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This thesis is presented in partial fulfilment of the degree of

Master of Science (Human Biology)

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Abstract

The first trimester of pregnancy is a dynamic time of change involving the implantation and development of the fetus, together with a wide range of related endocrine changes arising from the ovary and feto-placental unit, as well as other changes in maternal and fetal physiology. The present longitudinal study has investigated two main areas of endocrinology in circulating maternal serum from positive pregnancy test to the detection of a fetal heartbeat, namely (i) AMH and (ii) thyroid function. Women attending Fertility North and conceiving during fertility investigation or treatment underwent phlebotomy twice weekly and AMH, TSH, fT3 and fT4 levels were measured, in addition to the reproductive hormones oestradiol (E2), progesterone (P4) and human chorionic gonadotropin (hCG).

AMH is a well-established biomarker for assessing the age-related decline of the oocyte pool in healthy women, but has many other clinical uses including the assessment of polycystic ovary syndrome (PCOS), estimating response to ovarian stimulation with exogenous gonadotrophins, detection of granulosa cell tumours and identification of premature ovarian insufficiency. AMH also influences follicle growth, but the precise mechanism by which it does this is unknown and its role during pregnancy has not been adequately characterised.

In this study the key findings regarding AMH were the differences between pre-ovulatory and luteal phase AMH levels in pre pregnant women and the three distinct patterns of changing AMH noted in women once pregnancy was confirmed. Prior to pregnancy, mean AMH levels peaked at time of ovulation (gestation week 2) and then dropped at mid-luteal phase (gestation week 3). 86.1% of women had their maximum AMH level at or before ovulation, with 13.9% of women having their maximum AMH at mid-luteal phase. However, once pregnancy was achieved, AMH levels for each woman did not remain constant, but moved significantly away from their first non-pregnant measurement, with a trend in viable pregnancies of an AMH level that either consistently increased or decreased from gestation week 4 (time of first positive hCG measurement) through to week 7. The difference between women with rising AMH compared to those with falling AMH significantly increased ($p=0.000$) during early pregnancy. In general terms women with rising AMH levels, had continually rising AMH

levels and these levels became increasingly divergent from those women who had falling AMH levels and whose AMH levels continued to decrease. In contrast, AMH levels in women with non-viable pregnancies were very inconsistent, with no definitive rising or falling trend observed. Instead, AMH levels in women with non-viable pregnancies showed erratic and sporadic changes, both rising and falling in the same individual from gestation weeks 4 to 7. There was a significant negative correlation between baseline (day 2 of the menstrual cycle) AMH and patient age (r=-0.507, p=0.000). No associations were observed between AMH and patient body mass index (BMI), stimulation medications (follicle stimulating hormone dose), treatment type or fetal sex. There were weak negative correlations between AMH and P4 (r=-0.220, p=0.000,) and TSH (r=-0.155, p=0.001), but not E2, hCG, fT3 or fT4. This study was not designed to determine causative factors for the AMH changes observed and further investigations would be required to address possible causes of these reported changes.

The importance of normal maternal thyroid function in pregnancy and fetal development is well characterised and thyroid dysfunction can result in adverse effects on the unborn child, including a higher risk of miscarriage. Throughout the first trimester, the fetus is dependent on maternal thyroxine as the fetal thyroid is not fully functional until approximately gestation week 16. Despite this important role, the changes, if any, occurring during this vibrant period are poorly documented and often are limited to cross-sectional sampling.

In this study the key findings regarding thyroid function were of stable fT3, fT4 and TSH levels between gestation weeks 0 to 4 (pre pregnancy) with subsequent changes in thyroid function once pregnancy was established at gestation week 4 (hCG >25mU/ml). From gestation weeks 4 to 6.5 some significant changes in thyroid hormone levels were observed with a gradual decrease in fT3 (r=-0.104, p=0.005) and TSH levels (r=-0.123 p=0.013). In contrast, fT4 levels remained constant during early pregnancy (gestation weeks 4 to 6.5). No markers of thyroid function appeared to affect pregnancy outcome. Despite TSH levels ranging from 0.27 - 4.93mU/L, only 6 patients (7.0%) had TSH levels >4.0mU/L, of whom only 3 (3.5%) miscarried. Reference ranges were calculated for fT3, fT4 and TSH for gestation weeks 4 to 6.5 and were

found to be comparable to later first trimester ranges reported in other studies. There were two patients who tested positive for thyroid peroxidase antibody (TPOAb) who were excluded from the study (both were viable pregnancies with normal thyroid hormone levels).

In summary, this study was able to directly follow the changes in AMH and thyroid hormone levels within the same individual over time, resulting in the identification of unique changes in very early pregnancy.

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CHAPTER 1

INTRODUCTION

1 Introduction

A human pregnancy is a time of dynamic physiological change, and its success relies on the correct balance of hormones to support and maintain the growing fetus. Extensive research investigating these hormones has allowed clinicians to increase women's chances of conceiving and maintaining a pregnancy, by closely monitoring these changes and providing supportive care to improve the pregnancy outcome.

There are a growing number of couples who rely on assisted reproductive technology (ART) in order to conceive. There were 61,158 ART cycles performed in Australia in 2011, representing an increase of 8.3% over the previous year [1]. Couples are having children later in their lives and this can have a detrimental effect on their ability to conceive [2]. A woman's fertility starts to decrease from her early twenties and, as a result, many women are diagnosed with fertility issues when they begin trying to conceive. The median age of all mothers who gave birth to their first child was 28.9 years in 1993, rising to 29.5 years in 1998 and 30.8 years in 2013 [3]. Unfortunately, postponing childbirth to an age at which female reproductive capacity is reduced often leads to involuntary childlessness. In addition to an increased number of women having difficulties conceiving, there are also a large percentage of miscarriages or pregnancy losses during early gestation. Although there are many factors which can contribute to an increased risk of miscarriage, such as higher age, smoking and pre-existing medical conditions, a large number of pregnancy losses remain unexplained [4].

The monitoring of reproductive hormones in pregnancy is a useful tool for identifying women who may benefit from therapeutic interventions, for example progesterone supplementation to reduce the risk of miscarriage [5]. This is particularly relevant in patients undertaking IVF where the ovaries are manipulated via exogenous hormones to stimulate the uterus and support the pregnancy in the early stages [5]. Thyroid disorders have a great impact on pregnancy outcome, however monitoring of these hormones in early pregnancy is not routine due to the low percentage of women that are affected.

Routine reproductive hormone assays form part of a patient's treatment at Fertility North and these blood samples were further analysed to detect any changes in AMH and thyroid hormone levels during early pregnancy, in addition to the monitoring of the standard reproductive hormone levels (E2, P4 and hCG). Normal reference ranges for thyroid hormones fT3, fT4 and TSH were established for gestation weeks 4 to 7.

1.1 The Female Reproductive System

1.1.1 Folliculogenesis

The main components of the female reproductive system are the gonads (ovaries) and uterus. The primary functions of the ovaries are to produce mature oocytes in a process termed oogenesis, and produce hormones to prepare the uterus for incubation of one or more embryos [6-8].

The follicles are the primary functional units of the ovaries. In a developing embryo, germ cells populate the ovary and begin forming primordial follicles, which are comprised of small, non-growing functionally immature oocytes surrounded by a single layer of squamous granulosa cells [9]. Development of primordial follicles begins half way through gestation and completes shortly after birth, when there are approximately one million primordial follicles in each ovary [10, 11].

As women mature, their primordial follicles are released from dormancy and are recruited to the growing follicle pool, of which only about 400 will mature and ovulate [11]. The majority of a woman's follicles will degenerate prior to puberty at approximately 10-11 years of age, by a process called atresia [12]. Once a follicle is recruited, it goes through various stages of growth and development necessary for ovulation and potential fertilisation and this is termed folliculogenesis. Folliculogenesis is characterised by the proliferation and differentiation of granulosa and theca cells [13].

The pre-antral stage of folliculogenesis commences at puberty, when a cohort of primordial follicles is recruited during each menstrual cycle and the granulosa cells undergo rapid proliferation. Pre-antral follicles grow to between 0.1–0.2 mm in

diameter, are not regulated by gonadotrophins and have limited capacity for hormonal production. Instead, oocyte-derived growth factors including two members of the transforming growth factor (TGF)β super family, growth and differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) have been implicated in the regulation of early follicle growth [14, 15].

The antral stage of follicular development is characterised by follicles growing to 0.2 - 0.4mm in diameter [13]. This phase of follicle recruitment is dependent on the hypothalamic–pituitary–gonadal endocrine axis [16]. The granulosa cells at this stage develop receptors for follicle-stimulating hormone (FSH) and oestrogen, while the thecal cells develop receptors for luteinizing hormone (LH) [13, 17]. Most of the growing follicles will undergo atresia, unless they are rescued by FSH [18-20]. Among the group of rescued follicles, usually only one follicle is selected to become the dominant follicle. This follicle is characterized by a fluid-filled cavity or antrum and is called an antral or Graafian follicle [13]. One study postulated that the future dominant follicle contains more granulosa cells and therefore FSH receptors, making it more sensitive to FSH, compared with the remaining subordinate follicles that become atretic [21]. This dominant follicle will then release its oocyte at ovulation under the influence of LH [22]. The cyclic recruitment of follicles continues until the growing follicle pool is exhausted, folliculogenesis ceases and this time coincides with the onset of menopause.

1.1.2 Reproductive Hormones in the Menstrual Cycle and Pregnancy

The menstrual cycle describes the changes that occur in the uterus and ovary that enable a fertile female to reproduce. Menarche is the term used to describe a female's first menstrual cycle, which usually occurs around the age of 12 [23]. The menstrual cycle can vary in length, but on average takes 28 days [24] and is regulated by a complex interplay of endocrine factors and hormones that results in ovulation and the maintenance of an embryo through to a foetus if there is a successful fertilisation.

In the absence of an implanted embryo, the endometrium undergoes apoptosis and sheds its inner lining. This stage of the menstrual cycle is termed menses and this

menstrual fluid is composed of inflammatory exudate, red blood cells, and proteolytic enzymes [25]. This 'bleeding' typically takes around four days and marks the start of each menstrual cycle [26]. The proliferative phase of the menstrual cycle lasts on average fourteen days and is characterised by the build-up of the stratum functionalis of the endometrium of the uterus, driven by follicle growth and recruitment in the ovary [25]. Increasing amounts of oestrogen are secreted by growing follicles, resulting in a negative feedback on FSH secretion from the pituitary gland [27].

Gonadotrophin-releasing hormone (GnRH) is secreted by the hypothalamus which triggers the anterior pituitary to release LH and FSH. These hormones work in conjunction with each other to stimulate follicle development, assist in their recruitment and maturation and contribute to the production of oestrogen and triggering the final release of the egg from the ovary [28]. It is thought that ovulation, which occurs around day 14-15 of the menstrual cycle, occurs due to an increased release of GnRH, causing a surge in LH [29]. The remnants of the follicular granulosal and thecal cells after the oocyte is released, form the corpus luteum (CL) in the ovary [30].

The luteal phase of the menstrual cycle occurs approximately from days 14-28 and during this time, the CL is responsible for secreting the hormones progesterone and oestrogen, along with relaxin and inhibin [31-33]. The release of progesterone triggers stromal differentiation and stimulates glandular secretions in conjunction with the build-up of basal vacuoles in the glandular epithelium to provide an environment that will maintain early embryonic development [34].

Once the CL has matured and reached its maximal potential for secretion of progesterone, luteal function is maintained for a few days and then if fertilisation of an oocyte and implantation of the resulting embryo does not occur, the CL regresses to become the corpus albicans, causing a drop in oestrogen and progesterone levels [30]. The unfertilised egg passes through the uterus during menses and the cycle begins again.

If implantation occurs, the trophoblast cells of the resultant embryo release human chorionic gonadotrophin (hCG) which directly stimulates the CL to continue to secrete progesterone and oestrogen and sustain the pregnancy for approximately the first three months, until the placenta takes over in synthesising and releasing these hormones [35]. A positive pregnancy can usually be determined at about gestation week 4, when a blood or urine test detects the presence of hCG, indicating a successfully implanted blastocyst and commencement of embryogenesis [36].

1.2 Anti-Müllerian Hormone (AMH)

1.2.1 AMH Physiology and Ovarian Function

AMH is a member of the transforming growth factor β family of peptide growth and differentiation factors [37] and is produced by granulosa cells of small ovarian follicles [38]. AMH plays an essential role in ovarian folliculogenesis, by inhibiting the recruitment of primordial follicles into the pool of growing follicles and decreasing the responsiveness of growing follicles to FSH [39].

AMH is expressed in the growing follicles of the ovary until they have reached the size and stage for dominant selection [40]. AMH expression in a follicle peaks once it reaches the preantral and small antral stages, then gradually diminishes as follicles grow to the larger antral and preovulatory stages [41].

1.2.2 AMH in a Woman's Life Cycle

AMH levels throughout a woman's life are strongly correlated with the different stages of follicle development and the numbers of non-growing follicles. One study [42] measured AMH levels in healthy females from birth to the end of their reproductive life. At birth, AMH levels were undetectable, followed by a small increase in the levels a few weeks after birth. This was then followed by a gradual increase in AMH levels during childhood until early adolescence, reaching a maximum level at 15.8 years old. Thereafter, AMH levels remained stable until a woman reached her early 20s, and then from the age of 25 years, AMH levels progressively declined throughout a woman's reproductive life until menopause.

1.2.3 AMH as a Marker of Ovarian Reserve and Ovarian Response in Assisted Reproduction

Serum AMH concentrations have been correlated with the number of antral follicles present in the ovary, such that low levels of AMH can often indicate low ovarian reserve whilst a high level may indicate polycystic ovary syndrome (PCOS) [43]. AMH has been used as a predictor of ovarian responsiveness to exogenous gonadotrophins in fertility treatments such as IVF, being a better predictor than FSH, inhibin B or oestradiol [44]. Many groups have since reported on the capacity of AMH to predict ovarian response and/or IVF/ICSI cycle outcomes, being a better marker of ovarian reserve than increasing age or antral follicle count (AFC) [45, 46]. Algorithms have been devised showing the changing levels of AMH at different stages of life [47, 48]. However, this presumes that the levels are a function of the maturing and senescent ovary, simply reflecting the ovarian reserve. Unfortunately, this is not the case and production of AMH has now been shown to vary in differing physiological states when the ovarian reserve is constant, such as during the menstrual cycle [49] and whilst using the contraceptive pill [50].

1.2.4 AMH in PCOS

PCOS is the most common endocrine disorder in women of reproductive age [51] and the primary cause of anovulatory infertility and hyperandrogenism [52]. Women with PCOS show markedly raised AMH levels compared to normoovulatory women [53, 54] which is largely attributable to the increased number of small antral follicles in the ovary and characteristics of the granulosa cells, resulting in anovulation [55]. AMH levels are thought to be directly related to the severity of PCOS, with higher levels indicating more problems in folliculogenesis and granulosa cell function [56].

1.2.5 AMH in Pregnancy

Despite extensive research into the roles of AMH in ovarian reserve, ovarian responsiveness to gonadotrophin stimulation and pregnancy outcomes in assisted reproductive methods, there are only a few studies on AMH levels during pregnancy and the findings are inconsistent. The first investigation was a cross-sectional study and only defined the subjects according to whether they were in the first, second or third trimester of pregnancy [57]. There were no changes seen but it is likely that the study design may have limited the likelihood of small changes being detected. AMH

during pregnancy has been studied again more recently using a longitudinal design to follow individual women, although again the sampling for each woman was only done once in each of the three trimesters [58]. There was a significant reduction in AMH as the pregnancy progressed, returning to baseline levels following delivery. A decline of AMH levels in the first trimester was also noted in another cross sectional study [59]. However, neither of these studies was able to examine any possible changes in AMH over the first few weeks of pregnancy when there are significant changes in the hormonal milieu.

1.2.6 Effects of Contraceptive Pill on AMH

The combined oral contraceptive pill (COCP) has been used by women as an effective means of preventing pregnancy for over four decades. A study in five European countries estimated that over 22 million women use the COCP yearly [60]. The COCP is comprised of synthetic oestrogen and progesterone which when taken in accordance with the manufacturer's directions, will suppress the hypothalamic-pituitary-ovarian axis to inhibit ovulation [61], by preventing follicular growth and maturation and also affecting the proliferation of the endometrium [62, 63].

The effect of taking the oral contraceptive pill on levels of AMH is debateable. In the past, women have been tested for ovarian reserve whilst taking the oral contraceptive pill, because AMH levels were thought to be unaffected [64-66]. However, there are conflicting reviews that show AMH levels are reduced whilst taking oral contraception [67-69] and increased after ceasing oral contraception [50]. This could indicate that AMH levels are not only an indicator of remaining follicle numbers, but also that AMH levels may be influenced by the state of follicles, which will be important to note when measuring AMH levels during pregnancy when follicle growth is suppressed.

The COCP is often used in conjunction with a GnRH agonist in IVF cycles to improve the outcome of controlled ovarian hyperstimulation and better time oocyte retrieval by preventing a premature LH surge [70]. Oral contraceptive pre-treatment has been used to improve the outcome in poor responders to ovarian stimulation by reducing the amount of gonadotrophins and period of time required for ovarian stimulation [71, 72]. Oral contraception is also used to reduce the incidence of ovarian hyperstimulation syndrome (OHSS) in high responders [73] and to avoid cyst formation after agonist administration [74]. It will be important to note in the present study

whether a patient has been taking oral contraception in their IVF or ICSI treatments, as AMH levels may be influenced.

1.2.7 Basis for Investigating AMH in Early Pregnancy

FSH and LH are supressed in pregnancy to prevent ovulation and risk of multiple conceptions [75]. FSH is important in development of the maturing follicle, including the formation of AMH-releasing granulosa cells. Since there is thought to be no follicular activity during pregnancy one might expect minimal changes in AMH. The same principal should apply during OCP use when gonadotropins are supressed, however there is evidence of a decrease in AMH levels [49]. There is also evidence that ovarian follicular development is not completely inhibited during OCP use [76]. A longitudinal study observed a decrease in AMH in each trimester during pregnancy and stated that there is a reduced follicular maturation during pregnancy (and consequently less AMH-producing follicles), however since AMH did not reach undetectable levels, there is likely some follicular activity present during pregnancy [58]. Therefore the benefit of this present longitudinal study investigating the changes in serum AMH concentrations during pregnancy will hopefully gain further insight into the possible changes in follicle growth and other factors that may affect AMH expression.

1.3 Thyroid Hormones

1.3.1 Thyroid Function in Pregnancy

Under the influence of thyrotropin-releasing hormone (TRH) from the hypothalamus, thyroid stimulating hormone (TSH), also known as thyrotropin, is secreted by thyrotrope cells in the anterior pituitary gland, stimulating the thyroid gland to produce T4 (thyroxine), which is then converted to T3 (triiodothyronine) - the active hormone responsible for cell metabolism [77]. Maternal thyroid hormones play a critical role in fetal development, since the fetal thyroid gland does not become a functional until the second trimester. In pregnancy, increased maternal T4 levels are vital to the developing fetus, particularly during embryonic and fetal nervous system development in the first twelve weeks [78].

TSH levels are known to decrease in pregnancy, particularly during the first trimester, correlated with an increase in human chorionic gonadotropin (hCG) [79]. The decrease in TSH is most likely due to a weak stimulatory effect of hCG on TSH receptors on the thyroid. The most commonly accepted reason for TSH reducing slightly in the first trimester is that there is homology between the alpha subunits of both TSH and hCG [80]. As such, hCG can act on TSH receptors at the thyroid and stimulate them, thereby increasing T4 and T3. This feeds back to the hypothalamus, decreasing TRH, TSH and, in turn, decreasing T3 and T4 as part of the natural negative feedback loop. TSH is therefore lowest when hCG is at its highest because hCG rises until 8-10 weeks of pregnancy and then plateaus and starts falling when TSH starts recovering. Such changes during pregnancy have resulted in the need for pregnancy-stage specific reference ranges to prevent the misdiagnosis of disease [81-83].

All studies on the change in thyroid function tests in pregnancy are cross-sectional in design, using blood obtained from pathology laboratories undertaking first trimester screening [81]. Whilst providing practical guidance on the interpretation of clinical screening tests, they do not describe the changes that may occur during early pregnancy, nor are there established reference intervals at this time during pregnancy.

1.3.2 Thyroid Disease in Pregnancy

Thyroid disease is the second most common endocrine disorder affecting women of reproductive age and when left untreated during pregnancy, can lead to a multitude of adverse effects including an increased risk of miscarriage, placental abruption, hypertensive disorders and fetal growth restriction [84].

Frank hypothyroidism, sometimes referred to as an underactive or low thyroid, is characterized by a low fT4 and raised TSH levels. In contrast, subclinical or asymptomatic hypothyroidism is characterized by slightly to moderately raised TSH only and normal fT4 levels. The incidence of hypothyroidism during pregnancy is approximately 0.3% to 0.5% and 2% to 3% for subclinical hypothyroidism [85]. Since high maternal TSH levels are known to increase the risk of miscarriage in women with subclinical hypothyroidism [86], this could be a contributing factor to supposedly unexplained miscarriages in women not displaying any symptoms of thyroid dysfunction.

Hyperthyroidism occurs with an overactive thyroid. Primary hyperthyroidism results in an increase of fT4 and fT3 and a suppressed TSH level, whereas subclinical hyperthyroidism is a milder form of hyperthyroidism and is characterized by a suppressed TSH level, but with a normal fT4 and fT3 level [87].

Graves' disease is the most common cause of hyperthyroidism. Hyperthyroidism occurs in about 0.2% of pregnancies [85]. Subclinical hyperthyroidism is not thought to be associated with adverse pregnancy outcomes [88] and therefore treatment is not recommended.

1.3.3 Thyroid Autoimmunity in Pregnancy

Thyroid autoimmunity is characterized by the presence of antithyroid antibodies. Of particular interest is the marker antithyroid peroxidase which is responsible for thyroid hormone synthesis [89]. Approximately 10-20% of euthyroid women (having TSH within a defined normal range) are positive for thyroid peroxidase antibody (TPOAb) [90, 91], of which 16% will develop a TSH that exceeds 4.0mlU/L by the end of the third trimester. About 50% of TPOAb positive women will develop postpartum thyroiditis, which is a consequence of the immunological changes that occur during pregnancy [92]. Therefore TPOAb is a strong risk factor for thyroid dysfunction both during and after pregnancy [93].

There is evidence that women who test positive for TPOAb during pregnancy tend to have higher TSH levels, which may indicate a slight impairment of thyroid function [94-97]. Numerous studies have reported that TPOAb positive women have an increased risk of miscarriage [98-103] as well as an increased risk of preterm birth [104]. Thyroid autoimmunity is associated with infertility [105], which may be attributed to a higher prevalence of endometriosis in infertile women [101, 106]. The association between thyroid autoimmunity and endometriosis is likely due to an autoimmune dysfunction in the pathogenesis of both diseases, although the exact mechanisms are unknown. There is evidence of increased secretion of pro-inflammatory cytokines and compromised function of cell-mediated natural immunity in endometriosis [107].

In summary, women who test positive for TPOAb should be excluded from thyroid hormone studies, as these women may have higher TSH levels and a greater risk of miscarriage. More importantly, inclusion of these women when calculating thyroid reference intervals could result in falsely high values.

1.3.4 Thyroid Disease Screening, Diagnosis and Treatment in Pregnancy

Adequate treatment of thyroid conditions in pregnancy, particularly during early gestation greatly minimises pregnancy risks and complications, resulting in significantly improved outcomes and highlighting the need for correct diagnosis. Despite this need for detection of thyroid dysfunction in pregnancy, diagnosis can prove challenging, mainly due to reference interval variations between methods, but also due to varying TSH levels at different stages of pregnancy, variations in TSH in different aged women and between populations of different ethnicity [108].

There have been numerous studies aimed at determining reference intervals for thyroid function in pregnancy using different assays [82, 97, 109-117], with significantly variable results. One study that compared the reproducibility of thyroid reference ranges in pregnant women between two different laboratories, observed up to 100% of maternal fT4 levels fell outside the other group's reference range despite similar TSH levels [118]. This further emphasises the need for assay-specific pregnancy reference ranges to minimise the misclassification of thyroid disease.

The symptoms of thyroid disease can often be mistaken for the usual symptoms of pregnancy, making it even more challenging to identify. Furthermore, the 2012 Endocrine Society guidelines recommend screening only pregnant women at high risk of thyroid disease, which includes women with a history of previous thyroid dysfunction, goitre, positive thyroid antibodies, cervical irradiation, thyroid surgery, family history of thyroid disease, presence of clinical signs or symptoms of hypothyroidism, diagnosis of type 1 diabetes mellitus or any other autoimmune disease, history of repeated abortions, prematurity, or infertility, morbid obesity, treatment with lithium, amiodarone, living in an area with moderate to severe iodine deficiency and also if a woman is over 30 years of age [103]. One study found that by selectively screening only high-risk patients, 30% of those with subclinical

hypothyroidism would be missed [119]. Despite this, another randomised controlled trial of 4,562 women did not show a reduction in adverse outcomes in those who were universally screened compared to case finding [120].

The clinical classification of thyroid dysfunction has been debated for over a decade. Only recently has a general consensus been reached that the upper limit for TSH in pregnancy is lower than in non-pregnant adults. A generally accepted upper reference interval for TSH in the first trimester of pregnancy is an upper limit of 2.5 mIU/L [92]. Researchers have investigated the treatment and pregnancy outcomes of pregnant women with TSH levels elevated above this cut-off range. Treating thyroid antibody-positive women with Levothyroxine results in a significant decrease in maternal and neonatal complications [121]. One study group found that pregnant women with first-trimester TSH levels between 2.5 and 5.0 mIU/litre were 70% more likely to have fetal loss than euthyroid women [120]. Abalovich and colleagues [122] showed that a successful pregnancy wasn't dependent on whether a hypothyroidism diagnosis was overt or subclinical, but mainly on the treatment received. Despite the known adverse effects of thyroid disease during pregnancy, the prevalence of the disease is low and further research is needed on pregnancy outcomes and treatment of women with thyroid disease.

The issue of whether to perform systematic screening for thyroid hormones in pregnancy remains largely controversial. Studies are now directing focus to the consequences to the mother and unborn child of untreated subclinical hypothyroidism and subclinical hyperthyroidism. Universally accepted reference ranges for thyroid function tests during pregnancy are still needed. Until then, laboratories need to adapt their own assay-dependent, gestational age specific and population specific reference ranges for thyroid testing in pregnant women [90].

1.4 Infertility

1.4.1 Definition and Causes of Infertility

Fertility is ultimately assessed by a successful pregnancy. However, definitions of infertility vary widely, impacting on estimated prevalence. The International

Committee for Monitoring Assisted Reproductive Technology and the World Health Organization (WHO) define (clinical) infertility as 'a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse' [123]. For couples who are diagnosed as infertile, further assessments are required to determine the underlying cause or causes and recommend possible treatment options. Worldwide there are approximately 72.4 million couples that experience fertility problems annually [124]. In Australia, approximately one in six couples do not conceive in their first year of trying [125].

There are many factors which contribute to infertility. Approximately one third of cases of infertility are the result of female related factors, one third to male related factors and one third are attributable to both partners or remain unexplained [126]. The most common causes of infertility are advanced maternal age [127], female reproductive factors such as endometriosis, polycystic ovary syndrome and menstrual cycle irregularities [126] and sperm production problems and blockage of sperm transport in men [128]. Lifestyle factors in both genders are known to increase the risk of infertility, some being drinking alcohol, smoking, higher body mass index (BMI) and sexually transmitted diseases [129].

Humans are not particularly efficient at reproduction compared to other mammals. The chance of getting pregnant (fecundity) is between 25 to 30% each month for the first 3 to 6 months of trying [127]. The chance of conceiving dramatically decreases once a woman reaches 31 years of age [130-132] due to a diminishing ovarian reserve, which poses a problem for many couples who do not try to have children until they reach their thirties.

1.4.2 Reproductive Ageing and Ovarian reserve

Reproductive ageing is associated with reduced fertility and the ovarian and hormonal changes that occur in the lead up to menopause, when the permanent cessation of menstruation resulting from the loss of ovarian follicular activity occurs [133]. Decreasing female fertility and ovarian ageing corresponds to a decrease in follicle

numbers and oocyte quality [134]. Depletion and resistance of the ovarian follicular pool leads to reduced inhibin B production, reduced negative feedback at both pituitary and hypothalamic levels, and elevated FSH and LH levels in the perimenopause stage [135, 136].

Ovarian reserve is the term used to describe the remaining follicles in the ovary and reflects the reproductive age of a woman [137]. A diminished ovarian reserve (DOR) is strongly correlated with advanced maternal age [138]. Assessing ovarian reserve is a useful tool in the management and treatment of patients undergoing assisted reproduction. In the past, FSH has been the primary hormone used to assess ovarian reserve and it is still routinely measured in the early follicular phase when investigating infertile women. Women who have regular menstrual cycles but exhibit raised basal FSH levels, will usually have a reduced ovarian reserve and are at a higher risk of infertility [139, 140].

AMH has recently been shown to be an earlier indicator of ovarian reserve compared to FSH, despite both hormones being associated with the changes that occur in menopausal women [141]. AMH levels are declining soon after a woman's menstrual cycle becomes irregular which can occur in her mid to late thirties, whereas elevated levels of FSH may not appear until after a woman reaches 45 years of age [135].

1.5 Assisted Reproductive Technology (ART)

1.5.1 Definition of ART and Procedures

Assisted reproductive technology (ART) is a term used to describe a group of procedures involved in the *in vitro* (in glass) handling of human oocytes, sperm or embryos for the purpose of achieving a pregnancy [123]. Approximately 4.1% of all women who gave birth in Australia in 2010 received some form of ART treatment [142].

The 2011 report from the Fertility Society of Australia (FSA), in collaboration with the University of New South Wales (UNSW), categorise ART treatments according to

whether a woman used her own oocytes or embryos in an autologous cycle, or alternatively if oocytes/embryos are donated by another woman/couple and whether the embryos are transferred soon after fertilisation (fresh cycle) or following cryopreservation [1]. In 2011 there were 66,347 ART treatment cycles performed in Australia and New Zealand, of which 95.1% of cycles were autologous and 33.7% of all cycles used embryos thawed from cryopreservation [1].

Most people associate ART with the typical *in vitro* fertilisation (IVF) procedure which encompasses controlled ovarian hyperstimulation, oocyte collection and fertilisation with sperm in the laboratory, embryo maturation and then transfer of the fresh embryo into the uterus [143]. A variation to this procedure is intracytoplasmic sperm injection (ICSI), which involves a single sperm injected directly into the oocyte [144]. Male factor infertility is one of the primary causes of difficulties achieving a pregnancy in couples [145] and ICSI has resulted in increased fertilisation and pregnancy rates in cases where male sperm motility and quality were involved [144, 146].

Embryo cryopreservation plays a significant role in ART treatments. Frozen embryo transfers (FET) are performed when embryos not transferred in the initial fresh treatment cycle are cryopreserved, stored and once thawed can be transferred in subsequent treatments. This provides more opportunities for patients to achieve a pregnancy if their first attempt is unsuccessful, thereby improving cumulative pregnancy rates [147, 148].

Other treatments which help infertile couples achieve a pregnancy include ovulation induction in which the ovaries are stimulated by administering drugs such as clomiphene or commercially produced FSH and LH injections in conjunction with timed intercourse, or alternatively coupled with intrauterine insemination whereby semen is prepared in the laboratory into a highly concentrated sample and then placed directly into the female genital tract (intracervical insemination [ICI] or intrauterine insemination [IUI]) [149].

Less common ART treatments include gamete intrafallopian transfer (GIFT), when mature oocytes and sperm are placed directly into a woman's fallopian tubes so that

fertilisation may take place in vivo (inside the body), zygote intrafallopian transfer (ZIFT), when fertilisation takes place in vitro then transferred back to the fallopian tubes and finally surrogacy arrangements, where a woman agrees to carry a child for another person or couple with the intention that the child will be raised by the intended person or couple [1].

1.5.2 Risks Associated with ART

Optimal evaluation of women and proper treatment are essential for a successful outcome of ART and to minimise risks to the mother and unborn child. One risk associated with ART is the increased risk of multiple gestation pregnancy, which in turn can increase the health risks to both mothers and babies. These risks include pregnancy and birthing complications, preterm delivery and low birthweight babies [150, 151]. The risks in Australia have greatly minimised, since the percentage of single embryo transfer cycles has increased from 63.7% in 2007 to 73.2% in 2011 [1]. Although there is a worldwide push to decrease the numbers of embryos transferred after IVF, there still remains significant differences in practice between countries. The policies regarding the number of embryos transferred in ART vary, therefore statistics on multiple births are highly variable [152, 153].

Controlled Ovarian Stimulation (COS) is a process which is required for ART treatments such as IVF and ICSI, where a high oocyte yield increases the chances of pregnancy by having more embryos available for cryopreservation for use in subsequent cycles if the first transfer is not successful [154] . COS involves priming the ovaries, usually by administering FSH and then a trigger to induce final follicular maturation. A serious complication or risk involved in COS is ovarian hyperstimulation syndrome (OHSS), which affects approximately 1.4 % of all cycles [155]. In OHSS, the protein-rich fluid shifts from the intravascular space (blood vessels) to the transcellular space in the abdominal cavity as a result of increased vascular permeability following artificial stimulation with human chorionic gonadotropin (hCG) or later in pregnancy when there is a natural rise in hCG levels [156]. Symptoms of OHSS range from mild abdominal distention due to enlarged ovaries alone or with an accompanying fluid shift into the abdomen, to ascites, liver dysfunction, renal failure [157] and potential

death as a result of haemoconcentration and reduced perfusion of vital organs such as the brain [158].

There are many precautions that are taken to minimise the risks of OHSS, some of which can include identifying high risk patients by measuring baseline AMH levels prior to treatment to predict responsiveness to ovarian stimulation [159, 160], measuring basal FSH, BMI, age and the number of follicles <11 mm [161], cycle cancellation[162], withholding gonadotropin administration [163], cryopreservation of all embryos [164], intravenous albumin administration [165] and the use of the dopamine agonist cabergoline to suppress the pituitary [166, 167]. More recently, administering gonadotropin-releasing hormone (GnRH) agonist as an oocyte trigger in GnRH antagonist cycles instead of an hCG trigger has been shown to reduce the risk of OHSS, but has also been associated with lowered live birth rates and ongoing pregnancy rates, so should only be used in women with a higher risk of OHSS [168].

CHAPTER 2

AIMS AND HYPOTHESES

2 Aims and Hypotheses

The proposed study uses automated immunoassay technology to measure concentrations of circulating AMH, thyroid (TSH, fT3, fT4) and reproductive (E2, P4 and hCG) hormones in women during early pregnancy (gestation week 4 to 7). The aims were structured to test a number of null hypotheses as described below.

2.1 Aims

The specific aims of the study were to:

1. Evaluate the performance of the immunoassays used to measure these hormones.
2. Compare the hormone concentrations in pregnancy to those seen before the pregnancy was diagnosed.
3. Describe changes in these hormone concentrations from the time of a positive pregnancy (two weeks after ovulation, or gestation week 4 using the obstetric convention of time from the last menstrual period) to gestation week 7.
4. Identify any association between these hormones in pregnancies that are either viable or result in miscarriage.

2.2 Hypotheses

This study will test the following null hypotheses:

1. The immunoassays used to measure these hormones do not perform well in the ranges of concentrations found in the study samples.
2. The reproductive hormones do not follow the classical patterns seen before and during pregnancy.
3. AMH and thyroid hormone levels do not change following diagnosis of pregnancy or during pregnancy.
4. There is no association between AMH or thyroid hormones in pregnancies that are either viable or result in miscarriage.
5. There is no association between any of the reproductive and study hormones during pregnancy.

CHAPTER 3

MATERIALS AND METHODS

3 Materials and Methods

3.1 Ethics

Ethical approval to undertake this research project was given by the Joondalup Health Campus Human Research Ethics Committee [JHC HREC, Reference 1414] and the Edith Cowan University Human Research Ethics Committee [ECU HREC, Project 12077] as shown in Appendices A and B respectively.

