The relationship between Anti-Müllerian Hormone (AMH) levels and pregnancy outcomes in patients undergoing *in-vitro* fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI)

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Submitted in partial fulfilment of the requirements for the degree of

MASTER OF HEALTH SCIENCES IN CLINICAL TECHNOLOGY

In the

Department of Biomedical &Clinical Technology

Faculty of Health Sciences

Durban University of Technology

AUTHORS 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 that has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

The research described in this dissertation was carried out in the Department of Biomedical and Clinical Technology, Faculty of Health Sciences, Durban University of Technology under the supervision of Professor J.K. Adam (IREC: Chairperson) and C.A.R.E Clinic, Westville, South Africa under the supervision of Dr A Ramdeo (Clinical Director).

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DEDICATION

I dedicate this work to:

My grandmother **Kamlapathy Tholsie** – without this incredible woman in my life I would not be the woman I am today, all her teaching and guidance throughout my life has given me the strength and motivation to strive towards this Masters, even though god has decided to call you home I know you are watching over me and will be proud of all I have achieved.

My husband, **Rishi Reddy** - thank you for all the understanding and support and for walking this journey this me. Without the encouragement I would not have been able to achieve this.

My parents, **Roy and Shakun Singh** - For being my greatest supporter and biggest critics whilst showering me with an abundance of love and support.

ABSTRACT

Introduction

Woman are born with a lifetime supply of oocytes, these oocytes gradually decrease in both quantity and quality with age. Anti-Müllerian Hormone (AMH) is a hormone secreted by cells in the developing oocyte sacs (follicles). AMH has become an important predictor of ovarian reserve. Low AMH levels can forecast reduced response to gonadotropins in *In-Vitro* Fertilization (IVF) cycles a well as pregnancy rate. Accurate and detailed tests should be conducted that can aid in predicting the chance of a pregnancy through IVF. The clinician together with the patients can make an educated decision about whether to continue with the treatment or review other options that may be viable. Many medical studies have found that a woman's AMH concentration in the blood can predict how many oocytes they can produce during IVF treatment. On this basis, tests have become available to measure AMH and other markers that indicate fertility changes and the state of advancement of ovarian ageing.

Aim and Objectives

The aim of the study was determine the relationship between Anti-Müllerian Hormone levels and pregnancy outcomes in patients undergoing *in-vitro* fertilization or intra-cytoplasmic sperm injection (ICSI).

The objectives were as follows:

(1) To determine whether there is a correlation in patients presenting with low AMH and low oocyte reserve;

(2) To examine if AMH levels affect the oocyte quality;

(3) To evaluate the correlation between AMH level and a positive pregnancy outcome.

Methodology

A total of 50 patients were recruited from C.A.R.E (Centre of Assisted Reproduction and Endocrinology) Clinic in Westville who were undergoing IVF treatment.

The blood samples were taken at room temperature. Serum was used to determine estrogen (E_2), progesterone (P_4), luteinizing hormone (LH), anti-müllerian hormone

(AMH), and follicle stimulating hormone (FSH) levels. Levels were determined using an ultra-sensitive enzyme-linked immunosorbent assays (ELISA) (Beckman Coulter).

Two stimulation protocols were used to harvest a maximum number of oocytes. The long protocol used Lucrin Subcut 10 units daily and Lucrin Depo 3.75mg for down regulation. Stimulation drugs were Gonal F®, Menopur®, Puregon®, Clomid®, Fertomid® and Fostimon®

The short protocol consisted of GnRH antagonist with Cetrotide 0.25mg primed with Logynon ED. No down regulation was required. The patient started on day 2 or day 3 of the menstrual cycle, Cetrotide 0.25mg is given to suppress the LH. The same drugs were used for stimulation as per the long protocol.

The patients' blood serum was tested to measure the amount of Estrogen in her body prior to the collection of oocytes. In all patient's ovulation was induced using 5000 – 10 000 IU hCG (Pregnyl®, Ovitrel®) trigger injection, provided the lead follicle had reached a diameter of 19mm. Oocyte retrieval was performed trans-vaginally under ultra sound guidance 36 hours after the administration of the hCG.

The fluid containing the oocytes was aspirated using an oocyte recovery needle and forwarded to the IVF laboratory where the oocytes were identified, rinsed in culture media and were incubated at 37°C in IVF incubators.

After 2-4 hours incubation period the cumulus complex was removed from the oocyte using the enzyme hyaluronidase and glass pipettes. Following denudation, the oocytes were placed back into the incubator until the ICSI was performed. Maturation and morphological features of the oocytes were noted before the ICSI. The features of each oocyte were evaluated using an inverted microscope. Fertilization was assessed 19-21 hours after the ICSI was performed and was characterized by the presence of two pronuclei to show the result of the union between the male and female genetic material to form a zygote cell. Embryos were grown up to Day 3 (8 cell stage), Day 5 (blastocyst stage) or Day 6 (hatching blastocyst) and transferred into the patient.

A pregnancy test was performed 14 days post transfer.

Results

50 patients that met the inclusion criteria were recruited for the study. From the initial sample size of 50, 42 presented with data that could be analysed whilst 8 patients had oocytes that where abnormal and did not result in a transfer. The data from these 8 patients were not included in the study due to poor embryo development.

According to the AMH levels, 52.4% of patients were in High Category, 40.5% were in the Normal and 7.1% were in the Low to Normal Category.

A cross-tabulation of the number of oocytes retrieved; the number of oocytes mature, and the number of oocytes fertilized was done. Not all eggs obtained were at the metaphase 2 stages and had to be matured in the incubator overnight and injected with sperm the following day. A Chi-square test for Independence was performed to check whether there is an association between the number of oocytes fertilized and the AMH category. A Chi-squared value of 18.5, degrees of freedom = 12, with a $p = 0.10$ was found. Therefore, showing no statistically significant relationship between the numbers of embryo's fertilized versus AMH category ($p > 0.05$).

A Chi- squared test were done of AMH category and Number of embryos transferred was done resulting in a value of 6.384 with df = 4 and a cross tabulation ensued a p-value of 0.172. There was thus not a significant association between AMH category and No. of embryos transferred.

A cross-tabulation and a Chi-square test were done of AMH category and the day of embryo transfer was done. A Chi-square value of 14.117, 6 degrees of freedom and $p = 0.028$ was observed. There was a statistically significant relationship between the AMH category and the day of embryo transfer ($p< 0.05$).

A cross-tabulation of AMH category and Pregnancy outcome was calculated. It can also be seen that of the 22 cases reported in the High category, 6 resulted in a positive pregnancy, 17 cases where the AMH category was "Normal", 6 resulted in a positive outcome (6/12 = 50.0%), while out of the 3 cases where the AMH category was "Low to Normal" there were no pregnancies reported.

The Chi-squared test for independence of AMH category and Pregnancy outcome gave a Chi-Squared value of 0.502, 2 degrees of freedom and $p = 0.778$.

Race and pregnancy outcome were calculated using a cross tabulation and a Chi-square test for independence gave a Chi-squared value of 2.246, with 3 degrees of freedom and $p = 0.532$ ($p > 0.05$).

To determine if a statistical significance exists between AMH and age, E_2 and FSH a Pearson Correlations was performed. Table 12 shows the Pearson analysis between E_2 and AMH. The Pearson Correlation coefficient of 0.151 with $p = 0.341$ ($p<0.05$) indicates a very weak/ no statistically significant relationship between E_2 and AMH.

AMH and age produced a coefficient of -0.028 thus showing a weak, negative correlation with $p = 0.859$ ($p> 0.05$). A stronger relationship between these two variables was expected as it is known that as age increase, AMH should decrease. Pearson Correlation between the AMH and FSH produced a coefficient of -0.185 thus showing a weak, negative correlation with $p = 0.240$ ($p> 0.05$).

The Pearson Correlation between FSH and age also showed that there was no statistical significance, $p = 0.583$ ($p > 0.05$) but a very weak negative correlation (Pearson Correlation -0.087). Pearson correlation between the number of oocytes and age also did not show any statistically significant relationship ($p = 0.082$; $p<0.05$). Pearson Correlation value of -0.271 shows a weak negative relationship.

No significant relationship was shown between AMH and number of oocytes using a Pearson Correlation test ($p = 0.191$), number of mature oocytes ($p = 0.300$) and number of oocytes fertilized ($p = 0.146$). The number of oocytes, mature oocytes and oocytes fertilized all showed a weak positive relationship to AMH (0.206, 0.164, and 0.228, respectively).

Conclusion

In conclusion, while appropriate reference values are being created per age category and until the consequences of having a low or high AMH for one's age are being established, AMH should only be determined in the context of clinical studies. At present, the most important clinical role of AMH at this stage is to serve as a red-flag for reduced ovarian reserve in women of reproductive age who must undergo further diagnostics. As per the study conducted, we can deduce that AMH can accurately predict ovarian reserve but cannot predict the oocyte quality or a positive pregnancy

outcome. The more oocytes obtained, increases a patient's chance of more viable embryos and therefore, improving chances of a healthy pregnancy and ultimately a live birth. This thesis has established a definite role for AMH as a forecaster for both current and future individual fertility.

ACKNOWLEDGEMENTS

I wish to extend my sincere appreciation to:

Professor JK Adam, my internal supervisor. My sincere appreciation is given for your commitment, encouragement and guidance in the study.

Kinnon K Naidoo, my colleague and friend. My sincere appreciation for all your support and time during this study, without you I would not have had the courage to persevere.

Lindi Theron, my best friend, no words can express your support and help, I will forever be thankful.

Shrinav Dawlat, thank you my friend, all your encouragement got me to the end.

To the Clinical Director of C.A.R.E Clinic, **Dr Anil Ramdeo** for allowing me access to conduct the study, and help in overseeing the work at the clinic.

Dr Glenda Mathews, Head of Department (Statistics), Durban University of Technology for her immense help in understanding and interpreting the statistical results.

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LIST OF ABBREVIATIONS

%	-	percentage
°C	-	degree Celsius
<	-	less than
>	-	greater than
et al	-	*et alia* (and others)
AFC	-	Antral Follicle Count
AMH	-	Anti-Müllerian Hormone
ART	-	Assisted Reproductive Techniques
DSL	-	Diagnostic Systems Laboratory
E_2	-	Estrogen
EOR	-	Estradiol per oocyte ratio
FSH	-	Follicle Stimulating Hormone
GnRH	-	Gonadotropin Releasing Hormone
GV	-	Germinal Vesicle
hCG	-	Human Chorionic Gonadotropin
ICSI	-	Intra cytoplasmic Sperm injection
IVM	-	*In Vitro* Maturation
IOT	-	Immunotech
IVF	-	*In-Vitro* fertilization
LH	-	Luteinizing Hormone
M1	-	Metaphase 1
M2	-	Metaphase 2
ORT	-	Ovarian Reserve Test

P_4	--	Progesterone
PCOS	-	Poly Cystic Ovarian Syndrome
POI	-	Primary Ovarian Infertility
POF	-	Premature Ovarian Failure
POR	-	Poor Ovarian Response
WHO	-	World Health Organization

CHAPTER 1

INTRODUCTION

Infertility is defined as the inability of a couple to conceive within a year of regular unprotected intercourse. These couples can be separated into two groups: couples who are incapable of conceiving without treatment and those with reduced fertility who still have a considerable chance to conceive (Gunnell and Ewings., 1994). The underlying cause of infertility can be diagnosed using a fertility workup including tests to evaluate ovulation, sperm analysis, and tests for tubal patency. The couples with decreased fertility presenting with conditions such as endometriosis, oligozoospermia, or luteal insufficiency can be found (Forti and Krausz., 1998). In some couples visiting a clinician, a clear diagnosis explaining their decreased or absent fertility cannot be found (referred to as unexplained fertility).

More couples are seeking help than previously, management of the patient can only be appropriately provided once the cause of the problem is discovered, which in turn requires a proper history, physical examination and appropriate investigations. Investigations can be expensive and occasionally invasive, but it is essential that the cause is established so that treatment modalities that are available are outlined to couples (Kuroda *et al*., 2018).

In-vitro fertilization is an assisted reproductive technique that is otherwise referred to as IVF. It is a process of obtaining fertilization by extracting oocytes from the ovary and retrieving a sperm sample and physically merging the oocyte and the sperm in a petri dish. Patients who have blocked or damaged fallopian tubes, ovulatory disorders, premature ovarian failure, male factor infertility such as a decreased sperm counts or motility and unexplained infertility undergo IVF treatments to conceive (Bavister, 2002).

The announcement of the birth of Louise Brown in July 1978 was the beginning of an important milestone of *in-vitro* fertilization which is now an internationally recognized treatment option for some infertile couples. There are three major accomplishments that

have been achieved in successful IVF in humans. The first being the development of suitable media for culture of oocytes and sperm, the second to obtain suitable sperm which are acrosome-reacted and the third to obtain oocytes that were good enough to use before ovulation. Although assisted reproduction has come a long way since 1978 there is still much to be achieved. Implantation rates need to be improved, for even though embryo quality is good, still about 75% of embryos transferred into the uterus fail to implant (Zhao *et al*., 2011). We are now able to provide comprehensive tests for sperm function and ovulation. Today Ovum donation and IVF surrogacy gives hope to some couples who have had none. Although these techniques exist it is important to diagnose the full extent of the couple's problem to present professional advice (Brinsden and Rainsbury, 1992).

The hormonal control of ovarian function by the gonadotropins plays a key part in the physiological process of follicular growth (Richards, 2018). Over the last decade, in an article written by Gardner *et al* in 2001, the contribution of follicle stimulating hormone (FSH) and luteinizing hormone (LH) to follicular development has been better defined by clinical data obtained from assisted reproductive technique (ART) cycles performed using gonadotropin-releasing hormone (GnRH) agonist protocols. In all patients, the serum hormone levels of these hormones can be used to evaluate the endocrine environment of the follicles (Gardner *et al*., 2001).

A mature follicle provides the environment which is required in the final stages of maturation in preparation for ovulation. Likewise, the ampulla and fallopian tube during a mid-cycle present both chemical and physical conditions favourable for both sperm and oocytes, but also for a successful union and subsequent embryogenesis. The oocyte is well equipped for this journey. The assisted reproductive procedures need to ensure there is a safe and transitional environment for the oocyte from aspiration to insemination thus ensuring optimum growth and ultimately a viable pregnancy (Gardner *et al*., 2001).

AIMS AND OBJECTIVES

In assisted reproduction serum levels for several hormones are used to assess the ovarian reserve and to monitor the development of the follicles that have been stimulated by gonadotrophins. Traditional techniques used to predict ovarian stimulation have included the serum levels of hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH) and estrogen (E_2) together with ultrasonographic guides such as ovarian volume and the number of early antral follicles (Kunt *et al*., 2011). Recently Anti-Müllerian hormone (AMH) has been projected as a novel marker for predicting ovarian response to gonadotrophin stimulation.

The aim of the study is to determine the relationship between Anti-Müllerian Hormone (AMH) levels and pregnancy outcomes in patients undergoing *in-vitro* fertilization or intra-cytoplasmic sperm injection (ICSI). The ability of the ovary to respond to gonadotropins with adequate follicular development has been referred to as ovarian reserve (Penarrubia *et al*., 2005). Ovarian reserve declines with age; it has a biological as well as a chronological function (Scott and Hofmann, 1995). The diminishing ovarian reserve is noted in a woman during mid-late thirties and sometimes even earlier, reflecting a decline in the follicular pool and oocyte quality. Ovarian Reserve tests can aid in providing an estimation of a woman's remaining follicular pool (Jirge, 2011). During the early stages of development in mammals, foetuses of both sexes have two pairs of ducts; the Wolffian duct and the Mullerian duct. During the 1940s, Alfred Jost, an Endocrinologist and a scientist in the field of foetal endocrinology, presented that a testicular product different from testosterone was accountable for the deterioration of Mullerian ducts in the male foetus. This product was called "hormone inhibitrice." Thus the human gene for Anti-Müllerian Hormone (AMH) was isolated and sequenced (Cate *et al*., 1986).

Anti-Mullerian Hormone is strongly expressed in Sertoli cells from testicular differentiation up to puberty and to a much less degree in granulosa cells from birth up to menopause. AMH seems to only act on reproductive organs (Marca and Volpe., 2006). The AMH value, independent of age, to predict the live birth rate and oocyte quality remains debatable. Some studies show no association, whereas others demonstrate a small but useful association (Leader and Baker, 2014).

The objectives of the present study are as follows:

(1) To determine AMH level and assess whether there is a correlation between AMH level and oocyte reserve.

(2) To examine if AMH levels affect the oocyte quality.

(3) To evaluate the correlation between AMH level and a positive pregnancy outcome.

CHAPTER 2

LITERATURE REVIEW AND STUDY BACKGROUND

Worldwide infertility rates are tough to determine, due to the presence of both male and female factors which obscure the estimate which may only address the woman and an outcome of a pregnancy diagnosis or live birth. A WHO study, published at the end of 2012, has shown that the overall burden of infertility in women from 190 countries has remained similar in estimated levels and trends from 1990 to 2010. WHO evaluated data (2004), estimated that more than 186 million women of reproductive age in developing countries were maintaining a "child wish", translating into one in every four couples (Mascarenhas *et al*., 2012).

Infertility is a common feature in couples visiting fertility clinics. All reproductive centres perform Ovarian Reserve Tests (ORT's) as part of the assessment for women with infertility prior to Assisted Reproductive Techniques (ART). The primary aim is to achieve a live birth. It is important to know the factors that can influence the success of the treatment, not only for the decision of the patient but to determine the treatment protocol to be selected. Bloods tests and physical workups are easy to implement and the decisions based on their results can help distinguish woman with a normal or poor ovarian reserve. This can aid the clinician in counselling the couple about their chances of conception and to prevent expensive and repeated treatment (Broekman *et al*., 2006). Infertility assessment procedures are crucial before the initiation of ART. It is obviously not possible to count the ovary directly to determine the number of oocytes and evaluate the oocyte quality; therefore, indirect investigations can be conducted such as hormonal measurements (Guerif *et al*., 2009).

