* Human Reproduction, 20 (4): 923-927.

Ficicioglu C., Kutlu T., Baglam E., Bakacak Z. (2006). *Early follicular antimüllerian hormone as an indicator of ovarian reserve*. Fertility and Sterility, 85(3): 592-596.

Firouzabadi R., Tayebi N., Asgharnia M. (2008). *Serum Level of Anti-Mullerian Hormone in Early Follicular Phase as a Predictor of Ovarian Reserve and Pregnancy Outcome in Assisted Reproductive Technology Cycles.* Archives of Iranian Medicine, 11(4):371–376.

Frederic M., Michael T., Robert T. (2008). *Human Anatomy*, 6th Ed. Pearson/Benjamin Cummings.

Freiesleben N. L., Rosendahl M., Johannsen T., Lossl K., Loft A., Bangsboll S., Friis-Hansen L., Pinborg A., Andersen A. (2010). *Prospective investigation of serum anti-Mullerian hormone concentration in ovulatory intrauterine insemination patients: a preliminary study*. Reproductive BioMedicine Online.

Ganong W. (2003). *Review of Medical Physiology*, 21th Ed. McGraw-Hill Companies, San Francisco, USA.

Gnoth C., Schuring A., Friol K., Tigges J., Mallmann P. Godehardt E. (2008). *Relevance of anti-Mullerian hormone measurement in a routine IVF program.* Human Reproduction, 23, 1359-1365.

Gruber J., Tschugguel W., Schneeberger C., Huber J. (2002). *Production and actions of estrogens*. New England Journal of Medicine, 346(5):340-352.

Hasegawa T., Lee M.M., Donahoe P.K., Silverman B., Crist G.B., Best S., Hasegawa Y., Noto R.A., Schoenfeld D., and Maclaughlin D.T. (1996). *Mullerian inhibiting substance in humans: normal levels from infancy to adulthood*. Clinical Endocrinology and Metabolism, 81**,** 571-576.

Hirobe S., Donahoe P., Lee M. (1992). *mullerian hormone messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity*. Endocrinology, 131(2):854–862.

Jones E. (1997). *Human reproductive biology*, 2nd Ed. Academic Press New York.

Jorgensen N., Rajpert-De Meyts E., Graem N., Muller J., Cate R. and Skakkebaek N. (1999). *Expression of anti-Mullerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulosa cells*. Clinical Endocrinology and Metabolism, 84**,** 3836-3844.

Josso N., Picard J. (1986). *Anti-Mullerian hormone*. Physiological Reviews, 66**,** 1038-1090.

Kobayashi A., and Behringer R. (2003). *Developmental genetics of the female reproductive tract in mammals*. Nature Reviews Genetics, 4**,** 969-980.

Kosasa T. (1981). *Measurement of human luteinizing hormone*. Reproductive Medicine, 26, 201-206.

Krohmer R. (2004). *The reproductive system*. Chelsea House, 1st Ed. New York.

Kronenberg H., Melmed S., Polonsky K., Larsen P. (2008). *Williams Textbook of Endocrinology*, 11th Ed. Elsevier Philadelphia, Pennsylvania.

La Marca A., Giulini S., Tirelli A., Bertucci E., Marsella T., Xella S., Volpe A. (2006). *Anti-Mullerian hormone measurement on any day of the menstrual cycle strongly predicts ovarian response in assisted reproductive technology.* Human Reproduction, 22(3):766-771.

La Marca A., S.Giulini, Tirelli A., Bertucci E., Marsella T., Xella S. and Volpe A. (2007). *Anti-Mullerian hormone measurement on any day of the menstrual cycle strongly predicts ovarian response in assisted reproductive technology.* Human Reproduction, 22(3):766–771.

La Marca A., Sighinolfi G., Radi D., Argento C., Baraldi E., Artenisio A., Stabile G., Volpe A. (2010). *Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART).* Human Reproduction, 16(2):113-130.

Laven J.S., Mulders A.G., Visser J.A., Themmen A.P., De Jong F.H., Fauser B.C. (2004). *Anti-Mullerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age.* Clinical Endocrinology and Metabolism, 89, 318-323.

Lee M.M. and Donahoe P.K. (1993). *Mullerian inhibiting substance: a gonadal hormone with multiple functions*. Endocrine Reviews, 14, 152-164.