3.2 Study Participants

3.2.1 Selection and Recruitment

Women who attended Fertility North for fertility treatment were recruited on the basis of a positive pregnancy blood test, namely if the serum hCG concentration was >25 IU/mL. Women with a history of thyroid disease (ie pre-pregnancy baseline serum TSH concentration outside the normal range 0.4-4.0 mU/L or outside the specific pathology reference range for normal thyroid levels), as well as women taking thyroid medication such as thyroxine were excluded from the study. Women who tested positive for TPOAb were excluded prior to the analysis of thyroid hormone reference ranges. All women were confirmed to be non-smokers, of an acceptable weight range to commence ART in the clinic (BMI <35) and negative for HIV, Hepatitis B or C and Syphilis, and positive for Rubella immunity.

The clinic's electronic diary (Genie; Genie Solution Pty Ltd, Indooroopilly, Queensland) was used to obtain each patient's medical history (oral contraceptive use prior to recruitment, fertility medications during their treatment cycle and starting body mass index or BMI) and keep track of their scheduled phlebotomist appointments on any given day.

Once a woman was identified as being pregnant, a note was put on her next phlebotomist appointment on Genie, at which time the patient was presented with the project information sheet (Appendix C) and invited to participate in the study. Once the patient signed the consent form (Appendix D), her name was added to an Excel spreadsheet of recruited patients.

3.2.2 Treatment Cycles

All women recruited had regular pregnancy monitoring, however patients were divided into four main groups based on the type of treatment they received leading up to the time of conception. The first group of women were tracked during their natural menstrual cycle for timed intercourse at the time of ovulation and were not prescribed any medications. There were 15 women recruited in the ''Natural pregnancy cycle" group.

The second group of women received high doses of stimulation medication, including recombinant FSH, to produce multiple follicles for collecting and subsequent *In-vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) treatment. There were 25 women recruited in the "IVF/ICSI pregnancy cycle".

In the third group of women, one or more embryos were implanted back into their uterus via a Frozen Embryo Transfer (FET) if the first fresh embryo transfer from an IVF or ICSI cycle was unsuccessful. FETs were performed in either natural or controlled medicated cycles (used to better time the embryo transfer). There were 25 women recruited in the "FET pregnancy cycle".

Finally, the fourth group of women were administered low doses of stimulation drugs to produce one follicle (OI or Ovulation Induction) for either timed natural intercourse or artificial insemination (AI), where the semen was produced and prepared by the laboratory before being administered into the uterus at the time of ovulation. There were a total of 7 "AI pregnancy cycles" and 13 "OI pregnancy cycles".

3.3 Hormone Assays

3.3.1 Sample Collection and Processing

All patient blood samples were taken at Fertility North by the clinic's phlebotomists between 7:00am and 9:00am according to the clinic's Standard Operating Procedure (SOP) "Venepuncture – The Collection of Blood Samples" (Appendix E) and processed according to "Patient Identification, Blood Sample Acceptance and Preparation for Analysis" SOP (Appendix F)." Blood samples were centrifuged for 10 minutes at 3,700 rpm using a Beckman Coulter Allegra X-30 centrifuge.

Bloods samples were collected twice weekly from gestation week 4 until fetal heartbeat was seen by ultrasound (approximately gestation week 7). If a patient had a chemical pregnancy or miscarried, blood samples were collected once weekly thereafter, until the patient's hCG <5 IU/mL. In addition to the post-pregnancy blood samples that were collected, a pre-ovulatory or ovulatory sample (gestation week 2), mid-luteal (gestation week 3) sample as well as a baseline (day 2) sample (where available) were collected from each patient.

3.3.2 Reproductive Hormone Assays and Sample Storage

Patient serum was measured for the standard reproductive hormones (E2, P4, and hCG) using the Siemens Centaur XP Automated Analyser (Siemens, Bayswater 3053, Victoria Australia) at Fertility North, adhering to the clinic's SOP "Operation of the Biochemistry Analyser (Siemens Advia Centaur XP)" (Appendix G) and "Assay Protocol for Siemens Advia Centaur eE2, LH, P4, FSH and ThCG" (Appendix H). These hormones formed part of the patient's regular cycle tracking or pregnancy monitoring and were requested by their doctor at Fertility North. All serum samples were stored in their original collection tubes at 4°C for one week, then at -20°C until all the samples obtained for each patient over their pregnancy monitoring were collected.

3.3.3 AMH Assays and Sample Storage

The blood samples from each patient/pregnancy were thawed at room temperature and analysed together on the same day in one batch to minimise technical variability. AMH concentrations were measured at PathWest Nedlands using the AMH GEN II enzyme linked immunosorbent assay (ELISA) (Beckman Coulter) following the manufacturers guide (Appendix N). A set of 7 calibrators for each AMH assay were provided in the manufacturer's kit to plot a calibration curve of absorbance versus AMH concentration (Appendix O). Sample AMH concentrations were then able to be calculated from this calibration curve. Patient samples were then stored for a second time at -20°C for further hormone testing at a later stage.

3.3.4 Thyroid Function and Oestradiol Assays

Patient samples were thawed at room temperature for a second time and analysed together in batches to minimise technical variability. Each patient sample was assigned a new barcode in numerical order so as to efficiently record, print and transfer the

hormone results to an excel spreadsheet. TSH, fT4, fT3 and E2 were measured in each patient sample using the Siemens Centaur XP Automated Analyser (Siemens, Bayswater,Victoria 3053, Australia) at Fertility North, adhering to the clinic's SOP "Operation of the Biochemistry Analyser (Siemens Advia Centaur XP)" (Appendix G) and "Assay Protocol for Siemens Advia Centaur eE2, LH, P4, FSH and ThCG" (Appendix H). One sample from each batch of patient pregnancy samples (gestation week 4 - 7) was tested for thyroid peroxidase antibody (TPOAb) using the same protocols. Two patient serum samples of known concentrations, one negative and one positive control were used in the TPOAb assay.

3.4 Quality Assurance

3.4.1 AMH Stability

The Beckman Coulter AMH GEN II ELISA kit has specific recommendations by the manufacturer for the pre-analytical preparation and storage of blood samples. Due to the patient's pre-pregnancy samples being collected retrospectively, they had already been stored for one week at 4°C in their original collection tubes before being frozen at -20°C (this is the clinic's storage protocol). Therefore the effect upon AMH concentration by storing patient serum in the refrigerator in the primary collection tube for up to one week prior to assay, as opposed to the recommended maximum 48 hours then transferring to a secondary storage tube, was investigated.

Serum samples (n=20) were stored at 4°C for 7 days in either the original/primary 5ml Vacutainer SST™ collection tube (Becton Dickinson, UK) or after transfer to a secondary storage tube. 100µl aliquots were removed daily for 7 days from the original serum sample and frozen at -20°C. All samples were stored for a further week at -20°C prior to thawing at room temperature on the day of measurement. Results were expressed as the mean (µ) ± standard error of mean (sem) or as a proportion/percentage (%) of the original value. AMH concentration over time was assessed using repeated measures ANOVA, and differences between tubes by paired student t-test. Differences were considered significant if $p<0.05$. The concentration of AMH did not differ significantly between the primary and secondary tubes ($p=0.603$) and the relationship between the two is illustrated in Figure 1.

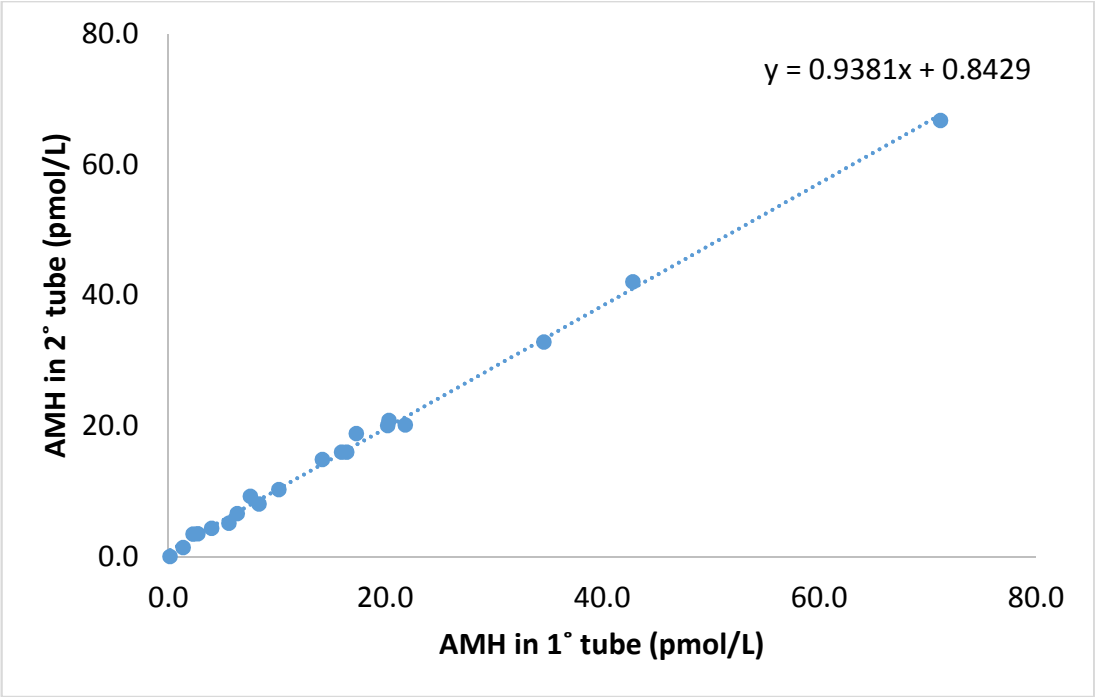

Figure 1. Serum AMH concentrations (pmol/L) following short-term storage at -20˚C in primary vs secondary tubes.

Serum AMH concentrations did not change significantly (p=0.811) (Table 1) over the 7 days in the collection tubes stored in the refrigerator (Table 2), decreasing by a mean of only 2.8±2.6% (Table 3).

Table 1. Repeated measures ANOVA for AMH stability over 5 days.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
days	0.400	4	1	0.811	0.615	1.599	0.059

Table 2. Serum AMH (pmol/L) stability over 7 days at 4°C.

	Sample					
Day	**A**	**B**	**C**	**D**	**E**	**μ ± sem**
1	5.0	10.0	14.2	30.1	47.0	21.3 ± 7.7
2	6.8	11.0	15.9	28.5	38.4	20.2 ± 5.8
3	8.3	10.9	15.1	28.2	39.5	20.4 ± 5.9
4	7.8	10.6	15.9	29.5	40.5	20.9 ± 6.2
5	8.1	10.1	14.3	27.9	44.8	21.0 ± 6.9
6	7.9	10.2	14.3	26.2	38.0	19.3 ± 5.6
7	7.3	10.6	14.5	23.6	37.7	18.7 ± 5.5

Table 3. Serum AMH expressed as a percentage of the original sample tube.

	Sample					
Day	**A**	**B**	**C**	**D**	**E**	**μ ± sem**
1	100.0	100.0	100.0	100.0	100.0	100.0 ± 0.0
2	136.9	110.6	112.0	94.7	81.7	107.2 ± 9.3
3	121.2	98.5	94.5	98.8	102.8	103.2 ± 4.7
4	93.6	97.4	105.6	104.8	102.6	100.8 ± 2.3
5	104.3	95.3	89.6	94.4	110.5	98.8 ± 3.8
6	97.6	101.3	100.6	93.9	84.8	95.7 ± 3.0
7	91.9	103.6	101.3	90.1	99.2	97.2 ± 2.6

3.4.2 AMH Assay Precision

The precision of the Beckman Coulter AMH Gen II ELISA kit was assessed using both commercial quality control material and pooled patient serum. Intra-assay variation was determined by analysing 20 replicates of 4 serum pools at know varied concentrations (low, medium, high and very high). These pools were then run in each of the 23 separate assays to indicate between-assay variability. Quality control material (1 high and 1 low) provided by the kit manufacturer were also included in each assay. Results were expressed as μ ± sem and a coefficient of variation (CV) calculated as standard deviation (SD)*100/μ.

The mean AMH concentration (pmol/L) in 20 replicates of Pools A-D is shown in Table 4. A precision curve was plotted (Figure 2) to demonstrate the reproducibility of AMH measurements at varying concentrations within one assay. The AMH Gen II assay performed well at concentrations between 13.4pmol/L and 35.7pmol/L with CVs of 5.6% and 5.2% respectively. The average patient serum AMH concentration was 21.4±0.8pmol/L which fell within this range. Samples with low AMH levels (<5.4pmol/L) had a higher CV of 10.5%.

Table 4. AMH (pmol/L) intra-assay variability of serum pools.

Pool	μ ± sem	CV (%)
A	5.4 ± 0.02	10.5
B	13.4 ± 0.02	5.6
C	35.7 ± 0.06	5.2
D	94.9 ± 0.27	9.0

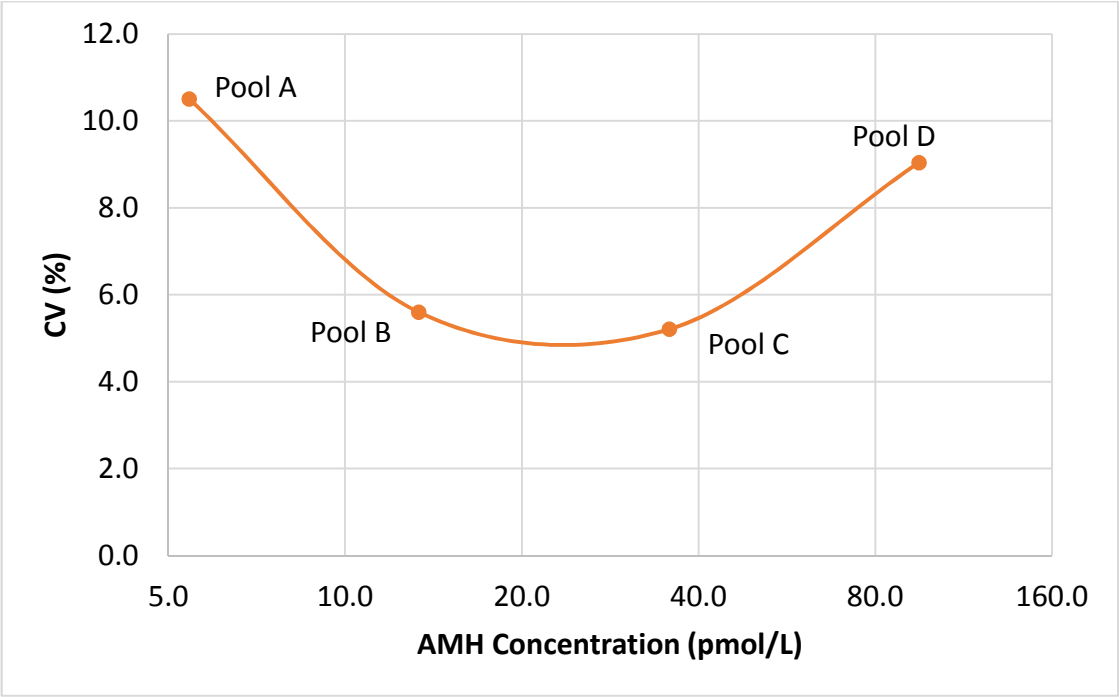

Figure 2. Beckman Coulter AMH Gen II intra-assay precision profile.

The variation in AMH measurements between assays was evident, both in pooled serum (Table 5) and commercially prepared internal controls (Table 6). A precision curve of the variability between assays is shown in Figure 3. Serum Pool A had the highest variability between assays, with a CV of 15.9% at an AMH level of 4.9±0.2pmol/L. The manufacturer's internal control 2 had the best reproducibility with a CV of 5.1% at mean AMH concentration of 57.7±1.1pmol/L, compared to Pool C that had the lowest CV of all the Pools (8.0%) at mean AMH concentration of 30.5±0.5pmol/L. The effect of inter-assay variability was therefore eliminated for each woman by analyzing all samples for one cycle in one assay.

Table 5. AMH (pmol/L) inter-assay variability of serum pools.

Pool	μ ± sem	CV (%)
A	4.9 ± 0.2	15.9
B	11.7 ± 0.3	11.1
C	30.5 ± 0.5	8.0
D	92.2 ± 2.1	11.2

Table 6. Inter-assay variability of AMH Gen II internal controls (pmol/L).

Control	μ ± sem	CV (%)
1	19.4 ± 1.8	8.5
2	57.7 ± 1.1	5.1

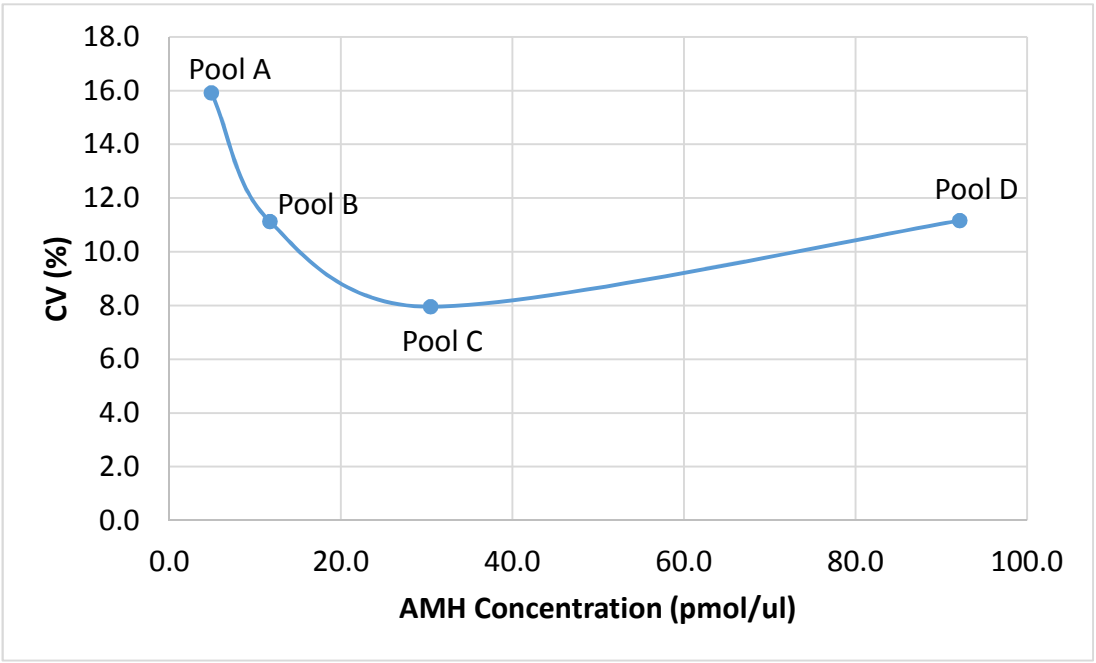

Figure 3. Beckman Coulter AMH Gen II inter-assay precision profile.

3.4.3 Thyroid Assay Precision

The precision of the Siemen's thyroid hormone reagents (fT3, fT4 and TSH) were analysed using both commercial QC material (Bio-rad Lyphochek Immunoassay Plus Control 1, 2 and 3, Irvine, CA) and pooled patient serum. Intra-assay variation was determined for each thyroid hormone by analysing 20 replicates of 3 serum pools at concentrations representative of the mean levels found in the patient sample population. Two of these serum pools were then run in each of the 12 separate assays to indicate between-assay variability and reflect the nature of the patient samples being measured. Quality control material (three levels) provided by the manufacturer were also included in each assay. Results were expressed as μ ± sem and CV. Analysis of three different pooled sera samples at three different concentrations revealed excellent reproducibility for the fT3, fT4 and TSH assays (Tables 7 - 9 and Figures 4 - 6), with the average CV of each assay being <5.0%. The concentrations of each pooled sample were close to the average levels of thyroid hormones typically found in each patient.

Table 7. Within-assay performance of fT3 (pmol/L) using pooled sera.

	μ ± sem	CV (%)
fT3 A	4.59 ± 0.03	3.16
fT3 B	4.94 ± 0.02	2.12
fT3	5.04 ± 0.03	2.39

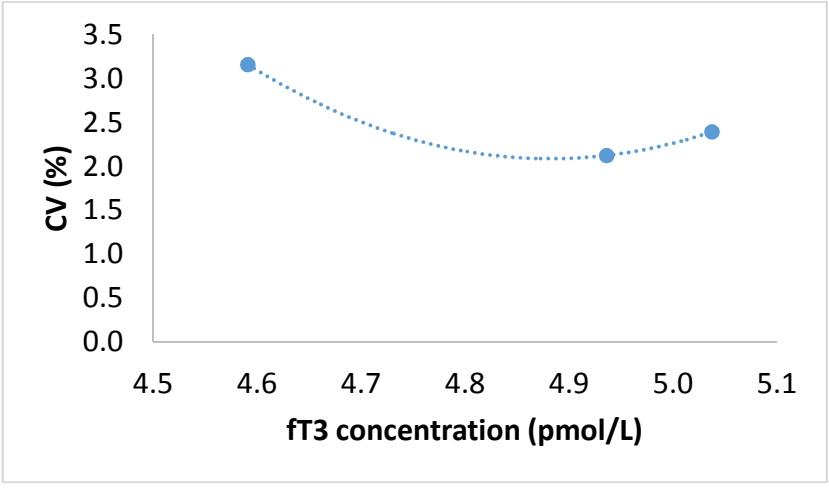

Figure 4. Siemens fT3 intra-assay precision profile.

Table 8. Within-assay performance of fT4 (pmol/L) using pooled sera.

	μ ± sem	CV (%)
fT4 A	15.04 ± 0.11	3.23
fT4	16.26 ± 0.17	4.75
fT4 B	17.46 ± 0.20	5.23

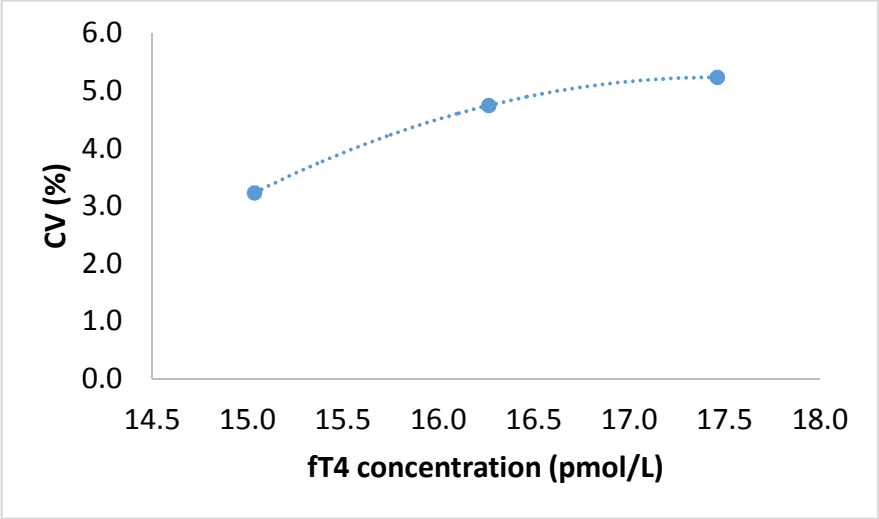

Figure 5. Siemens fT4 intra-assay precision profile.

Table 9. Within-assay performance of TSH (mU/L) using pooled sera.

	μ ± sem	CV (%)
TSH A	1.77 ± 0.01	2.75
TSH	1.96 ± 0.01	1.60
TSH B	2.64 ± 0.02	2.60

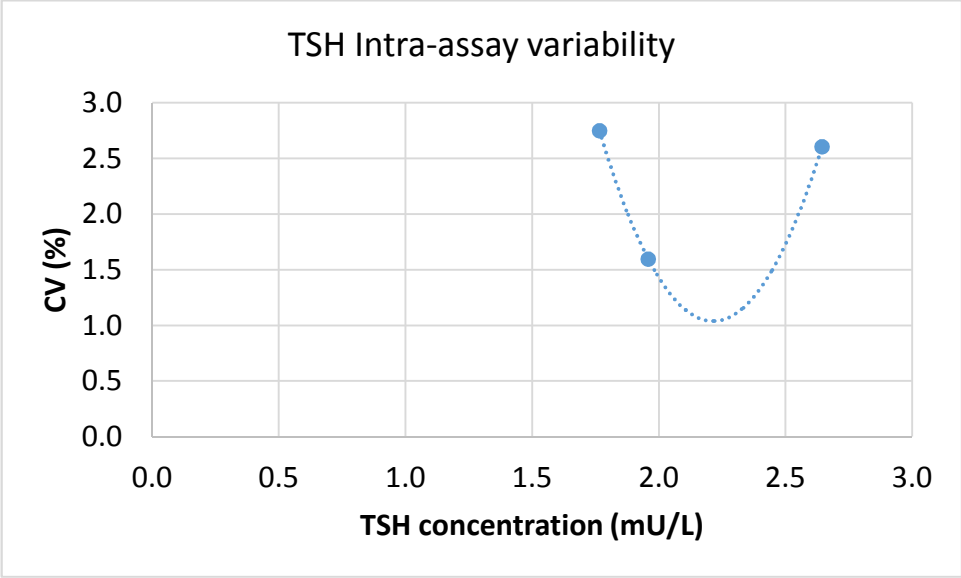

Figure 6. Siemens TSH intra-assay precision profile.

Two external controls (derived from pooled patient sera) and three internal quality controls (provided by the manufacturer) were run in each of 12 different assays to establish between-assay variation for fT3, fT4 and TSH as shown in Tables 10 - 12 and Figures 7 - 9. The effect of inter-assay variability for all thyroid hormones was eliminated for each woman by analyzing all samples for one cycle in one assay.

Table 10. Inter-assay variability of fT3 (pmol/L).

	μ ± sem	CV (%)
fT3 QC1	3.72 ± 0.03	2.87
fT3 A	4.62 ± 0.03	2.53
fT3 B	4.99 ± 0.04	2.77
fT3 QC2	10.33 ± 0.08	2.56
fT3 QC3	16.80 ± 0.19	3.90

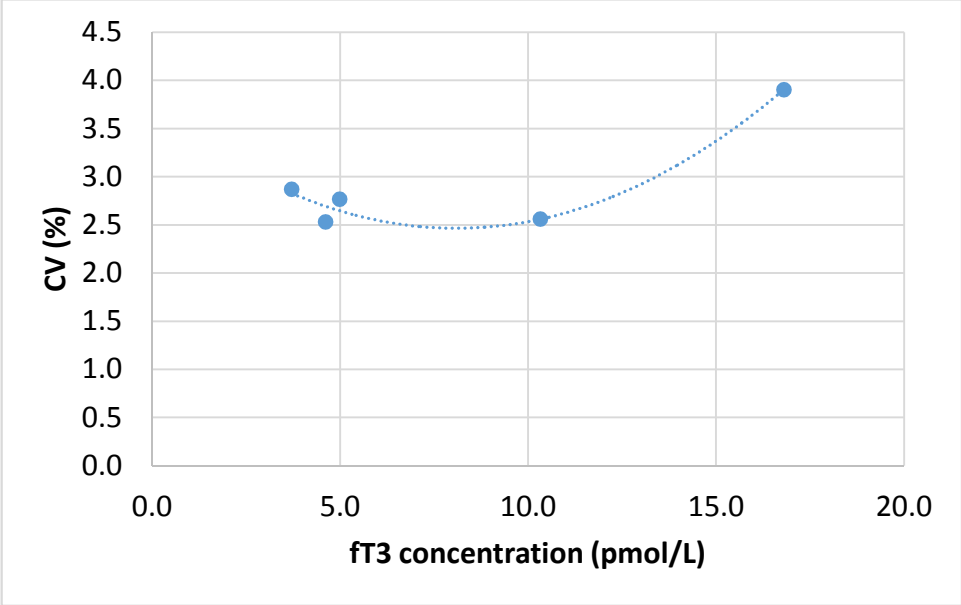

Figure 7. Siemens fT3 inter-assay variability.

Table 11. Inter-assay variability of fT4 (pmol/L).

	μ ± sem	CV (%)
fT4 QC1	9.80 ± 0.19	8.91
fT4 A	16.41 ± 0.29	6.06
fT4 B	17.99 ± 0.39	7.48
fT4 QC2	24.43 ± 0.29	4.06
fT4 QC3	47.88 ± 0.68	4.93

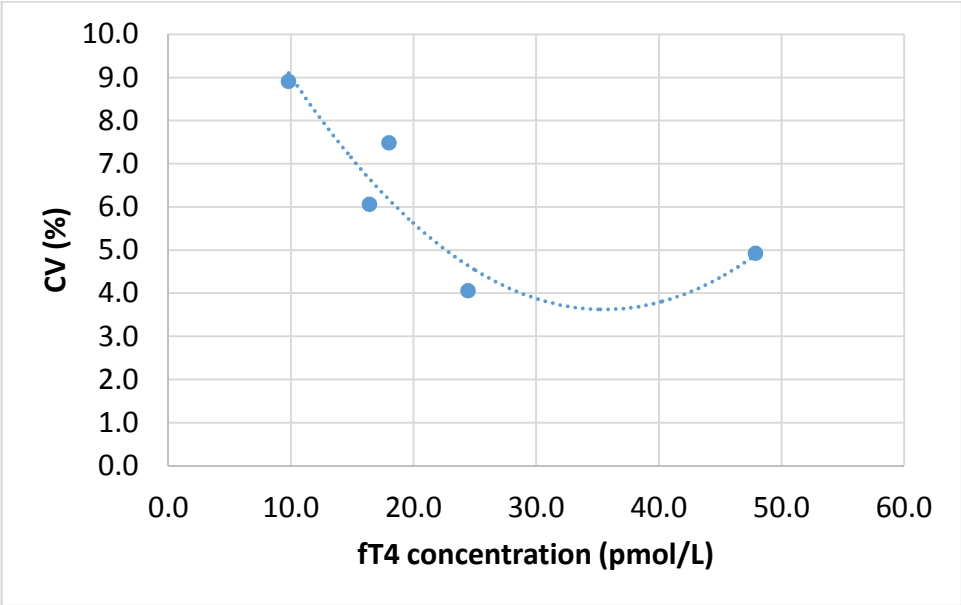

Figure 8. Siemens fT4 inter-assay variability.

Table 12. Inter-assay variability of TSH (mU/L).

	μ ± sem	CV (%)
TSH QC1	0.35 ± 0.00	3.41
TSH A	1.61 ± 0.05	9.94
TSH B	2.44 ± 0.05	6.81
TSH QC2	4.86 ± 0.05	3.61
TSH QC3	32.05 ± 0.35	3.80

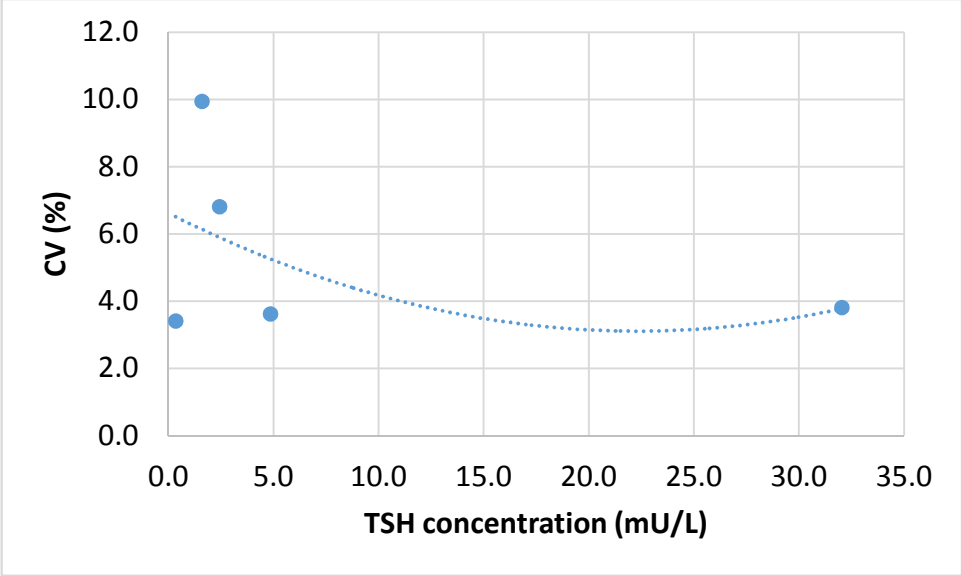

Figure 9. Siemens TSH inter-assay variability.

3.4.4 Reproductive Hormone Quality Controls

To monitor hormone assay performance and maintain a high level of precision in the laboratory, Internal Quality Controls (Bio-rad Lyphochek Immunoassay Plus Control 1, 2 and 3, Irvine, CA) were used in every run. Fertility North is also enrolled in two External Quality Assurance Schemes for the measurement of five reproductive hormones, including E2, P4 and hCG (Appendix L). A patient sample repeat from a previous assay was also measured for all hormones on each run.

3.4.5 Measurement Uncertainty

There is a degree of variability between the reproductive hormone concentrations (Oestradiol, P4 and hCG) measured on different occasions (refer to Appendix M), therefore care must be taken when interpreting such results. Because E2 was not routinely measured, it was measured in all patient samples in batches to minimise technical variability. The P4 and hCG levels were measured routinely on the day of each sample collection and recorded.

3.4.6 Reproductive Hormone Linearity

The reproductive hormone assays (oestradiol, progesterone and hCG) were further validated by demonstrating the linearity of hormone measurements in selected samples because the concentrations often extended beyond the range of the standard curve. Serial dilutions (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) were performed on the reproductive hormones E2, P4 and hCG in patient blood serum samples with known high concentrations. One patient sample from each of the four different cycle types (Natural, OI, IVF/ICSI and FET) that had been identified as having a positive pregnancy test were used to measure each reproductive hormone. Samples were diluted manually using a transfer pipette and adding the required amount of each hormone's specific diluent (Appendix J).

Samples were measured on the Siemens Centaur XP Automated Analyser, adhering to the clinic's SOP "Operation of the Biochemistry Analyser (Siemens Advia Centaur XP)" (Appendix G) and "Assay Protocol for Siemens Advia Centaur eE2, LH, P4, FSH and ThCG" (Appendix H). The observed concentrations of each test were plotted against the expected concentrations to determine the gradient, correlation and intercept values (Tables 13 - 15 and Figures 10 - 12). The sample used to plot the linearity graph for each hormone is marked with an asterix. In essence, all three assays showed good

linearity with correlations ≥0.997, and gradients close to 1.000. E2 and P4 had intercepts close to the origin and hCG appeared to have the largest shift from the intercept, but this was acceptable given the high values measured.

Table 13. Mean E2 difference (%) following serial dilutions in difference treatment cycles.

	Patient Cycle		
Parameter	IVF/ICSI*	FET	Natural
gradient	1.012	1.020	1.018
correlation	0.999	0.999	0.997
intercept	-86.766	-98.572	-88.831

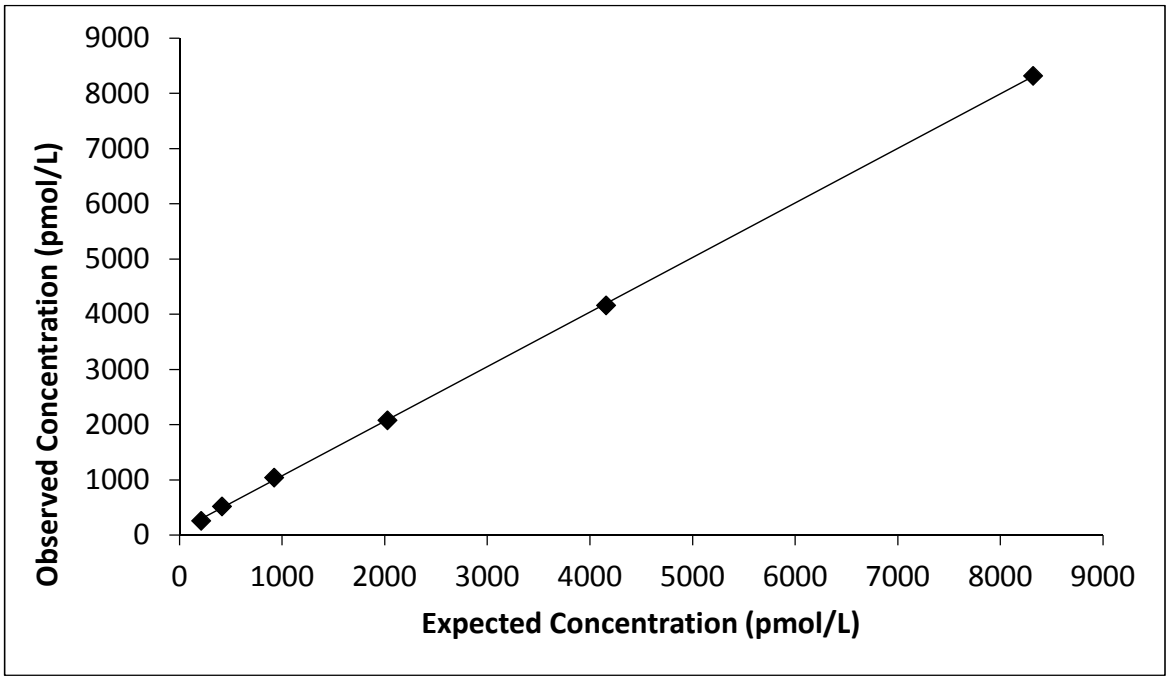

Figure 10. Serial Dilution of E2 in an IVF/ICSI patient serum sample.