Appropriate clinical assessment and suitable treatment of woman are essential for a positive outcome in ART cycles. To produce good results it is necessary to measure the ovarian reserve before scheduling treatment. Biomarkers are needed to provide an ovarian frame for the onset and the end of menopause transition, as well as indicate the

proximity to the final menstrual period, and to contribute to the decision making. Studies showing FSH and LH have been used to assess ovarian response to ART (Coccia and Rizello., 2008, Franchin *et al*., 2003 and Van Rooij *et al*., 2002). However, it has been reported that an increase in FSH levels occurs late in the sequence of events associated with ovarian ageing (Klein *at al*., 1996). Recently, several investigators reported the effectiveness of antral follicle count and ovarian volume in predicting ovarian response to hormonal stimulation (Bancsi *et al*., 2002, Bancsi *et al*., 2000 and Chang *et al*., 1998). They stated that AFC provides better prognostic information on the poor ovarian response. Recently, a new endocrine marker anti-müllerian hormone (AMH) was evaluated and is said to be a more sensitive marker of ovarian response (De Vet *et al*., 2002, Patrelli *et al*., 2012 and Broer *et al*., 2014).

To obtain a higher success in ART, it is essential to improve factors such as the oocyte quality and endometrial receptivity. Similarly, the number of oocytes retrieved, oocyte quality (nuclear and cytoplasmic maturity) is also an imperative parameter. Do the women presenting with low AMH levels produce oocytes with poor quality? Several theories have been formulated to explain the decline in oocyte quality with age. In the production line, oocyte quality is established during foetal life, and oocytes that have abnormal chromosomes ovulate first, leaving the poor-quality oocytes to be ovulated later in life (Eichenlaub-Ritter, 1998). Beckers *et al*. (2002) and Tarlatzis *et al*. (2006), believe that an increase in the number of oocytes improves the ability to select the most suitable embryo and that the fewer number of oocytes represent a weak clinical pregnancy outcome and ovarian ageing. In contrast, there is a publication that argues the that a low number of oocytes represent a higher pregnancy rate (Hohmann *et al*., 2006), or that there is no correlation between the number of oocytes collected and the pregnancy outcome (Vail and Gardener., 2003). Despite the advances in technology, assisted reproduction is not only expensive but also tiresome and demanding for patients because of the medical effects of the treatment as well as the emotional stresses. Therefore, the knowledge of the factors that can assist in predicting the success of the treatment is crucial.

Due to advancements in technology, we can now have a better understanding of reproductive ageing. Faddy *et al*., (1992), has shown that recent IVF cycles present with an accelerated decline in infertility and an early onset of menopause is triggered by several ovarian follicles dropping lower than the threshold value (25000 follicles), the rate of follicles decline from -0.097 to -0.237, therefore, suggesting that this decline takes nearly 13 years to result in menopause. A woman who becomes menopausal by the age of 45 years will have begun a decline at age 32 years. A lot of these women are asymptomatic and have a regular menstrual cycle making it hard to diagnose, however, their fertility is reduced compared to that of their peers with normal ovarian ageing and progressively reach lower levels in infertility. In the years to follow they take longer to conceive or present with unexplained infertility. This is known as premature ovarian ageing or primary occult insufficiency, therefore showing a shift in the normal ageing process. It has been estimated that 10% undergo menopause before the age of 46 and 10 out of those women in the overall population might be in jeopardy of early ovarian ageing (Treloar *et al*., 1998).

The European Society of Human Reproduction and Embryology issues the following agreement on the criteria for poor ovarian response. At least two of the three criteria are needed to define poor ovarian reserve (POR). This poor ovarian reserve in the early thirties is synonymous with the early ovarian response. These can be characterised by the following criteria (Ferraretti *et al*., 2011):

1. Advanced maternal age (>40 years)
2. A previous POR (<3 oocytes with a conventional stimulation protocol)
3. An abnormal ovarian reserve test (i.e. AFC or AMH value)

The following risk factors are also likely to determine the risk of early ovarian ageing:

1. Genetic: a family history of premature menopause, mosaics, deletions, inversions and translocations (Cramer *et al*., 1995)
2. Autoimmune factors: thyroid autoimmunity and autoimmune oophoritis (de Bruin *et al*., 2001)

3. Acquired modifiable factors: chemotherapy, radiotherapy, pelvic surgery, pelvic infection or tubal disease and sever endometriosis (Lass *et al*., 1998 and Tulandi *et al*., 2002)

Couples postpone childbearing due to busy schedules and career orientations; therefore, trying to conceive at a more advanced age contributes to a rise in the occurrence of infertility. Most women are unaware that fertility starts to decline after the early thirties in some individuals. Even with ART protocols, some woman with a poor ovarian reserve can still get disheartening results. There is on-going investigation into the likelihoods of altering ovarian reserve, however, it still is difficult to assess and detect ovarian reserve which may enable the clinician to recommend an appropriate form of treatment to couples to achieve a healthy pregnancy or help with preservation in women who are at risk (Broekmans *et al*., 2008).

2.1 HORMONE ASSESSMENT

In Assisted Reproductive Technology (ART), it is necessary that together with the AMH indirect measurements are also conducted. These include Estrogen (E_2), Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). These hormone levels have been used to support the larger role of AMH.

2.1.1 FOLLICLE STIMULATING HORMONE

Follicle Stimulating Hormone (FSH) is an essential part of the reproductive system. FSH is a hormone that is released by the anterior pituitary gland. It helps manage the menstrual cycle and stimulates the ovaries to produce oocytes (Hiller, 2001).

As per the World Health Organization (WHO), ovarian dysfunction is classified based on FSH and E_2 serum level. FSH fluctuates with the menstrual cycle. Therefore, samples are collected on Day 3 of the cycle to measure the basal level. But there has, however, been evidence shown to indicate that measurements may fail to be correct on Day 3 (Perloe *et al*., 2002). As woman and their follicles age, FSH rises in reaction to decreased ovarian responsiveness (Jamil *et al*., 2016).

The accuracy of FSH to predict reduced response to ART stimulation has been influenced by the identification of high serum levels. Ashrafi *et al*. (2005), observed that woman with FSH levels >15 IU/mL had less aspirated oocytes and a larger number of cancelled cycles than a woman with a lower FSH. The same gonadotropin doses where administered. Creus *et al*. (2000), demonstrated mean FSH levels to be significantly higher amongst patients with ART cycles and Al-Azemi *et al*. (2004) showed that FSH could interpret poor oocyte yield. Klinkert *et al.* (2005), suggested that a pregnancy occurrence was less frequent amongst woman with FSH levels >15 IU/L when associated to those with lower levels. It has also been confirmed that pregnancy rates in a woman aged <35 years with an elevated basal FSH were higher than those of older woman with normal levels, therefore, reinforcing age as a main ovarian reserve marker (Luna *et al*., 2007).

Table 1: Normal values for FSH adapted from Daniel (2015).

Category	Value
Before puberty	0 to 4.0 mIU/ml
During puberty	0.3 to 10.0 mIU/ml
Women who are still menstruating	4.7 to 21.5 mIU/ml
After menopause	25.8 to 134.8 mIU/ml

Increased FSH levels are the earliest sign of human reproductive ageing (Reame *et al*., 1998 and Scheffer *et al*., 2003). Inhibin B is presumed to overpower pituitary FSH secretion. By reducing the production of Inhibin B from growing follicles FSH secretion elevates (Klein *et al*., 1996). A reduced outcome in assisted reproduction can be expected in a woman with high basal FSH levels. However, the cut off values differ in different studies, ranging from 8 to 15 IU/mL (Watt *et al*., 2000, Ashrafi *et al*., 2005 and Klinkert *et al*., 2005). FSH levels are raised in patients who are receiving hormonal therapy, are on oral contraceptive pills, have pituitary tumours and in patients with Turners Syndrome (Master-Hunter and Heiman., 2006).

Lower levels of FSH are observed in a woman with Polycystic Ovarian Syndrome (PCOS) and non-functioning pituitary tumours (Jamil *et al*., 2016). FSH level continues to be a thought-provoking assessment on ovarian reserve studies since it is easily available and is a low-cost marker that can be beneficial in pre-treatment evaluation in a woman (Luna

et al (2007), recommended that careful counselling be provided for patients 35 years and older who are more prone to reduced ovarian response to stimulation and higher rates of cancelled ART cycles. High FSH levels should not be used to exclude a woman from proceeding with ART provided they are adequately counselled.

2.1.2 LUTEINIZING HORMONE

Luteinizing hormone (LH) is a glycoprotein secreted by the anterior pituitary gland. In the first one to two weeks of the menstrual cycle, LH is required to stimulate the ovarian follicle to produce Estrogen (E_2). Around day 14, a surge in the LH levels causes the ovarian follicle to release a mature oocyte otherwise known as ovulation. If fertilization occurs LH stimulates the corpus luteum to produce progesterone which is required to support the early stages of pregnancy (Jamil *et al*., 2015).

The secretion of LH from the anterior pituitary is regulated through a system called the hypothalamic-pituitary-gonadal axis. The hypothalamus releases Gonadotropin-releasing hormone which binds to the receptors in the anterior pituitary gland to stimulate both the synthesis and the release of the LH. LH is carried in the blood stream where it binds to the receptors in the ovary to regulate their hormone secretions and the production of oocytes. LH levels are normally low during childhood. LH levels peak even higher around the middle of the menstrual cycle (Jamil *et al*., 2015).

Table 2: Normal values for LH adapted from Daniel (2015).

Category	**Value**(IU/L)
Before menopause:	5 to 25 IU/L
Women after menopause	14.2 to 52.3 IU/L

The physiological mechanism has prompted its use in the assessment of ovarian function. In 1998 basal LH levels of less than 3 mlU/ml were reported to be the earliest evidence of prediction of poor ovarian response (Noci *et al*., 1998). Interestingly, since then, researchers have found a lack of association between LH concentrations and the prediction of ovarian reserve (Chun *et al*., 2014).

Excessive LH can be an indication of infertility. High levels of LH can indicate decreased sex steroid function (i.e., premature ovarian failure). Polycystic Ovarian Syndrome (PCOS) is a common condition due to an imbalance between the LH and FSH resulting in the inappropriate production of testosterone (Dunaif, 1997).

Genetic conditions such as Klinefelter's Syndrome (male only disorder which results from carrying an extra X chromosome; so, men have XXY instead of XY) and Turners Syndromes (female only disorder caused by a partial or full deletion of the X chromosome so woman have XO instead of XX) can also result in high LH levels (Anon, 2018).

2.1.3. ESTROGEN HORMONE

Estrogen (E_2) is the female sex organ produced by the ovaries and is an important hormone during a female's reproductive year. The main function of E_2 is during the menstrual cycle to cause an oocyte to mature and be released, in addition the uterus starts to thicken so that when the oocyte has fertilized the growing embryo can be implanted. E_2 levels fluctuate during the menstrual cycle, the highest is at ovulation and the lowest during menstruation. E_2 levels reduce slowly with age, with a large decrease occurring during menopause. E_2 levels continue to rise in older woman who still have menstrual cycles due to follicular development and the selection of the dominant follicle which is forced upon by the rising FSH levels. Higher rates of cancelled cycles have been demonstrated with E_2 levels <20 pg/mL or >80 pg/mL (Frattarelli *et al*., 2000).

Too much of Estrogen can have several side effects. In mild cases, acne, constipation, loss of libido and depression are observed. More severe side effects include uterine and breast cancer, infertility, weight gain, stroke and heart attack. In the cases of too little Estrogen females can come across difficulties during puberty such as delay in, or failure of, breast development, a disrupted or absent menstrual cycle and infertility.

The ovulatory follicles mainly produce Basal serum E_2 and therefore can only be used to estimate the number of ovulatory follicles. The levels produced are also reliant on the LH levels during the cycle. It is an indirect marker of ovarian reserve as it is anticipated to predict ovarian response in assisted reproduction cycles (Evers *et al*., 1998).

Table 3: Normal values for E_2 adapted from Daniel (2015).

Category	Value
Female (premenopausal)	30 to 400 pg/mL
Female (postmenopausal)	0 to 30 pg/mL

Studies by Smotrich *et al*. (1995), Ficicioglu *et al*. (2006), and Carvalho *et al*., (2009), favour the use of E_2 as an ovarian reserve marker however it has been unable to demonstrate the ability to predict the occurrence of a pregnancy. It has also been demonstrated that similar basal E_2 levels have been recorded in the blood levels of good and poor responders to gonadotrophic stimulus in ART. E_2 may be used to direct the clinician as to whether the stimulation with gonadotrophins should be started, but it does not have value as an IVF prognostic tool (Carvalho *et al*., 2012).

2.1.4 ANTI-MÜLLERIAN HORMONE HORMONE

Diagnostic Systems Laboratory (DSL) and Immunotech (IOT) have simultaneously been developing assays to measure AMH. The AMH concentration produced by IOT was approximately 40% higher when compared to DSL resulting in the trial assays being problematic (Freour *et al*., 2007).

In 2010 both these companies merged to form Beckman Coulter, whereby, a more stable assay was produced. Several studies were conducted by different companies ultimately resulting in the uncertainty concerning the stability of the AMH assay. Zuvela *et al*. (2013), compiled a study using 10 laboratories in which 20 serum samples were tested using the assay, but the results showed a wide range of normal values comparative to the agreed value. They concluded that the differences could have been as a result of different storage and shipping conditions or in the work-up of the assay.

Studies were done by Rustamov *et al.* (2012) and Fleming and Nelson (2012), who also concluded that several factors could have affected the results. Schipper *et al.* (2012), stated that one must be careful in using AMH cut-off levels from studies into their clinical practice since it remained unclear if they would be able to directly translate the AMH

values research projects into daily practice. Since the test has not been a routine test for many years the “normal” levels are yet to be clarified and agreed upon by experts. Table 4 is used as a guideline from fertility literature to interpret AMH levels.

Table 4: Clinical interpretation of AMH levels adapted from Sherban (2016).

Interpretation	AMH Blood Level
High (often PCOS)	Over 3.0 ng/ml
Normal	Over 1.0 ng/ml
Low Normal Range	0.7 – 0.9 ng/ml
Low	0.3 – 0.6 ng/ml
Very Low	Less than 0.3 ng/ml

De Vet *et al*. (2002), concluded that there is a reduction in the number of antral follicles with age, the AMH production appeared to be reduced and became undetectable after menopause. In the absence of AMH, the primordial follicles are recruited at a faster rate thus resulting in an exhausted follicular pool at a younger age (Durlinger *et al*., 1999). Ovarian reserve comprises the size of the stock of the primordial follicles and the quality of the oocytes produced. From the primordial follicle pool, the primary follicles will mature through preantral follicles from which the monthly follicle is selected for ovulation in that cycle.

2.1.5 OVARIAN PHYSIOLOGY OF AMH

Anti-Müllerian Hormone (AMH) is a dimeric glycoprotein produced by the granulosa cells of the pre-antral (primary and secondary) and small antral follicles (AF’s) in the ovary. The production of AMH starts following the follicular transition from the primordial to the primary stage and it continues until the follicle reaches the antral stages, with diameters of 2-8 mm (Weenen *et al*., 2004).

Females are born with a fixed number of primordial follicles, resting in a dormant stage of meiosis ii until puberty. The quality as well as the quantity of the primordial follicle constitutes the ovarian reserve. Due to the size and the placement of these follicles, it is

difficult to measure the pool of follicles as these dormant primordial follicles do not secrete AMH. When these follicles are recruited for development it has been reported that AMH is secreted (Broer *et al*., 2010).

AMH is shown by the small and large pre-antral follicles (broken arrows) and small antral follicles (solid arrow) which contribute to the serum level (Figure 1). Early recruitment takes place as a continuous process, whereas recurring recruitment is driven by a rise in FSH serum levels at the end of the previous menstrual cycle.

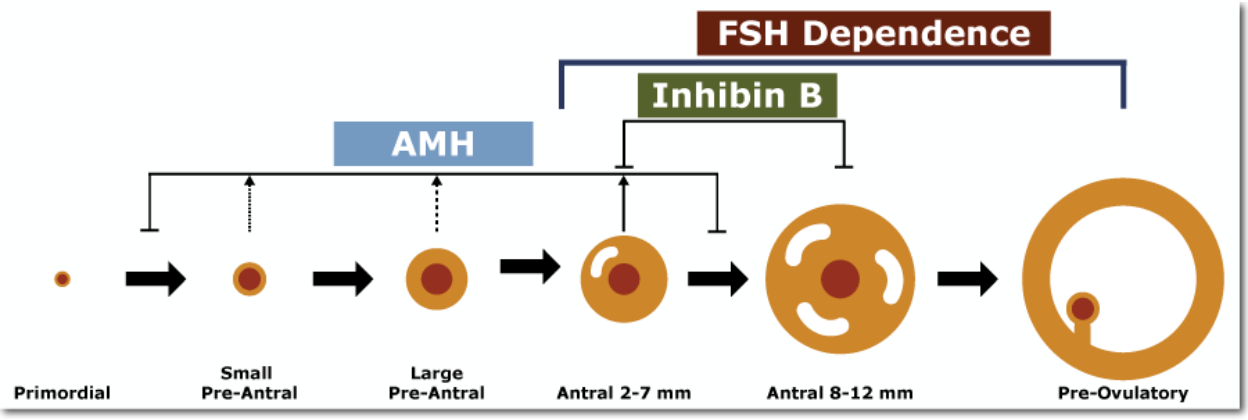

Figure 1: Schematic representation showing AMH secretion by pre-antral and antral follicles adapted from Reproductive Medicine Associates of New Jersey.

AMH levels can be almost undetectable at birth, with a subtle increase within the first 2-4 years of age, and then AMH seems to be stable until adulthood. Serum AMH levels have been measured at different times in the menstrual cycle, with extremely subtle or non-existent fluctuation. Slight variations in serum AMH levels may be consistent with the continuous non-cyclic growth of small follicles. Hence AMH is relatively useful to regulate especially as it seems to be stable throughout the cycle making it a contributing factor of ovarian activity (Grujters *et al*., 2003 and Marca *et al*., 2004).

Although AMH is stable throughout the cycle it does not mean AMH production is fully gonadotrophic independent. The production of AMH by the small antral follicle indicates that AMH production is likely to be FSH regulated, as these follicles are sensitive if not

reliant on FSH. Currently, it is unclear which follicle size within the 2-10mm range contribute to the AMH concentration. It has, however, been shown that continued gonadotrophin suppression by GnRH analogue administered bring about a progressive decline in AMH (Anderson *et al*., 2006). This, however, does take many months to develop with a considerably slower decline in E_2. The prolonged use of the pill may motivate the observation that there is little effect on AMH secretion, although there has been data that suggests a degree of suppression (Van den Berg *et al*., 2010). Data suggests that there is suppression of AMH during pregnancy (Nelson *et al*., 2010).