Lenton E., NealL. and Sulaiman R. (1982). *Plasma concentration of human gonadotrophins from the time of implantation untile the second week of pregnancy*. Fertility and Sterility, 37, 773-778.

Maheswaran S., Teixeira J. and Donahoe P.K. (2001). *Mullerian inhibiting substance: an instructive developmental hormone with diagnostic and possible therapeutic applications*. Endocrine Reviews, 22, 657-674.

Marieb E.N. (2006). *Essentials of human anatomy and physiology*, 8th Ed Pearson/Benjamin Cummings,. San Francisco, USA.

Munsterberg A. and Lovell-Badge R. (1991). *Expression of the mouse anti-Mullerian hormone gene suggests a role in both male and female sexual differentiation*. Development 113(2):613–624.

Muttukrishna S., Suharjono H., McGarrigle H., Sathanandan M. (2004) *Inhibin B and anti-Mullerian hormone: markers ofovarian response in IVF/ICSI patients. Obstetrics and Gynaecology*, 111, 1248–1253.

Parmentier M., Libert F., Maenhaut C., Lefort A., Gerard C., Perret J., Vav Savd J., Dumont J. and Vassart G. (1989). *Molecular cloning of the thyrotropin receptor*. *Science,* 246**,** 1620-1622.

Purves D., Augustine G., Hall W., LaMantia A., McNamara J. and White L. (2008). *Neuroscience,* 4th Ed. Sinauer Associates.

Scanlon V., Sanders T. (2007). *Essentials of anatomy and physiology*. F, 5th Ed. A. Davis Company, Philadelphia, USA.

Seifer D., MacLaughlin D., Christian B., Feng B., Shelden R. (2002). *Early follicular serum Mullerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles*. Fertility and sterility, 77, 468 – 471.

Shahin A. (2007). *The problem of IVF cost in developing countries: has natural cycle IVF a place*? Reproductive Bio-Medicine Online, 15**,** 51-56.

Siiteri P.K., Murai J.T., Hammond G.L., Nisker J.A., Raymoure W.J. and Kuhn R.W. (1982). *The serum transport of steroid hormones*. Clinical Endocrinology and Metabolism*,* 38**,** 457-510.

Silberstein T., MacLaughlin T., Shai I., Trimarchi J., Seifer B., Keefe L., Blazar S. (2006). *Mullerian inhibiting substance levels at the time of HCG administration in IVF cycles predict both ovarian reserve and embryo morphology.* Human Reproduction, 21(1): 159-163.

Smeenk J., Sweep F., Zielhuis G., Kremer J., Thomas C. and Braat D. (2007). *Antimullerian hormone predicts ovarian responsiveness, but not embryo quality or pregnancy, after in vitro fertilization or intracyoplasmic sperm injection.* Fertility and sterility, 87(1): 223-226.

Snell S.R. (2007). *Clinical anatomy,* 8th Ed Baltimore. Lippincott Williams and Wilkins.

Szkudlinski W., Fremont V., Ronin C. and Bruce W. (2002). *Thyroid Stimulating Hormone and Thyroid Stimulating Hormone Receptor Structure-Function Relationshipe*. Physiological Reviews, 82, 473.

Te velde R. and Pearson L. (2002). *The variability of female reproductive ageing*. Human Reproduction, 8, 141-154.

Tietz N.W. (1992). *Clinical guide to laboratory tests,* 3rd Ed. W. B, London.

Tietz N.W. (1995). *Clinical guide to laboratory tests*. W . B Saunders, Philadelphia. 3rd Ed, 216-217.

Van Rooij I.A., Broekmans F.J., Te Velde E.R., Fauser B.C., Bancsi L.F., De Jong F.H. and Themmen A.P. (2002). *Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve*. Human Reproduction, 17**,** 3065-3071.

Van Rooij A.J., Frank J.M., Gabrielle J., Caspar W. N., Bart J.M., Axel P. N.(2005). *Serum anti-mullerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: a longitudinal study.* American Society for Reproductive Medicine, 83, 4.

Visser J.A., Durlinger A.L. & Themmen A.P. (2002). *Regulation of ovarian function: the role of anti-Mullerian hormone*. Reproduction, 124, 601-609.