Table 14. Mean P4 difference (%) following serial dilutions in difference patient cycles.

	Patient Cycle		
Parameter	IVF/ICSI*	FET	Natural
gradient	0.999	1.016	1.015
correlation	0.999	0.999	0.997
intercept	-3.638	-0.825	1.673

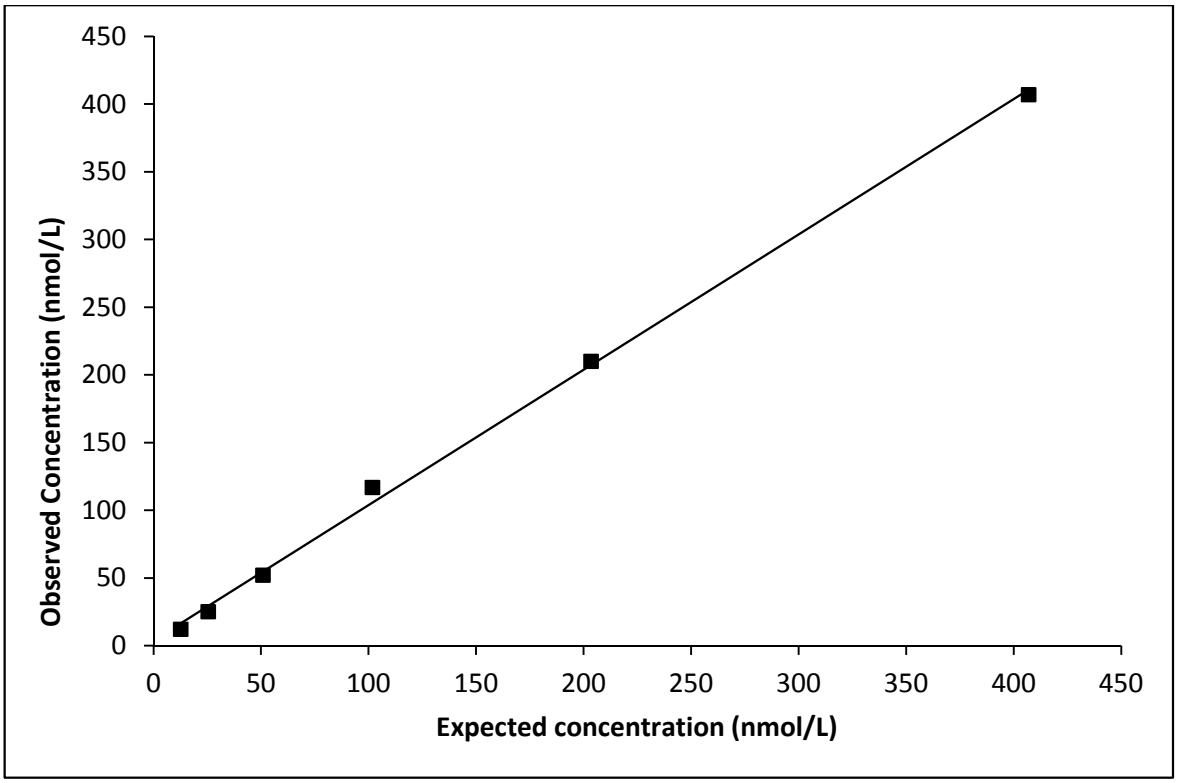

Figure 11. Serial Dilution of P4 in an IVF/ICSI patient serum sample.

Table 15. Mean hCG difference (%) following serial dilutions in difference patient cycles.

	Patient Cycle		
Parameter	IVF/ICSI	FET	Natural
gradient	1.003	1.018*	1.013
correlation	0.998	0.999	0.999
intercept	55.104	-1701.378	-469.458

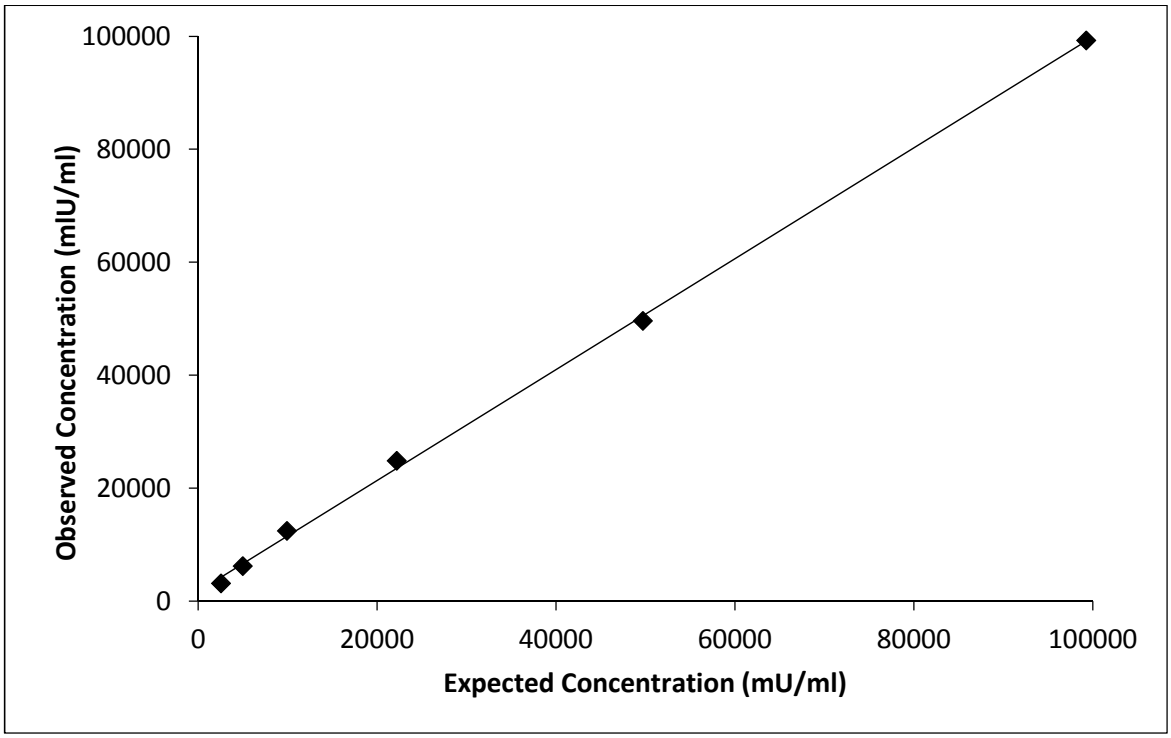

Figure 12. Serial Dilution of hCG in an IVF/ICSI patient serum sample.

3.4.7 Reproductive Reagents and Calibrations

Reagents and calibrators were closely monitored and recorded to keep track of lot numbers to ensure they are all used within their expiry dates (Appendix I). All the primary hormone reagents (E2, P4, hCG, TSH, fT3, fT4 and TPOAb) were calibrated prior to use, with their specific calibrator (Appendix J). Lyophilized calibrators were prepared by adding the required amount of deionized water (Ibis Technology, Mt Hawthorn) and all consumables stored at 2-8˚C in the fridge or on-board the Siemens Centaur XP for the recommended duration as recommended by the manufacturer (Appendix J).

3.5 Data Collection and Statistical Analysis

3.5.1 Recording and Storage of Hormone Results

All patient hormones that formed part of their routine fertility treatment (P4 and hCG) were automatically recorded on the Centaur XP, then printed and recorded in the clinic's main database program 'PRAGMA' (*ARTEMIS*, 1991) adhering to Fertility North's SOP "Recording Blood Test Results that are Collected and Analysed Internally" (Appendix K).

Once patients had completed their pregnancy hormone monitoring, these results were collected retrospectively and transferred to an Excel spreadsheet located within the clinic's network, which was password protected. Patient's AMH, E2, TSH, fT3 and fT4 hormone results were printed and entered manually into an excel spreadsheet also saved within the clinic's network.

Names were initially associated with these results so as to search for their medical history and treatment information within their records at Fertility North. Patient's names were de-identified upon data analysis and will not be released upon publication of any results.

3.5.2 Statistical Analysis of Hormones

De-identified information was analysed under the guidance of a suitably qualified statistician at Edith Cowan University. Quantification of within- and between-assay variability for all hormones was determined by calculating the mean (μ), standard error of mean (sem) and coefficient of variation (CV) using Microsoft Excel. The age of viable and non-viable women was expressed in mean ± sem and p-value calculated using 2-

tailed t-test (two sample, unequal variance). Analysis of the relationships between hormones and other variables (gestation week, treatment or cycle type, patient age, patient BMI, FSH dose, viability of pregnancies and fetal gender) were performed using IBM SPSS Statistics Version 22.

The relationships between E2, P4, hCG, AMH, TSH, fT3 and fT4, gestation week, age and BMI during pregnancy were analysed using bivariate correlations (2-tailed, Spearman) to determine the correlation coefficient (r) and p-value (significance). The relationship between FSH dosage (ovarian stimulation) and AMH levels at gestation week 4 were also investigated in this way.

The relationships between each of the hormones during pregnancy were further investigated by partial correlations, controlling for cycle type and cycle and week combined. Patterns between hormones were visualized by generating scatterplots for the partial correlations between AMH or Thyroid hormones and other hormones that showed a significant correlation.

General linear models - repeated measures ANOVA were used to measure the relationships between E2, P4, hCG, AMH, TSH, fT3 and fT4 and gestation week. Cycle type and pregnancy outcome (viability) were investigated as between-subject factors for AMH and thyroid hormones. Non-pregnant or baseline (gestation week 0) AMH and thyroid levels were compared to each gestation week before and during pregnancy. Hormone values from gestation week 7 were excluded from all ANOVA analyses due to the drop in patient numbers affecting the power of calculations.

Tests for normality were significant (ie not normally distributed) for all hormones besides fT3 and fT4; therefore the logarithm of variables were also analysed and Spearman's rank of p-value was reported as significant if $p=<0.05$. Results from ANOVA calculations were reported as $F_{(df1/H,\ df2/error)}$ (p-value) and Partial Eta (power). P-values were calculated using Wilks' Lambda, or Pillai's Trace if Levene's Test of Equality of Error Variances or Box's Test of Equality of Covariance Matrices were not satisfied ($p=>0.05$).

Reference ranges were calculated for thyroid hormone concentrations in early pregnancy by determining the 2.5^{th} and 97.5^{th} percentiles, minimum (min) and maximum (max), median and mean ± sem concentrations at each gestation time point. TSH concentrations were log transformed to define ranges within a normal distribution. Nonviable pregnancies and women who tested positive for TPOAb were excluded from the analysis.

CHAPTER 4

REPRODUCTIVE HORMONE RESULTS

4 Reproductive Hormone Results

4.1 Oestradiol

Table 16 shows the mean concentration and confidence intervals of E2 during early pregnancy. E2 increases significantly with advancing gestational age (p=0.000) (Table 17).

Table 16. E2 levels (mean ± sem) and confidence intervals for viable pregnancies from gestation weeks 4 to 6.5 (n=55).

Gestation Week	Mean (pmol/L)	Sem	95% CI	
			Lower	Upper
4	1205.9	154.1	896.9	1515.0
4.5	1608.7	227.3	1152.9	2064.5
5	1979.3	276.9	1424.2	2534.4
5.5	2183.1	311.3	1558.9	2807.3
6	2338.3	332.1	1672.5	3004.0
6.5	2436.4	299.5	1835.9	3036.8

Table 17. Repeated measures ANOVA for E2 gestation weeks 4 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	9.018	5	50	***0.000***	0.474	45.088	1.000

4.2 Progesterone

Table 18 shows the concentration and reference intervals of P4 during early pregnancy. Progesterone levels remain constant (p=0.211) (Table 19) between gestation weeks 4 to 6.5, ranging from the highest level of 199.7 ± 27.5 nmol/L at gestation week 4 to the lowest level of 177.0 ± 18.5 at gestation week 6.5.

Table 18. P4 levels (mean ± sem) and confidence intervals for viable pregnancies from gestation weeks 4 to 6.5 (n=57).

Gestation Week	Mean (nmol/L)	Sem	95% CI	
			Lower	Upper
4	199.7	27.5	144.6	254.7
4.5	198.6	23.9	150.6	246.6
5	194.1	21.2	151.6	236.6
5.5	187.2	19.7	147.7	226.7
6	181.1	18.9	143.3	218.9
6.5	177.0	18.5	140.0	214.1

Table 19. Repeated measures ANOVA for P4 gestation weeks 4 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	1.485	5	52	0.211	0.125	7.423	0.480

4.3 Human Chorionic Gonadotropin

Table 20 shows the changes in hCG concentration during early pregnancy. Levels of hCG increased significantly with increasing gestational age (p=0.000) (Table 21). hCG levels increased by a mean factor of 3.2 every 3 days, from the time of positive pregnancy test at gestation week 4 (251.9 ± 30.1 mU/ml) to 53844.4 ± 3525.2 at gestation week 6.5.

Table 20. hCG levels (mean ± sem) and confidence intervals for viable pregnancies from gestation weeks 4 to 6.5 (n=57).

Gestation Week	Mean (mU/mL)	Sem	95% CI	
			Lower	Upper
4	251.9	30.1	191.6	312.2
4.5	1384.1	182.2	1019.2	1749.0
5	5187.5	565.4	4054.8	6320.2
5.5	13852.5	1167.7	11513.3	16191.6
6	29686.4	2183.5	25312.4	34060.4
6.5	53844.4	3525.2	46782.6	60906.2

Table 21. Repeated measures ANOVA for hCG gestation weeks 4 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	48.093	5	52	***0.000***	0.822	240.466	1.000

CHAPTER 5

AMH RESULTS

5 AMH Results

5.1 AMH Levels in the Menstrual Cycle and Early Pregnancy

The AMH concentrations (mean ± sem) for each cycle type at baseline (day two of the menstrual cycle), ovulation and mid-luteal are displayed in Table 22. Only the women with measurements taken at all three time points were included in the analysis. The percentage AMH change (%) for each woman at the time of ovulation and mid-luteal (calculated as the percentage change from their baseline AMH measurement) is shown in Figures 13 - 16 for all cycle types, except IVF/ICSI cycles due to the very low concentrations. The AMH levels at baseline were strongly correlated with both ovulation and mid-luteal time points (Table 23).

Table 22. AMH concentrations (mean ± sem) for each cycle type at various stages of the menstrual cycle.

	Baseline	Ovulation	Mid-luteal
IVF/ICSI (n=2)	2.3 ± 0.8	2.4 ± 1.0	2.6 ± 0.4
Natural (n=14)	18.0 ± 3.8	21.3 ± 5.7	21.4 ± 5.4
FET (n=18)	23.3 ± 4.4	26.7 ± 5.6	18.9 ± 4.0
AIH (n=3)	25.8 ± 5.9	41.2 ± 9.0	30.6 ± 8.1
OI (n=6)	60.5 ± 17.2	59.0 ± 16.6	50.2 ± 14.4

Table 23. Correlation (r) for AMH levels at ovulation or mid-luteal compared to baseline levels for each cycle type.

	Baseline vs Ovulation	Baseline vs Mid-Luteal
AIH (n=3)	0.88	0.91
FET (n=18)	0.97	0.95
OI (n=6)	0.87	0.95
Natural (n=14)	0.95	0.92

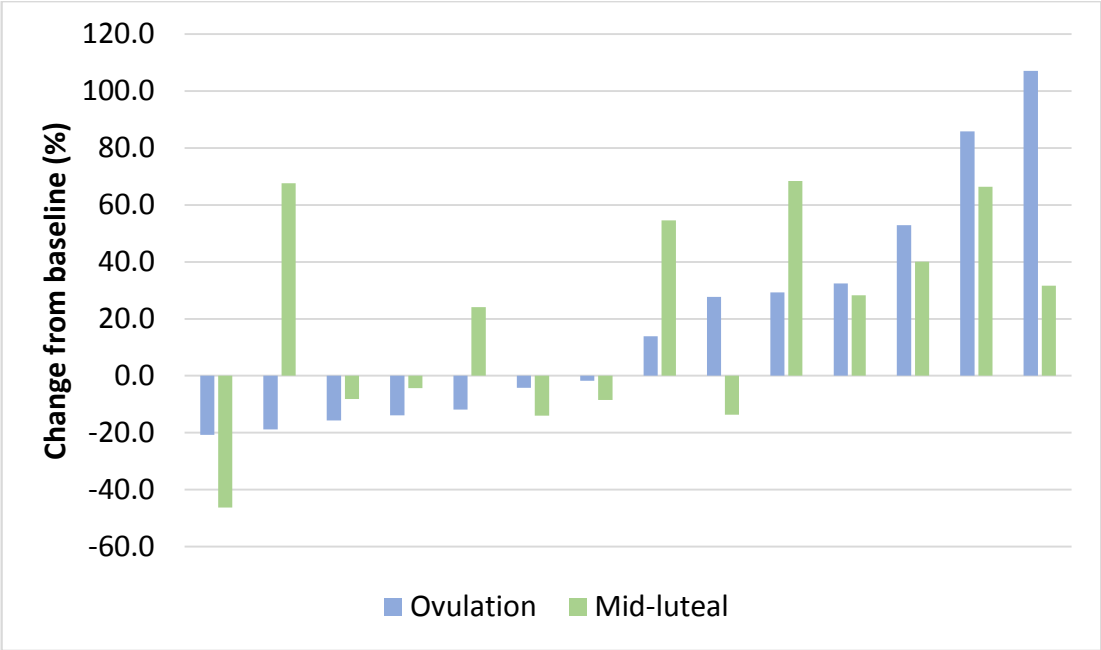

Figure 13. Percentage AMH change (%) from baseline for women in natural cycles (n=14).

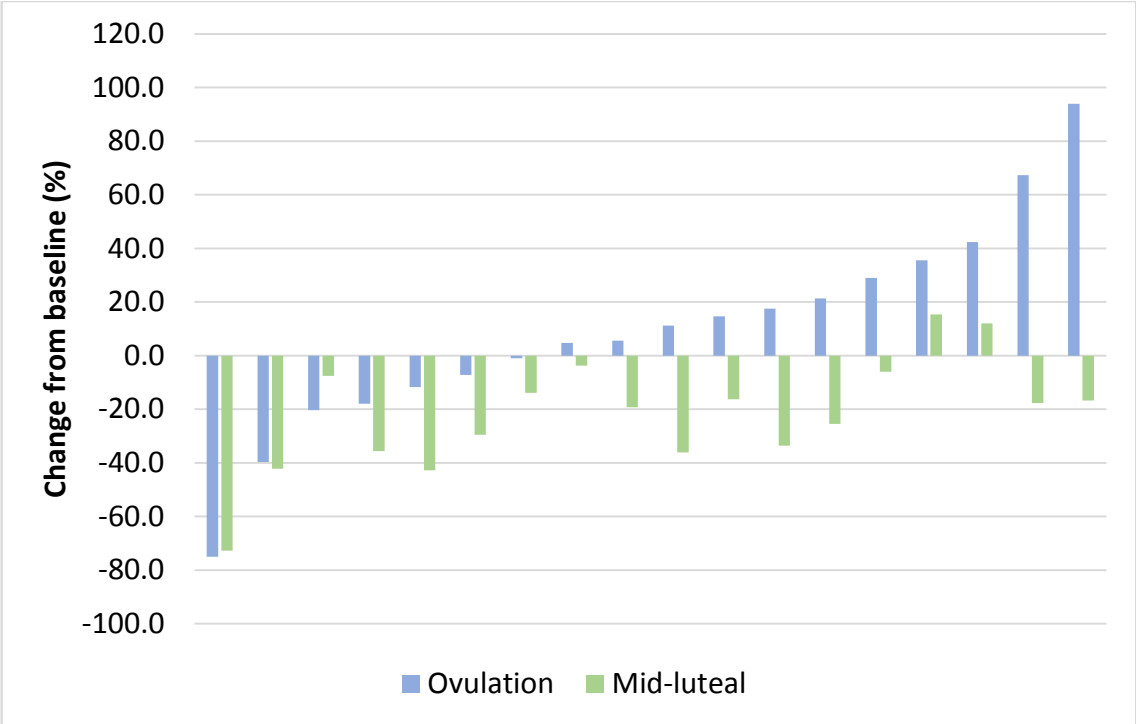

Figure 14. Percentage AMH change (%) from baseline for women in FET cycles (n=18).

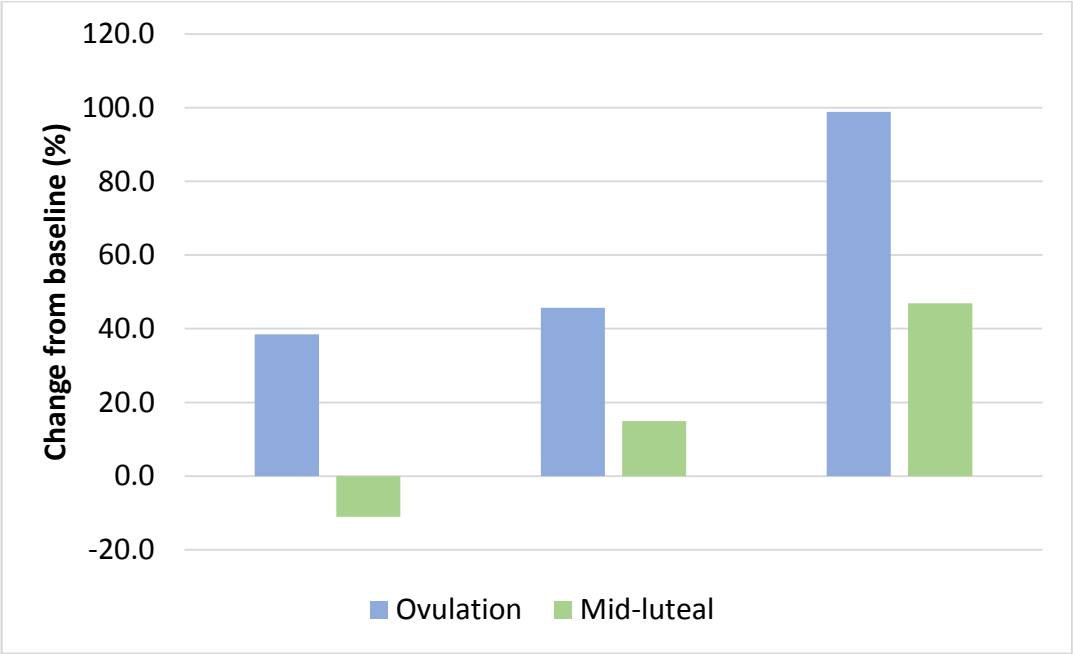

Figure 15. Percentage AMH change (%) from baseline for women in AIH cycles (n=3).

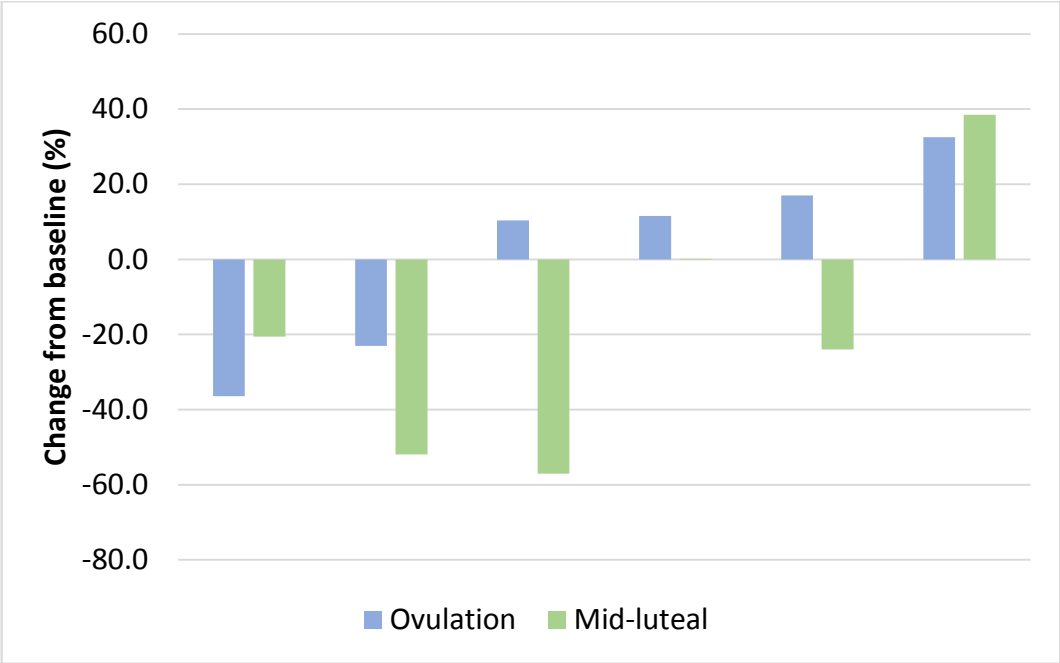

Figure 16. Percentage AMH change (%) from baseline for women in OI cycles (n=6).

Due to the small number of women with bloods taken at all three time points (baseline, ovulation and mid-luteal) in some cycle groups, power to detect statistical significance between time points within these groups was low, therefore p-values were not included in this analysis. However, when looking at all viable pregnancies (regardless of cycle type), there were significant differences in AMH levels observed at various time points before and during pregnancy (p=0.005) (Table 24). Table 25 shows the mean AMH levels for all cycle types before and during pregnancy (gestation weeks 0 to 6.5). The largest difference in AMH levels existed between time of ovulation (30.7 ± 5.5 pmol/L) and gestation week 6 (18.7 ± 3.1 pmol/L) with a mean difference of 12.07pmol/L.

Table 24. Repeated measures ANOVA for AMH gestation weeks 0 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	3.912	8	23	***0.005***	0.576	31.297	0.950

Table 25. AMH levels (mean ± sem) and confidence intervals for gestation weeks 0 to 6.5.

Week	Mean (pmol/L)	Sem	95% CI	
			Lower	Upper
0	25.8	4.3	17.0	34.6
2	30.7 [a, b]	5.5	19.6	41.9
3	24.6 [c]	4.2	16.0	33.1
4	21.6	3.4	14.7	28.5
4.5	21.1	3.2	14.5	27.7
5	19.6	2.8	13.8	25.4
5.5	20.4 [a]	3.2	13.8	27.1
6	18.7 [b, c]	3.1	12.3	25.1
6.5	20.3	3.5	13.2	27.4

Note – concentrations with same superscript are significantly different (p<0.05)

Although the mean AMH levels peaked at time of ovulation (gestation week 2), it is important to note that this was not the same for each woman. Only 53.5% of patients (with blood samples taken at gestation weeks 0, 2 and 3) had maximum AMH levels at gestation week 2, with 32.6% having their highest AMH level measured at baseline (gestation week 0) and 13.9% at mid-luteal (gestation week 3). The maximum (highlighted red), middle (yellow) and lowest (green) AMH level measured during the menstrual cycle for each woman is shown in Table 26.

Table 26. AMH levels (pmol/L) for each woman during the menstrual cycle.

Cycle	Baseline	Ovulation	Mid-luteal
AIH	17.18	23.79	15.27
AIH	23.08	45.90	33.89
AIH	37.04	53.98	42.57
FET	3.94	3.66	2.78
FET	7.96	6.34	7.36
FET	12.11	7.31	7.00
FET	18.68	18.49	16.09
FET	10.22	19.83	8.51
FET	15.43	20.91	17.81
FET	28.12	23.08	18.09
FET	15.00	25.10	12.34
FET	23.71	27.18	19.84
FET	27.94	29.25	26.90
FET	42.47	37.49	24.31
FET	40.10	44.59	25.63
FET	31.87	45.35	35.70
FET	2.51	0.63	0.69
FET	6.80	8.25	5.07
FET	79.03	101.94	74.25
FET	13.28	14.02	10.72
FET	39.63	46.60	26.31
ICSI	3.08	3.31	2.93
IVF	1.56	1.39	2.20
OI	28.39	37.63	39.31
OI	112.48	71.51	89.30
OI	71.03	79.24	71.11
OI	106.25	124.37	80.75
OI	25.15	19.35	12.08
OI	19.83	21.89	8.53
TRACK	4.01	3.26	6.73
TRACK	3.26	6.74	4.28
TRACK	9.87	8.33	9.07
TRACK	10.17	10.00	9.31
TRACK	11.62	13.24	17.96
TRACK	7.40	13.76	12.32
TRACK	11.07	14.13	9.56
TRACK	17.71	15.61	21.98
TRACK	20.06	17.27	19.19
TRACK	20.89	20.01	17.96
TRACK	29.27	23.22	15.75
TRACK	28.29	36.56	47.65
TRACK	57.81	88.38	81.00
TRACK	20.43	27.05	26.21

5.2 AMH Levels in Early Pregnancy

5.2.1 Overall Changes Over Time

The mean AMH concentrations (pmol/L) when measured for all patients at each time point (gestation weeks 4-7) did not show any significant differences (r=-0.012, p=0.784), with the lowest AMH level of 18.1pmol/L calculated at gestation week 7, compared to the highest AMH level of 20.5pmol/L calculated at gestation week 4 (Figure 17). The number of patients with serum available decreased at each gestation week due to some patients having earlier ultrasounds than routinely performed at week 7, after which time no further blood samples were required when a positive fetal heart was detected.

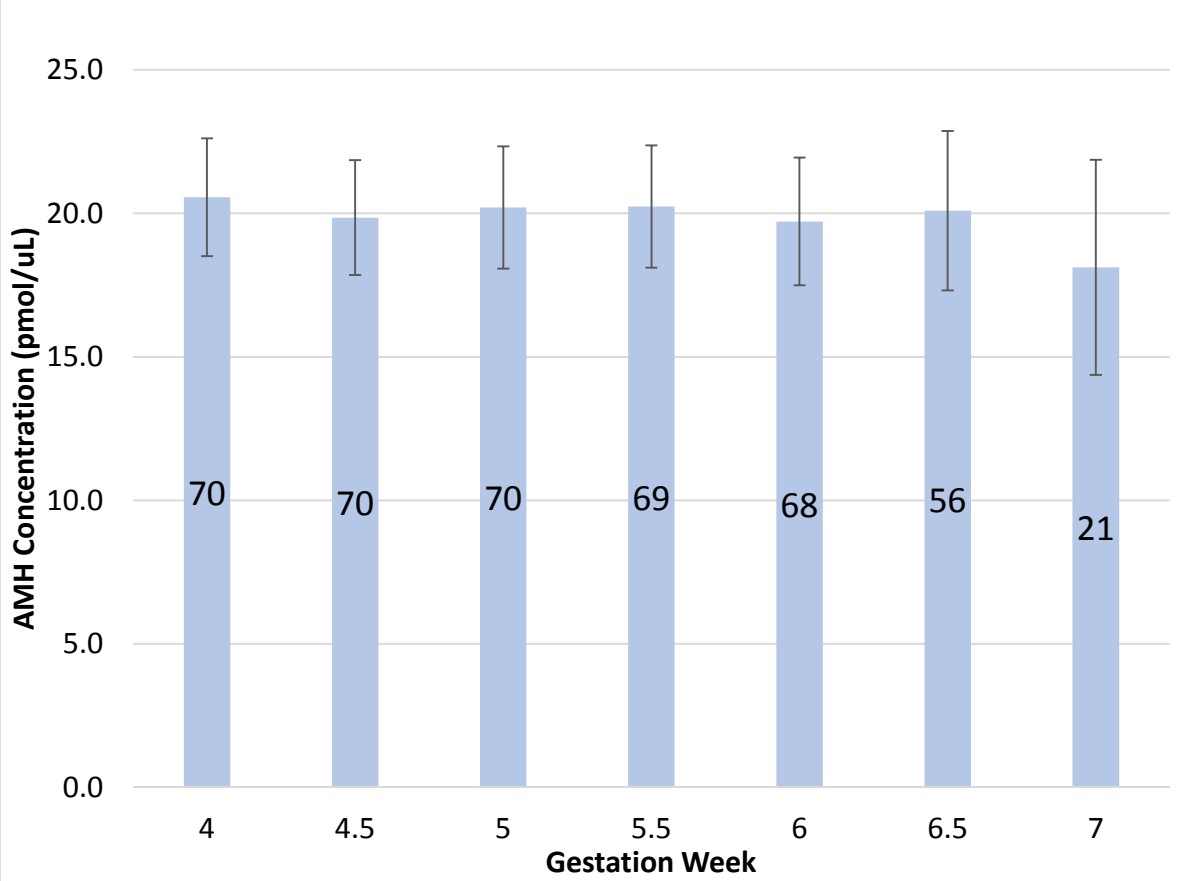

Figure 17. Serum AMH concentrations (mean ± sem) from gestation week 4 to week of fetal heartbeat detected by ultrasound. Number of patient samples available for analysis at each gestation week is shown within each column.

5.2.2 AMH Levels between Weeks

AMH levels were not significantly different between gestation weeks 4 to 6.5 for all viable pregnancies (p=0.278) (Table 27). AMH levels during pregnancy were also analyzed between gestation weeks 4 to 6.5 for each cycle type. There were no significant differences observed between gestation weeks 4 and 6.5 for IVF/ICSI (p=0.404), FET (p=0.677) or OI/AIH (p=0.820) cycles (Tables 28 - 30). However in natural cycles, there were significant difference observed (p=0.028) (Table 31), with AMH levels decreasing from 24.7pmol/L at gestation week 4 to 18.7 pmol/L at gestation week 6 (Table 32).

Table 27. Repeated measures ANOVA for AMH gestation weeks 4 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	1.302	5	50	0.278	0.115	6.512	0.422

Table 28. Repeated measures ANOVA for AMH levels gestation weeks 4 to 6.5 in IVF/ICSI cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	1.093	5	15	0.404	0.267	5.465	0.284

Table 29. Repeated measures ANOVA for AMH levels gestation weeks 4 to 6.5 in FET cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	0.634	5	17	0.677	0.157	3.170	0.179

Table 30. Repeated measures ANOVA for AMH levels gestation weeks 4 to 6.5 in OI/AIH cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	0.423	5	7	0.820	0.232	2.115	0.105

Table 31. Repeated measures ANOVA for AMH gestation weeks 4 to 6.5 in Natural cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	5.031	5	7	***0.028***	0.782	25.154	0.770

Table 32. AMH concentrations (mean ± sem) and confidence intervals for natural cycle patients in early pregnancy (n=12).

Gestation Week	Mean (pmol/L)	Sem	95% CI	
			Lower	Upper
4	24.7 [a]	5.0	13.6	35.8
4.5	22.5 [b]	4.5	12.7	32.4
5	21.1	4.5	11.2	31.0
5.5	21.6	5.2	10.1	33.0
6	18.7 [a, b]	4.5	8.7	28.6
6.5	20.8	5.9	7.7	33.9

Note – concentrations with same superscript are significantly different ($p<0.05$)

5.2.3 Between Patient Variability on Day of Positive Pregnancy Test

When comparing AMH levels between patients at any one given time point, there were substantial variations. For example, the AMH concentration for each woman at the beginning of the pregnancy (week 4 or the time of positive pregnancy test) ranged from 1.1pmol/L to 69.4pmol/L (Figure 18), and reflects the wide range of baseline values and ovarian reserves.