2.2 CLINICAL MARKERS

2.2.1 MENSTRUAL CYCLE

The length of the menstrual cycle is determined by the rate and quality of the follicular growth, consequently the duration of the follicular phase in overall menstrual cycles range from 23 to 32 days and has a mean interval of 28 days (Cole *et al*., 2009).

According to a study conducted by Brodin *et al*. (2008), conception rate was observed to be significantly reduced amongst woman with a menstrual cycle of shorter than 30 days. Also, the menstrual cycle had a significant association with ovarian response to gonadotrophin stimulus and embryo quality in IVF/ICSI cycles and even if the interference of age is excluded. They observed that among women with cycles >34 days pregnancy rates are almost twice as high when compared with those with cycles <26 days.

The menstrual cycle begins on the first day of menstruation when the blood starts to flow. The menstrual cycle is assumed to be 28 days in an average woman and is split up into four main phases (Figure 2).

Menstrual phase:

This is the first day of menstruation and lasts up to 5 days of the menstrual cycle. During this stage the uterus sheds its inner lining of soft tissue and blood vessels. Normal blood loss can be between 10 ml to 80 ml. during this time abdominal cramps are experienced

due to the contractions of the uterine and abdominal muscles to expel the menstrual fluid (Reed and Carr., 2015).

Follicular phase:

Follicular phase starts on the first day of menstruation and lasts for at least 13 days. During this time the pituitary gland stimulated the oocytes to grow and only 1 oocyte will mature. While the oocyte matures, the follicle secretes a hormone (progesterone) to stimulate the uterus to develop a lining of blood vessels and soft tissue called the endometrium (Reed and Carr., 2015).

Ovulation phase:

On the 14^{th} of the cycle, the pituitary gland secretes a hormone (luteinizing hormone) that causes the ovary to release the mature oocyte. The mature oocyte is swept into the fallopian tube by the wave like action of the cilia of the mucosal epithelium aided by contractions of smooth muscle cells. (Reed and Carr., 2015).

Luteal phase:

Luteal phase starts on the 15th day and lasts until the end of the cycle. The oocyte is released during the ovulation phase and stays in the fallopian tube for 24 hours if no fertilization occurs the oocyte disintegrates to form the corpus luteum. The hormone used to maintain the endometrium decrease thus the lining will start to fall away and the menstrual phase starts again (Reed and Carr., 2015).

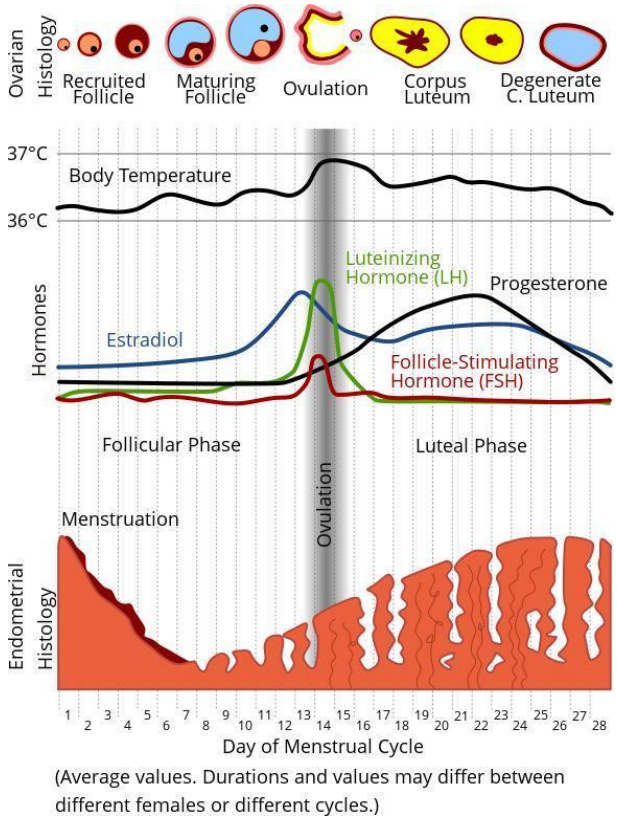

Figure 2: The menstrual cycle and hormones (Reed and Carr., 2015).

2.2.2 AGE

One of the utmost important factors in determining quality and quantity of ovarian reserve is age (Figure 3). The quality and quantity of ovarian follicles significantly decrease as a woman advances in age. Fecundability deteriorates significantly since the early 30s (Faddy *et al*., 1992) and the prevalence of infertility increase significantly after the age of 35 years. Ninety-nine percent of patients are expected to be infertile by the age of 45 (Menken *et al*., 1986).

Numerous factors attribute to fertility decline associated with advanced age, including changes in the oocyte quality, frequency and efficiency of ovulation, sexual function, uterine disease, and the threat of pregnancy complications such as diabetes and hypertension. Also, environmental and genetic factors such as smoking, infections and adnexal surgery can diminish ovarian reserve in an older woman (Rowe., 2006).

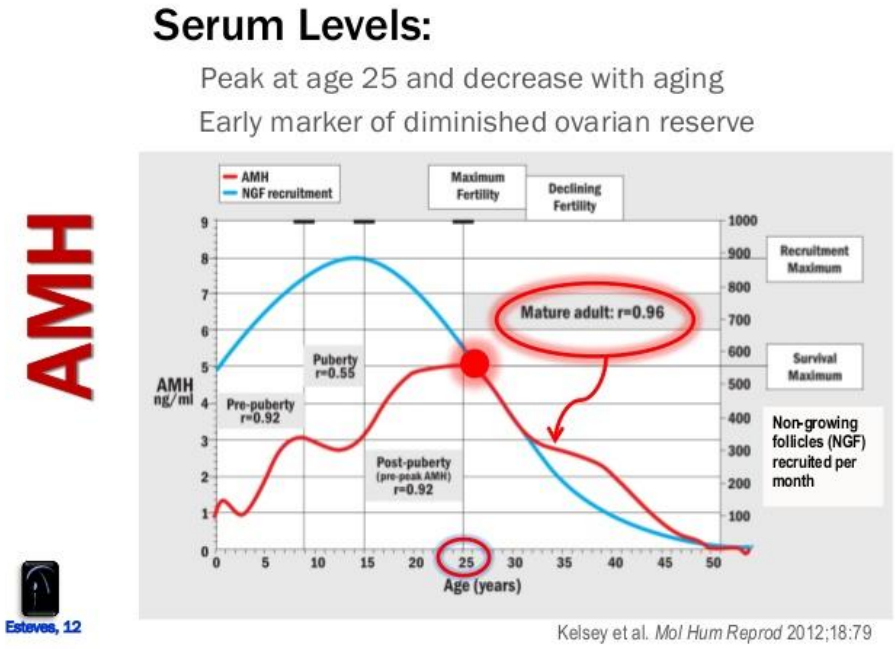

Figure 3: Comparison of serum AMH concentrations (Kelsey *et al*., 2012).

In the past decade, numerous indicators have been suggested to evaluate the ovarian reserve, the increase in FSH and decrease in antral follicle count seem to be the most consistent markers (La Marca and Volpe, 2006). However, in a study conducted in 2002 by de Vet and his colleagues, serum levels on day 3 of the menstrual cycle presented a progressive decline with age, which correlated with the AFC. Serum AMH levels have been shown to decrease over time in a young normal ovulating woman. The group of women were studied twice and the interval between the two visits ranged from 1.1 years to 7.3 years and a reduction of 38% where observed, although the quantity of antral follicles and levels of FSH did not change (de Vet *et al*., 2002).

In 2005 another study looked at 81 women who were studied over a 4-year period (mean age 39.6 years and 43.6 years); it was found that the AFC did not vary over time whereas the AMH and FSH altered significantly AMH was the only marker of ovarian reserve presenting a deterioration over time both in woman younger than 35 years and in a woman older than 40 years (Van Rooij *et al*., 2005). With respect to other acknowledged indicators, AMH appeared to better imitate the continuous deterioration of the oocyte/follicle pool with age. The decline in AMH with advancing age indicates that serum

AMH levels may be the best marker for ovarian ageing and menopausal transition (Van Rooij *et al*., 2002).

2.3 ULTRASONOGRAPHIC MARKERS

2.3.1 THE OVARY

The ovary (Figure 4) is a ductless reproductive gland in which the female's reproductive cells are produced. Females have a pair of ovaries, held by a membrane beside the uterus on each side of the lower abdomen. The ovaries are small, oval-shaped and grey in colour, with an uneven surface. The actual size of the ovary is dependent on the age and hormonal status of the woman. The ovaries are covered by a peritoneum and are roughly 3-5 cm in length during child bearing age and become much smaller and atrophic during menopause. A cross section of the ovary reveals many cystic structures that vary in size; these represent the ovarian follicles at different stages of development and degeneration. The ovary is divided into two sections: the outer cortex and inner medulla. The cortex is where the follicles and oocytes are found at various stages of development. The stroma of the cortical connective tissue is composed of spindle-shaped fibroblasts that respond to hormonal stimulation. The medulla is where the ovarian vasculature is found. The ovarian follicles are found in the stroma of the cortex. A follicle consists of an oocyte surrounded by follicular cells called granulosa cells. Approximately 20 follicles begin maturation every month with the goal of a mature oocyte being released for fertilization and reproduction. If no fertilization occurs the oocyte goes through degeneration (Sargis, 2015).

The ovaries are responsible for the production of Estrogen and Progesterone. There are three types of estrogen known as estradiol, estrone and estriol and all these elements work simultaneously to promote the healthy development of female sex characteristics for the duration of puberty and to ensure fertility. Estradiol specifically is contributory in breast development, fat distribution in the hips, leg and breast and development of the reproductive organs. Estrone is the least abundant of the three and Estriol is produced during pregnancy by the placenta (Sargis R, 2015).

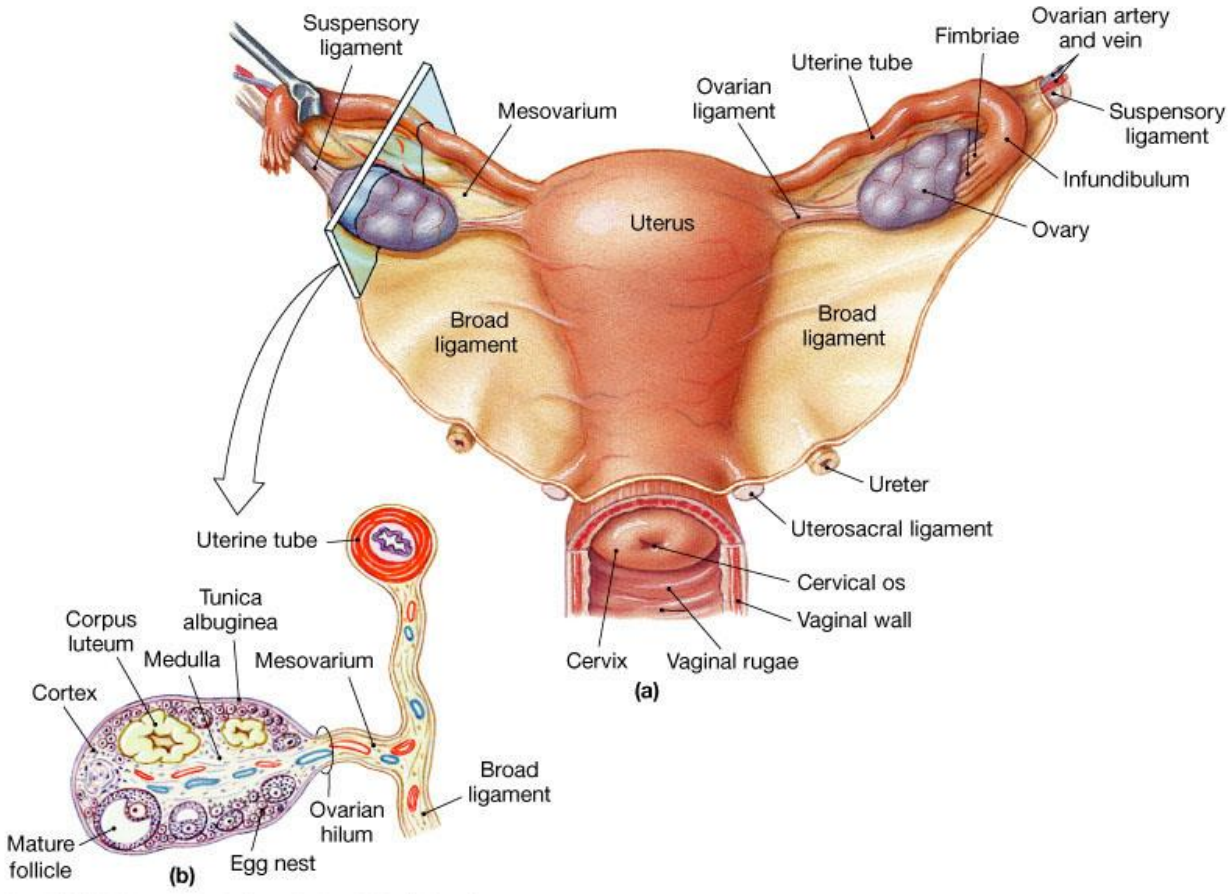

Figure 4: Anatomy of the ovary (Martini 2004).

2.3.2 ANTRAL FOLLICLE COUNT (AFC)

Antral follicles are small follicles (2-9mm in diameter) that are visible within the ovarian cortex. Antral follicles are also referred to as “resting follicles”. The antral follicle count has been shown to be an excellent predictor of ovarian reserve and ovarian response; it demonstrates a significant correlation between the AFC and the hormone assessment. AFC is measured by transvaginal ultrasonography in the early follicular phase. The numbers of follicles in both the ovaries are added for the total AFC. AFC has long been used as a predictor of ovarian response (Anon., 2016).

The number of antral follicles presented on ultrasound is suggestive of the quantity of microscopic primordial follicles remaining in the ovary. Each primordial follicle contains

an immature oocyte that can possibly develop and ovulate sooner or later. Only a few antral follicles are visible as women age, they have fewer oocytes (primordial follicles) remaining and they have fewer antral follicles. Antral follicle counts are a good predictor of mature follicles that can be stimulated in the ovary when stimulation medication is administered. Counting antral follicles using an ultrasound can be a subjective process. An ideal antral follicle count depends on the age of the woman; older women are not expected to have the same antral follicle count as women who are 30. A count around 20 indicates high ovarian reserve and a strong likelihood of success, and counts higher than 30 could indicate PCOS, which won't necessarily interfere with a healthy and successful pregnancy (Table 5). Age is the best prognosticator of the quality of the oocytes, so the antral follicle count must be taken into deliberation with the age of the woman as both are significant to foresee the chance of pregnancy (Anon., 2016).

Table 5: Correlation of antral follicle counts (Anon., 2016).

Antral Follicle Count	Interpretation	Expected Response to FSH	Anticipated Cancellation with IVF	Anticipated Pregnancy Rate with IVF
<4	Very low	Very poor	Very high	Very low
4-6	Low	Poor	High	Low
7-10	Reduced	Reduced	Increased	Decreased
11-30	Normal	Good	Low	Excellent
>30	Above Normal(PCOS)	Increased risk of hyperstimulation	Low	Good

2.4 AMH AS A MARKER FOR OVARIAN RESERVE

Ovarian reserve is defined as the existent quantitative and qualitative supply of follicles which are found in the ovaries that can potentially develop into mature follicles which in effect determines a woman's reproductive potential (Deb *et al*., 2008). Ovarian reserve is also used as a term to determine the capability of the ovary to provide oocytes that are capable of fertilization ensuing in a healthy positive pregnancy. Since true ovarian reserve cannot be determined directly, an ovarian biopsy would need to be done in order to establish the number of primordial follicles. The ovarian response is dependent on ovarian reserve which can be predicted with the use of markers (Broekmans *et al*., 2006).

The role of AMH as a prognosticator of the ovarian reserve has been deliberated in a limited number of papers. Long term studies have been conducted where several factors were assessed. Two studies were conducted over a period of 9 to 12 years whereby, it was concluded that woman with low age-specific AMH will have menopause earlier and *vice versa* (Tehrani *et al*., 2009; Broer *et al*., 2011). A third study by Freeman *et al*. (2007), confirmed these findings in a group of women of late reproductive age.

De Vet *et al.* (2002) and Van Rooij *et al*. (2002), confirmed that AMH is an excellent marker for ovarian ageing. The quantity of follicles in the ovary of a woman at the completion of her reproductive period is the most important determining factor of the timing of both her period prior to menopause and menopause itself. The AMH level of a woman with a regular cycle decreases over time and there is a strong association between AMH and the quantity of antral follicles. It gives the impression that the size of the recruited follicles is associated with the primordial follicle pool. Post-menopausal woman are infertile due to the exhaustion of the primordial follicle pool, whereas woman in years before menopause, who still comprise of follicles in their ovaries and have reduced fertility of which the cause is unknown (Richardson and Nelson., 1990; Te Velde and Pearson., 2002).

Dewally *et al*. (2014), have stated that genetic factors play a major role in defining when menopause can occur. Next, to genetic factors several environmental and lifestyle factors such as smoking, BMI, use of alcohol, have been claimed to impact menopause.

The biological activity of AMH in a woman is not entirely understood, but data advocate that it may act as a modulator of follicle recruitment and a regulator of ovarian steroidogenesis. AMH is identified to have an inhibitory effect on the pool of primordial follicles, acting on the pregranulosa cells in order to limit the number of viable follicles and later, as a pivotal feature in allowing the FSH dependent growth of ovarian follicles. AMH is considered to be an indicator that can evaluate the quantity and activity of retrievable follicles in early stages of maturation, thus being more dependable for the expectation of ovarian response and reproductive potential (Rooij *et al*., 2002, Franchin *et al*., 2003, Visser *et al*., 2005, and Muttukrishna *et al*., 2005). Other studies support this affirmation by demonstrating a decreased rate in AMH levels with age (de Vet *et al*., 2002, Van Disseldorp *et al*., 2008 and Nelson *et al*., 2010). It was mentioned in an article written by Disseldorp *et al*. (2010), that during the menstrual cycle it is observed that AMH demonstrates mild fluctuation whereas in more recent articles by Randolph *et al*. (2014) and Tal and Seifer (2013), it has been reported that fluctuations are observed at random times and can be measured throughout the cycle. Mild fluctuations were enlightened by the fact that dormant follicles lack AMH production resulting in a slight decline during the late follicular stage.