Vitt U.A., Kloosterboer H.J., Rose U.M., Mulders J.W., Kiesel P.S. (1998). *Isoform of human recombinant follicle-stimulating hormone: comparison of effects on murine follicle development in vitro*. Reproduction of Biology, 59, 854-861.

Weenen C., Laven J.S., Von Bergh A.R., Cranfield M., Groome N.P., Visser J.A., Kramer P., Fauser B.C., Themmen A.P. (2004). *Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment*. Molecular Human Reproduction, 10, 77-83.

Wunder D.M., Bersinger N.A., Yared M., Kretschmer R., Birkhauser M.H. (2008). *Statistically significant changes of anti m mullerian hormone and inhibin levels during the physiologic menstrual cycle in reproductive age women*. Fertility and Sterility, 89(4): 927-933.

Annex 1

Questionnaire about Antimullerian Hormone as a Predictor of Ovarian Reserve and Ovarian Response in IVF candidates

Demographic information:

Name:
Age:
Occupation:
Location: North ( )
Gaza ( )
Middle ( )
South ( )

Hormone related questions:

1. Number of sample:
2. This day corresponds to: from menstrual cycle
3. Do you have regular menstrual cycle?
Yes ( )
No ( )
4. Do you have any children?
Yes ( )
No ( )
5. Infertility is due to:
Male ( )
Female ( )
Both ( )
Idiopathic ( )
6. Have you undergone IVF before this time?
Yes ( )
No ( )

If yeas how many time
2 ( ), 3 ( ), 4 ( )

7. Have you been subject to surgical operation in your reproductive system before?
Yes ( )
No ( )
8. level of FSH(mIU/mL):
9. level of LH(mIU/mL) :
10. level of E2 (pg/mL) :
11. level of AMH (pM/mL):
12. level of PRL:
13. level of TSH:
14. Number of total oocytes:
15. Number of mature oocytes:
16. Number of immature oocytes
17. Number of embryos
18. Result of IVF
pregnancy occurred ( )
no pregnancy ( )

أنا الباحث/ ***محمد مروان لقان*** أرجو المساعدة في تعبئة هذا الاستبيان من أجل إجراء دراسة لإيجاد علاقة بين مستوى هرمون AMH ومخزون المبيض من البويضات لكي تستخدم لتعرف على مدي نجاح عملية الزراعة (أطفال الأنابيب).

البيانات الشخصية:

الاسم :
العمر :
المهنة:
مكان السكن: الشمال ( )
غزة ( )
الوسطى ( )
الجنوب ( )

أسئلة خاصة بالهرمون

1. رقم العينة:
2. اليوم يوافق كم: من الدورة
3. هل الدورة الشهرية منتظمة؟
   نعم ( )
   لا ( )
4. هل لديك أطفال؟
   نعم ( )
   لا ( )
5. أسباب العقم لها: علاقة
   بالرجل ( )
   بالمرأة ( )
   كلاهما ( )
   غير مفسر ( )
6. هل تم إجراء عملية زراعة (أطفال الأنابيب)قبل هذه المرة ؟
   نعم ( )
   لا ( )
   إذا كان نعم كم مرة
   2 ( )، 3 ( )، 4 ( )
7. هل تعرضت لعملية جراحية في التناسلي؟
   نعم ( )
   لا ( )
8. مستوى FSH :
9. مستوى LH :
10. مستوى E_2 :
11. مستوى AMH:
12. مستوى PRL:
13. مستوى TSH:
14. عدد الكلي للبويضات:
15. عدد البويضات الناضجة:
16. عدد البويضات الغير الناضجة
17. عدد الأجنة
18. نتيجة عملية التلقيح
    حدث حمل ( )
    لم يحدث حمل ( )

Annex 2

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

Name:

التاريخ:2010/3/23

الاسم : محمد مروان لقان

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

Anti Mullerian Hormone as predictor of ovarian reserve and ovarian response in IVF candidates.

In its meeting on March 2010

و ذلك في جلستها المنعقدة لشهر 3 2010

and decided the Following:-

و قد قررت ما يلي:-

To approve the above mention research study.

الموافقة على البحث المذكور عاليه.

Signature
توقيع

Member
عضو

Member
عضو

Chairperson

Conditions:-

- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.