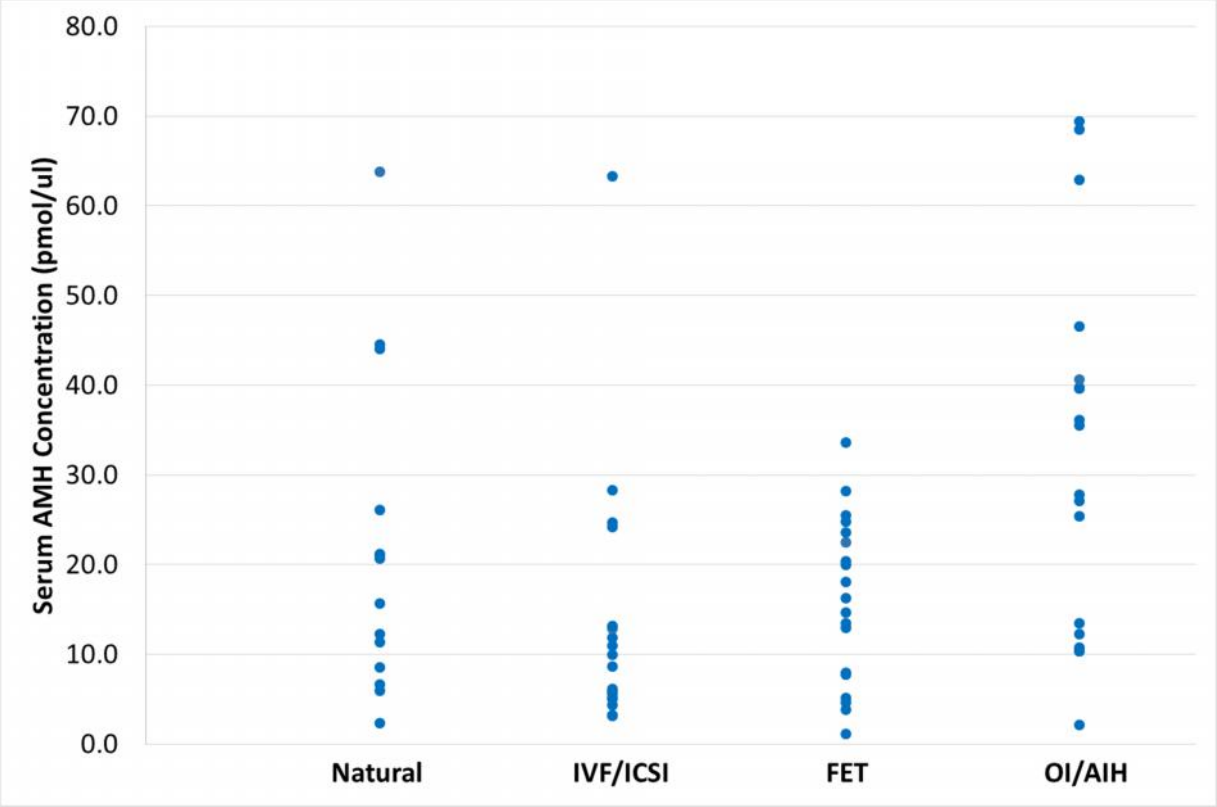

Figure 18. Inter-woman serum AMH concentrations (pmol/L) at gestation week 4 for different cycle types.

5.2.4 Proportional AMH Change

Due to the high variation in AMH concentrations between each patient, levels were then expressed as the mean percentage change over time (from gestation week 4 to week of fetal heart detected by ultrasound), to more accurately determine individual AMH changes that occurred. The AMH concentrations during each patient's pregnancy did not remain stable, but consistently changed and either increased or decreased (Figure 19). The largest increase in AMH levels during early pregnancy was seen in an OI patient where her AMH level increased by a mean of 114.1% in the small time of 2.5 weeks, increasing from 40.6pmol/L at gestation week 4 to 111.2pmol/L in gestation week 6.5. In contrast, a woman who fell pregnant in a natural cycle had AMH levels decrease by a mean of 72.4%, dropping from 6.6pmol/L at gestation week 4 to 0.6pmol/L at gestation week 6.5.

When dividing the patients into two sub groups that had either a mean increase or decrease in AMH (AMH trend), there was a statistically significant difference between these groups (p=0.000) (Table 33). Mean AMH variance between increasing or decreasing trend was almost seven times larger than the variance within the weeks, with a very strong effect size (Power = 0.996). The pattern between pregnancies that increased in AMH compared to those that decreased was discernible across gestation weeks 4 to 6.5 (Table 34 and Figure 20), where the estimated means follow opposite lines, crossing over between gestation weeks 4 and 4.5 and the dispersion between the two groups increased as the pregnancy progressed.

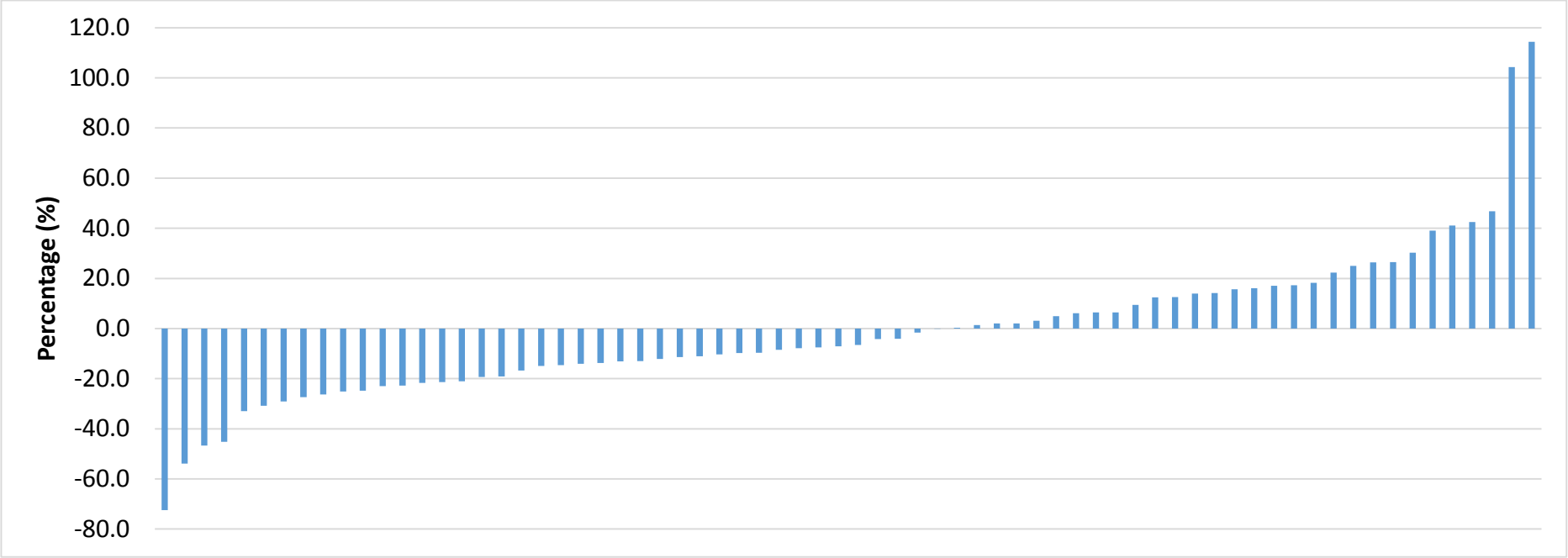

Figure 19. Percentage AMH change (%) of viable pregnancies from gestational week 4 to fetal heart detected by ultrasound (n=70).

Table 33. Repeated measures ANOVA for AMH gestation weeks 4 to 6.5 with AMH trend as between subject factor.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
trend	6.712	5	50	***0.000***	0.406	6.512	0.996

Table 34. AMH levels (mean ± sem) and confidence intervals in patients that increased compared to those that decreased.

AMH Trend	Week	Mean	Sem	95% CI	
				Lower	Upper
Decrease	**4**	21.9 [a, b, c]	2.9	16.1	27.8
	4.5	19.5	3.0	13.4	25.6
	5	17.8 [a]	2.9	12.0	23.7
	5.5	17.4 [b]	3.0	11.4	23.4
	6	17.1 [c]	3.2	10.8	23.4
	6.5	17.1 [c]	3.5	10.0	24.2
Increase	**4**	18.7 [a, b, c]	3.7	11.2	26.2
	4.5	22.0	3.9	14.2	29.7
	5	22.7	3.7	15.2	30.1
	5.5	23.2 [a]	3.8	15.6	30.8
	6	23.3 [b]	4.0	15.2	31.3
	6.5	25.6 [c]	4.5	16.5	34.6

Note – concentrations with same superscript are significantly different ($p<0.05$)

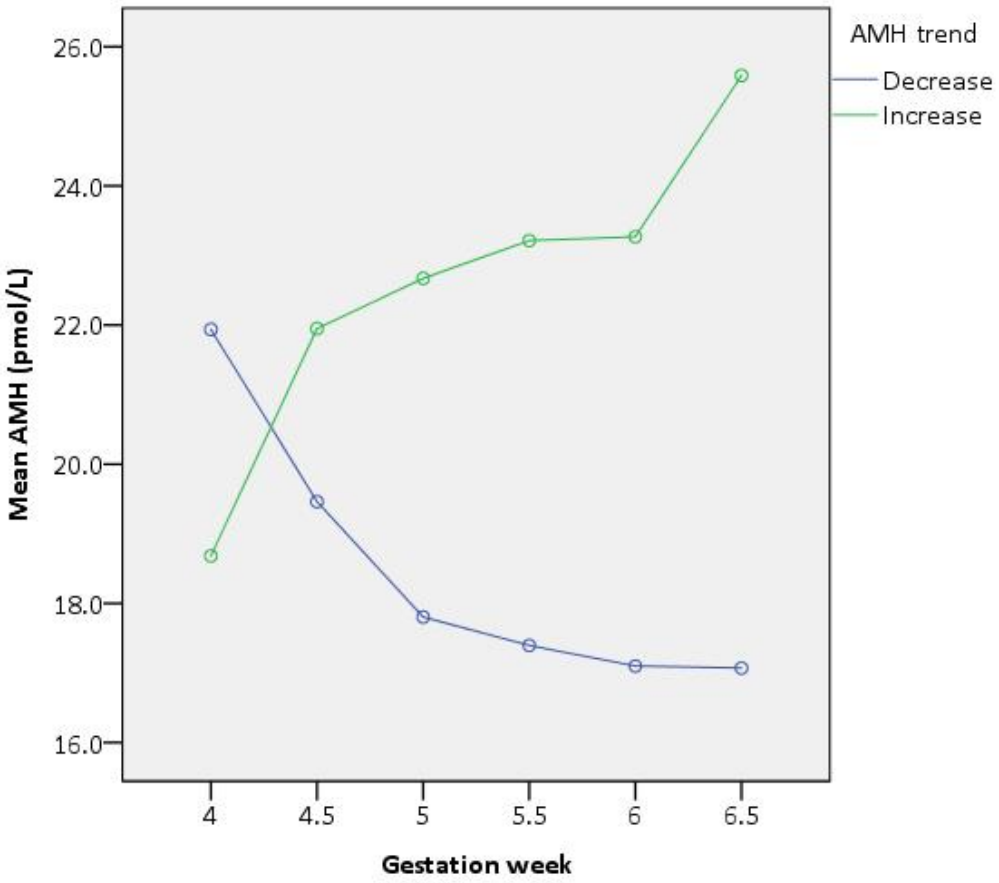

Figure 20. Mean AMH in patients that increased compared to those that decreased.

5.2.5 AMH Levels between Treatment Modality

AMH concentrations were compared between each cycle type at gestation week 4 (time of positive pregnancy test) and baseline (gestation week 0) for those women who had both bloods collected (Table 35). OI patients exhibited the highest AMH levels at the time of positive pregnancy test (32.4± 5.6pmol/L), compared to IVF/ICSI patients who had the lowest AMH levels (3.2pmol/L) at the same time point, probably reflecting the allocation of treatment modality according to ovarian reserve.

Table 35. AMH concentration (mean ± sem) and confidence intervals for each cycle type at gestation week 0 and 4.

Cycle	Week	Mean (pmol/L)	Sem	95% CI	
				Lower	Upper
IVF/ICSI (n=2)	0	2.3	16.5	-31.0	35.6
	4	3.2	12.5	-22.1	28.4
FET (n=19)	0	24.1	5.3	13.3	34.9
	4	17.0	4.1	8.9	25.2
OI/AIH (n=10)	0	44.2	7.4	29.3	59.1
	4	32.4	5.6	21.2	43.7
NATURAL (n=14)	0	18.0	6.2	5.4	30.6
	4	20.0	4.7	10.5	29.6

There was a significant difference in AMH levels between cycle types (p=0.009) (Table 36). Pairwise comparisons revealed significant differences between IVF/ICSI and all other cycle groups (FET, OI/AIH and Natural) (Table 37). Games-Howell was the post hoc test used as variances were not equal between groups.

Table 36. Repeated measures ANOVA for AMH gestation weeks 0 vs 4 with cycle type as between subject factor.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
cycle	4.377	3	41	***0.009***	0.243	13.130	0.838

Table 37. AMH levels (mean ± sem) and confidence intervals between cycle types.

		Mean difference	Sem	Sig.	95% CI	
					Lower	Upper
IVF/ICSI	**FET**	-17.8	3.6	***0.001***	-28.1	-7.6
	OI/AIH	-35.6	10.1	***0.027***	-67.0	-4.1
	NATURAL	-16.3	4.0	***0.006***	-28.0	-4.5
FET	**IVF/ICSI**	17.8	3.6	***0.001***	7.6	28.1
	OI/AIH	-17.8	10.7	0.387	-49.8	14.3
	NATURAL	1.6	5.4	0.992	-13.1	16.2
OI/AIH	**IVF/ICSI**	35.6	10.1	***0.027***	4.1	67.0
	FET	17.8	10.7	0.387	-14.3	49.8
	NATURAL	19.3	10.8	0.328	-12.9	51.6
NATURAL	**IVF/ICSI**	16.3	4.0	***0.006***	4.5	28.0
	FET	-1.6	5.4	0.992	-16.2	13.1
	OI/AIH	-19.3	10.8	0.328	-51.6	12.9

AMH levels decreased significantly from gestation week 0 to 4 in OI/AIH and FET patients (Table 38 and Table 39 respectively), however, there were no significant differences between gestation weeks 0 and 4 for patients in IVF/ICSI or Natural cycles (p=0.497 and p=0.251 respectively) (Tables 40 and 41) but there were only two IVF/ICSI patients with AMH levels tested at gestation week 0, so this analysis did not accurately reflect the patients in this cycle group as a whole.

Table 38. Repeated measures ANOVA for AMH levels at gestation weeks 0 vs 4 for OI/AIH cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	5.038	1	9	***0.050***	0.359	5.038	0.517

Table 39. Repeated measures ANOVA for AMH levels at gestation weeks 0 vs 4 for FET cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	16.038	1	18	***0.001***	0.471	16.038	0.966

Table 40. Repeated measures ANOVA for AMH levels at gestation weeks 0 vs 4 for IVF/ICSI cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	1.017	1	1	0.497	0.504	1.017	0.073

Table 41. Repeated measures ANOVA for AMH levels at gestation weeks 0 vs 4 for Natural cycles.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	1.443	1	13	0.251	0.100	1.443	0.200

5.2.6 AMH Levels in Viable vs Nonviable Pregnancies

There were a total of 85 patients recruited in this study, of which 81.2% (n=69) were single viable pregnancies and 17.6% (n=15) were nonviable pregnancies (consisting of 3 chemical pregnancies, 11 miscarriages and 1 blighted ovum) and there was 1 twin viable pregnancy (1.2%). The cycle groups that had the highest nonviable pregnancy rate of 24.0% were the FETs and IVF/ICSI, compared to the AIH group that had no failed pregnancies (Table 42). There was 1 natural (6.7%) and 2 OI (15.4%) patient cycles that resulted in miscarriage or chemical pregnancies (Table 38). The AMH concentrations of all nonviable pregnancies are shown in Table 43. There was no significant difference in age between women that had viable (37.0 ± 0.5 years) or nonviable pregnancies (35 ± 1.3 years) (p=0.214).

Table 42. Number of nonviable pregnancies in each cycle type.

	NATURAL	IVF/ICSI	FET	AIH	OI
Total	15	25	25	7	13
Nonviable	1	6	6	0	2
Percentage (%)	6.7	24.0	24.0	0.0	15.4

Table 43. AMH levels (mean ± sem) and confidence intervals in nonviable pregnancies.

Week	Mean (pmol/L)	Sem	95% CI	
			Lower	Upper
4	22.6 [a]	5.3	12.0	33.1
4.5	25.2	5.6	14.0	36.4
5	23.9	5.5	12.9	34.8
5.5	23.2	5.5	12.2	34.2
6	24.4	6.1	12.3	36.6
6.5	28.1 [a]	6.7	14.8	41.5

Note – concentrations with same superscript are significantly different ($p<0.05$)

There were clear differences observed in the patterns of AMH change between viable and nonviable pregnancies. Patients that had nonviable pregnancies had inconsistent levels of AMH from time of positive pregnancy test (gestation week 4) to their final blood test (when hCG ≤ 5 IU/mL). AMH levels in nonviable pregnancies were unstable, with rapid or sporadic increases and decreases (Figure 21). In contrast, AMH levels in viable pregnancies followed either an increasing or decreasing trend from gestation week 4 to detection of a fetal heartbeat, irrespective of their treatment cycle (Figures 22 - 25). When viability was analysed as an independent factor/between subjects effect, it was shown that the mean AMH concentration was not significant between weeks 4 to 6.5 ($F=0.540$, $p=0.465$, Partial Eta = 0.008, Power=0.112). However, the power to detect significant change was limited by the smaller number of nonviable pregnancies (n=15) compared to viable pregnancies (n=70).

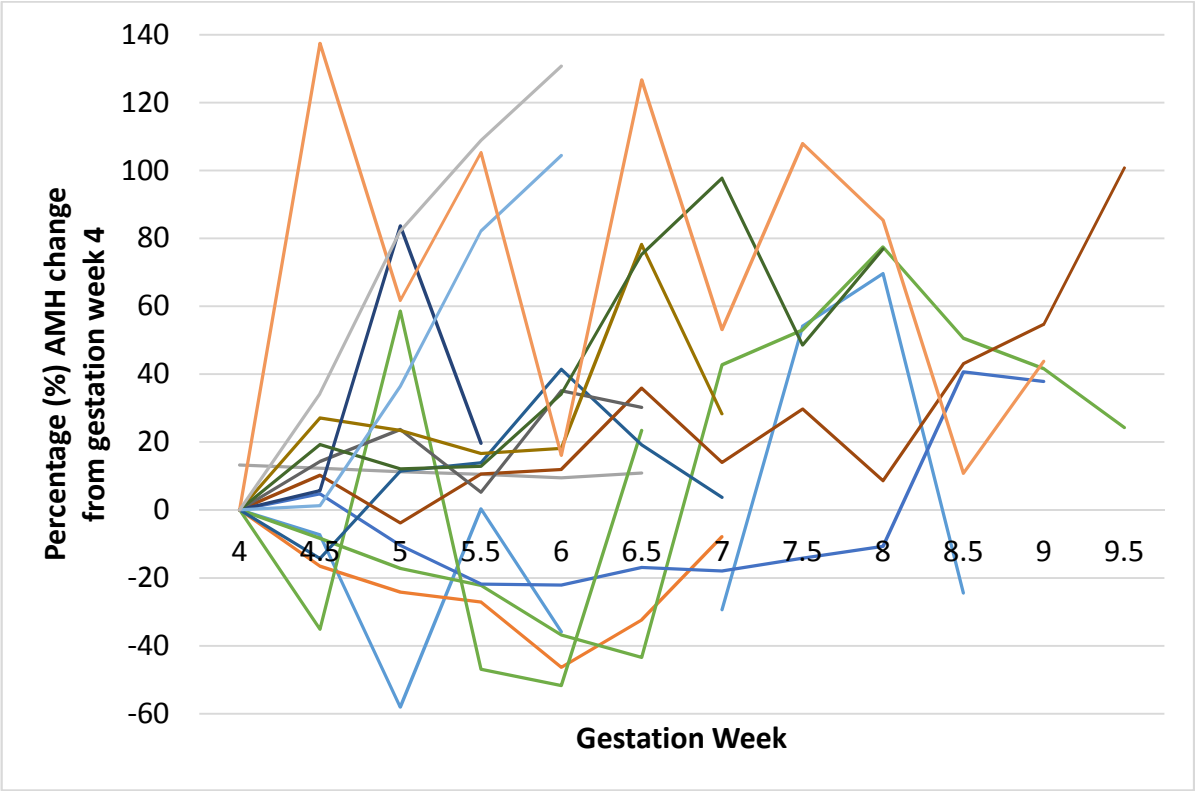

Figure 21. Percentage AMH change in nonviable pregnancy cycles.

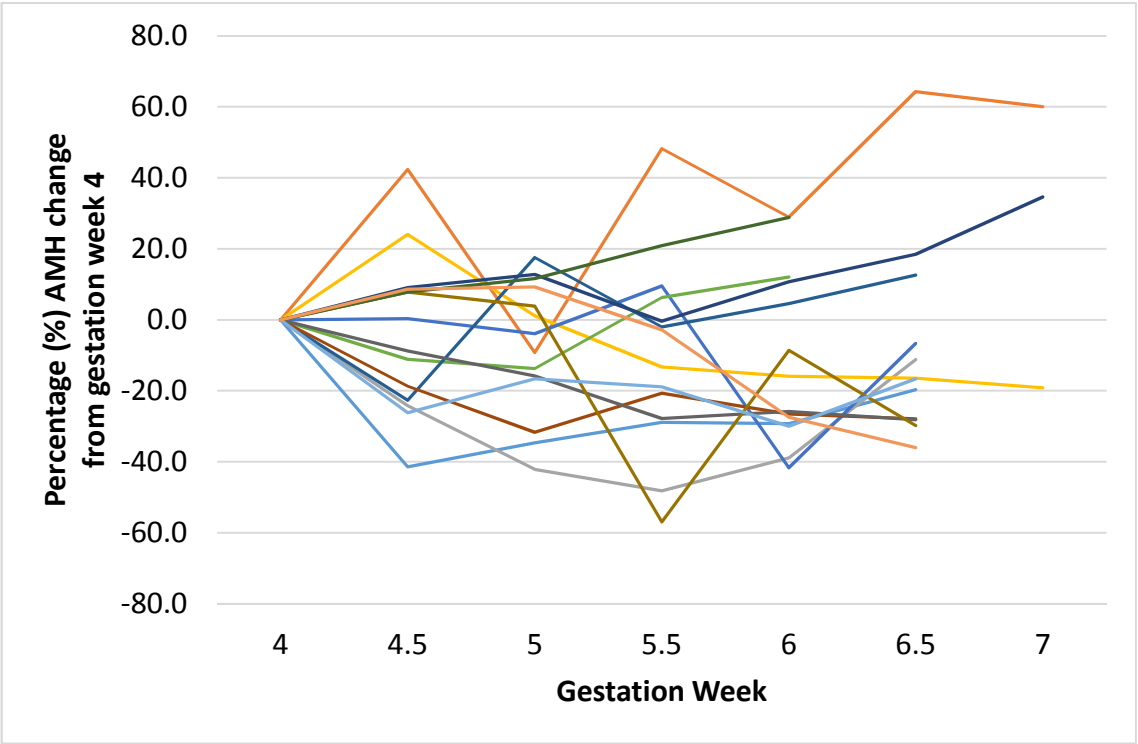

Figure 22. Percentage AMH change in Natural viable pregnancy cycles.

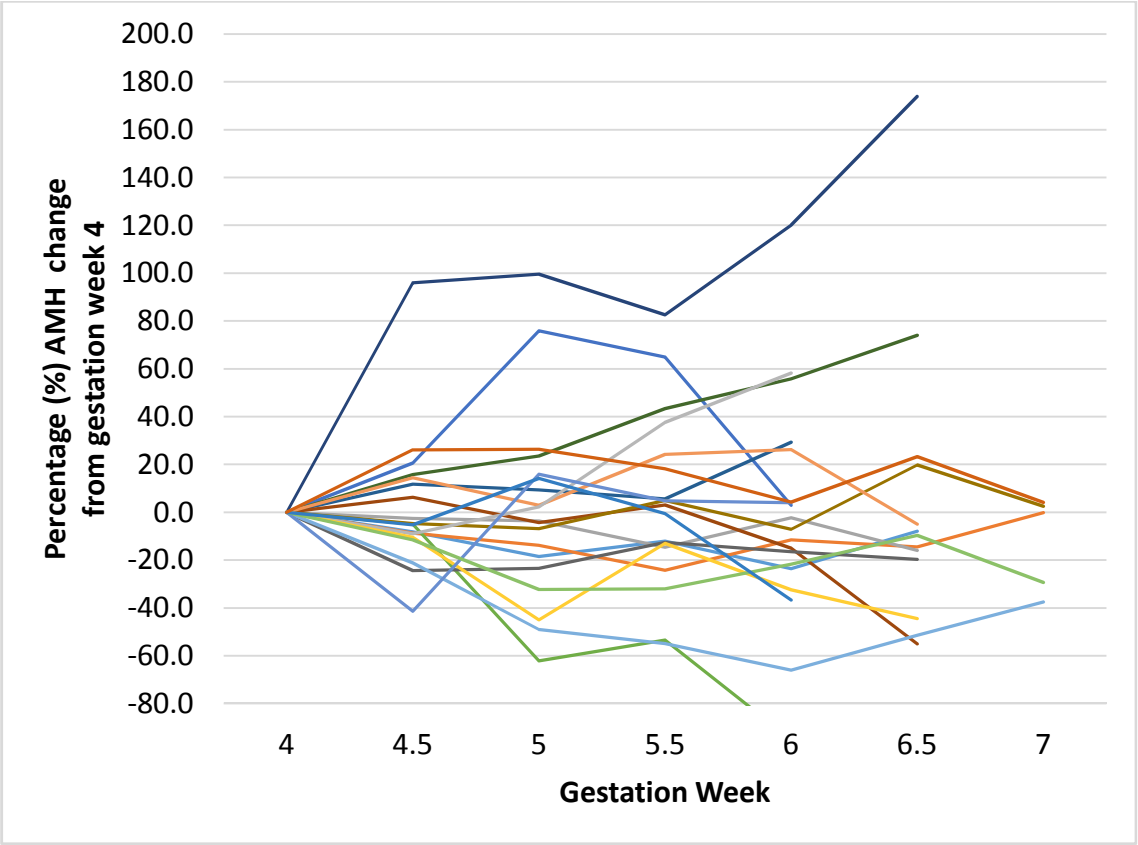

Figure 23. Percentage AMH change in viable pregnancies following IVF/ICSI cycles.

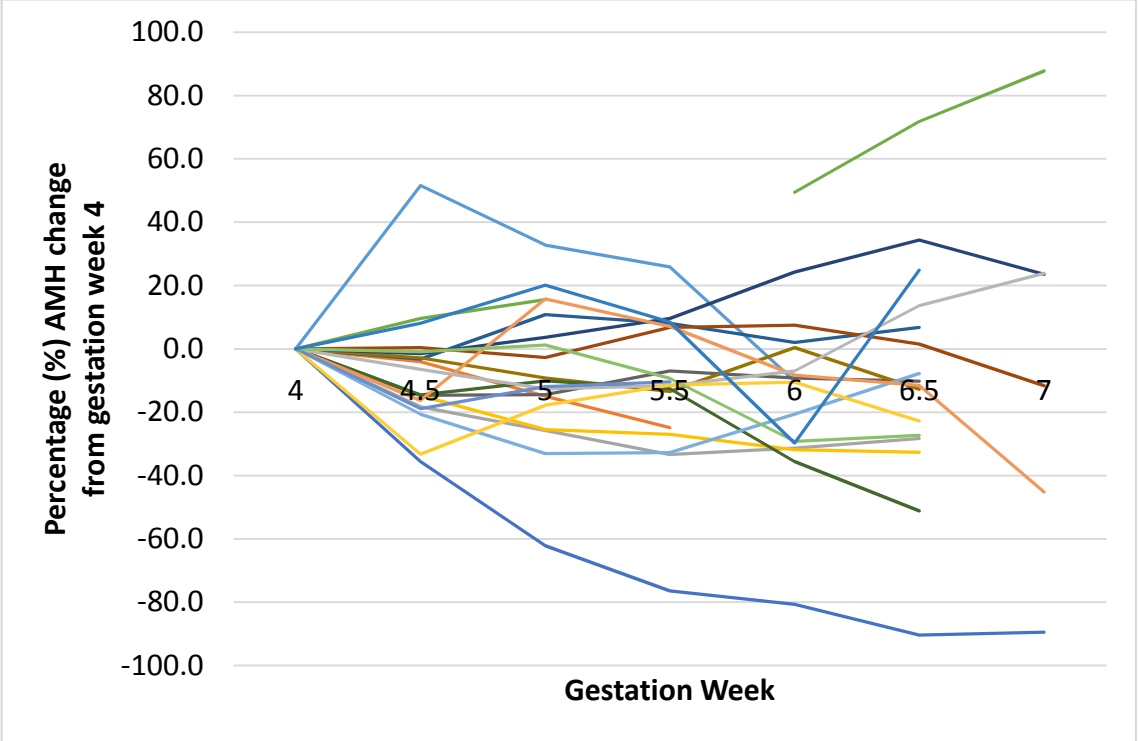

Figure 24. Percentage AMH change in viable pregnancies following FET cycles.

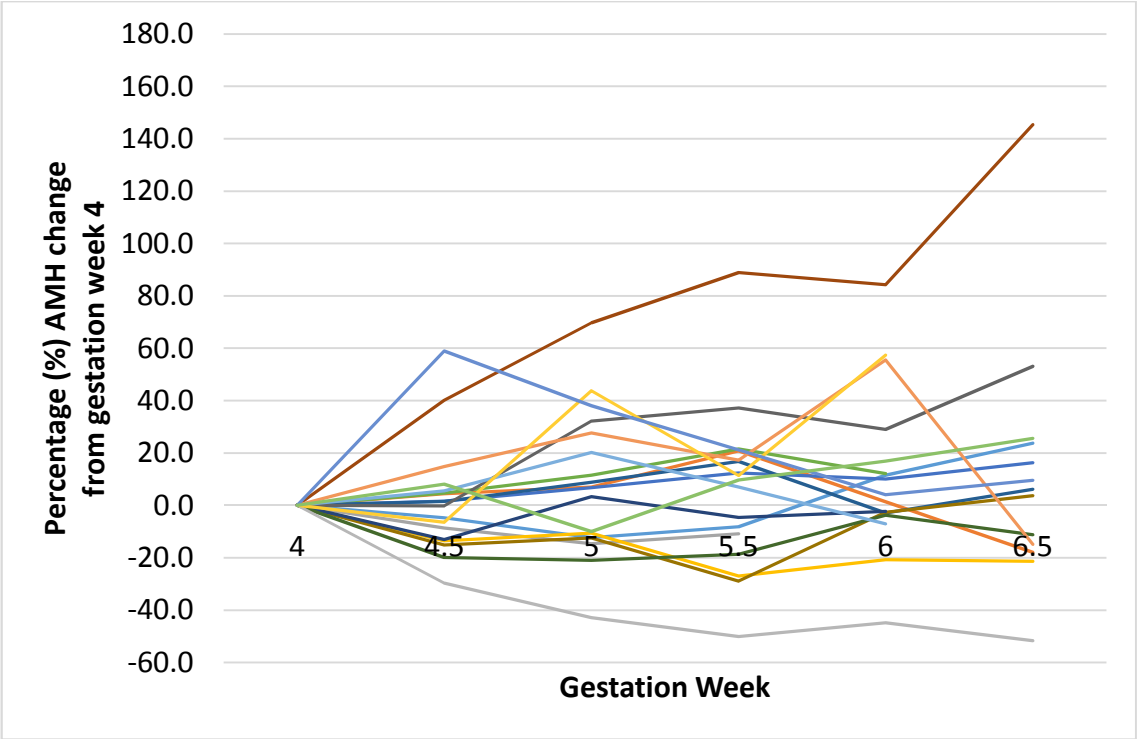

Figure 25. Percentage AMH change in viable pregnancies following OI/AIH cycles.

5.3 Relationship between AMH and other Factors

5.3.1 AMH and Age

The average age of all women (n=85) in this study was 35.4 ± 0.5years. Baseline (day two of the menstrual cycle) blood samples were collected for 45 women and assessed for AMH concentration. Women were divided into four age groups at the time of their first blood collection (26-30 years, 31-35 years, 36-39 years and 40 years and older) and there was a significant difference in AMH levels between all age groups (p=0.000). The mean AMH concentration determined for each group is shown in Table 44. It was shown that there was a significant negative correlation between baseline AMH and age (r=-0.507, p=0.000) (Figure 26), despite the wide range in AMH levels within each group.

Table 44. Effect of women's age on AMH levels.

Patient age (years)	Mean ± Sem	Range
26-30 (n=6)	48.9 ± 15.9	12.1 - 112.5
31-35 (n=17)	30.5 ± 6.4	3.26 - 106.3
36-39 (n=14)	17.3 ± 2.6	6.8 - 42.5
40+ (n=8)	12.9 ± 5.7	1.6 - 39.6

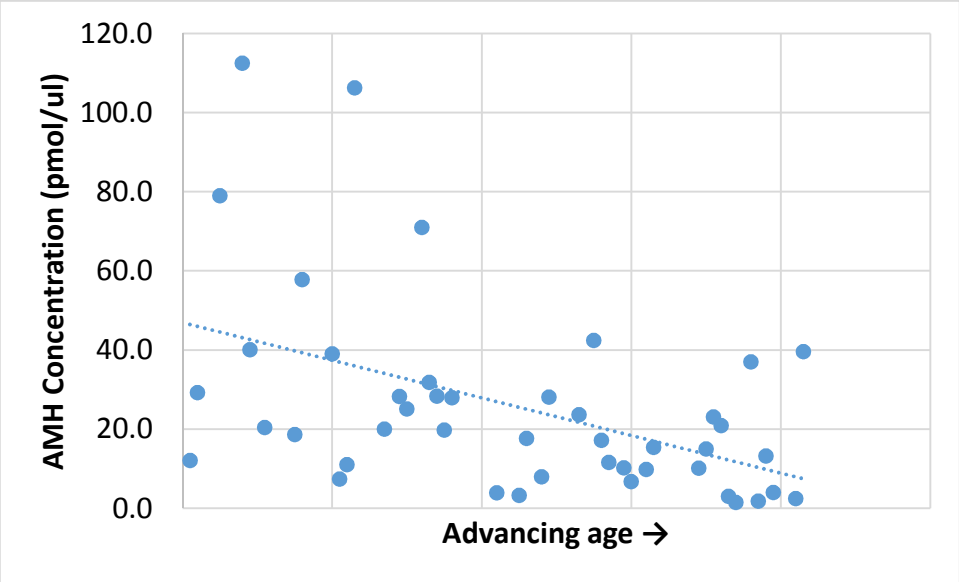

Figure 26. Changes in AMH levels with advancing age (dotted line representing the linear trend line).

5.3.2 AMH and BMI

No correlation (r=-0.102, p=0.537) was present between patient BMI and their baseline (day 2 menstrual cycle) AMH concentration (pmol/L) as shown in Figure 27. Patient BMI was calculated using patient's day 2 weight and height. Not all patient BMIs were available for analysis (n=39). The average patient BMI for all patients that achieved a successful pregnancy was 24.7, with a highly variable range between 18.0 and 36.1.