Van Rooij *et al*. (2002), state that the AFC gives the best prognostic information about poor responders in IVF. Moreover, the combination of the AFC and FSH tests appeared to improve the response prediction. AMH was found to have predictive performance comparable to that of AFC. The investigation on the prediction of reduced ovarian response revealed that AMH can contribute independently in the prediction but only if the AFC is removed from the analysis. This study confirmed the findings of a study that was conducted by Seifer *et al.* (2002), where lower serum AMH were found in patients having six or fewer oocytes retrieved compared with patients having eleven or more oocytes.

There are advantages of the use of AMH over AFC in the prediction of ovarian response since all the information is obtained with blood sampling. Since there is no change to AMH levels in response to gonadotropins, AMH can be measured throughout the cycle in contrast to the other parameters, which can only be determined during the early follicular phase. In view of all the above studies that have been discussed the on-going pregnancy

appears to be limited since they only represent a quantitative aspect of ovarian reserve, whereas pregnancy is also dependent on oocyte quality. Never the less the ability to predict a poor response can still be a valuable tool for patient counselling.

Recently, AMH has been proposed as an ideal test for ovarian testing (Table 6) due to the following characteristics:

1. Decreases continually during the fertile life
2. Not influenced by gonadotropin feedback mechanism
3. AMH levels shows very low inter and intra-cycle variability thus can be measured independent of the day of cycle
4. Best correlating marker with AFC

Table 6: Comparison of characteristic of the most widely used markers of ovarian reserve adapted from Dr Lal Pathlabs (2012)

Characteristics for a good marker	Age	AMH	FSH	AFC
Prediction of poor response	+	+++	++	+++
Prediction of hyper response	+	+++	-	++
Low inter-cycle variability	+++	++	-	++
Low intra-cycle variability	+++	++	-	++
Blinded to the operator	+++	+++	+++	-
Application to all patients (a)	+++	+++	+	+
Cheapness	+++	-	-	-

(a) FSH and AFC are not informative in patients on hormonal contraception of GnRH agonist treatment. AFC counts maybe difficult in woman with ovarian cysts of previous pelvic surgery.

2.5 ANTI-MÜLLERIAN HORMONE AS A MARKER FOR PATIENTS UNDERGOING ASSISTED REPRODUCTIVE TECHNOLOGY (ART)

It is important to be able to evaluate the outcomes following assisted reproduction treatment. The ovarian response in ART includes outcomes such as the number of stimulated follicles following controlled ovarian stimulation, the number of oocytes retrieved, the number of mature oocytes, fertilization rate and the development of embryos. The ability to predict a poor response has resulted in some clinics withholding a cycle if the AMH value is low.

A study conducted by Lekamge *et al*. (2006), provided evidence that patients presenting with a high AMH value had a higher reproductive potential as opposed to the patients presenting with a low AMH. The high AMH patients produced more oocytes, had a higher fertilization rate, generated more embryos, and had a lower incidence of miscarriages from a fresh transfer. Patients with a low AMH produced a fewer number of oocytes and presented with a diminished oocyte quality as evaluated by the fertilization rates. The study further went on to prove that patients with a low AMH produced poor fertilization rates due to oocyte cytoplasmic deficiencies such a mitochondrial dysfunction and poor cytoplasmic competence. When oocytes from a low AMH group where subjected to IVF (i.e. good quality sperm) the fertilization rates were inferior to those obtained from patients presenting with a higher AMH group. Interestingly when oocytes from the low AMH group where subjected to ICSI, thus cancelling out any male contributions and cumulus barrier to prevent fertilization failure, the fertilization rates were still inferior to the high AMH group. This led them to suggest that there could be intrinsic deficiencies in the cytoplasm of the oocyte that could be responsible for the low fertilization rates (Lekamge *et al*., 2006).

Studies conducted by Seifer *et al*. (2002) and Van Rooij *et al*. (2002), demonstrated that AMH is an excellent marker to determine ovarian responsiveness. Their studies revealed that patients with a low AMH level presented with reduced ovarian response than in a woman with a normal ovarian response. The AMH levels were shown to be highly correlated with the number of antral follicles before treatment and the number of oocytes

retrieved via stimulation. The AMH levels showed a higher predictive value when compared to serum levels of E_2, FSH and inhibin B.

To study the value of AMH as the predictor of treatment outcomes Smeenk *et al*. (2007), reviewed the number of follicles, the number of embryos, embryo characteristics and the probability of an on-going pregnancy in 112 participants undergoing IVF/ICSI treatment. The results showed the AMH levels where found to be varying in association with an ovarian response but not with embryo quality or the ability to achieve a pregnancy. They concluded that although the AMH level correlated with ovarian reserve the predictive capacity is too limited for clinical value. The number of oocytes retrieved, and the number of oocytes could be predicted but not the chance of a pregnancy.

In a group of woman undertaking IVF treatment, the use of AMH serum levels as a measure of the ovarian reserve was tested. Hormonal parameters and AFC were obtained under ultrasonography on the 3rd day of the menstrual cycle in 119 patients, three months before commencing on IVF treatment. The parameters measured were studied after separating the patients into two groups based on the number of oocytes retrieved after IVF treatment: normal responders (four or more oocytes retrieved) and poor responders (fewer oocytes and cancellations). Ovaries of normal responding women contained significant more growing antral follicles than ovaries of woman with a poor response to the management. AMH serum levels were lower in low responders than in normal responding woman. Serum AMH levels correlated with the AFC, number of follicles retrieved, age and FSH. Logistic regression analysis in predictive models of poor and normal response showed that both AFC and AMH levels were equally important for prediction (van Rooij *et al*., 2002).

In a separate study, serum AMH levels on day 3 may likewise be of particular significance in predicting clinical pregnancy outcome in an IVF cycle. AMH levels were stated to have superior predictive value than age, FSH and E_2 (Wu *et al*., 2009). 109 women undergoing IVF underwent a retrospective study, basal AMH levels <1.1ng/ml were associated with IVF failure. Regression analysis indicated that AMH explained 26% of the variance for success or failure whereas FSH and age were 7% and 6%, respectively (Hazout *et al*., 2004). This study was confirmed by a larger prospective study conducted on 238 women.

The cut off value of 1.13 ng/ml was used. AMH assessment was revealed to predict ovarian reserve with a sensitivity of 80% and specificity of 85% (Tremellen *et al*., 2005).

2.6 OOCYTE QUALITY

2.6.1 THE OOCYTE

The oocyte is the female gamete which plays a vital role in determining embryo competence and therefore IVF outcomes. Oocyte quality is influenced by the nuclear and mitochondrial genome, but also by the microenvironment provided by the ovary and the pre-ovulatory follicle (Rienz *et al*., 2012). In 1992, Van Blerkom and Henry speculated that some morphological irregularities, which can effortlessly be evaluated at the light microscope level, may mirror a compromised developmental ability of the oocytes and could, therefore, represent a beneficial tool for choosing competent oocytes prior to fertilization.

For mature oocytes, the cumulus corona mass should appear expanded, following the removal of the cumulus corona cells in preparation for the ICSI, oocyte evaluation is more precise and is based on nuclear maturation status, the morphology of the ooplasm and on the appearance of the extra cytoplasmic structures. The presence of the first polar body is normally considered to be an indicator of oocyte nuclear maturity. However, studies have revealed that oocytes showing a polar body can still be immature (Reinzi *et al*., 2005). Only those displaying a meiotic spindle can, in fact, be considered as mature. An ideal mature human oocyte (Figure 6), based on morphological characteristics, should have a ‘normal looking’ cytoplasm, a single polar body, an appropriate zona pellucida (ZP) thickness and proper perivitelline space (PVS).

Figure 5: A mature oocyte, with a polar body in the 12 o' clock position (C.A.RE. Clinic, 2016)

2.6.2 ANTI-MÜLLERIAN HORMONE AND OOCYTE QUALITY

To obtain a higher success rate in IVF, various factors including oocyte quality is essential. Traditionally embryo selection is performed by using embryo morphology as a guideline. Further techniques include oocyte and zygote morphology, blastomere symmetry and blastocyst culture. Overall good quality, cleaving embryos show specific cell division; have blastomeres of equal size with few to no fragments (Ciray *et al*., 2006).

Only a small percentage of oocytes collected lead to the birth of a child. Ebner *et al*. (2006), was the first author to indicate the association between the actual AMH level and the quality of the oocytes. Roughly half of the oocytes derived from patients with a normal AMH where found to be unaffected compared with one third in the low and high AMH groups. Because AMH is produced by the granulosa cells at early follicular stages, decreased levels of the hormone may be linked with a failure in granulosa cell expression, which could permanently damage the oocyte. This was consistent with the discovery that the principal negative feature in the low AMH group is the dark central granulation of the cytoplasm, a phenomenon that is thought to arise in early oocyte maturation as found by Blerkom and Henry in 1992.

With advanced age the primordial follicle pool reduces, resulting in a reduction in oocyte quality. The number of chromosomal abnormalities increases in oocytes obtained after ovarian stimulation in a woman older than 37 years. This suggests that with increasing age there is a decrease in oocyte quantity as well as quality (Pellestor *et al*., 2003). In 2000, Huisman *et al.* suggested that the embryos are carefully chosen based on individual standards, according to an extensively used morphology score. While embryos rated as having a higher quality due to its morphological assessment are associated with higher implantation and pregnancy rates, successful implantation and on-going pregnancy cannot be forecasted with conviction. To obtain a higher success rate in IVF various factors including oocyte quality is essential.

2.7 ANTI-MÜLLERIAN HORMONE AND PREGNANCY OUTCOMES

In a study conducted in 2008 by Takashi *et al*., it was observed that oocytes were more likely to be fertilized when the follicle they were in was able to produce more AMH, as follicular fluid AMH levels with follicles with fertilized oocytes were more than three times higher than from follicles with no fertilization. Morphology of the first polar body can be a steadfast indicator of oocyte age and the presence of a well-shaped, non-fragmented polar body was associated with increased pregnancy rates (Seifer *et al*., 2011). According to Hazout *et al*. (2004), the serum AMH levels showed a confident connection with the number of metaphase ii oocytes during an IVF cycle. Furthermore, the number of oocytes obtained also has a significant positive correlation with serum AMH levels.

Three different articles used the patients mean AMH values measured to predict pregnancy outcomes. The AMH measurement was performed on the day of ovulation induction, a time when AMH values usually decline because of the presence of growing follicles and may thus fail to reflect the actual competence of the oocyte (Franchin *et al*., 2003, Elda-Geva *et al*., 2005 and Seifer *et al*., 2011).

In 2008, Wunder *et al* performed a study in 276 women and have shown that the concentration of AMH in both serum and follicular fluid were significantly higher in the group of women who became pregnant in the treatment cycle as opposed to the women who did not conceive. Whereas in a study by Irez *et al*. (2011), who tried to evaluate the

association between different basal levels of Anti-Müllerian Hormone and oocyte quality and IVF outcomes failed to draw a decision on the on-going pregnancy rate as the number of patients in each group was unequal and it was difficult to find patients with AMH levels of >10 and <1 ng/mL.

It is a renowned fact that fertility is diminished with increasing age; according to some statistics the atresia of the follicle pool is enhanced in woman over 37-38 years of age. As per IVF results published by Human Fertilization and Embryology Authority (HFEA), the life birth rate for the age range 40 – 42 years is 12.7%, 43 – 44 years is 5.1% and 1.5% in woman 45 years and older (Human Fertilization and Embryology Authority., 2010). The success of IVF procedures depends on many factors which are independent such as age and serum AMH concentration (Broer *et al*., 2009, Lukaszul *et al*., 2015 and Heidar *et al*., 2015). AMH can strongly predict a poor response to controlled ovarian stimulation. Despite results presented, there is still data, that shows that a woman over the age of 40 with very low AMH still have a chance of pregnancy (Iliodromiti *et al*., 2014).

Muttukrishna *et al*. (2004), in their prospective study, have investigated whether FSH, AMH and inhibin B could be valuable in foreseeing the ovarian response to gonadotrophin stimulation in patients who have reduced response. 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. 17 of these patients had to abandon the cycle due to 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 AMH, FSH and Inhibin B is modestly better than AMH on its own.

Various studies indicate similar or different results which could be explained by different assays being applied or even different kits, based on data available the pregnancy

chances for a woman over the age of 42 are very low but still the available methods fail to predict who will become pregnant. Now there is a strong inclination to delay parenthood; therefore, oocyte factors (decrease in oocyte quantity and quality) are the leading reasons for reduction of fertility in woman of late reproductive age. AMH seems to be the superior endocrine marker for evaluating the deterioration of the ovarian pool in healthy woman (Meczekalki *et al*., 2016).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 BACKGROUND

For the purpose of this study 50 patients (n=50) were randomly sampled from the C.A.R.E Clinic in Westville, Durban Kwa-Zulu Natal. Permission for accessing the database and patient recruitment where granted by the practitioner of the C.A.R.E Clinic (Appendix A).

3.1.1 Inclusion Criteria

- Female patients ranging between the ages of 20–45
- Woman of good physical health
- The presence of both ovaries
- No evidence of Endocrinological disorders
- All patients used for the study were on the IVF/ICSI program from December 2016 to September 2017
- Patients need to understand and speak English

3.1.2 Exclusion Criteria

- Patients on cancer therapy
- Patients on immune suppressant drugs

3.1.3 Informed Consent

Patients attending the C.A.R.E Clinic were approached by the investigator during their consult at the C.A.R.E Clinic. Patients who met the inclusion criteria where given an information leaflet (Appendix B) and an informed consent (Appendix C). Patients were informed of the nature of the study and were made aware that study participation was voluntary, and participation can be withdrawn. Patients who were willing to participate in the study signed the consent in the presence of the investigator.

This study was a prospective, observational investigation. Ethical approval was obtained from the DUT ethics committee (Appendix F).

3.2 CLINICAL APPROACH

3.2.1 Stimulation Protocols

Two stimulation protocols were used to harvest a maximum number of oocytes.

Protocol 1 - Long protocol:

Gonadotrophin Releasing Hormone (GnRH) Agonist - downregulation

This part of the treatment where the clinician “turns off” the ovaries temporarily, by doing so the clinician can better control ovulation and oocyte maturation during treatment. Drugs used to down-regulate the ovaries are GnRH antagonist and GnRH agonists. These drugs work in different ways but both suppress the body’s production of FSH and LH. GnRH agonist mimic the body’s natural GnRH. During the IVF program, the GnRH is started 21 days before the IVF cycle starts. At first, the body responds by producing high levels of FSH and LH. This overwhelms the cells in the ovaries and pituitary gland. In response, the receptor sites on the cells decrease and become desensitized. The body stops producing FSH and LH (Gardener *et al*., 2001).

Drugs given during downregulation are:

1. Lucrin Subcut 10 units daily;

2. Lucrin Depo 3.75mg

Stimulation Phase is an important phase in the IVF cycle during which patients receive a daily injection of gonadotrophins which stimulate the ovaries to produce multiple oocytes. Stimulation can vary from 8-12 days. At each visit, blood was drawn to note changes in the hormone levels and a vaginal ultrasound was performed to note the progression of the ovarian follicles (Gardener *et al*., 2001).

Drugs given during stimulation are:

1.Gonal F®;2. Menopur®;3. Puregon®;4. Clomid®;5. Fertomid®;6. Fostimon®

Protocol 2 – Short Protocol:

GnRH antagonist with Cetrotide 0.25mg primed with Logynon ED

Cetrotide 0.25mg is administered while GnRH agonist acts like the body's natural GnRH leading to the increase in hormones. The GnRH antagonists work against the GnRH in the body. The body becomes "deaf" to the GnRH hormones and it stops the production of FSH and LH. With GnRH antagonists down regulation occurs almost immediately. The stimulation is the same as per the long protocol (Gardener *et al*., 2001).

Once the follicles (at least 3 or more) reach a diameter equal or above 17 – 18mm, the endometrial thickness reached at least 7mm by ultrasound and E_2 levels were about 1500-1800 pmol/L then Human Chorionic Gonadotropin (hCG) was administered. All patients received 5000 – 10 000 IU hCG (Pregnyl®, Ovitrelle®). Oocyte retrieval was performed 36 hours after the administration of the hCG.

3.2.2 Blood Sample Collection

A registered nurse obtained blood samples from all patients in sterile and ambient conditions. Blood samples were obtained every 3-4 days on commencement of the program. The blood was thereafter centrifuged at 3000rpm for 10 minutes using a Biofuge centrifuge (Biofuge Primo – Heraeus) to obtain the blood serum. The volume of blood serum used to analyse all hormone levels was 1.0ml. AMH and FSH levels were recorded once, upon the first visit. E_2 and LH levels were monitored throughout the program until a peak E_2 and LH level were reached. It was at this peak level of E_2 and LH that determined when the trigger was administered. Ultra-sensitive enzyme-linked immunosorbent assays (ELISA) (Beckman Coulter) was used to analyse hormone levels from the blood serum (Gardener *et al*., 2001).

3.2.3 ULTRASOUND AND HORMONE MONITORING

Serum hormone was used to determine the E_2 and LH levels. Dosage and type of gonadotropin were administered according to the levels on day 2 in order to achieve an adequate response. If E_2 levels were low then the gonadotropin was increased. E_2 levels correlate with the number of mature follicles. Ultrasound was used to determine the number of antral follicles which then determines the dosage of gonadotropins administered. Transvaginal ultrasound scanning (Nemio Ultrasound Scanner) was used to view the ovaries, indicating the size and number (Figure 7) of developing follicles (Gardener *et al*., 2001).