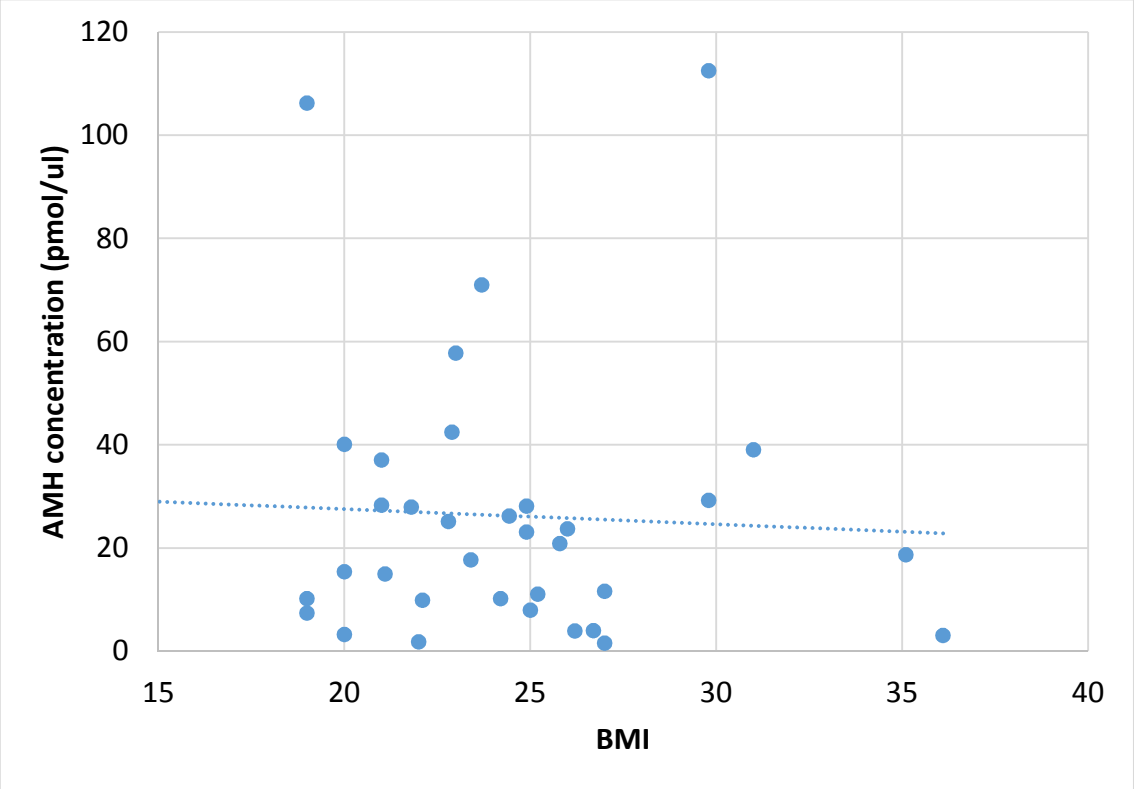

Figure 27. Patient BMI compared to baseline AMH concentration (dotted line represents the linear trend).

5.3.3 BMI and Age

To confirm that the significant relationship between AMH and age was not due to BMI influence, patient age was plotted against their BMI (Figure 28) and only patients that also has day 2 AMH levels were included in the analysis (n=39). No relationship existed between age and BMI (r=-0.006, p=0.972).

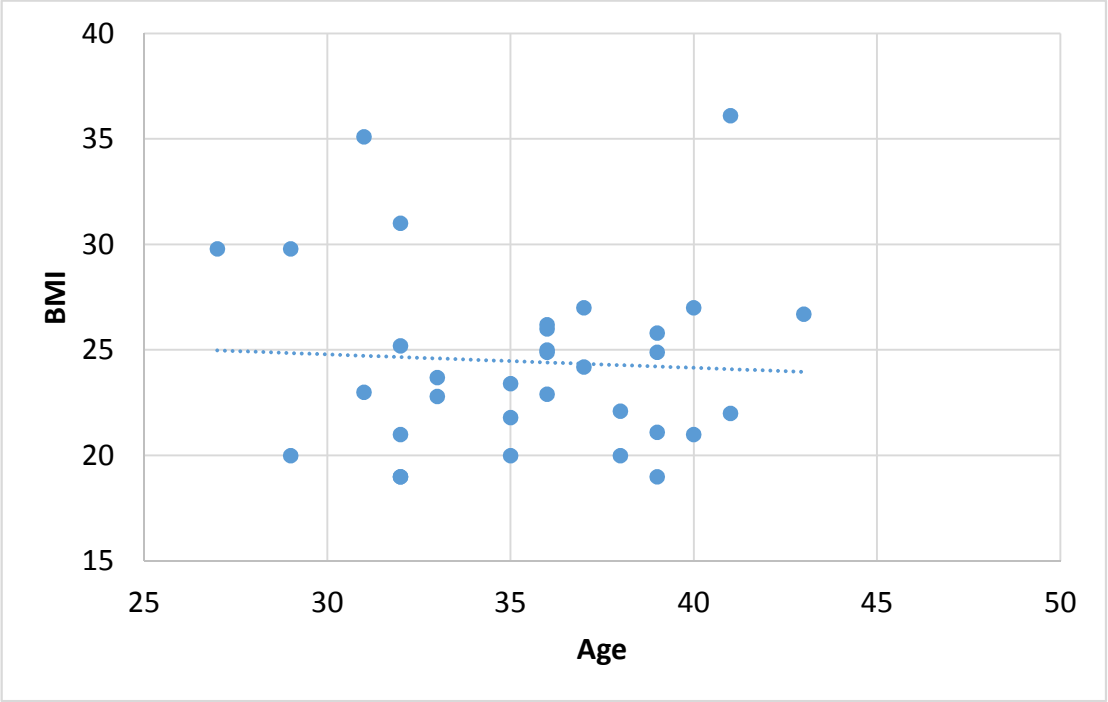

Figure 28. Patient age compared to BMI (dotted line represents the linear trend).

5.3.4 AMH and Medications

A table of medications taken by each patient in the cycle prior to their positive pregnancy is listed in Appendix P. There were no trends between AMH levels and any of the medications, including oral contraception. There were only two patients that were taking oral contraception the month directly prior to their fertility treatment (gestation week 0) and all other patients had been actively trying to conceive (had not been taking the contraceptive pill).

To demonstrate the non-existent effect of stimulation drugs on AMH, the daily dose of follicle stimulatory drug *follitropin alfa* (FSH) administered in IVF/ICSI, FET and OI/AIH cycles, was compared against the mean percentage AMH change between gestation week 4 and week of fetal heart detected by ultrasound (Figure 29). There was no correlation between dosage of FSH and percentage AMH change in early pregnancy (r=0.063, p=0.568). The average dose of FSH in each cycle group and the AMH levels at gestation week 4 (after administration of this drug) is shown in Table 45.

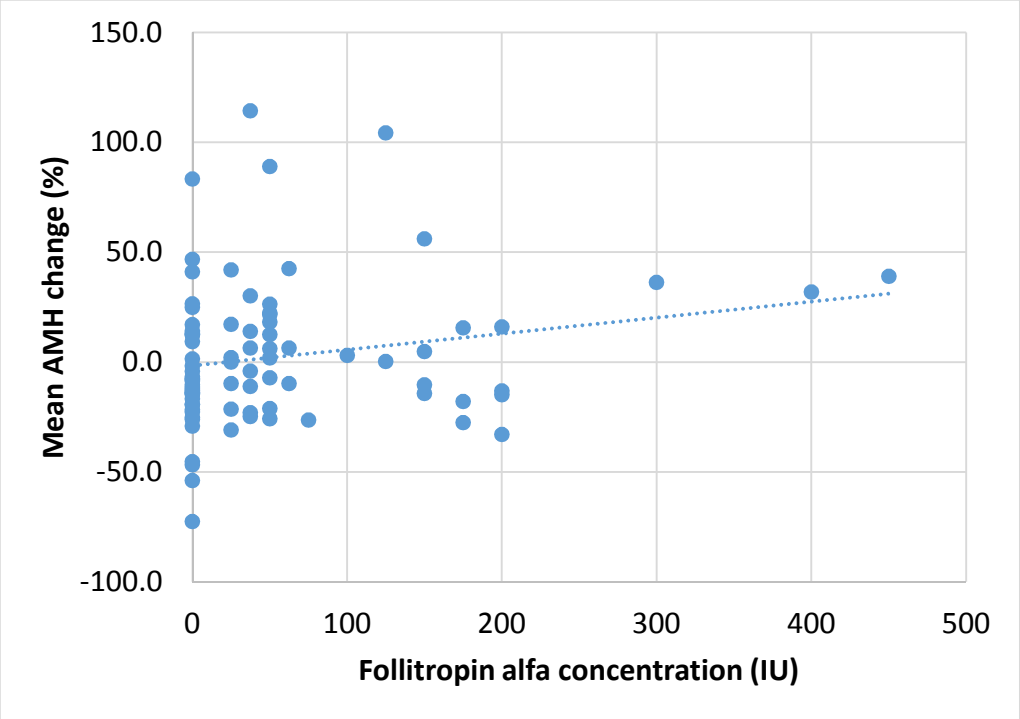

Figure 29. Daily f*ollitropin alfa* levels (IU) administration compared to mean AMH change (%) from gestation week 4 to week of fetal heart detected by ultrasound (dotted line represents the linear trend).

Table 45. Mean daily *follitropin alfa* dosage and the effect on serum AMH concentrations (mean ± sem) in different cycle types at gestation week 4.

Cycle Type	FSH Dose (IU)	AMH (pmol/L)
Natural	0.0 ± 0.0	21.7 ± 4.7
IVF/ICSI	207.8 ± 23.7	13.3 ± 3.3
FET	43.8 ± 3.2	16.0 ± 2.1
AIH	39.6 ± 6.8	24.1 ± 5.4
OI	54.2 ± 10.5	37.2 ± 6.9

5.3.5 AMH and Fetal Sex

Of the 70 patients that achieved a successful pregnancy, a total of 30 fetal or baby genders were identified at the time of data analysis, from either the 20 week ultrasounds or births respectively. There was no relationship between the fetal/baby gender and the changes in the mother's AMH concentration during early pregnancy (Figure 30). 54.5% (6/11) boys showed a decrease in mother's AMH levels, compared to 45.5% (5/11) that increased. Of the 19 girls, 12 (63.2%) had a decrease in the mother's AMH levels, compared to 7 (36.8%) that increased. Percentage AMH change was calculated as the mean percentage change from gestation week 4 to 6.5. Analysis of AMH within this gestational time revealed gender was not a significantly contributing factor (p=0.809) (Table 46).

Table 46. Repeated measures ANOVA for AMH gestation weeks 4 to 6.5 with gender as between-subject factor.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
gender	0.602	10	96	0.809	0.059	6.018	0.295

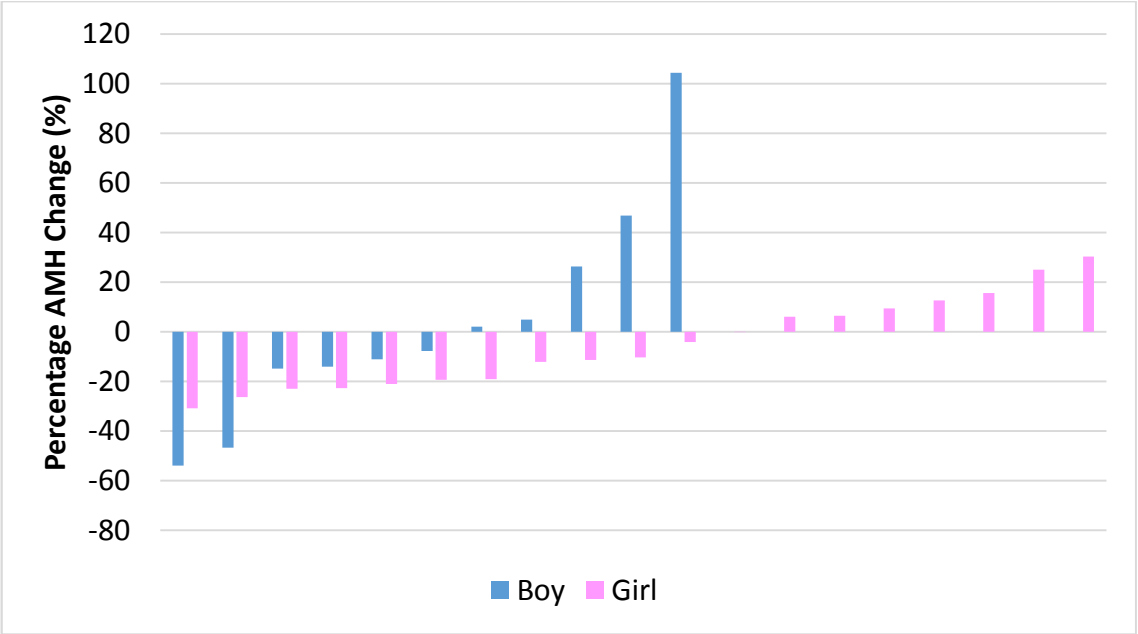

Figure 30. Percentage change in mother's AMH levels during early pregnancy compared to gender of fetus/baby.

5.3.6 AMH and Other Hormones

AMH was negatively correlated to P4 (r=-0.220, p=0.000,) and TSH (r=-0.155, p=0.001) during gestation weeks 4 to 6.5 (Table 47).

Table 47. Hormone correlations during early pregnancy (n=472 - 488).

Correlation	AMH	hCG	P4	E2	fT3	fT4	TSH
AMH	1.000	0.072	-0.220*	-0.023	-0.003	0.076	-0.155*
hCG	0.072	1.000	0.032	0.234*	-0.129*	-0.098#	-0.133*
P4	-0.220*	0.032	1.000	0.098#	-0.014	-0.024	0.073
E2	-0.023	0.234*	0.098#	1.000	-0.121#	0.098#	-0.072
fT3	-0.003	-0.129*	-0.014	-0.121#	1.000	0.276*	0.217*
fT4	0.076	-0.098#	-0.024	0.098#	0.276*	1.000	0.196*
TSH	-0.155*	-0.133*	0.073	-0.072*	0.217*	0.196*	1.000

*p<0.05, # p<0.005

AMH was negatively correlated with E2 (p=0.005), P4 (p=0.000), fT3 (p=0.026) and TSH (p=0.000) when accounting for cycle types (Table 48). In addition, AMH was positively correlated with hCG (p=0.001) when accounting for both cycle type and gestation week (Table 48). However, since approximately half of the women had increasing AMH

levels and half had decreasing AMH levels as gestation (and hCG) increased, the weak correlation (r=0.142) between these two hormones was expected. Scatterplots were generated to visualise any trends between those hormones that showed significant interactions (Figures 31 - 36).

Table 48. AMH vs other hormones when accounting for cycle type and cycle type and gestation week combined (n=674 - 728).

Correlation	AMH (cycle)	AMH (cycle & week)
E2	-0.108#	-0.087*
P4	-0.171#	-0.15*
hCG	0.039	0.142*
fT3	-0.082#	-0.091#
fT4	-0.025	-0.023
TSH	-0.160#	-0.162#

*p<0.05, # p<0.005

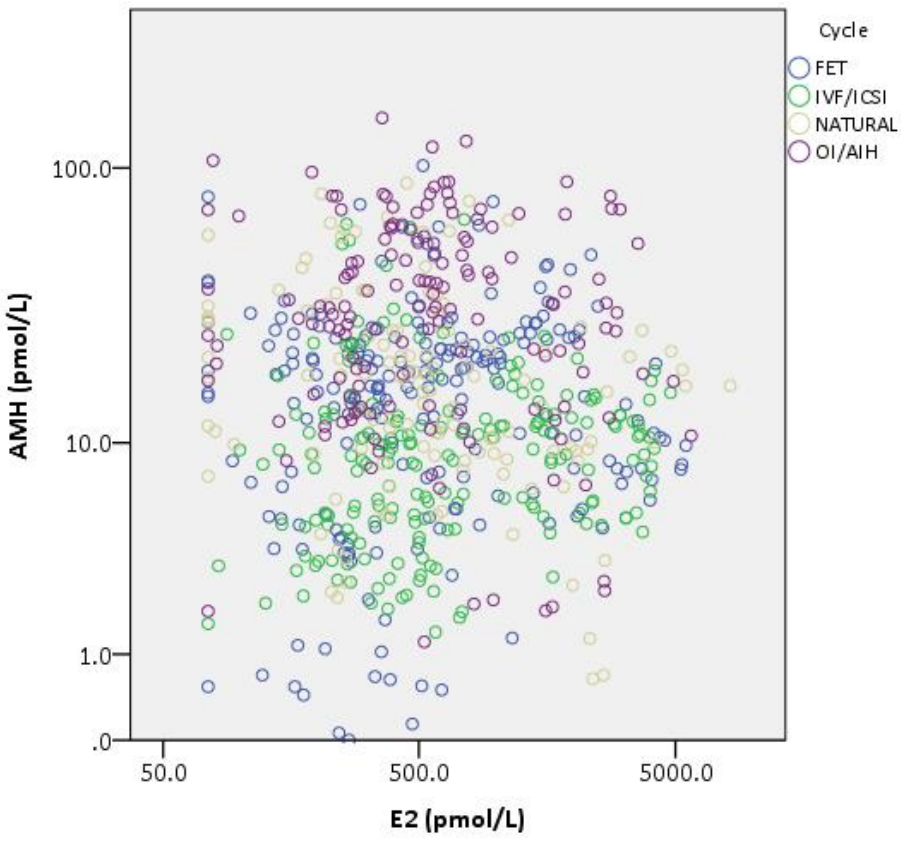

Figure 31. AMH vs E2 accounting for cycle type.

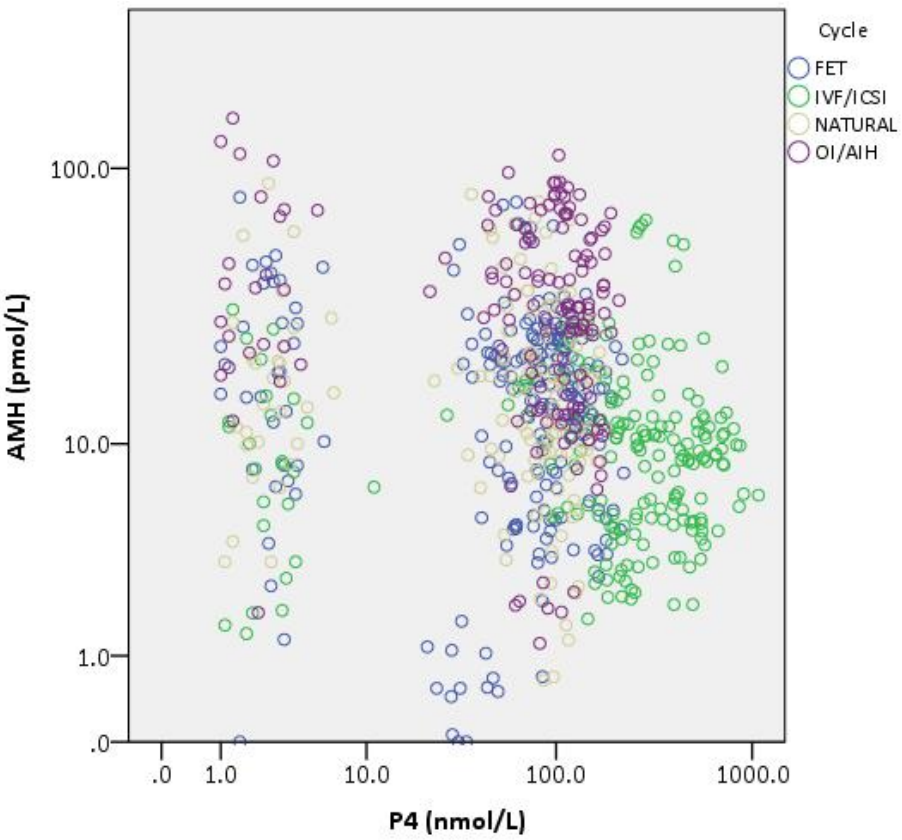

Figure 32. AMH vs P4 accounting for cycle type.

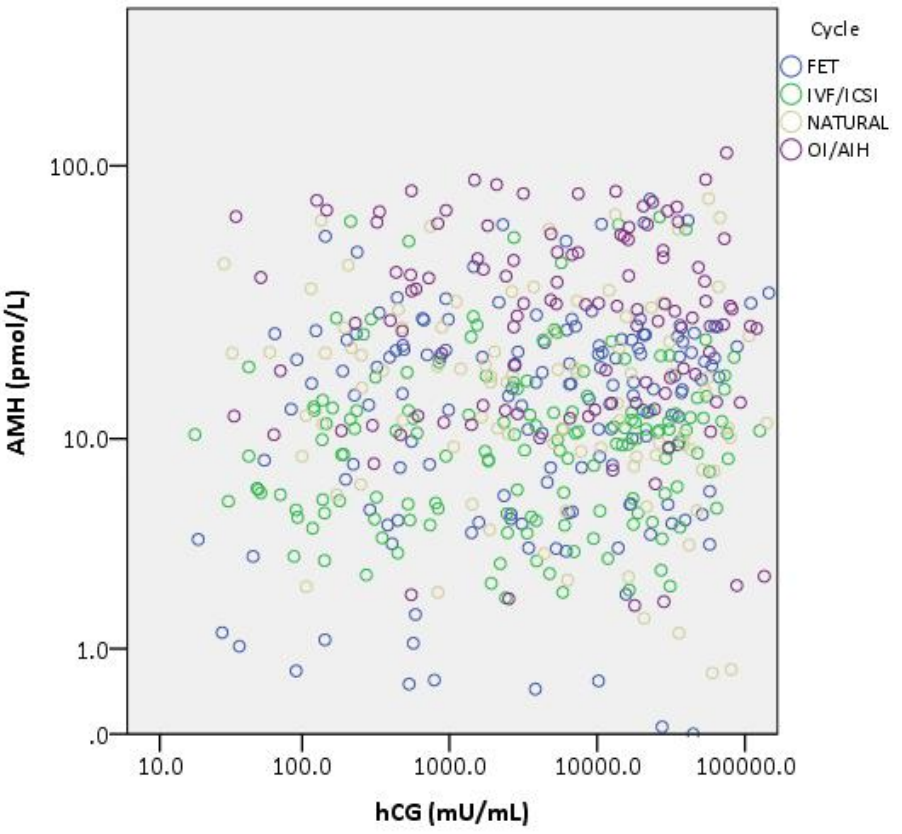

Figure 33. AMH vs hCG accounting for cycle type.

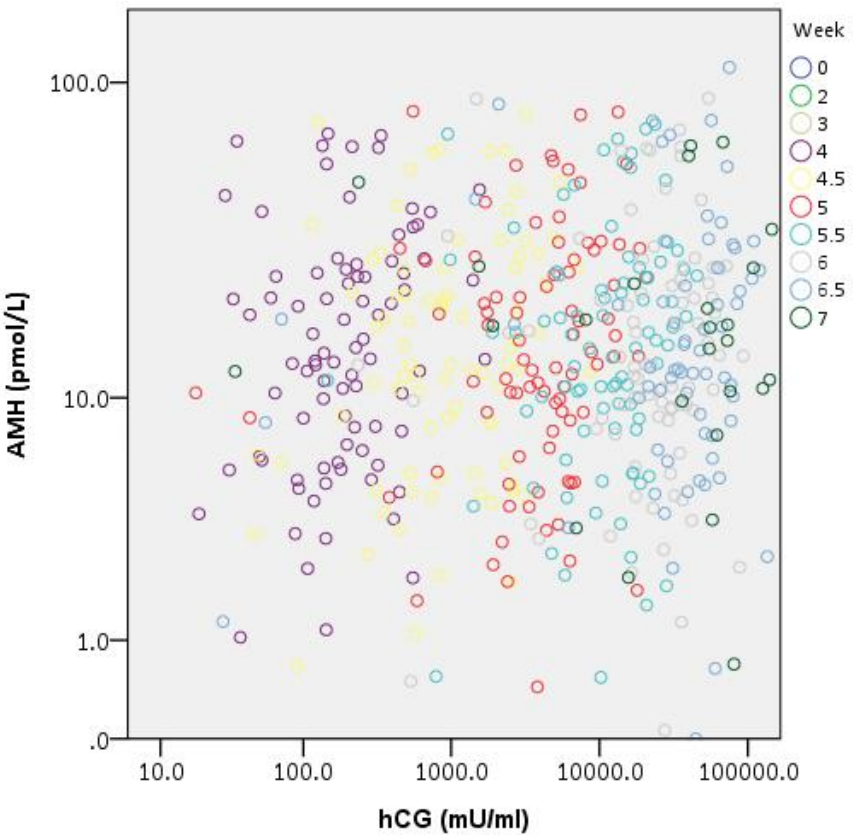

Figure 34. AMH vs hCG accounting for gestation week.

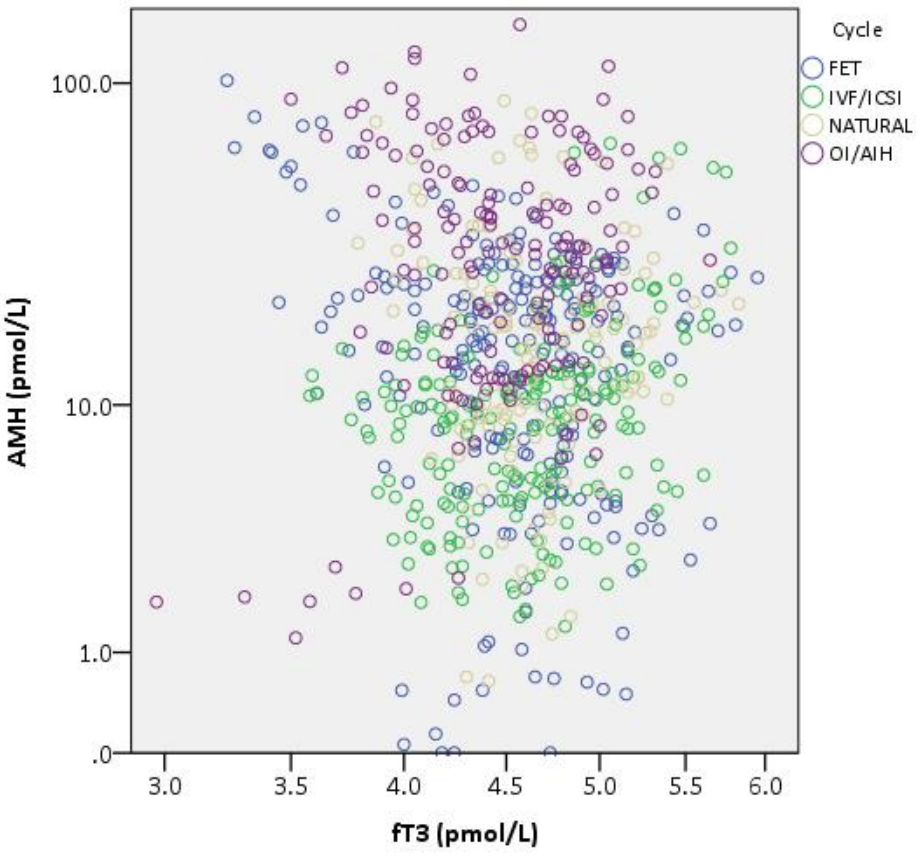

Figure 35. AMH vs fT3 accounting for cycle type.

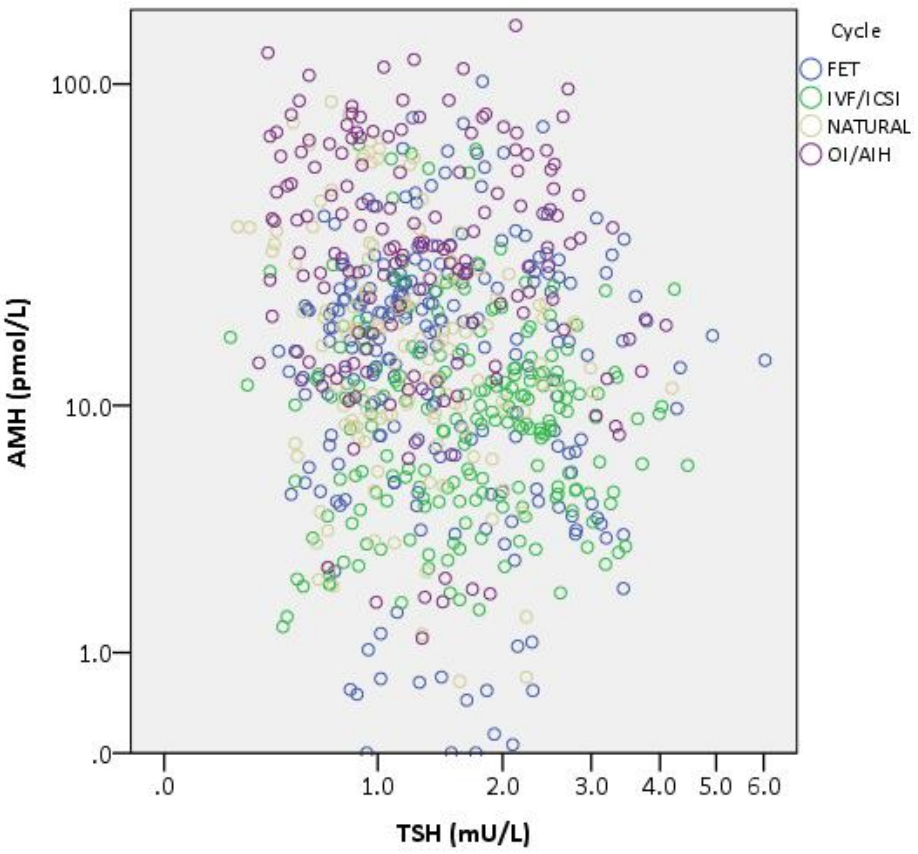

Figure 36. AMH vs TSH accounting for cycle type.

CHAPTER 6

THYROID HORMONE RESULTS

6 Thyroid Hormone Results

6.1 Thyroid Levels Before and During Early Pregnancy

6.1.1 fT3 Levels Before and During Early Pregnancy

The mean fT3 concentration in patients during early pregnancy (gestation weeks 4 to 7) was 4.58 ± 0.02pmol/L, which was similar to the non-pregnant mean concentration at gestation week 0 (4.61 ± 0.07pmol/L). Comparison of fT3 concentrations before pregnancy (gestation week 0) to day of pregnancy test (gestation week 4) revealed no significant difference (p=0.292) (Table 49). However, the concentration of fT3 decreased gradually during early pregnancy (gestation weeks 4 to 6.5) (Figure 37), (r=-0.104, p=0.005). There was a significant difference noted in fT3 levels (p=0.003) when accounting for week using ANOVA (Table 50). The fT3 levels (mean ± sem) and confidence intervals for patients with serum samples measured at all gestation weeks between 4 and 6.5 (n=55) are shown in Table 51.

Table 49. Repeated measures ANOVA for fT3 gestation weeks 0 vs 4.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	1.142	1	37	0.292	0.030	1.142	0.180

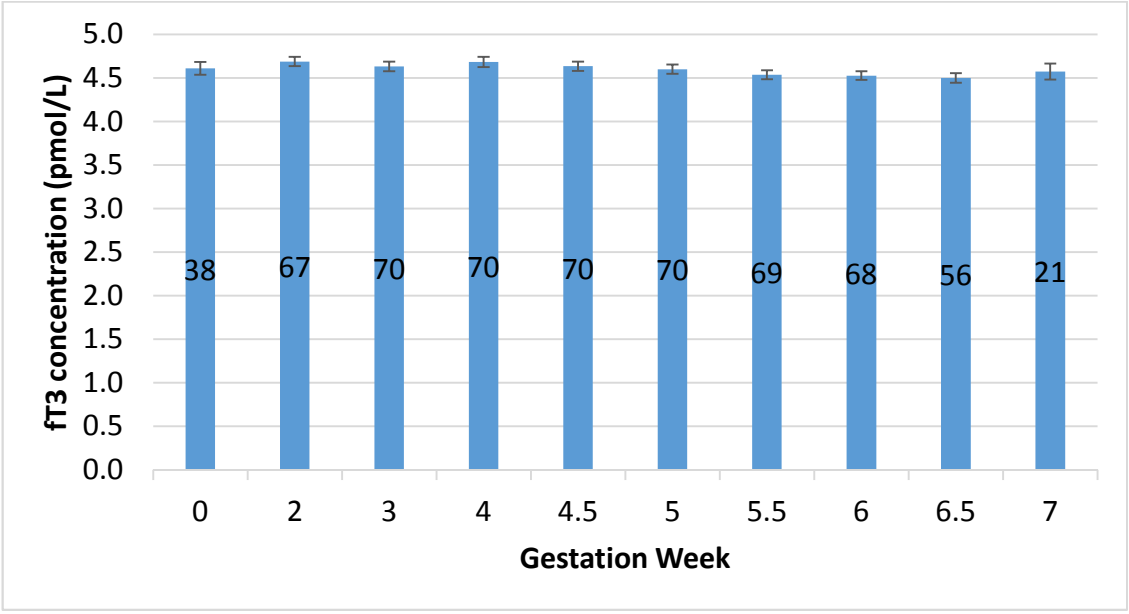

Figure 37. fT3 concentration (mean ± sem) from gestation weeks 0 - 7. Number of patient samples available for analysis at each gestation week is shown within each column.

Table 50. Repeated measures ANOVA for fT3 gestation weeks 4 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	4.180	5	50	***0.003***	0.295	20.899	0.937

Table 51. fT3 levels (mean ± sem) and confidence intervals during early pregnancy.

Gestation Week	Mean (pmol/L)	Sem	95% CI	
			Lower	Upper
4	4.69 [a, b]	0.07	4.54	4.83
4.5	4.64	0.06	4.52	4.77
5	4.61	0.06	4.48	4.74
5.5	4.55	0.06	4.42	4.67
6	4.53 [a]	0.06	4.41	4.65
6.5	4.50 [b]	0.06	4.39	4.61

Note – concentrations with same superscript are significantly different ($p<0.05$)

6.1.2 fT4 Levels Before and During Early Pregnancy

The mean fT4 concentration in patients from gestation weeks 4-7 was 14.9 ± 0.09pmol/L, which was comparable to the non-pregnant mean concentration at gestation week 0 (15.1 ± 0.35pmol/L). Comparison of fT4 concentrations before pregnancy (gestation week 0) to day of pregnancy test (gestation week 4) revealed no significant difference ($p=0.269$) (Table 52).The concentration of fT4 did not vary significantly over gestation weeks 4 to 6.5 (Figure 38) ($r=0.023$, $p=0.526$), however when analysing fT4 levels between weeks using ANOVA (Table 53), there was a significant difference noted ($p=0.018$) and this was due to a significant difference between gestation week 4 and 5.5. The mean fT4 levels (±sem) and confidence intervals for patients with serum samples measured at all gestation weeks between 4 and 6.5 ($n=54$) are shown in Table 54.

Table 52. Repeated measures ANOVA for fT4 gestation weeks 0 vs 4.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	1.260	1	37	0.269	0.033	1.260	0.194

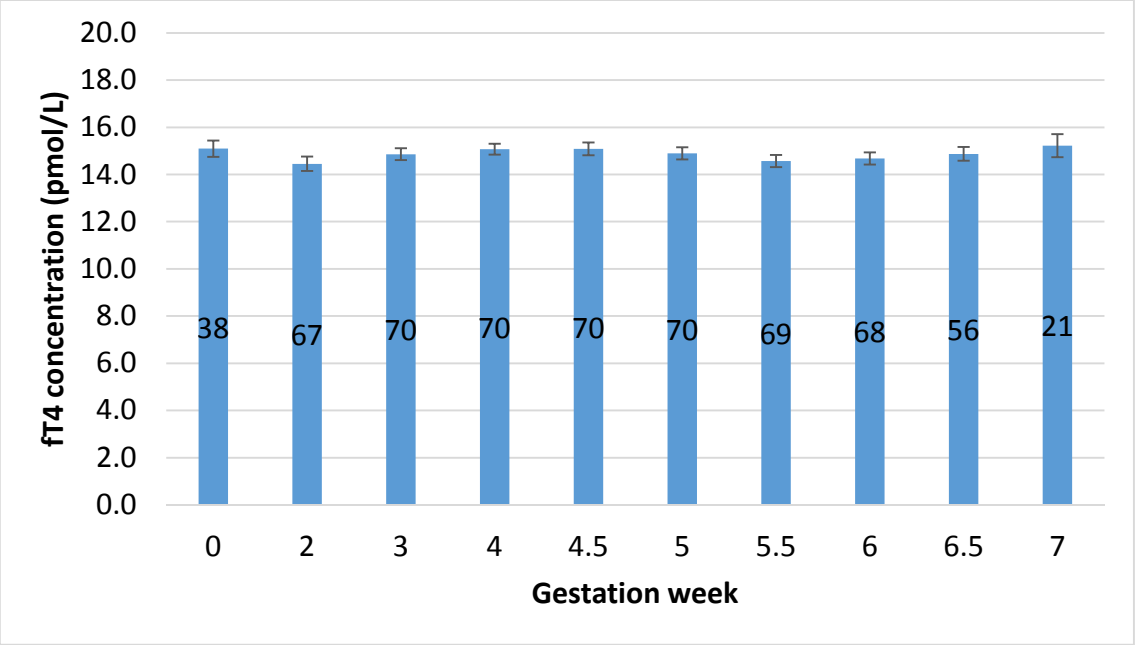

Figure 38. fT4 concentration (mean ± sem) from gestation weeks 0 - 7. Number of patient samples available for analysis at each gestation week is shown within each column

Table 53. Repeated measures ANOVA for fT4 gestation weeks 4 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	3.026	5	50	***0.018***	0.232	15.129	0.825

Table 54. fT4 levels (mean ± sem) and confidence intervals during early pregnancy.

Gestation Week	Mean (pmol/L)	Sem	95% CI	
			Lower	Upper
4	15.21 [a]	0.25	14.70	15.71
4.5	15.11	0.28	14.55	15.67
5	14.95	0.29	14.36	15.54
5.5	14.59 [a]	0.28	14.03	15.15
6	14.79	0.28	14.23	15.34
6.5	14.87	0.30	14.27	15.47

Note – concentrations with same superscript are significantly different ($p<0.05$)

6.1.3 TSH Levels Before and During Early Pregnancy

The mean TSH concentration in patients from gestation weeks 4-7 was 1.50 ± 0.04mU/L, which was comparable to the non-pregnant mean concentration at gestation week 0 (1.45 ± 0.13mU/L). Comparison of TSH concentrations before pregnancy (gestation week 0) to day of pregnancy test (gestation week 4) revealed no significant difference ($p=0.485$) (Table 55). However, the mean TSH concentration decreased significantly during early pregnancy (gestation weeks 4 to 6.5) (Figure 39) ($r=-0.123$ $p=0.013$). There was a significant difference noted in TSH levels ($p=0.009$) when accounting for week using ANOVA (Table 56). The mean TSH levels (± sem) and confidence intervals for patients with serum samples measured at all gestation weeks between 4 and 6.5 (n=55) are shown in Table 57.

Table 55. Repeated measures ANOVA for TSH gestation weeks 0 vs 4.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	0.498	1	37	0.485	0.013	0.498	0.106

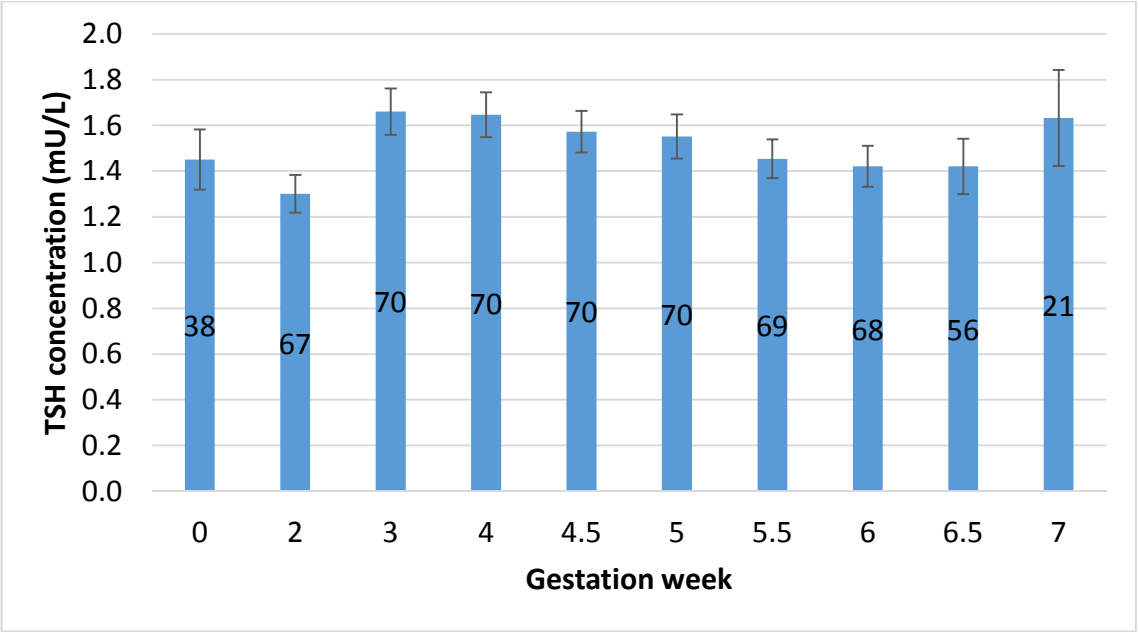

Figure 39. TSH concentrations (mean ± sem) from gestation weeks 0 - 7. Number of patient samples available for analysis at each gestation week is shown within each column**.**

Table 56. Repeated measures ANOVA for TSH gestation weeks 4 to 6.5.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
week	3.476	5	50	***0.009***	0.258	17.380	0.881

Table 57. TSH levels (mean ± sem) and confidence intervals during early pregnancy.

Gestation Week	Mean (mU/L)	Sem	95% CI	
			Lower	Upper
4	1.71 [a, b]	0.12	1.48	1.94
4.5	1.65	0.11	1.43	1.87
5	1.57	0.11	1.35	1.79
5.5	1.54	0.10	1.34	1.73
6	1.45 [a]	0.10	1.26	1.65
6.5	1.42 [b]	0.12	1.17	1.66

Note – concentrations with same superscript are significantly different ($p<0.05$)

6.2 Thyroid Markers and Other Hormones

TSH, fT3 and fT4 were all negatively correlated to hCG. TSH and fT3 were negatively correlated to E2 and fT4 positively correlated to E2, although this relationship was extremely weak (r=0.098). fT3, fT4 and TSH were all positively correlated with each other (refer to Table 47). All significant correlations were weak and were further investigated by accounting for cycle type (Table 58).

A weak positive correlation existed between TSH levels and P4 when accounting for cycle type (r=0.223, p=0.000). fT3 and TSH were negatively correlated with hCG, although neither were strong correlations (r=-0.123 and r=-0.131 respectively). All thyroid markers remained positively correlated with each other (p=0.000) when accounting for cycle type, although still a weak effect ($r \leq 0.290$). fT4 was no longer significantly correlated to hCG when accounting for cycle type. fT3 and fT4 were also not significantly correlated to E2 when accounting for cycle type.

Table 58. Thyroid vs other hormones accounting for cycle type (n=485 – 728).

Correlation	fT3	fT4	TSH
E2	-0.034	0.063	-0.008
P4	-0.081	0.082	0.223#
hCG	-0.123#	-0.037	-0.131#
fT3	1.000	0.290#	0.186#
fT4	0.290#	1.000	0.175#
TSH	0.186#	0.175#	1.000

# $p<0.005$

When the same partial correlations were performed, accounting for both cycle type and gestation week (Table 59), there was only one discernible change (from the significant relationships between hormones when accounting for cycle type alone) – fT3 was significantly negatively correlated to P4, but only a very weak effect size (r=-0.090).

Table 59. Thyroid vs other hormones accounting for cycle type and gestation week (n=485 – 728).

Correlation	fT3	fT4	TSH
E2	-0.021	0.058	-0.013
P4	-0.090*	0.080	0.218#
hCG	-0.091*	-0.012	-0.172#
fT3	1.000	0.293#	0.186#
fT4	0.293#	1.000	0.175#
TSH	0.186#	0.175#	1.000

*p<0.05, # p<0.005

Scatterplots were generated to visualise any trends between those hormones that showed significant interactions (Figures 40 -46).

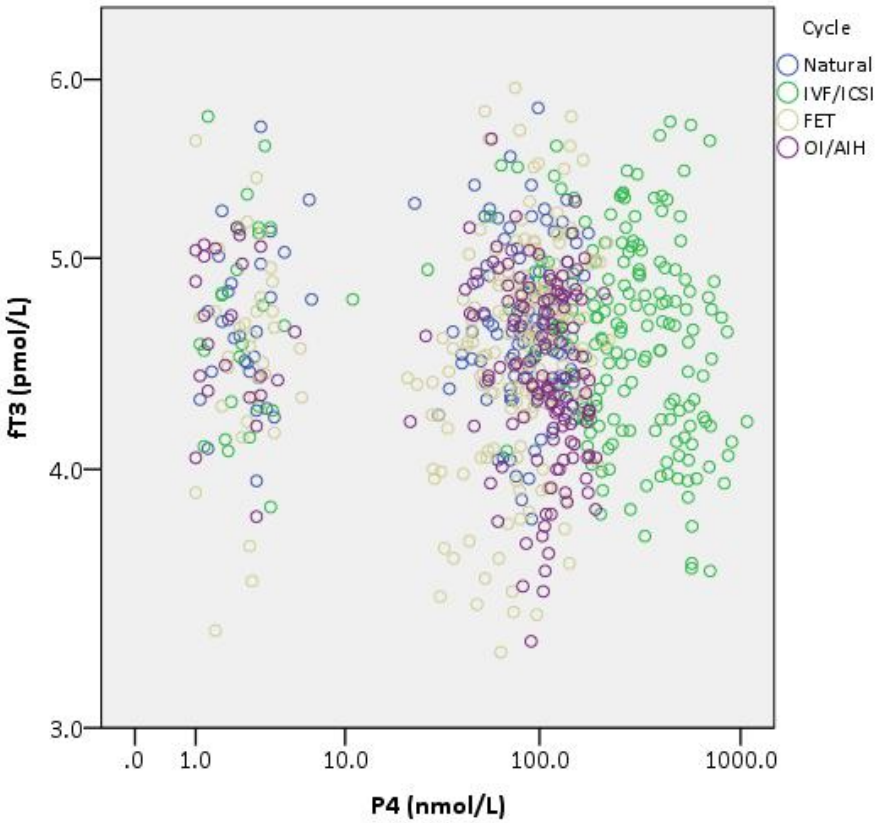

Figure 40. fT3 vs P4 accounting for cycle type.

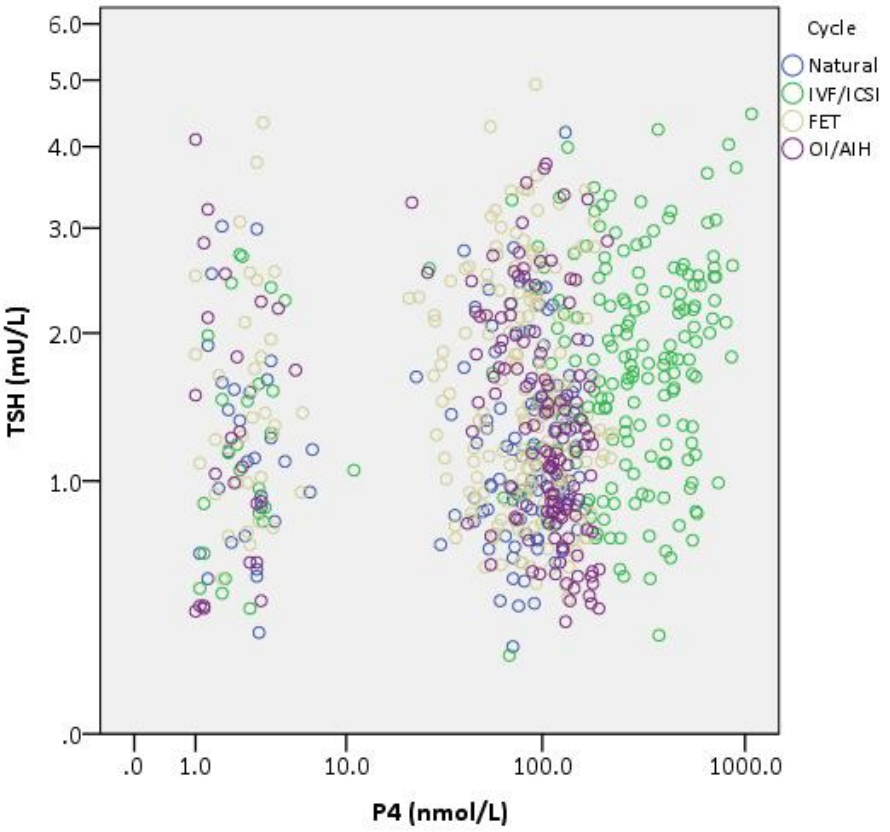

Figure 41. TSH vs P4 accounting for cycle type.

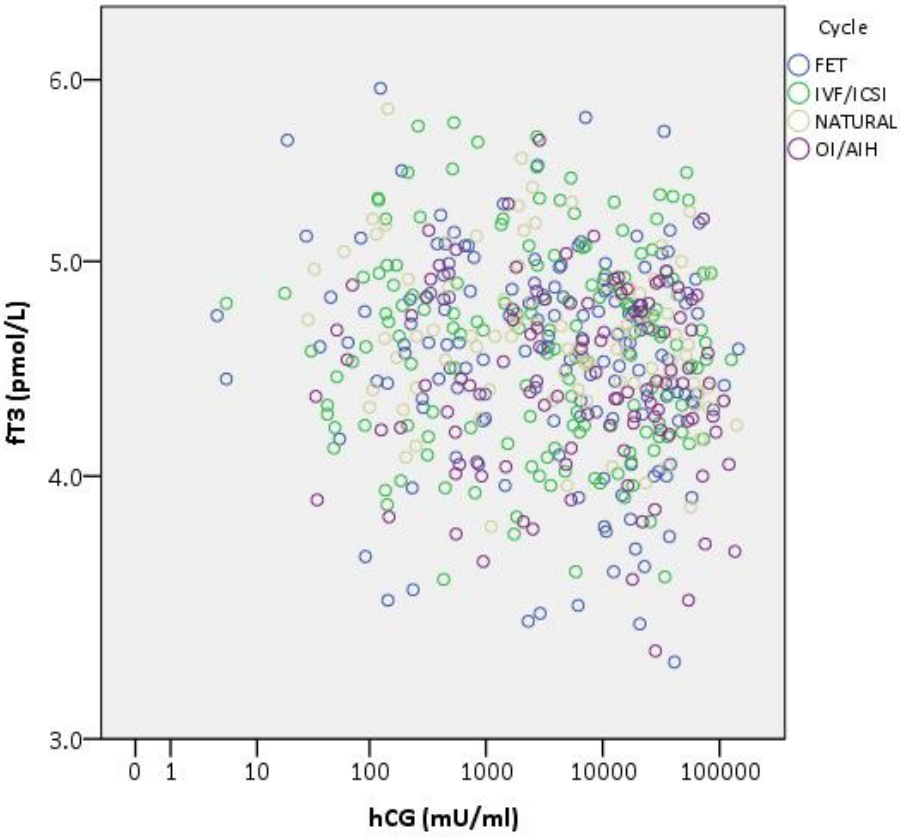

Figure 42. fT3 vs hCG accounting for cycle type.

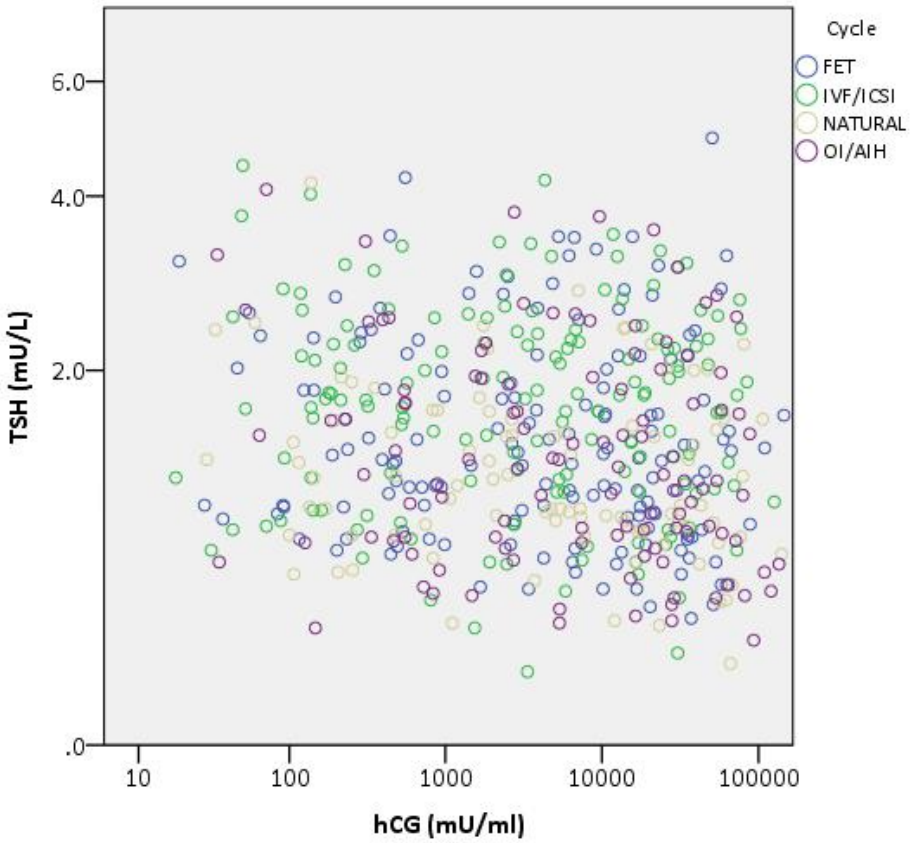

Figure 43. TSH vs hCG accounting for cycle type.

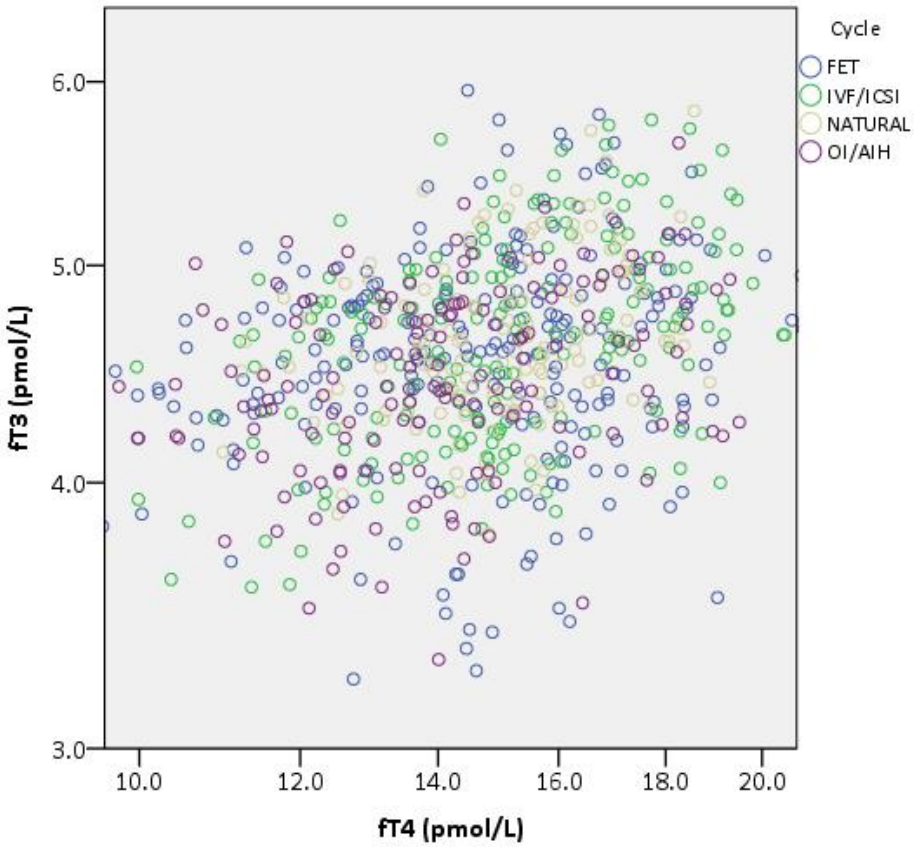

Figure 44. fT3 vs fT4 accounting for cycle type.

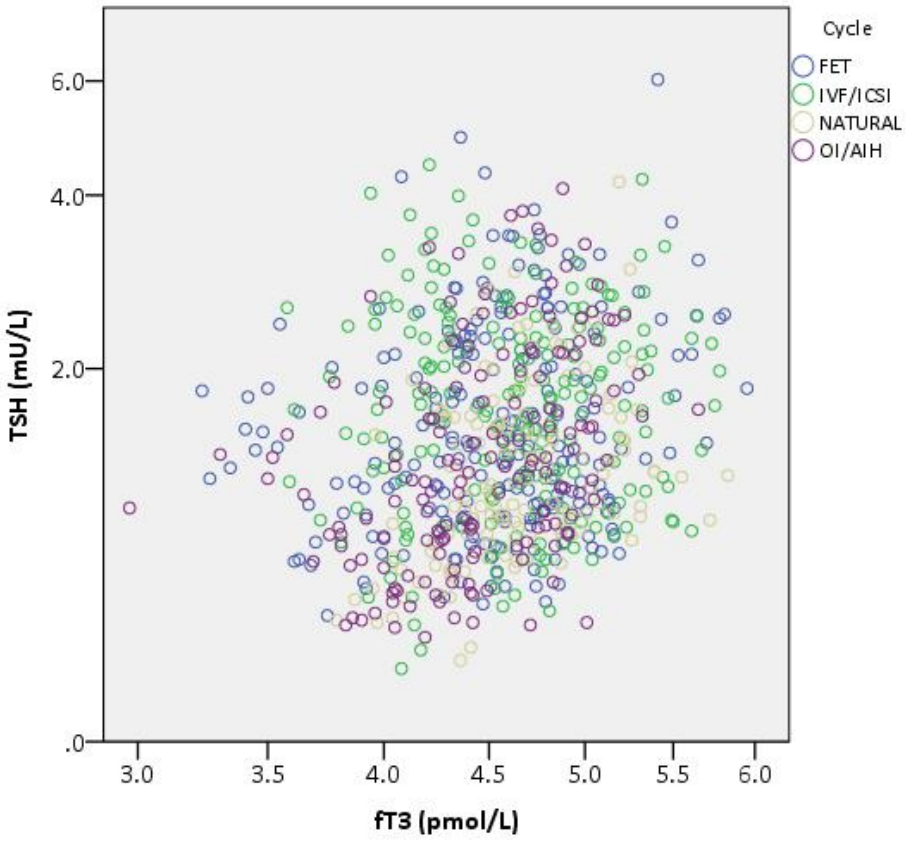

Figure 45. TSH vs fT3 accounting for cycle type.

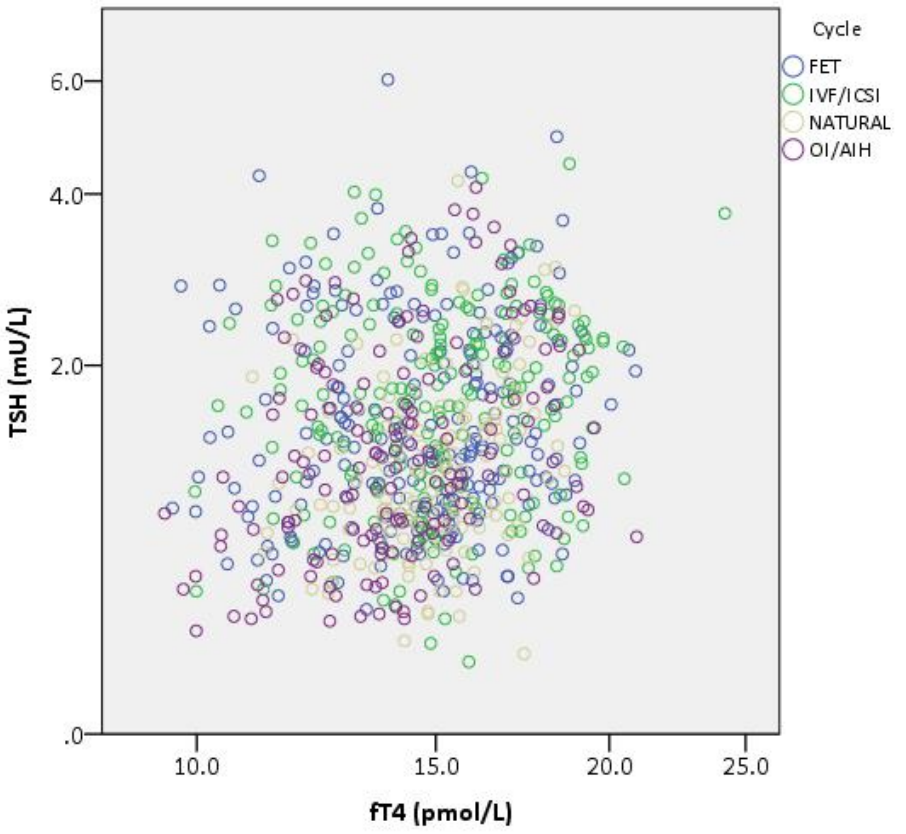

Figure 46. TSH vs fT4 accounting for cycle type.

6.3 Thyroid Levels in Viable vs Nonviable Pregnancies

The levels of all thyroid hormones were not discernible across gestation weeks 4 to 6.5 when accounting for pregnancy outcome. Viability was analysed as an independent factor/between subjects effect and the variances in thyroid hormones between viable and non-viable pregnancies were non-significant (Tables 60 - 62).

Table 60. Repeated measures ANOVA for fT3 gestation weeks 4 to 6.5 with viability as between-subject factor.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
viability	0.275	5	60	0.925	0.022	1.374	0.113

Table 61. Repeated measures ANOVA for fT4 gestation weeks 4 to 6.5 with viability as between-subject factor.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
viability	0.819	5	60	0.541	0.064	4.093	0.273

Table 62. Repeated measures ANOVA for TSH gestation weeks 4 to 6.5 with viability as between-subject factor.

Effect	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
viability	0.330	5	60	0.893	0.027	1.652	0.127

28 patients (32.9%) in this study at one or more points during their pregnancy had a TSH measurement >2.5mU/L and of these patients, 6 (7.1%) also had at least one TSH measurement >4.0mU/L. 3 patients (3.5%) had viable pregnancies and 3 patients (3.5%) miscarried with elevated TSH levels (>4.0mU/L). There were not sufficient numbers for a proper statistical analysis, although this data showed a notable miscarriage trend with 50% of patients having a TSH value over 4.0mU/L.

6.4 Thyroid Reference Ranges Before and During Early Pregnancy

There were two women who tested positive for TPOAb, with levels of 85.2U/ml and 607.9U/ml. Both women had normal fT3, fT4 and TSH levels (within the reference ranges determined for each gestation week in Tables 63 – 65) and viable pregnancies. These two patients were excluded from all thyroid hormone analyses and prior to calculating reference ranges. All other women in the study tested negative for TPOAb (<28U/ml) and serum samples from these women were used to calculate reference ranges for fT3, fT4 and TSH for gestation weeks 0 – 7 (Tables 63 – 65 and Figures 47 – 49).

Table 63. Maximum, minimum, median, mean and 2.5th – 97.5th percentile ranges for fT3 (pmol/L) from gestation weeks 0-7.

Week	0	2	3	4	4.5	5	5.5	6	6.5	7
n	37	66	69	69	69	69	68	67	55	21
Maximum	5.43	5.78	5.81	5.95	5.78	5.67	5.35	5.70	5.47	5.49
97.5th	5.32	5.67	5.61	5.76	5.68	5.52	5.30	5.26	5.21	5.42
Median	4.65	4.71	4.64	4.66	4.66	4.58	4.63	4.54	4.48	4.52
Mean	4.61	4.68	4.62	4.67	4.63	4.59	4.52	4.52	4.49	4.57
2.5th	3.72	3.91	3.92	3.89	3.79	3.76	3.61	3.93	3.71	4.15
Minimum	2.97	3.84	3.52	3.83	3.58	3.58	3.31	3.81	3.59	4.14

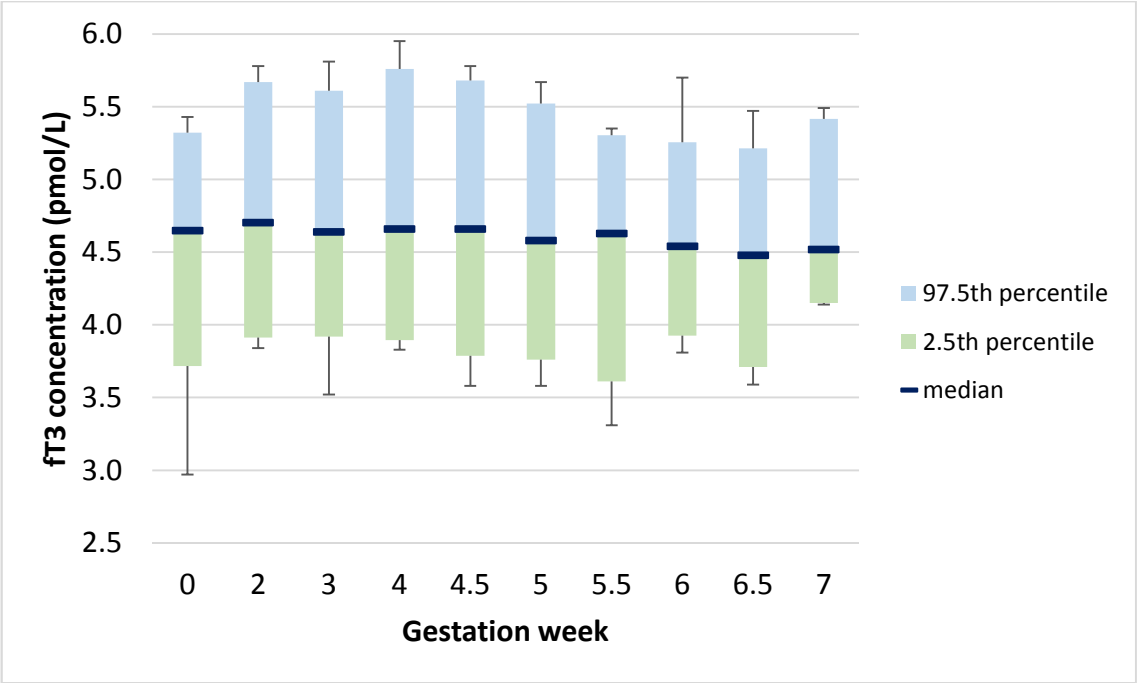

Figure 47. 2.5th – 97.5th percentile ranges for fT3 from gestation weeks 0 - 7 (error bars represent maximum and minimum values).

Table 64. Maximum, minimum, median, mean and 2.5th – 97.5th percentile ranges for fT4 (pmol/L) from gestation weeks 0-7.

Week	0	2	3	4	4.5	5	5.5	6	6.5	7
n	37	66	69	69	69	69	68	67	55	21
Maximum	20.88	20.06	20.92	19.24	20.66	19.45	19.28	19.80	20.50	18.52
97.5th	19.09	19.37	18.59	18.51	19.37	18.59	19.05	18.93	18.25	18.52
Median	14.96	14.48	15.12	14.90	15.15	14.75	14.66	14.58	14.99	15.14
Mean	15.09	14.46	14.91	15.09	15.11	14.90	14.58	14.70	14.92	15.22
2.5th	11.62	10.28	11.23	11.20	11.31	11.13	10.18	11.76	11.02	10.89
Min	10.98	9.97	10.75	10.42	9.46	9.99	9.73	9.99	10.22	10.40

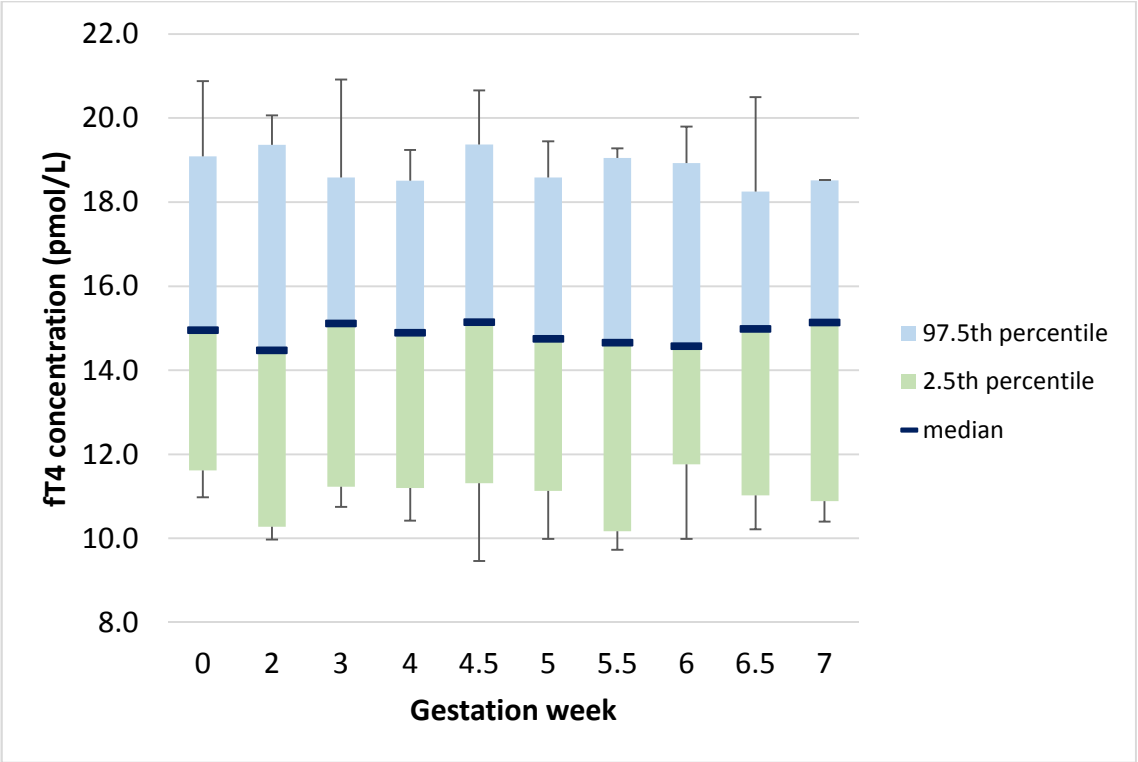

Figure 48. 2.5th – 97.5th percentile ranges for fT4 from gestation weeks 0 - 7 (error bars represent maximum and minimum values).

Table 65. TSH reference ranges (mU/L) from gestation weeks 0-7.

Week	n	geometric mean (limits of 1sd)	Range (min-max)
0	36	1.27 (0.74 - 2.20)	0.49 - 3.79
2	64	1.14 (0.67 - 1.93)	0.32 - 2.99
3	67	1.48 (0.88 - 2.42)	0.51 - 3.99
4	67	1.48 (0.89 - 2.44)	0.41 - 4.20
4.5	67	1.40 (0.86 - 2.36)	0.43 - 4.24
5	67	1.37 (0.81 - 2.25)	0.43 - 3.44
5.5	66	1.30 (0.79 - 2.20)	0.44 - 3.19
6	65	1.28 (0.76 - 2.15)	0.42 - 3.47
6.5	54	1.17 (0.62 - 1.85)	0.27 - 4.93
7	21	1.37 (0.75 - 2.11)	0.53 - 3.62

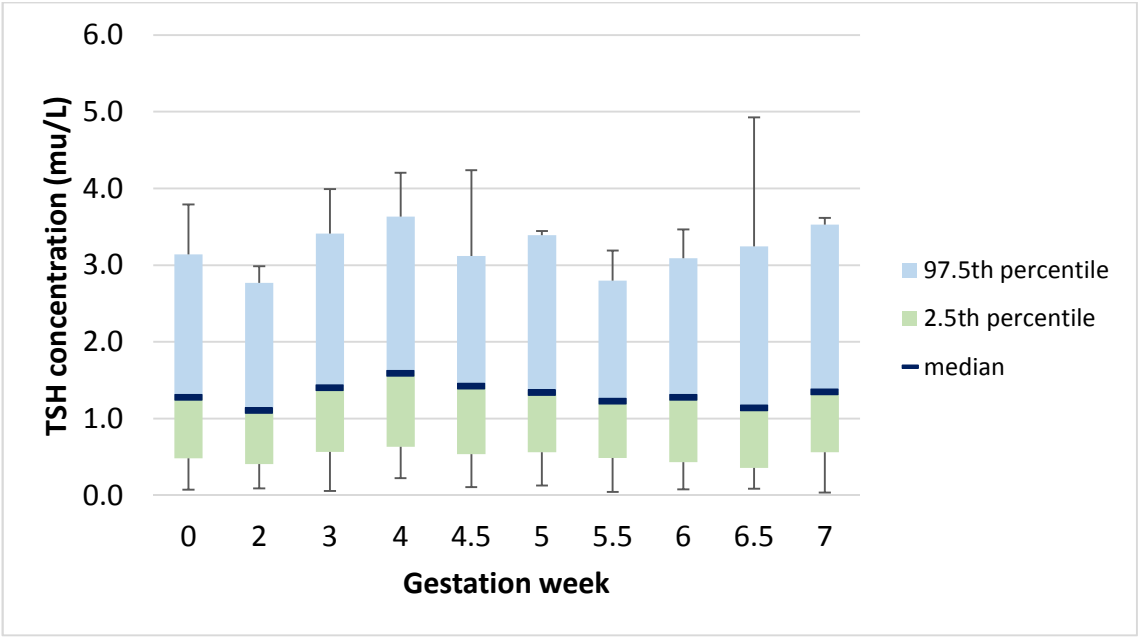

Figure 49. 2.5th – 97.5th percentile ranges for TSH from gestation weeks 0 - 7 (error bars represent maximum and minimum values).