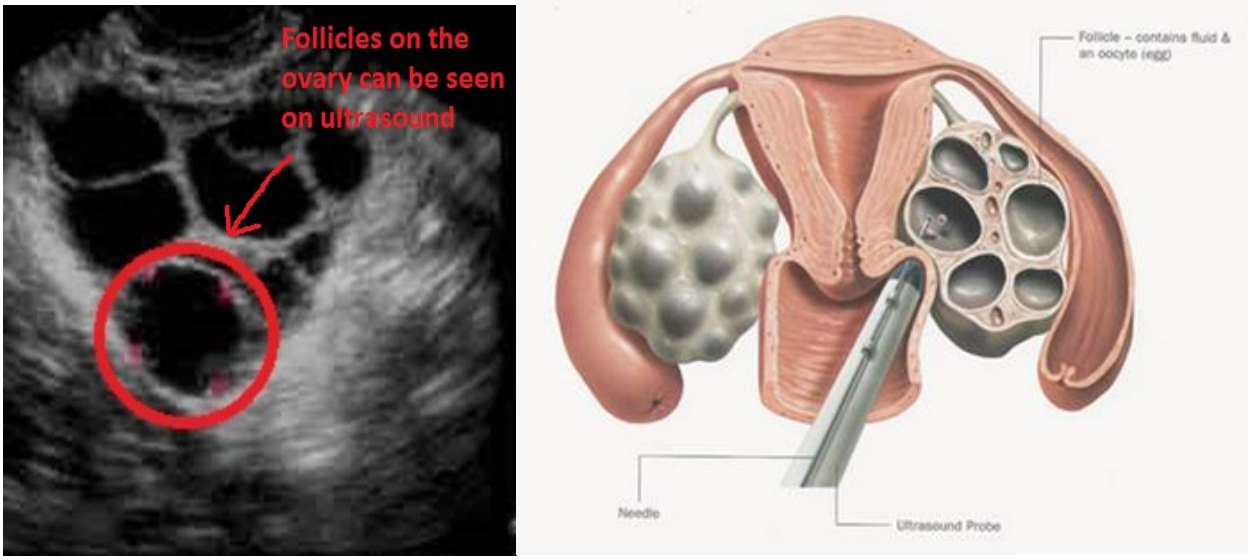

Figure 6: Mature follicles as seen on a transvaginal ultrasound (Thamari Fertility centre., 2015)

3.2.4 MEDIA PREPERATION AND OOCYTE RETRIEVAL

A day before oocyte collection the embryologist starts by preparing media. The media used for oocyte retrieval must meet minimal nutritional requirements in terms of carbohydrates, amino acids, vitamins and proteins; was compatible with the culture systems selected for insemination and embryo growth; and maintained the appropriate pH stability, buffer action and osmotic needs of the oocyte (Gardener *et al*., 2001).

Global Total Media was set at 37.0ºC as the oocytes are temperature sensitive in a humidified incubator (Thermo Incubator 150i) with 6% CO^2. The pH was in the range of 7.2-7.4. The osmolality was between 275-290 mosmol/kg. Three centre well dishes (BD Falcon) with 1.0ml inside and 2.0ml in the outer well, 1 x petri dish containing 5ml of Lite oil for the ICSI and 2 x flush media were placed in the incubator at the above-mentioned conditions overnight (Gardener *et al*., 2001). Flush is a medium that contains Heparin that is used during retrieval to hold and wash oocytes of follicular fluid.

The oocytes were retrieved through a surgical procedure transvaginally by ultrasound guidance. Ultrasound-guided retrieval is the gold standard for almost all the procedures. The female patients were placed in lithotomy position. The process took about 30 minutes and was done under conscious sedation or general anaesthesia. The primary goal was to provide safe and effective analgesia and a fast post-operative recovery (Wikland *et al*., 1990).

The procedure was done with a vaginal transducer that carries an aspiration needle (CASMED Aspiration needle). A 16-gauge double lumen aspiration needle was used. All movements were guided by ultrasound (Nemio Ultrasound Scanner). Once the needle was inside the ovary, suction was applied to aspirate the follicular fluid out through a tube and into a collection tube.

Follicular aspirates were passed to the embryologist by the circulating nurse and the contents were poured into a 100 x 15mm plastic petri dish. Oocytes were located and transferred using a sterile glass Pasteur pipette into a 60 x 15mm dish containing Flush medium. The oocytes were held in the dish containing the flush for the duration of the collection and were then transferred using another sterile glass Pasteur pipette into the

culture dish (global media) and was placed in the incubator at 37^{o}C (Gardener *et al*., 2001).

3.2.5 OOCTE GRADING

Hyaluronidase was set up after the oocyte collection by the embryologist; 1.0ml hiase was aliquoted into a centre well dish and incubated at 37.0^{o}C. After 2-4 hours incubation period the cumulus complex was removed from the oocyte using the enzyme hyaluronidase and glass pipettes. The cumulus-oocyte complexes were transferred into the hyaluronidase and repeatedly aspirated through the Pasteur pipette for up to one minute. The dissociation of the cells was initially observed. Once most of the cells were loosened the oocytes were transferred using the Pasteur pipette into a second dish of Global media where further mechanical denudation was carried out using a flexipet (Marcus Medical Flexipet) with the diameter of 140um until all coronal cells were removed. This procedure was carried out very gently as to avoid any damage to the oocyte (Gardener *et al*., 2001).

The embryologist grades the oocytes (Figure 7) as described by Bongso and Trounson that incorporates a germinal vesicle stage (grade 1), a metaphase 1 stage i.e. no polar body (grade 2), two metaphase stage 2 stages i.e. with a polar body (grade 3 –mature and grade 4 – very mature) and a post-mature stage (grade 5). Following denudation, the oocytes were placed back into the incubator until the ICSI was performed (Gardener *et al*., 2001).

Figure 7: Oocyte Maturity: A- GV, B- M1 and C- M2 (C.A.R.E Clinic., 2017).

Maturation and morphological features of the oocytes were noted before the ICSI. The features of each oocyte were evaluated using an inverted microscope. The morphological evaluations were as follows: a. normal oocytes showing clear cytoplasm with homogenous fine granularity; b. granular oocytes, dark with granularity either homogenous in the whole cytoplasm or concentrated in the central portion of oocyte; c. cytoplasmic intrusions comprising of vacuoles; d. anomalies of the zona pellucida (ZP); e. fragmented polar body of different sizes; f. non-spherical shape of the oocyte; and g. large/wide PV (perivitelline space) (Khalili *et al*., 2005).

3.2.6 INSEMINATION AND INTRA CYTOPLASMIC SPERM INJECTION

3.2.6.1 Semen Sample Preparation

Semen samples were produced in a sterile bottle and are collected via masturbation after 2-3 days of abstinence and allowed to liquefy for 20 minutes at 37.0^{o}C before an analysis. Semen concentration and motility was assessed on the Makler chamber and recorded. The sample was washed by centrifugation (Heraeus Centrifuge).

Gradient preparation:

80%→5ml supra sperm + 1ml prep
40% → 2ml 80% + 2ml sperm prep

1ml 80% was placed into a tube, 1ml 40% was placed on this, followed by 1ml of the sample. The layers were not allowed to mix. The sample was spun for 20 minutes @ 2000rpm. The supernatant was removed and re-suspended in 2ml of sperm buffer and spun for 10 minutes @1000rpm – the pellet was done twice. After the last spin the supernatant was removed and the pellet was re-suspended in 1ml of sperm buffer (Gardener *et al*., 2001).

3.2.6.2 ICSI (Intra cytoplasmic sperm injection)

An ICSI dish was prepared as demonstrated below by the embryologist. Each oocyte was placed into a 20µl droplet and approximately 3µl of sperm was transferred into the middle droplet containing the PVP. This was done with a Stereo microscope (Zeiss Stereo

Microscope) on a heated stage. The dish was then transferred to an Olympus inverted microscope using Hoffman Modulation Contrast optics. The holding and injecting pipette were both made from glass capillary tubes.

The embryologist immobilizes the sperm by kinking the tail to stop the sperm from swimming. The spermatozoa were then sucked into the injecting pipette. The oocyte was held in place by the holding pipette. Only mature oocytes containing a polar body can be injected. The oocyte was aligned with the polar body in either the 6 o' clock or 12 o' clock position. The injection pipette was lowered and aligned with the oolemma in a 3 o' clock position. The pipette was pushed against the zona; and as the pipette reached the middle of the oocyte a break in the membrane occurred. This was then slowly ejected back into the cytoplasm together with the sperm. The pipette was carefully removed. Once the pipette is removed the breached area was observed, forming a funnel shape with a vortex into the oocyte (Figure 8).

Figure 8: ICSI (C.A.R.E Clinic., 2017)

3.2.7 FERTILIZATION AND CLEAVAGE

The embryologist will assess fertilization 19-21 hours after the injection and was characterized by the presence of two pronuclei. Failure to have moved together 16-18 hours post fertilization could indicate some disruption and no fertilization. The size number and distribution of the nucleoli form the central aspect of zygote scoring Z1, Z2, Z3, Z4 as shown in Figure 9 below.

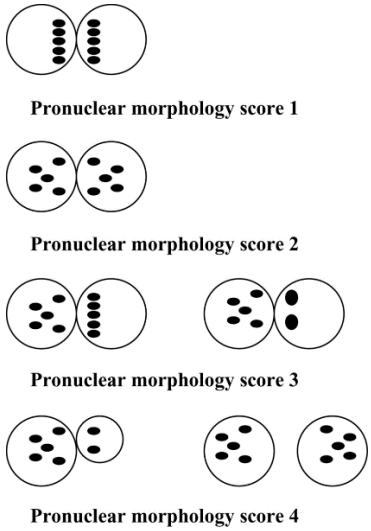

Figure 9: Different Fertilization Scores (Nicoli *et al*., 2013)

Day 2 (2-4 cells) embryo development was assessed by the number of blastomeres and the percentage of fragmentation 42-44 hours after fertilization. It was important to note uneven blastomeres or multinucleated blastomeres. Day 3 (8 cell or multicellular) morphology was assessed 66-70 hours after injection, Day 4 (morulla or compacting) 90-100 hours and Day 5 (blastocyst formation) 114-120 hours after injection (Figure 10).

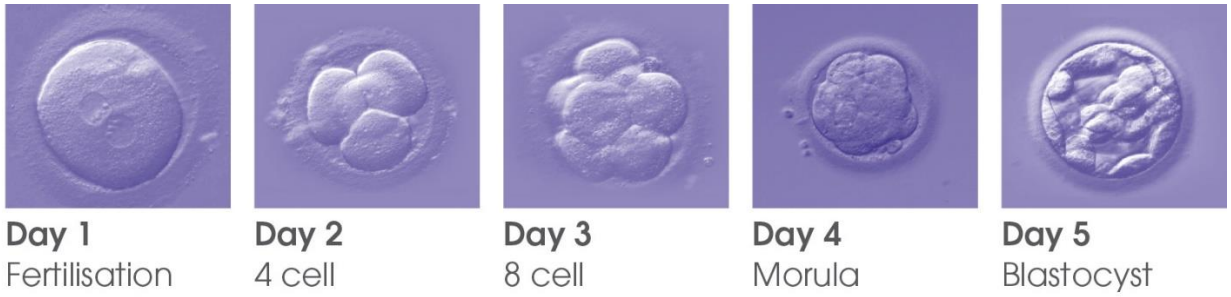

Figure 10: Embryo Morphology from Day 1 to Day 5 (Deba *et al*., 2017)

3.2.8 EMBRYO TRANSFER

The embryo transfer was the last procedure of the process. During the transfer there was minimal pain, no sedation or drugs were required. The patient should have a full bladder as it provides good ultrasound imaging and it places the uterus at a more cooperative angle. The embryo transfer catheter (Rocket Embryo Transfer Catheter) was loaded by the embryologist with the embryos and the clinician passed it through the cervix opening to the lining if the uterus. Abdominal ultrasound was used simultaneously to ensure the catheter reached the correct location. Once the lining had been located, the embryos were “transferred” into the lining of the uterine cavity. The catheter was carefully removed and taken back to the lab to be checked for any retained embryos. A bHCG test was done 12 days after embryo transfer to determine if there was a pregnancy.

3.3. Statistics

3.3.1. Statistician

Consultation, analysis and interpretation of the statistics were performed with the assistance of an institutional biostatistician (Appendix D)

3.3.2. Software and methodology

The statistical significance of the data was assessed and evaluated using the IBM SPSS. A *p* value of < 0.05 was considered statistically significant. A non-parametric Pearson's correlation was used to determine the direction, strength and significance of the correlation between X and Y variables between the different semen parameters. A parametric multiple linear regression analysis was used to evaluate the relationship between AMH and other available endocrine markers. ROC curves were used to assess predictive value for E_2 and AMH and assessing cut off values to optimise sensitivity and specificity.

3.4. Confidentiality

A strict and high degree of patient anonymity was maintained in the study. Patients were assigned study numbers which were recorded in a patient recruitment list. All patient details were known solely by the investigator and stored in an electronic format with encryption. Encryption passwords were known only by the investigator.
All material and documents related to the study and patient details, i.e., hard copies and electronic backups were stored in a secure restricted lockable cupboard which the investigator had access to. All documents will be stored for a period of 5 years and thereafter disposed of appropriately, i.e., hard copies will be shredded, and electronic backups deleted, and storage devices reformatted.

CHAPTER 4

RESULTS

4.1. Introduction

The objectives of this study were to determine if there was a correlation between AMH level and oocyte reserve, whether AMH levels affect oocyte quality and to evaluate if there is a correlation between AMH level and pregnancy outcome.

For all patients who underwent the study, the raw data recorded was consistent and compliant as per the standard operating procedure of the C.A.R.E Clinic for patients undergoing the IVF/ICSI program.

4.2 Sample Size

50 patients that met the inclusion criteria were recruited for the study. From the initial sample size of 50, 42 presented with data that could be analysed whilst 8 patients had oocytes that where abnormal and did not result in a transfer. The data from these 8 patients were not included in the study due to poor embryo development.

Three categories were classified according to the AMH concentrations (Table 7)

Table 7: Categories showing the distribution of AMH values

Category	AMH Blood Level Concentration	Frequency	% Patients
High (often PCOS)	$\geq$ 3.0 ng/ml	22	52.4
Normal	$\geq$ 1.0 ng/ml	17	40.5
Low Normal Range	≤ 0.3 – 0.9 ng/ml	3	7.1

According to the AMH levels, 52.4% of patients were in High Category, 40.5% were in the Normal and 7.1% were in the Low to Normal Category (Figure 11)

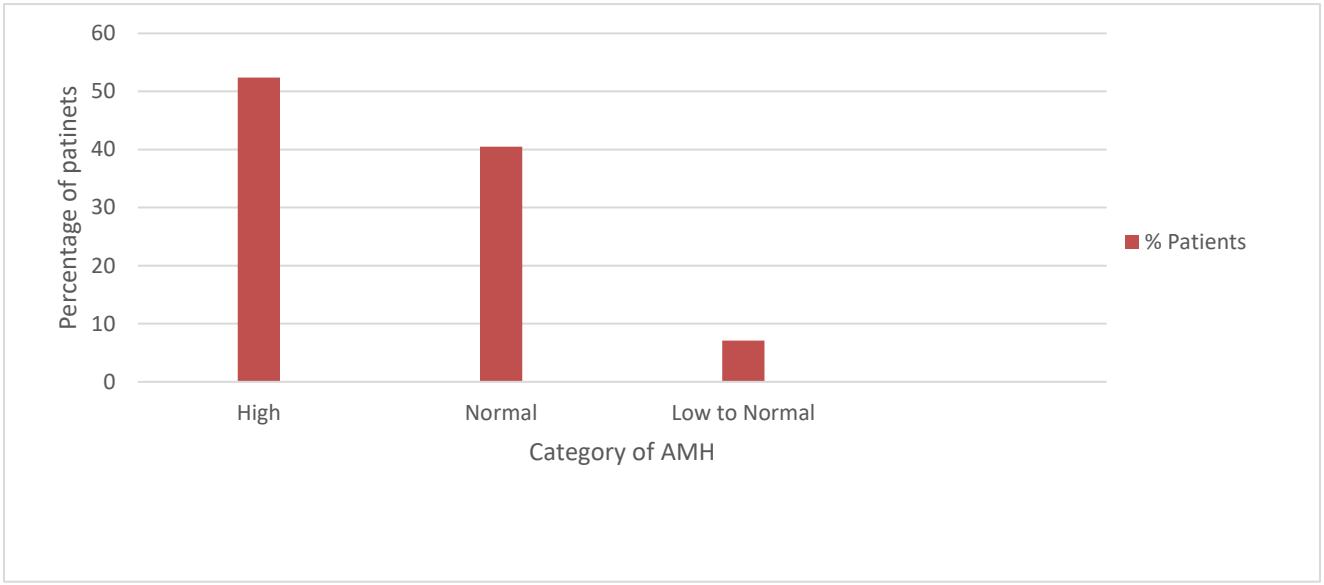

Figure 11: Percentage of patients in the respective AMH categories.

Amongst the 42 patients analysed, 4.76% were between 20-24 years, 9.52% were between 25-29 years, 40.47% were between 30-34 years, 35.7% were between 35-39 years and 9.52% were between 40-44 years (Figure 12).

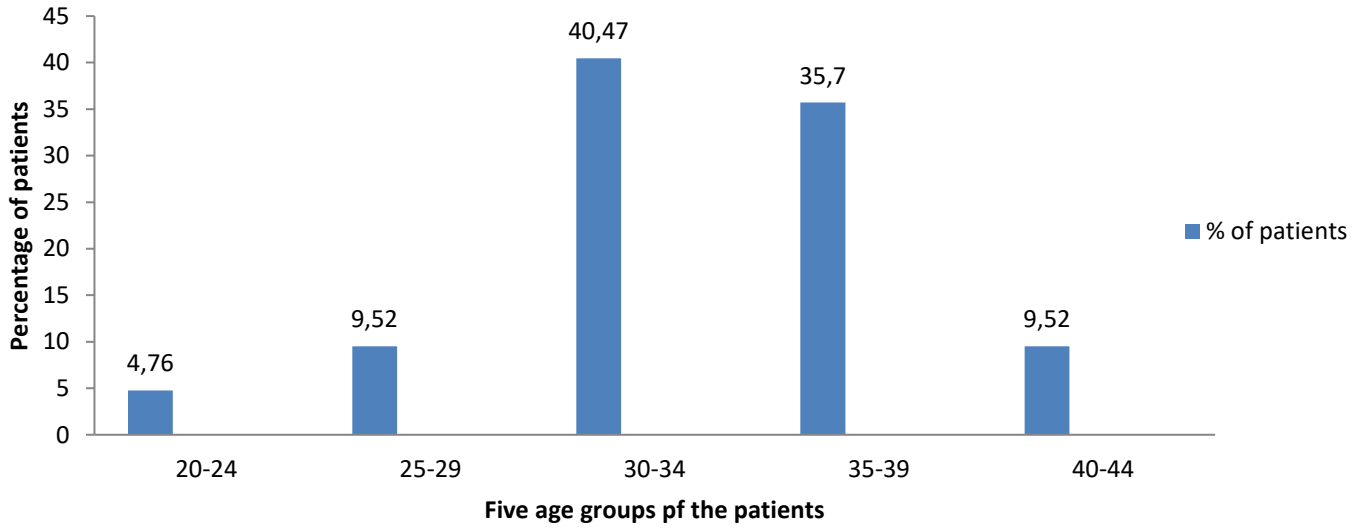

Figure 12: Age range of patients that participated in the study.