CHAPTER 7

DISCUSSION

7 Discussion

7.1 Reproductive Hormones in Early Pregnancy

Reproductive hormones such as oestradiol (E2), progesterone (P4) and human chorionic gonadotropin (hCG) are measured routinely during ART to assess ovarian responses to stimulation drugs, time ovulation and evaluate the normal biological processes during pregnancy. By evaluating the levels of these hormones in all women in this present study, these important events could be tracked and the progress of each pregnancy monitored to distinguish between an ongoing or viable pregnancy and a nonviable pregnancy or miscarriage.

The Siemens XP reproductive hormone assays were all shown to be reliable in the range of hormone concentrations measured, when performing serial dilutions. Oestradiol had the highest variability, particularly at very low or high concentrations. Therefore care must be taken when interpreting patient results by referring to the Siemens XP measurements uncertainty for each hormone.

7.1.1 Oestrogen

Oestrogen is released by developing ovarian follicles and by the corpus luteum and placenta during pregnancy. Although oestrogen is an essential steroid hormone which works in conjunction with progesterone to prepare the endometrium for embryo implantation, little is understood of its function during pregnancy [169, 170]. The three main naturally occurring oestrogens in women are oestrone (E1), oestradiol (E2), and oestriol (E3). E2 is the predominant oestrogen in terms of its activity and serum levels [171]. There is limited available data on maternal serum oestradiol concentrations prior to gestation week 10 [172], however the data available from the first trimester (gestation week 12) through to term documents a steady rise [173, 174]. The mean oestradiol levels measured during this study, showed levels rising significantly with increasing length of gestation, starting from 1367.7pmol/L at gestation week 4 and reaching a level of 3644.1pmol/L at gestation week 7, which is consistent with the trend in the later stages of pregnancy.

7.1.2 Progesterone

It is well known that adequate progesterone production by the corpus luteum is vital to the maintenance of early pregnancy. In a naturally occurring pregnancy, endogenous production of progesterone from the corpus luteum up to gestation week 7 is usually sufficient to support the growing fetus [175]. Of the 14 naturally occurring viable pregnancies observed in this present study, 50% were not given luteal and early pregnancy progesterone support and had a mean concentration of 121.4nmol/L at gestation week 4. Women who were having blood samples taken to track their natural cycle, or after a frozen embryo transfer or artificial cycle (AI or OI), were offered progesterone support if their levels were below 70nmol/L at gestation week 3 as it is known to improve pregnancy outcome [176, 177]. The clinic where the present study was undertaken has a policy for all women following transfer of an embryo in IVF/ICSI cycles to supplement with progesterone until at least gestation week 7. Progesterone concentrations remained constant during early pregnancy for all cycle types, with a mean of 177.1nmol/L from gestation week 4 to 7.

7.1.3 Human Chorionic Gonadotropin

During the first trimester of pregnancy, serum hCG levels follow an exponentially increasing pattern, typically doubling every 2 days [178] and reaching a plateau between gestation weeks 8 – 10 [179]. The function of hCG in early pregnancy is well known, maintaining corpus luteum progesterone production, thereby supporting the development of the embryo [180].

The current study tracked the classical pattern of hCG in individual women to establish an ongoing or viable pregnancy. The maternal serum levels of hCG in viable pregnancies increased significantly with gestational age, as expected, reaching levels of over 50,000mU/ml at gestation week 6.5. Most patients did not have a blood sample taken after this point following detection of a fetal heart at ultrasound. There were a total of 15 pregnancies (17.6%) that miscarried in the study, which displayed suboptimal hCG levels and were confirmed nonviable at ultrasound when no fetal heartbeat was present.

7.2 AMH

7.2.1 Sample Storage and AMH Stability

Beckman Coulter advises that reliable AMH results are only obtained by following the precise laboratory techniques and adhering to the package instructions for the Gen II ELISA. The instructions recommend transferring at least 500µL of cell-free serum to a secondary storage tube within two hours of centrifugation, storing the sample at 2 to 8°C and performing the assay within 48 hours - or alternatively freezing at -20°C.

The issue with collecting samples for this present study was that samples were collected retrospectively, following recruitment of the patient after a positive pregnancy test, by which time the samples had already been centrifuged and stored in their original collection tube for 7 days at 2 to 8°C, then frozen at -20°C (Fertility North's standard protocol for sample storage). Even though AMH levels have been shown to remain stable at room temperature over 5 days [181] and all samples were stored at 2 to 8°C within 4 hours of collection, protocol variations to the storage of patient serum samples needed to be validated to ensure the AMH concentrations were reliable in this study.

This study confirmed that storage of centrifuged blood in the original collection tube and subsequent storage in the refrigerator for up to 7 days does not affect AMH concentration as measured using the Beckman Coulter AMH GEN II assay. Other studies have also reported AMH to be stable for up to 7 days at 2 to 8°C [182] and up to 3 months at 2 to 8°C [183].

There was also no effect on serum AMH levels after freezing for 1 week, with no significant difference observed between levels before and after freezing of samples. Only a 1% variation in AMH levels has previously been reported between fresh serum samples and samples that have been frozen [182]. Further investigation may be warranted to determine AMH stability over extended storage periods at -20°C. Patient samples in this present study were frozen in batches so any differences seen in AMH levels were not due to varied storage conditions between samples.

Storage methods that deviated from the manufacturers recommendations had minimal effect on AMH concentrations. This validation allowed the collection of blood samples for routine patient monitoring to be stored simply in the laboratory for up to one week, whilst suitable patients were identified, invited and enrolled into the research project.

7.2.2 Assay Performance

The development of the new AMH Gen II assay by Beckman Coulter involved a merging of the Diagnostic Systems Laboratories (DSL) antibodies and the Beckman Coulter Immunotech assay calibration [184]. The interpretation of AMH values obtained from each of these assays has been compared and reviewed [185, 186], with a strong correlation between all three assays reported.

When using any immunoassay, assessing its variability is important to determine possible sources of technical error. This study used pooled serum samples as external controls in each of the patient assays (in addition to the manufacturer's artificial quality controls), to generate a precision profile for the Beckman Coulter AMH Gen II ELISA that reflected the nature of the samples being tested. Higher between-assay variability compared to intra-assay variability highlighted the importance of measuring AMH in serial samples from individual patients in one assay to minimise this source of error.

This current study confirms that the new Gen II assay performs well when measuring patient samples in one assay, even with deviations from the manufacturer's instructions regarding sample preparation. However, all laboratory methods have limitations when it comes to the reliability of measuring a hormone in patient samples. This study demonstrated that very low or very high AMH concentrations are subject to greater variability even when run in the same assay, indicating that care must be taken when assessing small changes between serial samples that fall into these ranges.

7.2.3 AMH in Early Pregnancy

There has been much debate over the last decade with regard to AMH levels in varying physiological states, such as different stages of the menstrual cycle and during pregnancy. The general consensus of AMH in the past is that it is a relatively stable, unchanging hormone and a reliable indicator of ovarian reserve. During pregnancy, serum AMH levels in the first trimester have been reported as comparable to non-pregnant levels [46, 57, 58]. This is consistent with the findings in this current study when baseline AMH levels were compared to gestation week 4 levels in natural cycle patients, showing no significant variation. Some studies have reported a decline in AMH levels with advancing gestational age [58, 59], however, in these studies AMH levels were measured only once in each trimester. Cross sectional studies of AMH are also limited by the fact that different women of the same age can show substantial variations in their AMH levels [57, 59, 187]. These studies report the mean AMH concentration at one particular time point and compare this mean to another time point between different groups of women. When analysed in this fashion, it is unsurprising that any true changes in AMH over time become undetectable. This study demonstrated this phenomenon by comparing the mean AMH levels of all patients at each gestation week, resulting in no apparent changes in AMH over time. This present study also demonstrated large variations in both baseline AMH levels and gestation week 4 (pregnant) levels between women in different age and cycle groups.

An important strength of this study was that we were able to follow changes in AMH in individual women across early pregnancy and this is the first study that we are aware of, that provides data of this nature. It showed that AMH levels in pregnant women do not remain stable but consistently change, moving up or down for different women, irrespective of treatment cycle (and FSH stimulation). These findings contradict the theory that AMH levels reflect the FSH-independent noncyclic growth of small ovarian follicles. This study suggests that ovarian quiescence may not be absolute during pregnancy, or that there may be a reduction in the number of follicles secreting AMH during pregnancy in some women but not in others. The hypogonadotropic status in pregnancy does not seem to affect AMH levels consistently. There could be other unidentified factors that are contributing to the increase in AMH observed during some pregnancies and not others. This study also demonstrated that a woman's

baseline (non-pregnant) AMH concentration had no effect on whether her AMH levels increased or decreased during pregnancy. It may be that the increase in AMH levels in some women are attributable to cyclic follicle growth and recruitment during pregnancy, therefore it may be useful to monitor this during early pregnancy via ultrasound.

In some respects these findings are not surprising as many of the previously accepted concepts about AMH and its "stability" and what physiological changes may affect AMH are also being challenged. For example it is now known that patients do have changes in AMH during normal menstrual cycles, with acute illness, smoking and that there are also minor circadian and seasonal changes. The findings from our study on the variability of AMH in early pregnancy, with some individuals' AMH levels rising and others falling, suggest that factors affecting AMH are as yet poorly understood. This study also suggests that further research into factors affecting AMH (including in pregnancy and miscarriage) are required.

7.2.4 AMH in ART cycles

Often a patient's prescribed treatment/cycle type will depend on a number of factors, not just AMH alone. However, despite the large range of AMH levels within each cycle type, the average level at baseline was indicative of the ovarian function of most women in the group. For example, older women with low ovarian reserve (AMH <5pmol/µL) typically undergo IVF or ICSI treatment, compared to women with large numbers of small follicles (eg PCOS patients) that exhibit high AMH levels (>30pmol/L) and are recommended for ovulation induction (to stimulate the maturation of a follicle for ovulation). Women with normal ranges of ovarian reserve (AMH ~20pmol/µL) typically undertake natural tracking cycles to correctly time ovulation, or possibly artificial insemination. This study confirmed significant differences in AMH levels between IVF/ICSI cycle patients and patients in all other cycle groups (FET, OI/AIH and Natural).

The changes in AMH levels between baseline and gestation week 4 also reflected the treatment or cycle type. For instance, an increase (although non-significant) in AMH levels in IVF/ICSI patients was noted in those women receiving ovulation stimulation

medication. This treatment is expected to result in high numbers of follicles developing for collection, and it is likely that each developing follicle releases AMH. However most women undergoing IVF usually have a lower ovarian reserve and hence small changes in AMH are trivial. In contrast, OI and FET patients showed a significant decrease in AMH levels between gestation weeks 0 and 4, which can be explained by one or more immature or small follicles growing to dominancy and ceasing to release AMH (AMH is only produced by the granulosa cells of preantral and small antral follicles, and not in primordial or atretic follicles [41]). Natural cycle patients typically show little change in AMH levels between gestation week 0 and 4, when there is no ovarian stimulation. However this study did note that AMH levels decrease more often in natural pregnancy cycles ($p=0.028$), with 70% (11/15) of women following this trend. This is consistent with other studies that have investigated AMH levels in normal (natural) pregnancies [58, 59] although this trend was seen in later stages of pregnancy. AMH has long been used as a predictive marker of ovarian response to IVF or ICSI treatment, with low responders (low AMH levels) typically assigned higher doses of FSH [188] to stimulate the growth of follicles.

7.2.5 AMH Patterns in Viable and Nonviable Pregnancies

With most of the viable pregnancies, AMH followed one of two patterns from the time of the positive pregnancy test to the detection of fetal heartbeat measured by week 7. Over this time period, AMH levels measured at each time point followed either a progressive upward or downward trend, and continued to increase or decrease as the gestation progressed.

A discernible difference between the pregnancies that were maintained (viable) and those that miscarried was the unpredictable nature of the AMH levels measured at each time point for the women that miscarried. However, when running posthoc analysis of gestation week with viability as a between subject factor, there were no significant differences in AMH levels between gestation weeks 4 to 6.5. It is difficult to predict whether the changes in AMH levels are in some way contributing to the pregnancy itself, or whether the levels are a consequence of an unknown factor during the pregnancy. It is very interesting that all 15 nonviable pregnancies had sporadic changes in AMH levels, constantly rising and falling from week to week. In contrast,

viable pregnancies followed a steadily increasing or decreasing trend of AMH. No other study has investigated the pattern of AMH change in individual women during a miscarriage, and the results of this study should be confirmed in a larger cohort of women to better understand what may be causing the irregular 'spikes' and 'dips' in AMH levels.

AMH has been investigated by other researchers to determine its usefulness in predicting oocyte and embryo quality and subsequent pregnancy rates [189, 190], however the results are still controversial. Quite often female age, which is strongly related to pregnancy rates, is omitted as a contributor to prediction models [191, 192]. This current study did not have high numbers of miscarriages, so the power to predict pregnancy outcome based on the changes in AMH levels was low. However, age was not seen as a contributing factor to pregnancy outcome, with only two years difference in the mean age between viable and nonviable pregnancies. In addition, there were miscarriages in all cycle groups apart from AIH cycles.

There have been numerous studies that have looked at AMH levels in predicting an ongoing pregnancy, with conflicting results. Although age is accepted as the best predictor of a successful (ongoing) pregnancy, some studies report that ovarian reserve testing such as AFC or AMH do not improve prediction [189, 193-195], whilst others note that levels of these markers can predict the risk of miscarriage [188, 196-199]. Since this study observed significant differences between viable and non-viable pregnancies, it would be worthwhile to investigate this further in a larger longitudinal cohort.

7.2.6 AMH and Age, BMI and the Menstrual Cycle

The relationship between age and female fertility is well established [131, 200, 201], with AMH levels declining with advancing age. This study reconfirmed the strong correlation between age and AMH, by comparing the mean baseline AMH (day two of the menstrual cycle) to patient age. Women were assigned to four age categories (26-30 yrs, 31-35 yrs, 36-39yrs and 40+ yrs) to demonstrate the significant decrease in mean AMH levels, despite the highly variable range of levels within each age group.

High inter-individual variability for AMH is not unexpected, since the hormone is widely used as a biomarker of ovarian reserve in women [202].

There are many factors that have been shown to affect a woman's ovarian reserve, such as PCOS [203, 204] and ethnicity [205, 206]. This present study reported no significant association between AMH and BMI, which is consistent with other studies [181, 207] and contradictory to others that have reported negative associations [208, 209]. BMI was not found to correlate with patient age, which is supported by a recent study [210], and therefore was excluded as a factor affecting the relationship between age and AMH.

The issue of whether or not AMH levels fluctuate during the menstrual cycle is a highly controversial one. One study found that AMH levels can vary as much as 80% in one cycle [49], with a significant decrease of the mean AMH concentration in the luteal phase compared to the follicular phase. Other studies have shown that AMH levels do not significantly vary in the menstrual cycle [211-214]. In the present study, a blood sample was collected on day two (follicular phase), day of ovulation and mid-luteal phase to assess the changes in AMH levels. All time points (between baseline and ovulation or mid-luteal) were significantly correlated. For example, a low baseline AMH would be indicative of a low AMH level at time of ovulation. AMH levels were observed to peak at ovulation (gestation week 2) and decrease at mid-luteal (gestation week 3) across all types, which is consistent with a recent study that reported significant variation during the menstrual cycle [49]. Although the difference in AMH levels between ovulation and mid-luteal was non-significant, there was a significant difference seen between levels at ovulation and gestation weeks 5.5 and 6, as well as between levels at mid-luteal and gestation week 6.

The limitation of this analysis was that the mean percentage change of all patient AMH levels did not reflect the pattern seen in each individual woman. In other words, when the percentage AMH changes for each woman were observed between baseline and ovulation and between baseline and mid-luteal, some women had increasing AMH levels whilst others had decreasing levels. Also, there is an important difference between the patients in this study compared to previous studies that have looked at

AMH levels in the menstrual cycle – and that is that fertilization has usually occurred a few days post ovulation – in fact, in the case of all patients excluding natural and artificial insemination patients, a precise day of embryo transfer is known. Therefore, the decrease seen in AMH levels at the mid-luteal time point from ovulation cannot be directly compared to the AMH changes at the same time-point in non-pregnant women.

7.2.7 AMH and Other Hormones

There has been much speculation as to whether AMH levels are regulated by paracrine or autocrine functions. This study found that AMH was independently associated with the reproductive hormone oestradiol during pregnancy, which is supported by other researchers [58, 215]. One study observed a correlation between E2 rising and AMH declining during IVF stimulation [216], which this present study did not observe in the follicular to luteal phase of the menstrual cycle in IVF/ICSI patients. AMH had a weak negative correlation with P4 and TSH. The weak relationship with P4 could be explained by the higher levels of support given to IVF/ICSI patients (who tend to have lower AMH levels to begin with) compared to those patients with relatively normal AMH levels in natural cycles that are not given any support (ie AMH is lower when P4 levels are higher, or P4 levels are lower when AMH levels are higher). However, most women in this study were given progesterone support and there were still some women who had increasing and some with decreasing AMH levels during pregnancy. TSH decreased significantly with pregnancy and given that about half of the patients also had a gradual increase in AMH levels, it is not surprising that there was a relationship noted. However, the weak relationship that was shown between these two hormones was not a large effect (only about 2.5% of the variability can be explained by one factor significantly affecting the other). AMH did not correlate with fT3 or fT4.

This study also suggests that AMH expression is not regulated by gonadotrophins alone, since AMH levels increased in some women and decreased in others, whilst FSH and LH levels are suppressed during pregnancy, and hCG increases in all pregnancies (no significant correlation exists between AMH and hCG).

7.2.8 AMH and Medications

Different fertility treatments involve various combinations and concentrations of medication, depending on the cycle type and individual response. Each woman has a tailor-made repertoire of drugs which is decided by their medical practitioner. This study found no significant correlations between the ovarian stimulation drug dosage and the trend in AMH levels, which is consistent with other findings [159, 217]. Despite large differences in the combinations and dosages of patient medications, there were still women within each treatment group that exhibited a rise in AMH levels, whilst some showed decreases.

One study showed that AMH levels were suppressed with the use of oral contraception and then rose immediately after its cessation [50], however this study was only performed on two women that took oral contraception for a long time period (over 12 years). Most patients in this study were not taking oral contraception in the month prior to their pregnancy - indeed most patients attending fertility clinics have issues conceiving and have been actively 'trying' for a long period (with no contraceptive use). However, there were a few patients who were prescribed oral contraception in the few months prior to their 'pregnancy cycle' in an attempt to regulate their cycles, but these patients did not exhibit the same trend in AMH levels. Similarly, some women who have never taken oral contraception exhibited increases and others had decreases in their AMH levels, so it can be assumed that oral contraception was not a contributing factor in these two cases. However, since there were only two patients in this study that used oral contraception, the numbers were too small to analyse whether there was any significant effect on AMH levels.

7.2.9 AMH and Fetal Gender

This study found that there was no association between the gender of the fetus and the trend in AMH levels. As a male embryo develops, the Sertoli cells secrete around 1000 fold higher AMH levels than in females, so it would have been interesting to note whether AMH crossed the placental barrier into the maternal blood to cause a rise in AMH if the fetus was male. However, comparison between gender and AMH levels proved insignificant. This result is consistent with other findings [218].

7.3 Thyroid Hormones

7.3.1 Assay Performance

The performance of direct free thyroid hormone assays (fT4 and fT3) and the use of dialysis or ultrafiltration methods have been debated [219]. Automated fT4 immunoassays in particular can be subject to interference, particularly in altered physiological states such as pregnancy, which is associated with altered protein binding [220]. Although results between various immunoassays and methods have been reported as comparable in one study [221], others have reported substantial differences [219, 220, 222]. However, direct free thyroid assays (fT4, fT3) are in common use and currently used for clinical management, therefore were utilized for this study.

The reliability of the Siemens XP automated fT3, fT4 and TSH assays were investigated to validate patient results and establish gestational-age specific reference ranges. Control pooled serum samples were used in all assay runs in addition to three manufacturers controls to compare inter- and intra-assay variability. The CVs of fT3 serum and artificial QCs were comparable between assays, whilst the CVs for fT4 and TSH serum controls were notably higher between assays than within the same assay (7.48% vs 5.23% and 9.94% vs 2.75% respectively). This highlighted the importance of running patient samples in one batch to minimize this source of analytical variation.

7.3.2 Maternal Thyroid Changes during Pregnancy

During the first trimester, maternal changes in thyroid hormones are well known – the rise in hCG results in very mild and transient increases in fT4 and fT3, which via a negative feedback, leads to a decrease in TSH [118, 223]. An increase in free T4 has been noted toward the end of the first trimester by some authors [224] , but not by all studies [225], and it is important to note that measurement of thyroid hormones in pregnancy is complex. Factors such as rising Thyroid Binding Globulin (TBG) and falling albumin levels can change binding dynamics [226] and there are unknown effects on direct free thyroid hormone assays. Furthermore it is known that levels of fT3 are largely determined by peripheral de-iodination of fT4 [77, 227]. Two main types of de-iodinase are found – Type I in the liver, kidney and thyroid and this enzyme determines measured serum levels of fT3. Type II de-iodinase is found in the pituitary and may be differently regulated from Type I. This differential regulation of the two de-iodinases

can explain apparent “discrepancies” in fT4, fT3 and TSH levels. These discrepant values are common in clinical practice and can occur in acute illness such as ‘sick euthyroid syndrome’ [228, 229] or in starvation [230], uraemia [231] or diabetes [232].

Despite the active thyroid changes that occur in the first trimester and the importance to fetal development, most published studies during this time are cross-sectional and do not look at any time points prior to gestation week 8. The current study focused on gestational weeks 4 to 6.5 (week 7 was eliminated from the analysis due to low patient numbers), with blood sampling twice weekly in individual women in order to establish longitudinal reference ranges in early pregnancy.

In this study, there were no significant changes documented in fT4 levels during the time period, with mean concentrations from gestation week 4 to 6.5 similar to the non-pregnant concentration at week 0. We did note however, gradual small but significant decreases in fT3 from gestation weeks 4 to 6.5. As expected, TSH concentration dropped significantly with gestational age, from gestation week 4 onwards. This strongly correlated with the rapid rise in hCG during this time, which is consistent with the trend in the first trimester of pregnancy [223]. The relationships between each of the hormones fT3, fT4 and TSH during gestation were demonstrated to be statistically significant. There were no significant differences between non pregnant (gestation week 0) and pregnant (gestation week 4) fT3, fT4 or TSH levels. However, there was a significant difference between TSH levels at gestation week 0 compared to gestation week 6.5.

The lack of change in fT4 is not surprising as many authors note that changes in thyroid hormones are minimal because the compensatory decrease in TSH occurs very rapidly to re-establish normal free thyroid hormones. The issues of the type of measurement of fT4 in pregnancy also need to be considered and to more exactly document changes to free T4, complex and expensive methods such as ultrafiltration or dialysis have been proposed. However these are not practical for clinical practice.

The reduction in fT3 initially appeared surprising. However when considering the changes that occur in activity of the Type I de-iodinase in states of starvation and in diabetes these changes are not dissimilar to those that occur in early pregnancy.

Early pregnancy is a state where fasting rapidly results in low glucose due to the high levels of insulin and activity of lipolytic hormones. In contrast, after a meal pregnant women are more likely to be hyperglycaemic (similar to diabetic women) as they have higher levels of insulin but concurrent insulin resistance. A reduction in Type I de-iodinase activity may result in reduced fT3 levels. However, in these situations, Type II de-iodinase which controls pituitary T3 levels, is not affected. As such, normal/high levels of fT4 in the pituitary are converted normally to fT3 and this results in reduction of TSH from the hypothalamic –pituitary axis. It is also interesting that fT3 levels decreased in early pregnancy, a time when women often experience nausea and/or vomiting which puts the body in a state similar to starvation, resulting in decreased peripheral conversion of T4 to T3.

7.3.3 Thyroid Markers in Viable and Nonviable Pregnancies

The incidence of miscarriage was investigated and no measures of thyroid function (fT3, fT4, TSH or TPOAb) appeared to have an effect on the pregnancy outcome, however numbers of miscarriages were small. There have been numerous studies that have investigated the role of thyroid hormones in maintaining early pregnancy or in recurrent miscarriage [233-240] and this literature suggests there is an increased association with miscarriage and abnormal levels of thyroid hormones. A relationship between elevated TSH serum levels and risk of miscarriage has been reported previously [86], however rates of subclinical hypothyroidism during pregnancy are very low, between 2-3% [85]. The results of this present study showed a slightly higher rate of hypothyroidism (7.1%), defined at TSH levels >4.0mU/L. Half of the patients (n=3) with raised TSH levels resulted in miscarriage and half (n=3) were viable pregnancies. So although not statistically significant due to small numbers, the data suggests that there is a trend present with regards to elevated TSH and risk of miscarriage. There were only two patients that tested positive for TPOAb and both women had a successful pregnancy. Thyroid hormones for both these women were within normal ranges during early pregnancy, however it is important to note that TPOAb positive women are at a higher risk for developing postpartum thyroiditis. Therefore, both of the women's treating doctors were made aware of their thyroid autoantibody results so that their thyroid hormones could be further monitored.

7.3.4 Thyroid Markers and Other Hormones

The weak positive correlation that existed between TSH and fT3 levels and P4 (accounting for cycle type and gestation week) can be attributed to the increasing levels of progesterone support that is administered in most ART pregnancies, especially in IVF/ICSI cycles. fT3 and TSH were negatively correlated with hCG, which is expected since hCG is well-known to increase as the pregnancy progresses [241] and both fT3 and TSH were found to decrease gradually during pregnancy. It is unlikely that these significant effects are a consequence of a direct interaction between the hormones, with very weak effect sizes (r^2<10%). All thyroid markers remained positively correlated with each other. There is no literature that examines the interaction between fT3, fT4 and TSH during this early gestation period, so it is difficult to establish what are considered expected changes in these levels. It is known that serum fT3 and fT4 levels increase slightly at 10-12 weeks when hCG is at its highest, but remain within normal limits [242], whilst TSH concentrations decrease, fT3 and fT4 levels also decrease later in pregnancy when hCG starts to decline. Furthermore, the changes in thyroid hormones that take place at different time points during gestation are a result of complex effects that may be seen only momentarily. Hence the gradual decrease in fT3 that was observed during gestation weeks 4 to 6.5 is not likely to have any effect on the pregnancy itself, especially when levels are within expected 'normal' limits.

7.3.5 TPOAb in Pregnancy

Thyroid autoantibody measurement is a common marker of autoimmune thyroid disease, therefore women who tested positive for TPOAb were excluded from all thyroid data analyses. This study found only 2 women (2.35%) tested positive for TPOAb, which is lower than another study that reported the prevalence of TPOAb as 5.4% in women undertaking ART [243], although this was in a larger cohort (n=688).

Of the two women who were TPOAb positive, one woman had undergone a FET cycle and the other an OI cycle. Both women had thyroid levels within the 'normal' ranges determined in this study and both pregnancies were viable (single fetal heartbeat detected). Interestingly though, the woman that had a successful FET cycle, had a history of recurrent miscarriage – three previous embryo transfers had failed to implant. There is a known elevated risk of miscarriage with the presence of TPOAb

[244]. In addition, this woman had also been tested as heterozygote for the MTHFR 677 C>T (A222V) and MTHFR 1298 A>C (E429A) mutations which are also known risk factors for recurrent miscarriage [245]. The other woman who undertook OI treatment, had only one failed cycle previously, but also had PCOS which is associated with an increased prevalence of autoimmune thyroiditis [246, 247].

Although only a small number of women tested positive for TPOAb in this study, each patient's medical history was a clear indicator of the association between reproductive disorders and thyroid dysfunction.

7.3.6 Thyroid Reference Ranges in Early Pregnancy

A woman's thyroid status during pregnancy is difficult to establish without gestational age-specific reference ranges. In addition to maternal thyroid changes during gestation [224], there are significant differences in reference intervals between immunoassays [248-250], highlighting the need for further research to establish reliable common limits to correctly diagnose thyroid dysfunction in pregnancy.

There is a current paucity of literature that examines thyroid reference ranges prior to gestation week 9, and most research is performed even later in the first trimester. Therefore the data obtained from this present study is invaluable, providing reliable thyroid reference ranges from gestation weeks 4 to 6.5.

TSH concentrations were log transformed to normalise the data before determining appropriate reference ranges. The mean for each gestation week fell below 1.5mU/L, with the highest upper range of 2.45mU/L at gestation week 4, which is just below the normal first trimester upper range of 2.5mU/L recommended by the Guidelines of the American Thyroid Association [92]. 32.9% of patients in this study at one point during their pregnancy had at least one TSH measurement >2.5mU/L and 7.1% of patients had at least one measurement >4.0mU/L. Elevated TSH levels did not affect pregnancy outcome. However, levels that are considered "normal" during this time are constantly debated. Mean thyroid hormones levels in this study did not differ between non-pregnant (gestation week 0) and early pregnancy (up to gestation week 7), however there were significant differences between some gestational weeks during early pregnancy. It is known that first trimester thyroid function is different to pre-pregnancy, in particular a decrease in TSH, which is the same trend observed in the

present study. Median levels of TSH in this study (1.34mU/L) were higher than published levels later in the first trimester (0.89mU/L) [250] indicating that levels of TSH would likely continue to decrease in later first trimester weeks and therefore would end up being significantly different to non-pregnant levels.

CHAPTER 8

SUMMARY

8 Summary

8.1 Clinical Relevance and Implications

The benefit of this present study was the repeated measures of hormones in the same patients, in contrast to using different groups of subjects in a cross sectional study design. The ability to directly compare each of the hormone levels within the same individual over time resulted in the discovery of unique changes in very early pregnancy that would otherwise remain unnoticed. Analysing serial samples in batches minimised between-assay variability in measurements. Precision of each of the hormone assays was useful to determine the reliability of results at varying concentrations.

No other study has investigated AMH changes prior to gestation week 7. This study has further contributed to the understanding of the mechanisms of AMH, by showing the patterns of change during pregnancy and the relationships this hormone has with other factors. In particular, there seems to be two distinct groups of women that show either an increase or decrease in AMH levels during pregnancy. Significant differences in AMH levels during the menstrual cycle and also in nonviable pregnancies were observed, which raises the issue that AMH levels are not constant and should be measured more than once in an individual when assessing their ovarian reserve. This would be particularly useful information for clinicians when diagnosing a patient with infertility and would be helpful when determining the best treatment. This study found that AMH is in fact variable and a gradual and consistent increase or decrease in levels did not impact on the viability of the pregnancy. This is an important reassurance to clinicians that AMH trending up or down should not cause alarm. In contrast, very erratic levels of AMH may need to be further considered in the context of a pregnancy‘s viability.

This study has also provided important thyroid reference range data for a critical time in pregnancy, when the growing fetus relies on maternal levels of thyroxine for neurocognitive development. We established that the average non-pregnant levels of all thyroid markers (gestation week 0) did not predict the levels during early pregnancy (from gestation week 4 to 6.5), and there were significant differences noted between

multiple time points during pregnancy for fT3 and TSH. It may be helpful for clinicians to be aware that thyroid levels may change week by week when observing reference ranges, despite all upper limits still falling within the “normal” range during the first trimester.

8.2 Limitations

A limitation of this present study was that a number of patients presented with varying fertility issues including PCOS, which was not accounted for in the analysis. Therefore the results of this study may not reflect a healthy pregnant population. Patients were also not grouped by ethnic background, and some authors have noted that ethnicity may affect AMH levels. The number of samples were large enough to accurately determine statistical significance in each of the analyses, except when comparing the hormones in viable vs nonviable pregnancies, when the patient numbers in the nonviable group were low and affected the power of calculations.

8.3 Future Research

There is not a lot known about AMH in pregnancy and therefore the main focus of this study was to document changes in AMH using in-depth analyses. Since there is not a lot of literature to explain the dynamics of AMH during pregnancy this study did not provide an in-depth discussion of what factors may be causing these changes. The fact that there are significant changes in AMH during early pregnancy is a novel and interesting discovery in itself, and definitely merits future studies to investigate possible causes.

The patterns of AMH change should be further investigated in a cohort of pregnant women that have not undertaken ART and do not present with any reproductive disorders. A larger cohort of women that experience recurring miscarriages may be worthwhile to validate the pattern of AMH change observed in this study in nonviable pregnancies. Other factors which may be contributing to the changes in AMH during early pregnancy need further investigation. A study to further examine AMH stability in serum samples frozen at -20°C over varying extended storage periods would be also be useful.

CHAPTER 9

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9 References

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CHAPTER 10

APPENDICES

10 Appendices

Appendix A – Joondalup Health Campus Ethics Approval Letter

JOONDALUP
HEALTH CAMPUS

Joondalup Hospital Pty Ltd trading as
Joondalup Health Campus
ABN 61 106 723 193
Cnr Grand Blvd & Shenton Ave
Joondalup WA 6027
PO Box 242
Joondalup WA 6919
Telephone: 08 9400 9400
Facsimile: 08 9400 9054
Web: www.ramsayhealth.com.au

19 May 2014

Mrs Kristina Hamilton
Fertility North
Suite 213, Specialist Medical Centre
Joondalup Health Campus

Dear Mrs Kristina Hamilton

RE: The serum concentrations of anti-müllerian hormone (AMH), thyroid-stimulating hormone (TSH), free triiodothyronine (fT3) and free thyroxine (fT4) during early pregnancy (ref 1414)

The Human Research Ethics Committee of Joondalup Health Campus is pleased to notify you that your proposal to undertake research at Fertility North has been approved. As the Committee is bound by NHMRC Guidelines, the following conditions apply:

- That the Committee be notified immediately of any substantial changes in the design, methodology, time line or intended subjects of the project,
- That the Committee be notified immediately of any unforeseen complications of the project,
- That the Committee be notified if the project does not commence within six months of approval,
- That the Committee receive annual/final reports on the study (you will receive a pro forma from the Committee in twelve months), and
- That the Committee be informed of any other matters which arise during the course of the project which may have ethical implications.

Your approval is initially for four years; after this period you may be asked to re-apply. You are also required to notify the Committee promptly of any changes in your contact details.

Our best wishes for a successful implementation of your research project.

Yours sincerely

Ann Y Hammer
Executive Officer, JHC HREC

Appendix B – Edith Cowan University Ethics Approval Confirmation

Kristina Hamilton

From: Research Ethics <research.ethics@ecu.edu.au>
Sent: Monday, 20 October 2014 3:52 PM
To: 'kristinb@our.ecu.edu.au'
Cc: Kristina Hamilton; Phillip MATSON; Peter ROBERTS; Research Assessments
Subject: Project 12077 ethics approval
Attachments: Conditions of approval - multicentre.pdf

Dear Kristina

Project 12077 - The serum concentrations of anti-müllerian hormone (AMH), thyroid-stimulating hormone (TSH), free triiodothyronine (fT3) and free thyroxine (fT4) during early pregnancy

Student Number: 6043245

The ECU Human Research Ethics Committee (HREC) has reviewed your application and has granted ethics approval for your research project. The Committee noted that the project has previously been approved by the Joondalup Health Campus Human Research Ethics Committee . In granting approval, the HREC has determined that the research project meets the requirements of the *National Statement on Ethical Conduct in Human Research*.