As demonstrated by this study the clinical pregnancy rate for patients 20 - 24 years was 100%, 25 -29 years was 50%, 30 – 34 years was 17.6%, 35 – 39 years was 26.6% and 40 - 44 years was 25% (Figure 13).

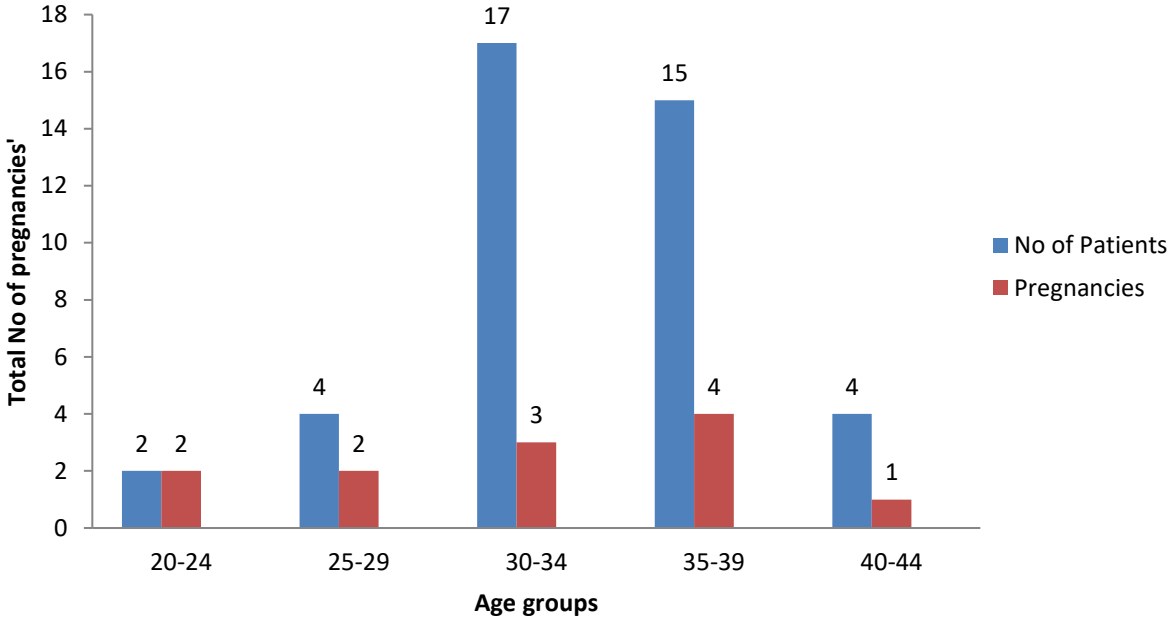

Figure 13: Number of patients compared with pregnancies in relevant age groups

4.3 Chi-Square Analysis and Cross-tabulation

A cross-tabulation (Table 8) of the number of oocytes retrieved, the number of oocytes matured, and the number of oocytes fertilized was tabulated into respective categories. Not all eggs obtained were at the metaphase 2 stages and had to be matured in the incubator overnight and injected the following day. The results shown were somewhat expected as AMH has been used an indicator of oocyte reserve in previous studies whereas the resulting fertilized or transferred embryo's may be due to a chance process based on many various factors such as the quality of the oocyte and sperm.

Table 8: A Cross-tabulation of Number of Oocytes Collected, Number of Oocytes Matured and Number of Oocytes Fertilised and AMH category

		Total no. of oocytes collected	Total no. of oocytes matured	Total no. of oocytes fertilized	% oocytes collected	% oocytes matured	% oocytes fertilized
AMH category	High	81	62	69	65.4%	60%	61.6%
	Normal	38	36	38	30.6%	35%	33.9%
	Low to Normal	5	5	5	4.0%	5%	4.5%
Total		124	103	112	100%	100%	100%

The Chi-square test for Independence was performed to check whether there was an association between the number of oocytes collected and the AMH category (Table 9). A Chi-squared value of 21.246, degrees of freedom = 8, with a $p = 0.007$ was found. There was a significant relationship between the numbers of oocytes collected versus AMH category ($p < 0.05$).

Table 9: Chi Square analysis of Number of oocytes collected and AMH.

	Value	Degrees of Freedom (df)	Asymptotic Significance (2-sided)
Pearson Chi Square	21.246[a]	8	.007
Likelihood Ratio	21.317	8	.006
N of Valid Cases	42		

a. 13 cells (86.7%) have expected count less than 5. The minimum expected count is 0.12

The Chi-square test for Independence was performed to check whether there was an association between the number of oocytes fertilized and the AMH category (Table 10). A Chi-squared value of 18.5, degrees of freedom = 12, with a p = 0.10 was found. There was thus no statistically significant relationship between the numbers of oocytes fertilized versus AMH category (p > 0.05).

Table 10: Chi Square analysis of Number of oocytes fertilized and AMH.

	Value	Degrees of Freedom (df)	Asymptotic Significance (2-sided)
Pearson Chi Square	18.504[a]	12	.101
Likelihood Ratio	17.736	12	.124
N of Valid Cases	42		

b. 18 cells (85.7%) have expected count less than 5. The minimum expected count is 0.02

A cross-tabulation of AMH category and the Number of embryos transferred was calculated (Table 11). In the High category out of the 22 patients a total of 43 embryos were transferred, the Normal category from the 17 patients a total of 31 embryos transferred and in the Low to Normal category out of 3 patients a total of 4 embryos were transferred.

Table 11: A Cross-tabulation of the Number of Embryos Transferred and AMH category.

		No. of embryos transferred			Total Number of patients	Total Number of embryo
		1	**2**	**3**		
AMH category	**High**	4	15	3	22	43
	Normal	3	14	0	17	31
	Low - Normal	2	1	0	3	4
Total					42	78

The Chi-squared test for independence of AMH category and No. of embryos transferred (Table 12) gave a Chi-squared value of 6.384 with df = 4 and p-value = 0.172. There was thus not a significant association between AMH category and No. of embryos transferred.

Table 12: Chi Square analysis of Number of embryos transferred and AMH.

	Value	Degrees of Freedom (df)	Asymptotic Significance (2-sided)
Pearson Chi Square	6.384[a]	4	.172
Likelihood Ratio	7.001	4	.136
N of Valid Cases	42		

a. 7 cells (77.8%) have expected count less than 5. The minimum expected count is 0.08

A cross-tabulation of AMH category and the day of embryo transfer was done (Table 13). Embryos were transferred from Day 3 through to Day 6. Embryos were transferred depending on embryo development and the number of embryos obtained. Most patients in the High and Normal categories resulted in a day 5 transfer.

Table 13: A cross tabulation of the Day of transfer and AMH category.

	Day of Transfer				
AMH Category	**3**	**4**	**5**	**6**	**Total N**
High	2	1	15	4	22
Normal	1	0	14	2	17
Low Normal	0	2	1	0	3
Total	3	3	30	6	42

The Chi-square test for independence between the variables AMH category and day of embryo transfer (Table 14) gave a Chi-square value of 14.117, 6 degrees of freedom and p = 0.028. There was a statistically significant relationship between the AMH category and the day of embryo transfer (p< 0.05).

Table 14: Chi Square analysis of AMH and the day of transfer.

	Value	Degrees of Freedom (df)	Asymptotic Significance (2-sided)
Pearson Chi Square	14.117[a]	6	0.028
Likelihood Ratio	6.432	6	0.377
N of Valid Cases	42		

a. 10 cells (83.3%) have expected count less than 5. The minimum expected count is 0.05

This is to be expected as AMH is used as a predictor for oocyte reserve. The higher the AMH, the greater the number of oocytes that should be retrieved on the day of oocyte collection. If a large number of oocytes are retrieved on the day of collection, there will be a greater chance of fertilization (greater sample size) and therefore more embryos will mature to a blastocyst stage.

A cross-tabulation of AMH category and Pregnancy outcome was done and is represented in Table 15. It can also be seen that of the 22 cases reported in the High category, 6 resulted in a positive pregnancy; 17 cases where the AMH category was “Normal”, 6 resulted in a positive outcome (6/12 = 50.0%); while out of the 3 cases where the AMH category was “Low to Normal“ there were no pregnancies reported.

Table 15: A cross-tabulation of Pregnancy outcome and AMH category.

	Pregnancy Result		
AMH Category	**Neg**	**POS**	**Total**
High	16	6	22
Normal	11	6	17
Low Normal	3	0	3
Total	30	12	42

The Chi-squared test for independence of AMH category and Pregnancy outcome (Table 16) gave a Chi-Squared value of 0.502, 2 degrees of freedom and p = 0.778. There was no statistically significant association between the pregnancy outcome and the AMH category (p > 0.05).

Table 16: Chi Square analysis of positive pregnancies and AMH.

	Value	Degrees of Freedom (df)	Asymptotic Significance (2-sided)
Pearson Chi Square	0.502[a]	2	0.778
Likelihood Ratio	0.774	2	0.679
N of Valid Cases	42		

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is .29.

4.4 Pearson Correlation

Pearson correlation coefficients were calculated to determine if statistical significance exists between AMH on a quantitative scale and age, E_2 and FSH. Table 17 shows the Pearson correlation coefficients between E_2 and AMH. The Pearson Correlation co-efficient of 0.151 indicates that a very weak positive relationship exists between E_2 and AMH, which is not significant (p = 0.341).

The Pearson correlation coefficient between the AMH and age produced a coefficient of -0.028 thus showing a weak, negative correlation p = 0.859 (p > 0.05).

The Pearson Correlation between the AMH and FSH produced a coefficient of -0.185 thus showing a weak, negative correlation p = 0.240 (p > 0.05).

Table 17: Pearson Correlation between basal AMH and E_2, Age and FSH.

		E_2	**Age**	**FSH**
AMH	**Pearson correlation coeff.**	0.151	-0.028	-0.185
	Significance value (2-tailed)	0.341	0.859	0.240
	No. in the sample	42	42	42

The Pearson's correlation coefficient between FSH and Age was equal to -0.087, indicating a weak negative correlation which was not significant (p = 0.583) (Table 18).

The Pearson correlation coefficient of -0.271 between the number of oocytes and age was not statistically significant (p = 0.082).

For most of the Pearson Correlation analysis, no significant relationships were found with the p values being greater than 0.05. This may be due to the small sample size used in this study of 42 patients. AMH being compared to age and number of oocytes showed a slightly negative correlation.

Table 18: Pearson Correlation between Age and FSH, number of oocytes.

		FSH	No. of oocytes
Age	Pearson Correlation	-0.087	-0.271
	Significance value (2-tailed)	0.583	0.082
	No. in the sample	42	42

The Pearson correlation coefficient showed no significant relationship between AMH and number of oocytes (p = 0.191), number of mature oocytes (p = 0.300) and number of oocytes fertilized (p = 0.146) (Table 19). The number of oocytes, mature oocytes and oocytes fertilized all showed a weak positive correlation with AMH (0.206, 0.164, and 0.228, respectively).

Table 19: Pearson correlation coefficients between AMH, number of oocytes, number of mature oocytes and number of oocytes fertilized.

		Number of oocytes	Number of mature oocytes	Number of oocytes fertilized
AMH	Pearson Correlation	0.206	0.164	0.228
	Significance value (2-tailed)	0.191	0.300	0.146
	No. in the sample	42	42	42

4.5 Logistic Regression and Classification Table

A logistic regression model was used to determine the possible predictor variables for the pregnancy outcome. The model was fitted to the data with the result of the pregnancy namely, “Positive” or “Negative” as the binary dependent variable and Age, E_2 LH, Basal FSH, Basal AMH and number of oocytes fertilized as the independent variables. In a logistic regression model, the odds ratio is given by Exp (B), (the last column in Table 20). The SPSS output for the model is given in Table 21.

Table 20: Parameter estimates (B), standard error (S.E.), p-values and odds ratios for Age, E_2, LH, Basal FSH and Basal AMH.

	B	Standard Error (S.E.)	Wald	Degree of Freedom (df)	p-value	OR = Exp (B)
E_2	.001	.000	3.396	1	.065	1.001
LH	-.556	.273	4.144	1	.042	.574
BasalAMH	-.335	.239	1.967	1	.161	.715
Age	-.146	.091	2.593	1	.107	.864
No_Fertilized	.150	.368	.166	1	.683	1.162
BasalFSH	-.102	.136	.559	1	.455	.903
Constant	4.451	3.452	1.663	1	.197	85.744

As can be seen in Table 21, LH has $p = 0.042$ ($p < 0.05$) and E_2 has $p = 0.065$, which is significant at a 10 % level. In this logistic regression model the remaining variables are not significant (p-values>0.10).

The classification table (Table 21) for the model above, shows that overall 73.8 % of the cases were correctly classified, while 5/12 =0.417 or 41.7 % of the positives were correctly classified, and 86.7% of the negative cases were correctly classified.

Table 21: Classification Table of Pregnancy Outcome.

Observed		Predicted		
		outcome		Percentage Correct
		Neg	POS	
outcome	Neg	26	4	86.7
	POS	7	5	41.7
Overall Percentage				73.8

A second logistic regression model with LH, Basal AMH, Age and E_2 was fitted to the data. The SPSS output is given in Table 22. E_2 has a p = 0.017 and LH has a p = 0.035. Both variables are significant at a 5% level. Age and Basal AMH play a role in the pregnancy outcome and the model is thus adjusted for these two variables. This means that the variables are included although their respective p-values are not less than 0.05. The column Exp (B) is the odds ratio, (OR).

Table 22: Parameter estimates (B), Standard error (S.E), p-values and odds ratio for Age, E_2, LH and Basal AMH.

	B	Standard Error(S.E.)	Wald	Degree of Freedom(df)	p-value	OR = Exp (B)
E_2	.001	.000	5.672	1	.017	1.001
LH	-.571	.271	4.450	1	.035	.565
Basal AMH	-.284	.224	1.607	1	.205	.753
Age	-.146	.090	2.624	1	.105	.864
Constant	3.680	3.100	1.409	1	.235	39.662

The interpretation for the variable, E_2 is that an increase of 1000 units in the E_2 - value will increase the odds of a positive result by

$$exp\,(1000 \times 0.001) = \ exp(1)\ = 2.72.$$

The interpretation for LH is that a 1 unit increase in LH gives a decrease of 0.565 in the odds of a positive result. The classification table for this model is represented in Table 23.

Table 23: Classification table of Predicted Pregnancies

		Predicted Outcome		
Observed		**Negative**	**Positive**	**Percentage Correct**
Outcome	**Negative**	25	5	83.3
	Positive	7	5	41.7
Overall Percentage				71.4

This model results in 71.4% of the values being correctly classified.

4.6 Area under the curve

A sensitivity and specificity analysis were performed to determine whether E_2 or AMH optimally predicted pregnancy in patients undergoing the IVF program. The area under the curve for E_2, AUC = 0.725 and for AMH, AUC = 0.497. E_2 is therefore a better single predictor of pregnancy outcome when compared to AMH. It has been shown that E_2 can better predict the number of oocytes obtained (Figure 14).

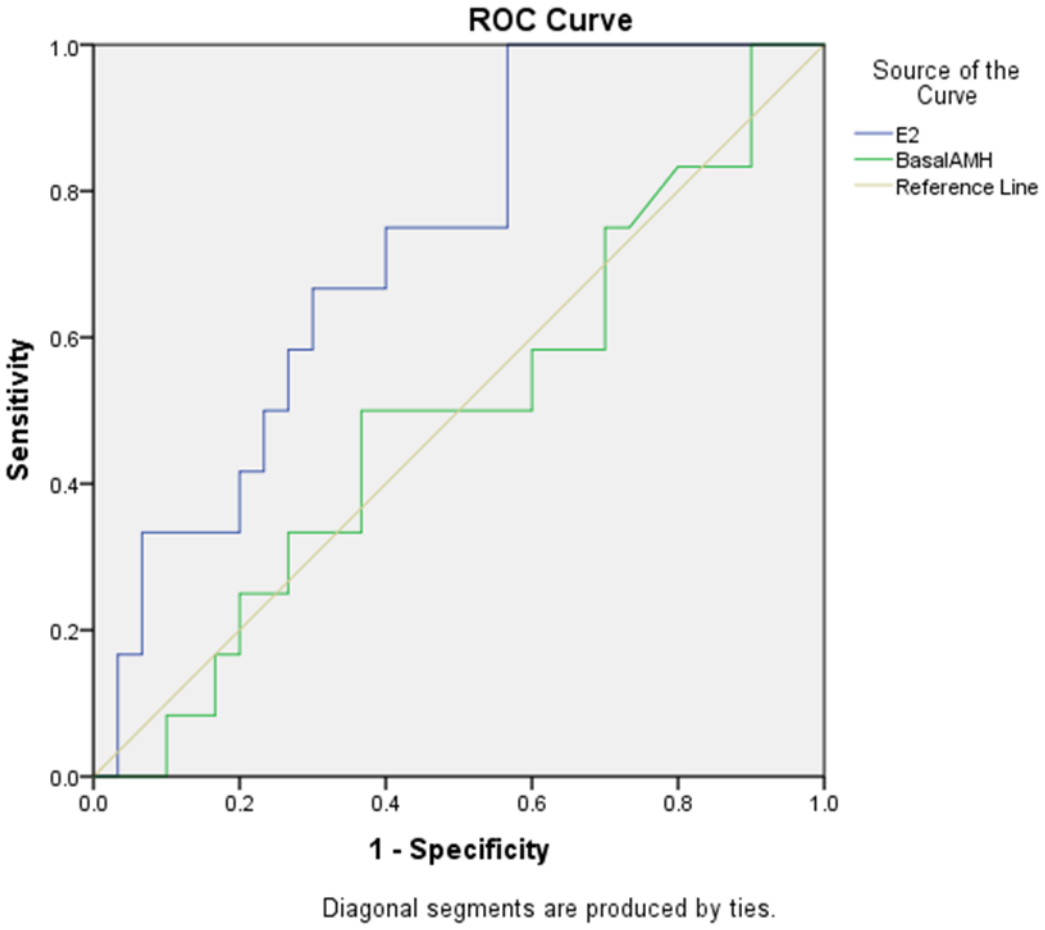

Figure 14: Sensitivity and specificity of E_2 and AMH in predicting pregnancy.

4.7 Supplementary Statistics

A cross-tabulation of race and pregnancy outcome (Table 24) was also done and a Chi-square test for independence (Table 25) gave a Chi-squared value of 2.246, with 3 degrees of freedom and $p = 0.532$ ($p > 0.05$). There was therefore no statistically significant association between race group and pregnancy outcome.

Table 24: A Cross-tabulation of AMH category and Race group

AMH category	Black	White	Indian	Coloured	Total
High	3	9	6	4	**22**
Normal	5	1	11	0	**17**
Low to Normal	0	2	1	0	**3**

Table 25: Chi Square analysis of Race and Pregnancies Outcomes and AMH.

	Value	Degrees of Freedom (df)	Asymptotic Significance (2-sided)
Pearson Chi Square	2.246[a]	3	0.532
Likelihood Ratio	2.217	3	0.529
N of Valid Cases	42		

a. 4 cells (50.0%) have expected count less than 5. The minimum expected count is 1.14.

CHAPTER 5

DISCUSSION

5.1. Basal AMH and E_2

The Pearson analysis between E_2 and AMH showed a Pearson Correlation co-efficient of 0.151 with $p = 0.341$ ($p < 0.05$) which indicates that a weak significant relationship exists between E_2 and AMH.

Initial studies (Licciardi *et al*., 1995; Smotrich *et al*., 1995 and Evers *et al*., 1998) have shown a relationship between a raised basal E_2 level and a reduced ovarian response using different values to express raised estrogen levels which replicated the findings in this study therefore showing that a low AMH can result in low estrogen levels.

The study by Licciardi *et al*. (1995), hypothesized that high E_2 levels on day 3 of IVF cycles without GnRH agonist (GnRH-a) are related to decreased oocyte numbers and pregnancy rates. They concluded for patients going through IVF without GnRH analogue that, oocyte numbers and PRs declined with increasing levels of day 3 E_2. E_2 and day 3 FSH where combined to enhance the predictive capability of either of these hormones used alone. This study reinforces this finding and can be concluded that a poor AMH value results in a poor ovarian reserve which means follicles produced will not correlated to a raised estrogen level, therefore indicating poor follicle growth, thus reducing the number of oocytes produced.

The objective of Smotrich *et al*. (1995), study was to assess the predictive value of day 3 E_2 levels, independent of day 3 FSH levels, on responses to ovulation induction and pregnancy rates (PRs) in IVF-ET patients. A higher cancellation rate was observed in patients who presented with a high day 3 E_2 (≥ 80 pg/mL) in a cycle before IVF-ET and a lower PR independent of FSH level. A day 3 E_2 level together with a day 3 FSH level appeared to be very helpful in prospectively counselling patients regarding cancellation risk and ultimate IVF-ET success. The weak relationship found between AMH and E_2 can

aid in unnecessary egg collection based on the blood results therefore reducing the trauma to the patient of going through the entire process with a fruitless outcome.

A large study conducted by Frattarelli *et al*. (2000), presented that a poor ovarian response was more commonly seen in those with <20 or >80 pg/ml of estrogen but did not show any association to the pregnancy rate.

It was concluded that poor response to stimulation in IVF, suggestive of a reduced ovarian reserve, is related with reduced baseline serum AMH concentrations (Van Rooij *et al*., 2002). Therefore, when women have normal ovarian reserve and good response, failure of IVF should be due to other infertility causes, 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 E_2 levels in these cases were higher than those of controls, they are still within the normal range of 25-100 pg/ml (Tietz, 1995), signifying that E_2 single-handedly is not adequate to predict the female reproductive potential.

5.2 Basal AMH and Age

The Pearson Correlation between the AMH and age (Table 17) produced a co-efficient of -0.028 thus showing a weak, negative correlation and with a $p = 0.859$ ($p > 0.05$). A stronger relationship between these two variables was expected as it is known that as age increases, AMH should decrease.

This inverse relationship agrees with that found by Van Rooij *et al*. (2005), whose study was to measure which of the basal ovarian reserve markers provided the best indication of the variations taking place in ovarian function over time and their reproductive age. Eighty-one women with normal reproductive management all through their lives were evaluated. In this select group of women, becoming older was measured as a variable. They concluded that serum AMH levels decline with age in normal women with proven fertility. They added that serum AMH represented as the better endocrine marker to gauge the age-related drop of reproductive capability.

A study conducted by Tremellen *et al*. (2005), analysed the practicality of AMH measurement as an instrument for determining ovarian reserve in an overall infertility population. The AMH levels of 238 women who were between the ages of 18–46 years during day 3–5 of their menstrual cycle. All 238 patients had FSH levels less than 10 i.u./L, signifying normal ovarian reserve on traditional FSH criteria. 87 of these patients gave their authorization to compare their AMH levels with IVF oocyte retrieval outcome. The patients who produced ≥ 8 oocytes were categorized as having normal ovarian reserve. Patients who produced ≤ 4 oocytes were categorized as having poor ovarian reserve. AMH levels remained relatively inactive (20–25 pmol/L) from between ages 18 to 29 years. By 30 years of age, AMH levels started rapidly decreasing, reaching only 10pmol/L by 37 years. AMH levels between the ages of 29 and 37 years showed a 50% fall, slight changes in FSH levels were detected. Using a cut off value of 8.1 pmol/L, plasma AMH assessment could predict poor ovarian reserve on a succeeding IVF cycle with a sensitivity of 80% and a specificity of 85%. It was decided that AMH assessments are superior to FSH in recognizing women with reduced ovarian reserve. Anti-müllerian hormone assessment should be considered as a beneficial aide to FSH and E_2 levels when assessing ovarian reserve. As per the results obtained in our study of the 42 patients analysed, a relationship was shown that AMH correlates with age. The older the patients the lower the AMH and the reduced chances of producing a viable oocyte to result in a positive pregnancy.

Anti-Müllerian hormone (AMH) is produced by the granulosa cells of preantral and small antral follicles and its levels can be measured in serum. Subsequently the number of ovarian follicles declines with increasing age, AMH levels might be used as a marker for ovarian ageing. Therefore, Van Rooij *et al*. (2002), studied the association between AMH levels and ovarian response during ovarian stimulation in which the data of 119 patients were analysed and concluded AMH is a marker for ovarian reserve. Our finding were suggestive that a diminished ovarian reserve, is related with reduced baseline serum AMH concentrations. It seems AMH can be used as a marker for ovarian ageing.

5.3 Basal AMH and FSH

The Pearson Correlation between the AMH and FSH (Table 11) produced a coefficient of -0.185 thus showing a weak, negative correlation and with a p= 0.240 (p> 0.05).

This coincides with a study conducted in the USA. 5354 women presented to fertility clinics from 30 different states established that in woman with FSH serum values together with AMH values which associated with decreased ovarian reserve where recognized in 20% of woman under the age of 35 years and in over 30% of woman by the age of 40 years. It was concluded that serum AMH and FSH values were normal and age-dependant using common clinical cut points, a bigger patient population, one laboratory and the same testing methodology (Leader *et al*., 2012). Therefore, testing AMH and FSH can be a significant way of recognizing many patients in the routine practice who are at a risk for faster decline in ovarian reserve.

Basal FSH was one of the first endocrine markers introduced into the ART program. Many clinics have used FSH together with other markers which are more reliable. A study was commenced to assess the influence of day 3 inhibin B and FSH and the woman's age on its own and as a combined prognosticator of ovarian response and pregnancy. It was determined that any two or all three of these variables studied did not improve the prognostic value of FSH alone. Woman's age was the only variable self-reliantly related with pregnancy rate. Patients undertaking their first IVF/ICSI treatment cycle their age and basal FSH was concluded to be a sturdier predictor rather than inhibin B. Basal FSH concentration was a better predictor of cancellation rate than age, but age was a stronger predictor of pregnancy rate (Creus *et al*., 2000). This study indicates a negative correlation, i.e., the higher the FSH the higher the chances the patient can present with a poor ovarian reserve and early menopause.

It is common practice to repeatedly test the basal FSH level in the cycle and to start IVF treatment only when the FSH level is below a certain threshold value. This was centred on the knowledge that these women will respond better to ovarian stimulation when the basal FSH level is lower at the start of the cycle. 39 women underwent two IVF cycles in a year. The basal FSH level prior to each of these cycles was known to have changed.

The treatment cycles were divided into two categories: High basal FSH (≥10 IU/l) and cycles with a low basal FSH (<10 IU/l). The results of this study divulged that women who were poor responders or had a reduced ovarian reserve had a poor outcome and repeated testing will add no value. Women who had a history of elevated FSH should be offered treatment without more delay. By delaying treatment for these patients, it could be counterproductive, as they are getting elder and more rapidly approaching menopause (Abdalla *et al*., 2006).

Results reported by de Vet *et al*. (2002), suggested that changes in serum AMH levels have been shown to occur moderately early in the classification of events linked with ovarian ageing. FSH serum levels that are elevated are not discovered until cycles became irregular (Burger *et al*., 1999). FSH level predicts poor responders during IVF (Jurema *et al*., 2003). It was acknowledged in a meta-analysis that, possibly due to inter-cycle variability, basal FSH is not an accurate predictor of IVF outcome (Broekmans *et al*., 2003).

5.4 Correlation between Age and FSH and Number of oocytes

The cross-tabulation and a Pearson correlation between the number of oocytes and age also did not show any statistically significant relationship ($p = 0.082$; $p<0.05$). The Pearson Correlation value of -0.271 shows a weak negative relationship. Again, due to the weak statistical relationship, the result of the analysis is regarded as not significant.

The Pearson Correlation between FSH and age (Table 18) also showed no statistical significance, $p = 0.583$ ($p > 0.05$). Also seen was a very weak negative correlation (Pearson Correlation -0.087) however, this is regarded as no significant relationship.

For most of the Pearson Correlation analysis, no significant relationships were found with the majority of the p-values, being greater than 0.05. This may be due to the small sample size used in this study of 42 patients. AMH being compared to age and number of oocytes showed a slightly negative correlation which is expected as it is shown in previous studies that AMH and number of oocytes decrease with maternal age (Van Rooij *et al*. (2005) and La Marca *et al*. (2007).

This inverse relationship agrees with that found by Van Rooij *et al*. (2005), who reported that serum AMH levels deteriorate with age in normal women with proven fertility. They stated that serum AMH signifies the best endocrine marker to assess the age-related decline of reproductive capability.

A 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 notion of female reproductive ageing suggesting that the reduction in the oocyte pool determines the age-dependent loss of female fertility (Te Velde *et al*., 2002). During the final stage of reproductive ageing (menopause) n distinct difference can be noted. Women differ regarding the status of the oocytes pool depending on their age. The change in the number of oocytes correlates with AMH change where AMH levels decrease with increased age.

If age and oocyte yield are related, a patient with a high AMH level would have a greater probability of having a normal oocyte retrieved than a woman with a low AMH level. If AMH together with other markers for ovarian reserve duplicate the quantity of remaining normal oocytes in the oocyte pool, it is then rational that the number of oocytes has an influence because a greater yield increases the chance that at least one oocyte retrieved may be euploid. Likewise, in patients with a high AMH resulting in low oocyte recovery is adequate because the proportion of euploid oocytes is likely to be higher (Verberg *et al*., 2009).

The AMH levels in our group who were high responders were over 3.0 ng/ml, normal responder over 1.0ng/ml and low responders to be below 0.9ng/ml. Oocytes were still retrieved even with the low AMH levels. Neither fertilization rate nor embryo quality could be estimated using basal AMH levels. This contrasts with the findings reported by Silberstein *et al*. (2006), who found embryos which had superior morphology and cleavage performance in patients with AMH levels > 2.7ng/ml as compared with patients with values below this threshold.

5.5 Correlation between AMH and the number of oocytes, number of mature oocytes and number of oocytes fertilized.

The second objective of the study was to examine if AMH levels affected oocyte quality.

After oocyte collection, the oocytes show different characteristics of maturation, integrity and viability. At present the best evaluation of oocyte quality remains to be a direct microscopic observation of morphology. Morphological assessment of human oocytes retrieved for ART remains to be a major tool in predicting *in-vitro* fertilization or intracytoplasmic sperm injection (ICSI) outcome and should be further advanced. There are no distinct and precise criteria for oocyte morphology evaluation as there are no commonly accepted categorizing systems in effect. It was decided that morphological assessment of the oocyte should be together with a detailed evaluation of the resulting embryo to reach a proper deduction regarding implantation and pregnancy (Balaban *et al.*, 2012).

In this study the Pearson Correlation test (Table 19) showed no significant relationship between AMH and number of oocytes ($p = 0.191$), several mature oocytes ($p = 0.300$) and number of oocytes fertilized ($p = 0.146$). The number of oocytes, mature oocytes and oocytes fertilized all showed a weak positive relationship to AMH (0.206, 0.164, and 0.228, respectively).

These findings agree with that found by La Marca *et al*. (2006 and 2007) and Dehghani-Firouzabadi *et al*. (2008), who conveyed that the mean amount of oocytes was suggestively lower in poor responding patients than in normal, good and high responding patients attending IVF programs. This therefore led to the deduction that the ovarian response can be viewed as a reflection of the ovarian reserve.

The Chi-square test for Independence was performed to check whether there is an association between the number of oocytes collected and the AMH category (Table 9). A Chi-squared value of 21.246, degrees of freedom = 8, with a $p = 0.007$ was found. There was a significant relationship between the numbers of oocytes collected versus AMH category ($p < 0.05$).

The Chi-square test for Independence was performed to check whether there is an association between the number of oocytes fertilized and the AMH category (Table 10). A Chi-squared value of 18.5, degrees of freedom = 12, with a p = 0.10 was found. There was thus no statistically significant relationship between the numbers of embryo's fertilized versus AMH category ($p > 0.05$).

This is somewhat expected as AMH has been used an indicator of oocyte reserve in previous studies whereas the resulting fertilized or transferred embryo's may be due to a chance process based on many various factors such as the quality of the oocyte and sperm.

The purpose of fertilization is the amalgamation of a single sperm nucleus with the female pronucleus within the activated oocyte. For this to occur successfully, numerous actions must become apparent, including the integration of the entire spermatozoon into the oocyte, the completion of meiotic maturation with the extrusion of the second polar body, the metabolic activation of the previously dormant oocyte, the decondensation of the sperm nucleus and the maternal chromosomes into the male and female pronuclei, respectively (Asch *et al*., 1995). Thus, this illustrates that fertilization or the lack thereof, is not only because of *in-vitro* conditions but due to the quality of the oocyte and sperm utilized in the fertilization process.

Ebner *et al*. (2006), demonstrated in their study that AMH serum levels were related with oocyte quality in stimulated cycles. The quality of the embryos was not assessed using baseline AMH which agrees with our evidence. However, the fertilization rate was not correlated with the serum AMH which differed with the results of the present study.

The study by Spandorfer *et al*., (1998), was to research any impact of maternal as well as paternal age on gamete physical characteristics and pregnancy results in intracytoplasmic sperm injection. 821 consecutive ICSI cases were analysed, retrospectively. A major decrease in the quantity of oocytes recovered and the number of mature oocytes developed was found with progressing maternal age. An increase in the event of digyny was noted with parental ageing, while no difference in single or bi-pronuclear fertilization was found. Older women had a decreased incidence of single pro-

nucleus formation and an increase in digyny, but no noteworthy change in the percentage of oocytes that underwent two-pronuclear fertilization was detected regarding maternal ageing. Pregnancy results were not affected by the age of the male partner, while a strong negative correlation was found with maternal ageing. To better analyse male partner ageing as a factor affecting pregnancy outcome, Spandorfer examined a subgroup of patients with a female partner aged <35 years who underwent ICSI. No paternal influence on ICSI pregnancy results were found in this subgroup of patients. It was suggested that the influence on pregnancy outcome after ICSI was connected for the most part to maternal and not paternal age.

Seifer *et al*. (2002), found that the mean serum AMH concentration was in the group with ≥11 collected oocytes as compared to the group with ≤ 6 oocytes. In addition, Dehghani-Firouzabadi *et al*. (2008), reported a significant increase in good responder women undergoing IVF (≥ 4 oocytes) as compared to poor responders (<4 oocytes). Accordingly, serum AMH levels can foresee ovarian response amid IVF treatment cycles. Rather than AMH, the total number of oocytes in different responsive classes increased with a significant decrease in the levels of E_2. 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 variation signifying that E_2 is a poor marker of ovarian response.

5.6 AMH category and the day of transfer

In this study, a Chi-Square (X^2) value of 14.117 (6 degrees of freedom) and a $p = 0.028$ (Table 14), showed that there was a statistically significant relationship between AMH and the day of embryo transfer ($p < 0.05$) and The Chi-squared test for independence of AMH category and number of embryos transferred (Table 12) gave a Chi-squared value of 6.384 with df = 4 and p-value = 0.172. This showed that there was not a significant association between AMH category and number of embryos transferred.

Pregnancy is dependent on embryo implantation. The number of embryos transferred result in a higher likelihood of pregnancy. This is to be expected as AMH is used as a predictor for oocyte reserve. The higher the AMH, the greater the number of oocytes should be retrieved on the day of oocyte collection. If a larger number of oocytes are

retrieved on the day of collection, there will be a greater chance of fertilization (greater sample size) and therefore, more embryos will mature to a blastocyst stage.

In 2006, Ebner *et al*. expressed that serum anti-müllerian hormone levels offer a powerful means for predicting ovarian response, which is reflected not only by the size of the primordial follicle pool but also the quality of the oocytes. Considering a conjoint association between AMH-expressing somatic cells and gametes, this prospective study evaluated whether extreme AMH levels indicate reduced oocyte quality and developmental incompetence. Overall, 141 patients were considered and split up into three groups as per their AMH levels. In these three groups, the morphology of all oocytes and fertilization rate, embryo quality and blastocyst formation was evaluated, and FSH, LH and Estrogen (E_2) levels were also measured. Cycle cancellation rate was associated with AMH levels $p< 0.05$. AMH groups 1 (<1.66 ng/ml) and 3 (>4.52 ng/ml) displayed oocytes of lower quality [dark central granulation, aggregation of smooth endoplasmic reticulum compared with the median group 2 (1.66–4.52 ng/ml). Basal serum FSH did not allow for a reasonable diagnosis to be made in relation to gamete appearance. Fertilization and further development up to blastocyst stage was not influenced by the AMH levels. AMH is by all accounts better than FSH in anticipating both oocyte number and quality.

The increase in the mean number of total oocytes in positive pregnancy coincides with increased mean levels of AMH, giving a better chance for a higher mean number of mature oocytes and embryos, and consequently higher likelihood for pregnancy to occur. A 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.

AMH concentrations decline with increasing age and produce an elusive indication for ovarian ageing. Furthermore, basal serum AMH concentrations predict ovarian response during IVF cycles. Alongside, oocyte quantity and embryo quality diminish with advancing age. Hence, it was theorized that AMH in serum constitutes a marker for embryo quality. Embryo morphology score and pre-implantation genetic screening was sued to assess embryo quality. AMH concentration on day 3 of the menstrual cycle correlated with the

number of oocytes retrieved. AMH and embryo morphology were correlated after moderate stimulation, but not after conventional ovarian stimulation. AMH and the chromosomal competence of embryos were not correlated. Serum AMH is predictive for ovarian response to stimulation. However, the lack of a consistent correlation with embryo morphology and embryo aneuploidy rate is not in favour of a direct connection between oocyte quantity and embryo quality (Fong *et al*., 2008).

5.7 AMH category and positive pregnancies

The final objective of the study was to determine if AMH can predict a positive pregnancy outcome. Embryo quality has been suggested to be of paramount importance to predict the occurrence of pregnancy after IVF. Various morphological factors have been proposed to identify embryos with the best chances of implantation. However, the influence of early embryo quality on embryo development after implantation and pregnancy outcome is poorly known.

A Chi-Square test was conducted to determine if AMH can predict a positive pregnancy outcome. (X^2) value of 0.502 (6 degrees of freedom) and a $p = 0.778$ (Table 16), there was no statistically significant relationship between the number of positive pregnancies versus AMH ($p > 0.05$).

In a regression model E_2 has a $p = 0.017$ ($p < 0.05$) and LH has a $p = 0.035$ ($p < 0.05$). Both variables are significant and Age and Basal AMH play a role in the pregnancy outcome and the model is thus adjusted for these two variables

In this study AMH value for predicting pregnancy outcomes does not exist because oocyte quality is not accounted for by ovarian reserve markers. As demonstrated by this study the clinical pregnancy rate for patients 20 - 24 years was 100%, 25 - 29 was 50%, 30 – 34 years was 18%, 35 – 39 years was 27% and 40 - 44 years was 25% (Figure 13). Even patients with a low AMH did not differ from those women with higher AMH concentrations in the same age group. A positive pregnancy was recorded across all age groups irrespective of the AMH level. These observations suggest that low ovarian reserve is not associated with low oocyte quality in patients and the prognosis remains the same despite low AMH concentrations.

Kini *et al*. (2010), evaluated the role of AMH in predicting cumulative pregnancy outcome during IVF treatment. 180 women undergoing IVF had their AMH levels tested on day 3 of ovarian stimulation. Patients who presented with a higher AMH level achieved ongoing pregnancy in the fresh IVF cycle and in patients showing ovarian hyper-response in the stimulated cycle. It was established that serum AMH concentration on day 6 of stimulation was significantly higher in participants who achieved cumulative ongoing pregnancy in IVF compared to those who did not. Serum AMH is a fairly suitable indicator of ovarian hyper-response.

Yao *et al*. (2015), conducted a meta-analysis to discover and approve the exact predictive value of serum AMH and the follicle fluid AMH (FF AMH) on the outcome of ART. They acknowledged all studies published by March 2014 with data related to *in-vitro* fertilization", "intracytoplasmic sperm injection", "assisted reproductive technology" and "antimullerian hormone" in Pubmed database. A sum of 26 studies were used for this meta-analysis. It was concluded that serum AMH, as an independent parameter, can predict pregnancy result after assisted conception and the positive correlation with serum AMH and non-pregnancy should not be ignored either.

Having accurate tests for predicting the chance of pregnancy through IVF is very important for doctors and patients to make an educated decision about whether to proceed with the treatment. Many medical studies have found that women's anti-müllerian hormone (AMH) concentrations in the blood can very accurately predict how many oocytes they can produce. However, the capability to ensure a positive pregnancy is always investigated. Wang *et al*. (2010), looked at the records of 1558 different couples who had completed IVF treatment and divided them into four groups based on the women's age (<34, 35–37, 38–41 and >42 years). In the youngest age group (<34), women with the lowest AMH blood concentrations did not have pregnancy rates lower than those of women with higher AMH concentrations. For women between 34 and 41 years of age, they observed very evidently that the higher the AMH concentration, the greater the chance of pregnancy during IVF treatment. As for women aged 42 years or older, high AMH concentrations did not result in increased pregnancy rates compared with those of women whose concentrations were normal; however, those women in this

age group with very low AMH concentrations had an extremely low chance of success through IVF treatment. Overall, AMH concentration is an important tool to predict IVF pregnancy rates for women aged between 34 and 41 years, but the test is not as useful for younger and older women.

5.8 Supplementary Statistics

Sensitivity and specificity analysis (Figure 14) were performed to determine whether E_2 or AMH optimally predicted pregnancy in patients undergoing the IVF program. The area under the curve for E_2 (AUC = 0.725) and AMH (AUC = 0.497). E_2 is therefore a better predictor of pregnancy outcomes when compared to AMH. It has been shown that E_2 can better predict the number of oocytes obtained.

Mittal *et al*. (2014), carried out an investigation to observe and assess the role of total serum E_2 on the day of injection of HCG, E_2 for each mature follicle, and E_2 for every oocyte retrieved (OR) on clinical pregnancy rate (CPR) and oocyte/embryo quality in assisted reproduction. 342 women who underwent the IVF and had normal ovarian reserve underwent long GnRH agonist protocol. The results measured included the number of oocytes retrieved, number of mature oocytes, number of oocytes fertilized, and fertilization rate, number of embryos cleaved, cleavage rate, number of Grade I embryos, number of cryopreserved embryo, and the clinical pregnancy rate. In conclusion, serum E_2 was detected to be an important aspect of IVF success. While serum E_2 did not show any confident or adverse influence on IVF outcome. On the other hand, estrogen per mature follicle and retrieved oocytes do have an influence. When E_2/follicle is between 200 and 299.99 pg/ml the pregnancy outcome was overall better. Also, increasing serum E_2/follicle positively associated with better oocytes and embryo quality. There was, however, a negative correlation with E_2/oocyte compared to oocytes and embryo quality parameters.

A retrospective analysis was conducted by Vaughan *et al*. (2016), to assess the serum E_2 per oocyte ratio as a role of selected embryology events and reproductive outcomes with IVF. This retrospective analysis included all IVF cycles where oocyte collection and fresh transfer occurred between January 2001 and November 2012 at a single institution. Patients were divided into three group depending on their age (<35, 36–39, and ≥40 years). Clinical pregnancy results were higher in patients with estradiol per oocyte ratio (EOR) of 250–750 and declined as this relationship increased, autonomous of patient age. No statistically significant correlation was seen in fertilization, cleavage rates or number of good quality embryos as a function of EOR.

In this study race group was analysed to determine if there was a relationship between people of different race groups and a positive pregnancy outcome. A cross-tabulation (Table 24) together with a Chi-Square (X^2) value of 2.246 (3 degrees of freedom) and a $p = 0.532$ (Table 25), showed that there is no statistically significant relationship between race group and positive pregnancies ($p > 0.05$).

Bleil *et al*. (2014), directed a study to determine whether reproductive age confirmed as a marker of ovarian reserve (AMH), between women of different race/ethnic backgrounds. The population consisted of Multi-ethnic sample of 947 (277 white, 237 African American, 220 Latina, and 213 Chinese) healthy and regularly cycling premenopausal women, ages 25–45. Inspection of the significance of race/ethnicity-by-linear age interaction among Latina women compared with white women across all ages showed that AMH levels were lower. African American and Chinese women compared with the white women at younger and middle age groups, respectively also showed AMH levels were lower. The AMH levels were higher among African American compared with Latina and Chinese women at older ages.

Begum *et al*. (2016), assessed whether the quality of early childhood settings amongst diverse groups of Bangladeshi women, adds to deviation in ovarian reserve and the rate of reproductive aging in later life. Levels of serum AMH hormone, inhibin B, FSH, and E_2, and anthropometrics resulting from biomarkers; reproductive, demographic, and health variables were obtained from controlled surveys. The study aimed to argue that there is significance in childhood development when considering variation in ovarian reserve across different ethnic groups. Scientific studies and investigation have emphasized the role of genes or race in defining inter-population variation in ovarian reserve. Early life developmental factors should be given due thought when assessing inter-group differences in response to assisted reproductive technology.

5.9 Limitations of this Study

An important limitation of the present study was the lack of AFC at time of oocyte collection. Another constraint was the different cut-off values for AMH and AFC. This is problematic as it hinders with the identification of a single threshold for AMH of AFC that could be predictive of an excessive response.

Currently, there is no consistent recognised definition of decreased ovarian reserve (DOR), as the term may refer to three related but distinctly different outcomes: oocyte quality, oocyte quantity, or reproductive potential. Obtainable evidence regarding the performance of ovarian reserve tests is partial due to small sample sizes, heterogeneity among study design, analyses and outcomes, and the lack of validated outcome measures.

CHAPTER 6

CONCLUSION

Assessment of ovarian reserve is mainly relevant in an IVF clinic, where AMH may be a predictor of ovarian response. AMH measurement is beneficial in refining the counselling of patients and enhancing the ability to enlighten them about the risk and associated consequences of a poor response. The more argumentative point is whether AMH measurements should be used to deny IVF treatment. Although there are several other markers together with AMH which aids in predicting ovarian response none of these markers are 100% consistent.

While appropriate reference values are being created per age category and until the consequences of having a low or high AMH for one's age are being established, AMH should only be determined in the context of clinical studies. At present, the most important clinical role of AMH at this stage is to serve as a red-flag for reduced ovarian reserve in women of reproductive age who must undergo further diagnostics. This study has established a definite role for AMH as a forecaster for both current and future individual fertility. Considering that there are many factors besides oocyte quantity and quality that determine on-going pregnancy such as uterine factors and sperm factors the most important message in this statement remained that women who conceive at a young age through natural reproduction have the best chance of a healthy child.

AMH has proven to be a valuable marker for ovarian reserve and may aid in the reproductive planning for a woman. Presently there is a strong tendency to delay motherhood. AMH hormone seems to be the best endocrine marker, however, the valuable role of AMH and its role in ovarian function should be looked at in relation to the other markers to assess the decline of the ovarian pool. It has been critically discussed but as with any hormone the interrelation needs to be made in a clinical context together with specific normal ranges and an in-depth patient history.

AMH has low predictive precision for live births. Pregnancies have even been reported with very low AMH, indicating its inability to reflect on the quality of oocyte. Hence its use in ART should be aimed for effective designing of protocol and counselling, at the same time keeping in mind that patients should not be deprived of treatment on the ground of very low AMH. Further research on the significance of varying levels of AMH within the follicular fluid may pave the way to establish it as a marker of “quality” besides a quantity of the growing follicles.

Many clinical studies confirm that adding serum AMH testing to a complete ovarian assessment provides a powerful tool to help provide better healthcare for women. The benefits of this testing can enhance fertility treatments; leads to earlier diagnoses of PCOS, pre-ovarian insufficiency (POI), pre-ovarian failure (POF), and certain autoimmune conditions. This allows couples to better plan for procreation and menopause and allow for improved medical decision-making by monitoring ovarian ageing. Although there are challenges with inconsistency in AMH cut-off results, it is advisable to use AMH as a prerequisite together with the other clinical biomarkers.

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 ageing.
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. Low AMH and high FSH levels should not exclude patients from treatment. Other parameters such as AFC can help select treatment modalities together with AMH and FSH.
5. AMH level can help determine treatment protocol.

CHAPTER 7

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APPENDIX A

Dr Anil Ramdeo
Unit Manager: C.A.R.E Clinic

Re: Permission to recruit subjects

Title of the Research Study:The relationship between Anti-Müllerian Hormone (AMH) levels and the pregnancy outcomes in patients undergoing *in-vitro* fertilization (IVF) or intra-cytoplasmic injection (ICSI)

Principle researcher: Shalini Umarsingh
Co-Investigator/s/supervisor/s: Supervisor: Prof J K Adam

I am currently registered as a Masters student in Clinical Technology – Reproductive Biology. I am interested in performing a study which evaluates AMH levels in correlation to oocyte quality and a positive pregnancy outcome
Participants will be recruited from the C.A.R.E Clinic to participate in the study.Participation is purely voluntary and participants may withdraw from the study at any given time. All procedures shall be kept in order to ensure complete patient confidentiality and no names will be revealed in the publication of the results. There will be no risks or discomfort to participants as there will be no change to their normal dialysis prescription and treatment regimen.

In light of the above-mentioned, I would like to request permission to recruit participants from your institution for the above-mentioned study.
Yours Sincerely
Shalini Umarsingh
(Clinical Technology student researcher)

APPENDIX B

LETTER OF INFORMATION

Title of the Research Study: The relationship between Anti-Müllerian Hormone (AMH) levels and the pregnancy outcomes in patients undergoing *in-vitro* fertilization (IVF) or intra-cytoplasmic injection (ICSI)

Principal Investigator/s/researcher: Shalini Umarsingh (BTech:Clinical Technology)
Co-Investigator/s/supervisor/s: Supervisor: Prof JK Adam (DTech:Clinical Technology)
Co-Supervisor: Dr A Ramdeo (MBCHB, BAO, MR.COG)

Brief Introduction and Purpose of the Study: Welcome and thank you for being a part of my study. My name is Shalini Umarsingh and I am studying for a Master's degree at the Durban University of Technology.Many couples are affected by infertility. There are many causes of infertility both in men and women. Majority of fertility clinics carry out ovarian reserve tests (ORT) as part of the assessment of woman presenting with infertility. ORT tests are easy to perform and the decisions based on their results can help woman with normal or poor ovarian response. This can in turn aid couples in their chances of conceiving to prevent expensive and repeated treatments. The measurement of Anti-Müllerian Hormone (AMH) is now being used as one of the insights to ovarian reserve and thus oocyte quality. For clinicians these parameters are important as it aids in counseling patients regarding their prognosis in terms of ovarian response. Similarly the oocyte retrieved and their quality is also an important factor in determining a positive pregnancy outcome. The ability to foresee a poor response can be a valuable tool since poor responders have a lower likelihood of a pregnancy.

Outline of the Procedures:

(1). The blood samples will be taken at room temperature. All AMH levels will be determined using an ultra-sensitive enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter).

(2). ICSI/IVF will be used to fertilize the oocytes therefore all patients will undergo stimulation protocols to harvest a maximum number of oocytes.

(3). Oocyte retrieval will be carried out 36 hours after hCG administration. After 2-4 hours incubation, oocytes will be stripped of their cumulus complex using hyaluronidase and glass pipettes. Maturation and morphological features of oocyte will be recorded before the ICSI. Fertilization will be determined 19-21 hours after injection by the presence of two pronuclei. Early embryo development will be assessed by the number of blastomeres and the percentage of fragmentation 42-44 hours after fertilization. Blastocyst formation can be seen 114-120 hours after fertilization. A pregnancy test will be performed 14 days post transfer.

Risks or Discomforts to the Participant: There will be no risks or discomfort to you as there will be no change to your treatment.

Benefits: This can in turn aid couples in their chances of conceiving to prevent expensive and repeated treatments. The measurement of Anti-Müllerian Hormone (AMH) is now being used as one of the insights to ovarian reserve and thus oocyte quality. For clinicians these parameters are important as it aids in counseling patients regarding their prognosis in terms of ovarian response.

Reason/s why the Participant May Be Withdrawn from the Study: Your participation in this research is completely voluntary. You may withdraw at any time and this will not affect your treatment.

Remuneration: There will be no form of remuneration. Participation is voluntary.

Costs of the Study: You will not be asked to cover any cost relating to the study.

Confidentiality: All the information collected will be kept confidential. You will be allocated a number and all your details will be recorded under that number. This means that anyone who looks at my records will not be able to trace it to you. This is done to protect your privacy. In addition, a statement of confidentiality will be signed by both my supervisors and me.

Research-related Injury: There will be no research-related injury as there will be no alterations made to your treatment.

Persons to Contact in the Event of Any Problems or Queries:
Please contact the researcher (031 266 7938), my supervisor (031 373 5291) or DUT ethics administrator (031 373 2900).

APPENDIX C

CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, Shalini Umarsingh, about the nature, conduct, benefits and risks of this study.
- I have also received, read and understood the above written information (Participant Letter of Information) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

_____ _____ _____ _____

Full Name of Participant **Date** **Time** **Signature**

I, _____ (name of researcher) herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

_____ _____ _____

Full Name of Researcher **Date** **Signature**

_____ _____ _____

Full Name of Witness (If applicable) **Date** **Signature**

APPENDIX D

Department of Statistics

30 May 2016

To whom it may concern,

This serves to confirm that I, Professor G. Matthews of the abovementioned department will assist with the statistical analysis of Shalini Umarsingh for the qualification Master of Health Sciences in Clinical Technology – Reproductive Biology.

The title of the research is:

The relationship between Anti-Mullerian Hormone (AMH) levels and pregnancy outcomes in patients undergoing in-vitro fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI).

Yours sincerely

G. Matthews

HOD Statistics.

The departmental rate for the consulting service is R200 per hour.

APPENDIX E

Institutional Research Ethics Committee
Faculty of Health Sciences
Room MS 49, Mansfield School Site
Gate 8, Ritson Campus
Durban University of Technology

P O Box 1334, Durban, South Africa, 4001

Tel: 031 373 2900
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www.dut.ac.za

17 November 2016

IREC Reference Number: **REC 128/16**

Ms S Umarsingh
11 Latina Place
No. 8 The Heanings
Westville

Dear Ms Umarsingh

The relationship between Anti-Mullerian Hormone (AMH) levels and pregnancy outcomes in patients undergoing in-vitro fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI)

I am pleased to inform you that Provisional Approval has been granted to your proposal REC 128/16 subject to:

- Obtaining and submitting the necessary gatekeeper permission/s to the IREC.

Full approval is subject to meeting the above condition.

The Proposal has been allocated the following Ethical Clearance number **IREC 122/16**. Please use this number in all communication with this office.

Approval has been granted for a period of two years, before the expiry of which you are required to apply for safety monitoring and annual recertification. Please use the Safety Monitoring and Annual Recertification Report form which can be found in the Standard Operating Procedures [SOP's] of the IREC. This form must be submitted to the IREC at least 3 months before the ethics approval for the study expires.

Any adverse events [serious or minor] which occur in connection with this study and/or which may alter its ethical consideration must be reported to the IREC according to the IREC SOP's.

Please note that any deviations from the approved proposal require the approval of the IREC as outlined in the IREC SOP's.

Yours Sincerely

Professor M N Sibiya
Deputy Chairperson: IREC

DUT DURBAN UNIVERSITY OF TECHNOLOGY
2016 -11- 17
INSITUTUTIONAL RESEARCH ETHICS COMMITTEE
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