The approval period is from 20 October 2014 to 28 July 2016.

The Research Assessments Team has been informed and they will issue formal notification of approval. Please note that the submission and approval of your research proposal is a separate process to obtaining ethics approval and that no recruitment of participants and/or data collection can commence until formal notification of both ethics approval and approval of your research proposal has been received.

All research projects are approved subject to general conditions of approval. Please see the attached document for details of these conditions, which include monitoring requirements, changes to the project and extension of ethics approval.

Please feel free to contact me if you require any further information.

Regards
Kim

Kim Gifkins, Research Ethics Officer, Office of Research & Innovation, Edith Cowan University, 270 Joondalup Drive, Joondalup, WA 6027
Email: **research.ethics@ecu.edu.au** Tel: +61 08 6304 2170 | Fax: +61 08 6304 5044 | CRICOS IPC 00279B

Appendix C – Project Information Sheet

RESEARCH PROJECT

PARTICIPANT INFORMATION

This research project is being conducted by Kristina Hamilton as part of the requirements of a Masters Degree (MSc) at Edith Cowan University.

The title for this project is:

The serum concentrations of anti-müllerian hormone (AMH), thyroid-stimulating hormone (TSH), free triiodothyronine (fT3) and free thyroxine (fT4) during early pregnancy.

Reproductive hormones such as hCG, progesterone and oestradiol are measured routinely in early pregnancy to monitor the progress of the implanted embryo. However, these are not the only hormones circulating at this time. The present study will measure the changes in circulating levels of a number of hormones over and above the ones measured routinely. This will provide novel endocrine information about events occurring in early pregnancies, and possibly identify some new biomarkers that may help in monitoring the progress of a pregnancy.

We are inviting you to participate in this study as a healthy female with a positive pregnancy test.

Aims of the project

a) Measure a number of hormones in early pregnancy of women attending Fertility North. These are AMH, thyroid stimulating hormone (TSH), and free triiodothyronine (T3) and thyroxine (T4).
b) Identify normal changes that occur over the next four weeks of your pregnancy from the time of the positive pregnancy test.

What we would like from you

Once we have finished analyzing your blood samples for hormones requested by your doctor, we would like to use what is left of the samples in the project rather than discarding them.

How will we use your samples?

We shall measure other hormones in your blood that do not form part of your routine pregnancy monitoring.

Can I access my test results?

You will be welcome to see your own results if you choose to ask, plus the summary data compiled for all patients at the end of the study.

Will the results affect my treatment?

Not unless the thyroid tests come back abnormal. However should this occur then your Doctor at Fertility North will be notified and will arrange any further investigations as required.

Will my test results remain confidential?

Absolutely! The samples will be labelled with a number and your name removed so that you will only be known to the staff at Fertility North.

What if I do not want to participate in the study or withdraw at a later date?

You are not obliged in any way to participate. If you do take part but change your mind, you are free to withdraw your consent at any time and the samples and results will then be discarded. Neither of these decisions will affect your pregnancy management by your doctor.

Who should I ask if there are any questions?

Dr Phillip Matson (Scientific Director), Dr Vince Chapple (Medical Director), Kristina Hamilton (Biochemist), Dr Narelle Hadlow (Pathologist at Sir Charles Gairdner Hospital) and Dr Peter Roberts (Senior Lecturer ECU) are the people involved in the project. These people, or any of the laboratory staff at Fertility North, will be happy to answer any questions or queries. If you require assistance, call Dr Matson on 9301 1075.

Appendix D – Participant Consent Form

FNC XX. CONSENT FOR THE USE OF BLOOD IN THE RESEARCH PROJECT ENTITLED:

The serum concentrations of anti-müllerian hormone (AMH), thyroid-stimulating hormone (TSH), free triiodothyronine (fT3) and free thyroxine (fT4) during early pregnancy.

Name: ……..

Address: ……..

DOB: ……..

By signing this consent form I have read the information sheet on the above research project and I agree to the following statements:

- I am happy for my blood samples to be stored and used to measure the reproductive hormones listed on the information sheet and any other hormones that may become relevant during the course of the project.
- I understand that I have the right to withdraw from this project at any time and refusal to participate will in no way affect my continuing medical care at Fertility North.
- I can obtain the results of any tests that are performed on my blood samples, but my results will not be made available to anyone not directly involved in the project.
- I can ask any questions in relation to this study by contacting the investigators listed on the information sheet.
- A copy of this consent form will be provided to me.

SIGNATURES:

Participant: …….. Date ……..

Witness: …….. Date ……..
(Must be FN member of staff)

The ethical aspects of this study have been approved by the Joondalup Health Campus Human Research Ethics Committee and the Edith Cowan University Human Research Ethics Committee. If you have any complaints or reservations about any ethical aspect of your participation in this research, you may contact the Joondalup Health Campus Committee through the Executive Office – phone 9400 9404 or Edith Cowan University's Committee through Kim Gifkins – phone 6304 2170. Any complaint you make will be treated in confidence and investigated, and you will be informed of the outcome.

Appendix E - Venepuncture – The Collection of Blood Samples

FertilityNorth
Fertility & Endometriosis Treatment Centre

SOP

Document Number: NP-S-783 Effective Date: **18-Dec-14**

VENEPUNCTURE – COLLECTION OF BLOOD SAMPLES

Purpose:
Provide guidance to phlebotomist and nurse when performing venepuncture.

Associated Documents:	
BC-S-787	Patient Identification, Blood Sample Acceptance and Preparation for Analysis
BC-F-607	Pathology Request Form
NP-F-1022	Patient Incident Form

Responsibilities:

Role or Department	Responsible for:
Phlebotomists	• Performing blood collection
Nurse Manager	• Ensuring all phlebotomists adhere to this procedure and have up-to-date venepuncture competency.

Table of Contents:

Venepuncture – The Collection of Blood Samples

1 Aim of Venepuncture

1.1 To obtain a blood specimen as skilfully as possible.

1.2 To collect enough blood to perform the desired tests.

1.3 When obtaining a blood sample, the patient's vein should be injured as little as possible.

1.4 You must demonstrate professionalism by using correct venepuncture procedures. All staff are required to have completed an authorised venepuncture competency that is re-evaluated annually.

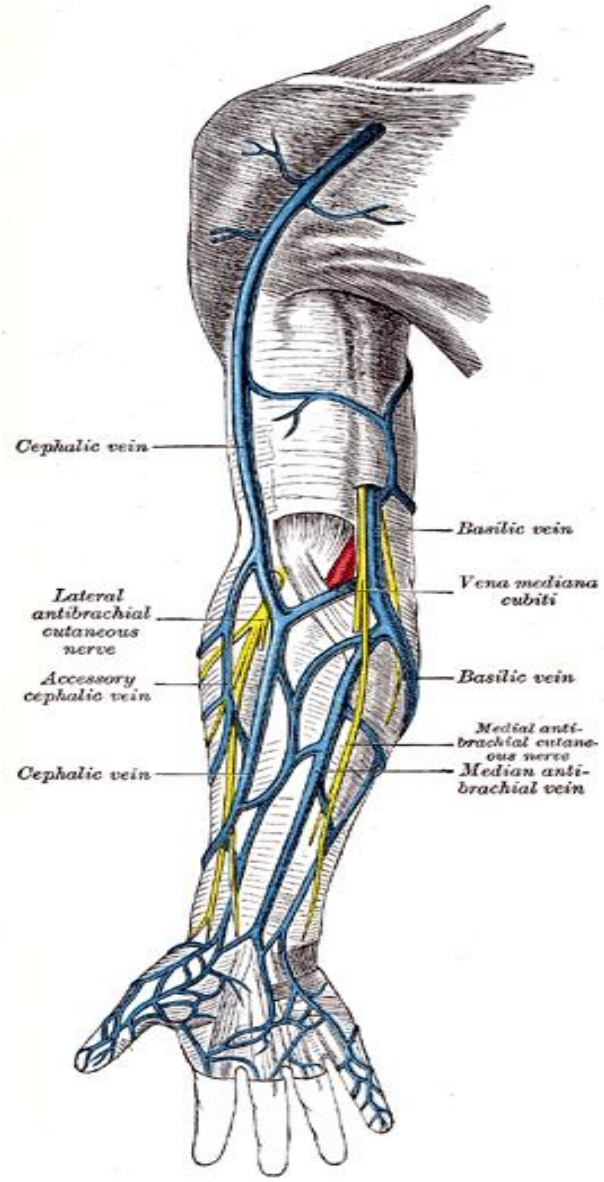


Figure 1 – Anatomy of a arm

The cubital (forearm/elbow) fossa (shallow depression) may also be referred to as the antecubital fossa. The cubital fossa is the triangular-shaped depression in the anterior region of the upper forearm, just below the elbow crease. The three veins most commonly recommended for venepuncture lie in the cubital fossa:

1. Median cubital vein
2. Cephalic vein
3. Basilic vein

Veins on the back of the hand and in the lower arm are often used for blood collection only when there are no accessible veins in the cubital fossa.

Venepuncture – The Collection of Blood Samples

2 Points to be Considered before Carrying-out a Venepuncture

The selection of the vein to be punctured is of the utmost importance. A vein should be chosen for

2.1 Accessibility;

2.2 Absence of underlying artery or tendons;

2.3 Volume of blood available - a surface or smaller vein has a decreased blood supply and although blood may be obtained from one of these veins the amount may be insufficient;

2.4 Stability - deeper veins are more stable than surface veins. However, in some patients the main veins are prominent and easily defined;

2.5 Patency - if a patient has had numerous venepuncture, a vein can become thrombosed and occluded. This can be detected by palpating the vein with the forefinger which will feel hard and fixed. Any attempt at venipuncture will be unsuccessful and this vein should be allowed to heal.

3 Equipment Required

3.1 Syringe- 5mL.

3.2 Needle- 23G, 25G or winged infusion (butterfly).

3.3 Swabs- alcohol and dry.

3.4 Tourniquet.

3.5 Blood tubes required

3.6 Pressure Dot

3.7 Sharps disposal box

The above items should be in a kidney dish prior to patient's arrival into the room.

4 Procedure

4.1 Approaching the Patient

4.1.1 Identify the patient. (refer to *BC-S-787 Patient Identification, Blood Sample Acceptance and Preparation for Analysis*)

4.1.2 Introduce yourself and build a rapport with the patient.

Venepuncture – The Collection of Blood Samples

4.1.3 Reassure the patient and gain their confidence. This can help relieve apprehension.

4.1.4 Collect the patients pathology form, Medicare card, and treatment card in order to verify the patients identity and credentials. (refer to *BC-S-787 Patient Identification, Blood Sample Acceptance and Preparation for Analysis*)

4.2 Positioning the Patient

4.2.1 This is done to readily access the vein and allow for a comfortable work position.

4.2.2 If the patient requests or there is evidence that the patient is very tense or has a tendency to faint, recline the phlebotomy chair.

4.2.3 Position the patient in the bleeding chair and ensure you and the patient are comfortable.

4.2.4 Ensure the arm is rested and supported.

4.2.5 Ensure any children are accompanied by another person (not staff member) or secured into a pram.

4.3 Procedure Preparation

4.3.1 Wash your hands.

4.3.2 Attach the needle to syringe, ensuring patient witnesses opening of sterile packaging of equipment.

4.3.3 If the patient has smaller veins you may need to select a smaller gauge. For a difficult vein or when collecting from the hand or wrist a winged infusion needle can be used.

4.3.4 Turn the needle so that the bevel is in line with the measurements marked on the syringe. Visually check the tip of the needle to ensure there are no burrs as a result of manufacturing.

4.4 Selection of a Vein and Performance of Venepuncture

4.4.1 Wash hands.

4.4.2 Personal Protective Equipment (PPE) including gloves, safety glasses and aprons are provided in each phlebotomy room.

4.4.3 Inspect the area you plan to use. In most cases patients themselves know where their best veins are. Apply the tourniquet about midway between elbow and shoulder firm enough to compress the vein but not the artery, and have the patient squeeze their hand into a fist, a ball can also be used. This enhances the veins and helps you locate them. Always palpate or feel the vein with the finger, whether you can see it or not the vein will feel like an elastic tube that gives under the pressure of your finger. Whilst doing this you will also be able to check its patency and the direction in which it runs. Avoid the vein if an underlying pulse can be felt or there is any evidence of thrombosis. You may need to select another vein if there is excess bruising. Check both arms thoroughly before making your decision.

Venepuncture – The Collection of Blood Samples

4.4.4 If the tourniquet has been applied for more than a minute while you search for a vein, release it for a moment, then reapply and relocate the vein. Prolonged obstruction of blood flow may cause changes to the quality of the specimen, which may affect test results.

4.4.5 Swab the area for venepuncture with an alcohol swab once in a downward motion and then wipe the area with the dry swab (excess of alcohol on skin may cause stinging when needle penetrates the skin).

4.4.6 Move the plunger back and forth in the syringe once, to ensure that it will move freely when drawing back and to exclude any unwanted air.

4.4.7 Place your finger about an inch below where the needle is to enter thus anchoring the vein preventing it from moving while inserting the needle.

4.4.8 When the needle has entered the vein, the back flow of blood will usually be visible in the plastic hub of your needle, this is known as flash back.

4.4.9 Anchor your hand against the patients arm and draw the plunger back slowly. If air bubbles appear in the blood cautiously push the needle a little further into the vein. Do not pull the plunger back too quickly as this may collapse the vein or cause the needle to be withdrawn from the vein. If no blood is obtained, gently withdraw the needle a little, you may have pushed the needle in too far and gone through the vein or the needle could be resting in a valve. If this does not succeed, gently rotate the barrel of the syringe so that the position of the bevel is moved from the edge of the vein to the centre of the vein. Lastly, withdraw the needle so that just the needle opening is under the skin. It is most important that you do not pull the needle completely out. With your forefinger, palpate the vein it may have moved to one side. With your finger still on the vein move the needle further into the vein, you will feel it move under your finger.

4.4.10 When the desired amount of blood has been obtained, steady the needle in the vein and release the tourniquet. The aim is to collect a full 5mLs of blood whenever possible. If that has not been achieved, check with a biochemistry member of staff to ensure the amount retrieved is sufficient before the patient leaves.

4.4.11 Place a dry swab over the needle and quickly withdraw the needle from the vein, and immediately apply pressure, ask the patient to press down hard on the dry swab. Pressure on the vein before removal of the needle may lead to the formation of a haematoma as well as being very painful to the patient.

4.4.12 Always hold the needle and syringe away from the patient. Remove the needle without replacing the cap via the needle removal device attached to the funnel of the sharps disposal bin. The needle must be removed from the syringe before placing the blood into the appropriate tubes in order to:

4.4.12.1 Prevent damage to blood cells - haemolysis.

4.4.12.2 Prevent spilling blood.

4.4.12.3 Prevent time wastage.

4.4.13 Remove the lid from required blood tube. Fill your tube keeping in mind that the moment the blood enters the syringe it's clotting, so the sooner it gets into the tube the better. When filling the tube run the blood gently down the inside wall. Avoid squirting the blood as this may damage the blood cells causing haemolysis.

4.4.14 Ensure the cap is on firmly and invert the bottle 5 times. Do not shake as this will cause haemolysis.

Venepuncture – The Collection of Blood Samples

4.4.15 Label the specimen bottle with the patients confirmed name, birthday, today's date, and your initials. If required also apply red dots to specimen bottle and pathology form. (refer to *BC-S-787*)

4.4.16 Place a pressure dot on the venepuncture site and advise the patient to leave it in place for at least an hour.

4.4.17 Patient to be advised to avoid heavy lifting with the arm where the blood has been taken and to not bend the arm at the elbow immediately after the blood has been taken. This is to avoid extra stress on the venepuncture site thus making sure there is no unnecessary bruising at the site.

5 Points to Remember

5.1 If you are having a problem getting a blood sample, do not attempt to perform a venepuncture on the patient more than twice, and only the second time if you are feeling confident.

5.2 Do not be afraid to seek help from another member of staff. Who will be more impressed with your professionalism if you recognise when you need assistance and the patient will also be grateful. The patient's comfort is a priority.

5.3 Haematomas can arise if:

5.3.1 You puncture right through the vein wall on a tourniquet arm, in which case a bluish swelling will appear. If this happens, release the tourniquet immediately, remove the needle and apply pressure to the area. A pressure bandage should be applied, and the patient alerted that a bruise will appear for a couple of days.

5.3.2 Bruising may also be a result of inadequate pressure on the venepuncture site after successful completion of the technique. You must ensure the patient is aware of the consequences of inadequate pressure.

5.3.3 Some patients faint when having blood taken, in most cases the patient will warn you that they have a tendency to faint. If you are forewarned advise another staff member and be prepared. If the patient begins to faint, call for assistance then quickly remove the tourniquet, needle and apply pressure to the puncture site. Try to prevent the patient from falling and/or injuring themselves if it is safe to do so and keep the patient's head between their knees if appropriate. Monitor patient closely until they recover and a member of clinical/nursing staff is satisfied they are able to resume their normal tasks. Complete a *Patient Incident Form (NP-F-1022*) detailing all actions required.

6 Procedure for Difficult or Failed Venepuncture

6.1 After two failed attempts at venepuncture, help and advice should be sought from the Senior Phlebotomist or Nurse Manager.

6.2 If the venepuncture attempt is a total failure, the staff member will contact the Nurse Manager or Senior Nurse on duty before the patient leaves the bleeding room.

END OF DOCUMENT

APPENDIX F - Patient Identification, Blood Sample Acceptance and Preparation for Analysis

FertilityNorth
Fertility & Endometriosis Treatment Centre

SOP

Document Number: BC-S-787 Effective Date: **4-May-15**

Patient Identification, Blood Sample Acceptance and Preparation for Analysis

Purpose:
This document provides explanation on the importance and high risk nature of correct patient identification, describes the criteria for sample acceptance for testing in the laboratory and gives instruction on how to prepare a blood sample for analysis. This SOP is designed to offer clear instructions to all staff at Fertility North who collect blood and/or prepare blood samples for analysis. This will ensure uniformity and compliance with the associated regulatory bodies is maintained across the clinic. It must be used for training and contains all the information necessary to complete regular competencies.

Associated Documents:	
QA-M-447	Quality Manual
LB-M-272	Laboratory Manual
BC-F-159	Sample ID Certification
BC-F-607	Fertility North Path Request Form
NP-F-014	Patient Waiting List for Bloods
	Genie Bloods Tab Daily Patient List
NP-S-783	Venipuncture – Collection of Blood Samples
BC-S-784	Producing Sample Identification (SID) Barcodes
BC-S-785	Operating the Biochemistry Analyser (Siemens Advia Centaur XP)
BC-S-786	Blood Samples Collected and Analysed Externally
Medicare Compliance information and PDF guidelines	http://www.medicareaustralia.gov.au/provider/business/audits/audit-guidelines.jsp
The Health Insurance (Pathology Services) Regulations 1989	http://www.comlaw.gov.au/Series/F1996B03648
BD Diagnostics SST Tube Package Insert	http://www.bd.com/vacutainer/pdfs/VDP40161.pdf
The Importance of Properly Processing a BD Vacutainer® SST™ Tube Fact Sheet	http://bd.com/vacutainer/pdfs/techtalk/TechTalk_November2005_VS7436.pdf

Definitions:	
DOH	Department of Health
FN	Fertility North
SOP	Standard Operating Procedure

Patient Identification, Blood Sample Acceptance and Preparation for Analysis

SST	Serum Separation Tube

Responsibilities:

Role or Department	Responsible for:
Phlebotomy	• To obtain complete and unambiguously identified samples and paperwork that comply with this protocol, which in turn assures compliance with the associated regulatory bodies.
Laboratory Scientist - Biochemistry	• To understand and apply the parameters of sample acceptance and identification. To understand and apply the compliance requirements as set out in this procedure and prepare samples accordingly.

Table of Contents:

1 Introduction

The claiming of Medicare rebates by the clinic is a serious issue and imposes a number of legal obligations and responsibilities. The inappropriate claiming of these rebates can be construed as being fraudulent with severe penalties. Compliance with this SOP is therefore essential.

By adhering to specific patient identification criteria, specimen reception procedure, sample acceptance and preparation requirements, the laboratory workload can be managed more effectively. This is turn minimises risk and helps ensure accuracy and reliability of the results.

2 Specimen Identification and Acceptance Requirements

2.1 General Information

2.1.1 Specimens analysed in biochemistry are serum taken from whole blood, collected in a 5ml gold capped SST (Serum Separation Tube). On occasion, follicular fluid may also be analysed.

2.1.2 Specimens are analysed for the hormones E2 (Estradiol), LH (Luteinising Hormone), P4 (Progesterone), FSH (Follicle Stimulating Hormone) and hCG (human Chorionic Gonadotropin).

2.1.3 Blood samples are collected at Fertility North every morning of business, between 7am-9am Monday-Friday and 7.30am-9am on Saturday and public holidays. Phlebotomists are primarily responsible for collection, although nursing staff also maintain their blood taking competencies.

2.1.4 There will inevitably be patients who attend for hormone analysis outside these times. It is up to the nursing and phlebotomy staff to determine the necessity of a 'late blood' and biochemistry staff to prioritise whether it can be run immediately or held until the following day.

2.1.5 All patients known to be attending for blood tests will be recorded on the Bloods tab in Genie. This is an alphabetised list according to the patient's surname. It indicates what type of treatment cycle she is undertaking, what day in her treatment cycle she is and what tests have been requested.

2.1.6 For a patient to be on the blood list, she must have

a) A valid Treatment Verification from her clinician
b) A Procedure Number/cycle nominated in Pragma that has been made active
c) Valid screening blood results.

A patient attending for a blood test who is not already on the list, or who does not fulfil the above criteria, will require administration and/or nursing staff's attention before biochemistry can proceed with testing.

2.1.7 Risk Assessment: **High**

The source of most errors in pathology blood collection is misidentification of the patient.

2.2 HIC Requirements

The Department of Human Services administers Medicare on behalf of the Department of Health (DOH), who is responsible for developing Medicare policy.

2.2.1 To comply with Medicare Australia requirements, we must receive from the doctor a written or verbal request to carry out the pathology tests ordered. This can be typed or hand written and must be dated and signed. This can be in the form of a:

a) Pathology Request Form (it does not have to be a Fertility North form, however the clinic's own forms are custom designed for our purposes)
b) Letter from Doctor
c) Treatment Verification Form
d) Day Sheet Report

A verbal request must be followed up with at least one of the above. If a request is received in some form other than that discussed above it should be brought to the attention of the Scientific or Medical Director who will decide if it meets Medicare Australia requirements.

2.2.2 The person collecting the specimen is also RESPONSIBLE for making sure the patient information is correct and legible. Information that must be checked:

a) Patients SURNAME and GIVEN NAME.
b) DATE of BIRTH.
c) ADDRESS and TELEPHONE NUMBER.
d) REFERRING DOCTOR.

If this information is not easily read then PLEASE REWRITE THE DETAILS IN LEGIBLE BLOCK CAPITALS.

FAILURE TO COMPLY WITH THIS PROTOCOL CAN LEAD TO INCORRECT INFORMATION APPEARING ON THE REPORT TO THE DOCTOR AND IS UNACCEPTABLE.

To help prevent this we audit a random sample of request forms on a monthly basis. The audit check is to see if the patient has been correctly identified and that the form is correctly filled out, signed and dated.

3 Patient Identification – Phlebotomist

3.1 On arrival, patients add their first name and current cycle card number to the Blood Collection List in the waiting room area (NP-F-014).

3.2 Patients complete a Pathology Request Form, filling in ONLY the shaded area titled, "only this section to be completed by the patient" (BC-F-607). This section includes surname, given name, address, phone number, date of birth (DOB), sex and a dated signature assigning the Medicare benefit of the requested tests to the Approved Pathology Practitioner (APP) (Fertility North).

3.3 The phlebotomist collects patient by calling the next name on the list and escorting into the collection room.

3.4 The Phlebotomist takes Pathology Request Form, Medicare card and treatment card from patient to validate their identification.

3.5 The phlebotomist asks patient to verbalise their name and date of birth. For example, "Can you tell me your full name and date of birth, please?" The phlebotomist checks the filled in details to make sure they are legible and accurate according to what the patient has said. They then tick the checkbox for the surname, given name and DOB fields to acknowledge the verbal check has been completed.

3.6 The phlebotomist checks the Medicare card. The surname and given name used on the Pathology Form must be EXACTLY the same as that displayed on the patient's current Medicare card. If they are not, the phlebotomist will ascertain why and correct the information on the form. For example, if a patient has married and is still using a Medicare card in her maiden name, she must be known by her maiden/Medicare name and use this for all her identification in the clinic. If she wishes to change this, she will need to do so directly with Medicare. When a patient changes her Medicare details, it is her responsibility to notify the clinic. If the phlebotomist becomes aware a patient's Medicare details are different from Fertility North's records, the patient should be asked to update these with the administration staff before leaving.

3.7 The phlebotomist completes the Medicare Assignment Form section, including Procedure Number, Medicare Number, Medicare card expiry date, Treatment Card check and Screening Blood check.

The phlebotomist then proceeds to collect blood (see *NP-S-783Venepuncture – Collection of Blood Samples*)

3.8 Once the sample has been collected, the phlebotomist writes legibly on the tube the patient's name, DOB, time taken, date taken and signs the tube. The tube must be labelled WHILE the patient is present, (ideally whilst sitting compressing the venepuncture site). AT NO TIME leave a specimen unlabelled. The Genie Blood List displays which tests have been requested and this information is added by the phlebotomist in the "Tests Requested" box on the form. The phlebotomist initials the Genie Blood List patient entry to signify that their sample has been taken, and signs the "Collected by" box legibly on the form. The tube is placed in the sealed section of a specimen carrier bag and the unfolded pathology request form in the sleeve, with the written side facing out through the clear section (easily read in biochemistry before the sample is removed from the bag).

3.9 Samples are placed in the designated collection box. If a blood sample is required urgently (it will be marked "STAT" on the Genie List). In this case the phlebotomist takes the completed sample directly to the biochemistry lab.

3.10 At the end of the patient list, when there are no more patients waiting for blood tests, or when morning blood taking time is finished, the collection box is CLOSED by the phlebotomists or biochemistry scientist on duty. This signifies any further blood samples collected must be taken directly to the biochemistry laboratory.

4 Patient Identification, Sample Acceptance and Preparation for Analysis – Biochemistry

4.1 Blood samples are retrieved from the collection box by the scientist and returned to the sample reception area of the biochemistry laboratory.

4.2 The scientist matches the patient name to the corresponding entry on the Genie Blood list. An initial check is done to visualise that the tube has been labelled, that it matches the form and that the tests requested match those listed in Genie whilst the sample is still contained in the specimen carrier bag. The patient is "coffee cupped", changing the entry print from black to red, indicating the sample has been received in biochemistry.

4.3 If the sample is labelled correctly, it may proceed to the bench for processing.

4.4 ***If a sample has missing information or is only partially labelled*** it can be used if it can be unambiguously identified. If the sample cannot be unambiguously identified, it must be discarded unassayed and a new sample requested.
All such instances should be logged in the Unacceptable Sample book, which should be reviewed monthly and trends dealt with accordingly. All instances of incorrect labelling should be brought to the attention of the collector who was responsible for collecting the sample. The name of the collector must be recorded in the log.

4.5 ***If the sample is of poor quality (for example significantly haemolysed)*** but was collected within the last 24 hours, then it may be assayed, as there is little evidence that results will be affected. In this instance, a cautionary note should be added to the final report by way of the Sample Integrity Field on the Pragma results worksheet, coding the sample "C" – sample compromised use result with caution. However, if the sample is older than 24 hours the results may be significantly altered and it should not be assayed and a second sample ordered. If the sample has been in an uncontrolled environment (for example in transit) for more than 24hours, it must be considered suspect and not assayed and a second sample ordered.
All instances of poor quality samples that have not been assayed must be recorded in the log with details of the problem.

4.6 At the work bench, the scientist removes the sample and corresponding form from the bag. The tube is checked again for name, DOB, time the sample was taken and a phlebotomist signature. Once the scientist

has confirmed these criteria, a unique SID barcodes are attached, ONE to the Pathology Request form, ONE to the sample tube and ONE around the sample cap (see *Production of Sample Identification (SID) Barcodes* BC-S-784) At no time should there be more than one sample and one request form on the bench that have not been barcoded.

4.7 The integrity of the blood sample is checked to assure there is enough volume to load directly on to the analyser (always leave the sample in the primary tube if possible). If the volume is less than the DOB field demarcation line on the label, a secondary cup will need to be prepared to transfer the sample for analysis. The sample cup must be labelled at the same time as the primary tube. Write the patient's surname, given name, date of birth and the day's date on the tube. Apply the remaining barcode of the series and self-check this matches the sample tube and form.

4.8 Once both form and sample have been tagged with a barcode, they may be separated – the sample to coagulate and the form added to a chronological pile for entry onto a worksheet.

4.9 The sample should remain in a vertical position for a minimum of 30 minutes from the Time of Collection before being checked for a dense, retracted clot. Insufficient clotting can result in the formation of fibrin. It is then centrifuged according to manufacturer's instructions (3300rpm min -4000 rpm max for an 8cm radius swing bucket rotor) for 10 minutes.

4.10 Once centrifuged, the tube is again inspected to ascertain its quality. If there is any floating debris or fibrin, remove with a plastic transfer pipette. If required, the sample is transferred at this time to a secondary tube to allow testing on the analyser.

4.11 Samples are placed into the biochemistry analyser for testing (see *Operation of the Biochemistry Analyser* BC-S-785)

4.12 Blood tubes are removed from the analyser and capped for storage. Samples remain in the refrigerator for seven days in racks labelled Mon – Sat. At the end of this time, they are transferred to the freezer where they are stored for a calendar month before being discarded.

Patient Identification, Blood Sample Acceptance and Preparation for Analysis

5 Blood samples received in Biochemistry outside the normal blood collection times

5.1 These samples are processed in exactly the same manner as above. They may not necessarily be run for testing until the next day, however they should, wherever possible, be separated within 2 hours of collection. If being held until the following day, all THREE barcodes and the date on the Pathology Request Form are highlighted with fluorescent marker to indicate the sample has a different collection and processing date. The samples are stored in the Biochemistry fridge's top drawer, labelled "Blood Samples Received outside Normal Hours".

6 Blood samples collected externally and analysed at Fertility North.

6.1 There are some patients who have their blood collected externally and brought to Fertility North for testing. This scenario is neither advertised nor encouraged, however, under certain circumstances, is allowed (for example, it is difficult or impractical to attend the clinic during blood taking times and their partner is a nurse).This is annotated in the patient's notes, nominating who will be taking and/or delivering the blood sample.

6.2 The patient or collector will be supplied with the appropriate needles, tubes and specimen bags.

6.3 When the sample is delivered to Fertility North, the biochemistry scientist on duty or a member of nursing staff will need to receive the sample personally.

6.4 A Sample Identification – Certification Form (159) must be completed for the sample to be accepted.

6.5 Refer to *BC-S-786 Blood samples collected AND analysed externally*.

END OF DOCUMENT

APPENDIX G – Operation of the Biochemistry Analyser (Siemens Advia Centaur XP)

FertilityNorth
Fertility & Endometriosis Treatment Centre

SOP

Document Number: BC-S-785 Effective Date: **03 Mar-15**

Operation of the Biochemistry Analyser (Siemens Advia Centaur XP)

Purpose:
This document provides concise instruction on the operation of Fertility North's biochemistry analyser, namely the Siemens Advia Centaur XP. It is designed to offer clear direction to all staff at Fertility North who are required to run patient blood samples, QC material and calibrations on the XP. This will ensure uniformity and compliance with the associated regulatory bodies is maintained and it must be used during biochemistry training. Additional information available on the operation of the Centaur XP is comprehensive and is located onboard the machine's computer (referred to as online help) and in the *Advia Centaur XP Quick Referencing Guide* provided by Siemens. This SOP must be used for training biochemistry staff and contains all the information necessary to complete regular competencies.

Associated Documents:	
LB-M-272	Laboratory Manual
BC-S-787	Patient Identification, Blood Sample Acceptance and Preparation for Analysis
BC-S-1048	Biochemistry Quality Control
BC-S-1043	Cleaning, Maintenance and Temperature Control in Biochemistry
Advia Centaur Quick Reference Guide	Written Manual available for trained operators. For more information refer to the Operators Guide on board the Centaur XP
Advia Centaur XP Operator's Guide	Available on board the Centaur XP via the help menu

Definitions:	
Biochemistry Analyser	Siemens Advia Centaur XP or XP
PPE	Personal Protective Equipment
QC	Quality Control

Responsibilities:

Role or Department	Responsible for:
Laboratory Scientist - Biochemistry	• To be able to follow the instructions provided to produce reportable results from the Advia Centaur XP for patients, QC material and Calibrators. • To be able to diagnose and troubleshoot machine errors through to resolution with the help of Siemens Technical Support Staff

Operating the Biochemistry Analyser (Siemens Advia Centaur XP)

Table of Contents:

1 System Description

The ADVIA Centaur and ADVIA Centaur XP systems are automated, direct chemiluminescent immunoassay analysers that offer optimal productivity and efficiency. Sample racks are loaded in the sample entry queue and the sample start button is pressed to activate the test sequence. The sample entry queue moves the sample racks to the inprocess queue, where the sample is aspirated and dispensed it into a cuvette in the incubation ring.

Reagents are dispensed into the cuvette, the reaction mixture is incubated, and then the cuvette is moved to the wash station where the magnetic particles are washed. Acid Reagent is dispensed into the cuvette and then the cuvette is moved into the luminometer. The addition of Base Reagent causes the chemiluminescent reaction to occur. The PMT measures the chemical light reaction that takes place.

Fast Stop

CAUTION

Do not disconnect the system. Disconnecting the system turns off the thermal components of the primary reagent compartment and ancillary queue. Shut down the system instead.

There are two fast stop buttons on the system (refer to Figure 1-2, page 7 of *Advia Centaur XP Immunoassay System Quick Reference Guide*) Use these buttons if there is an obstruction that could cause serious system or physical damage.

Operating the Biochemistry Analyser (Siemens Advia Centaur XP)

2 Signing In and Out

2.1 Staff must sign in before using any operative functions of the XP. It is important to note the analyser will allow a number of functions to be performed without the user signing in. The logging of each user operating the machine records a traceable account retrospectively to all data.

2.2 At the workspace, select **sign in.**

2.3 Choose **New Operator**, enter your initials and select **sign in**. No password is required to perform basic operating functions. For certain troubleshooting and some post analytic functions it may be necessary to sign in as a Level 1 Operator. To do so, use the **password** ROUTINE.

2.4 The sign in display on the workspace will now show the initials (and level if chosen) of the current user.

2.5 To sign out, return to the **sign in** workspace and select **sign out**

3 Loading and Managing Supplies

3.1 Check Onboard Supplies

3.1.1 All reagents and consumables should be onboard and ready to use. They must be checked at the beginning of each day and replaced at the end of each run (see BC-S-1043)

3.1.2 While the system is running, you can manage the following supplies without interrupting the run.

- Primary reagents
- Ancillary reagents
- System fluids
- Water
- Cuvettes
- Tips
- Cuvette waste
- Tip tray waste
- Tip waste
- Liquid waste

3.1.3 You can monitor the volumes of primary and ancillary reagents using the **Worklist – Reagent Inventory** window.

CAUTION: Mix all reagent packs by hand before loading them onto the system. It is important to minimize foaming. For detailed information about preparing reagents for use, refer to the ADVIA Centaur and ADVIA Centaur XP systems operating instructions.

Operating the Biochemistry Analyser (Siemens Advia Centaur XP)

3.2 Loading Primary Reagents

3.2.1 Mix the primary reagent pack by hand. Visually inspect the bottom of the reagent pack to ensure that all particles are dispersed and resuspended.

3.2.2 Open the primary reagent door. Identify a location for the pack by using the arrows as a placement guide.

3.2.3 Pull the reagent holder towards you. Load the pack on the reagent holder.

3.2.4 Press down on the top of the pack to ensure it is correctly seated on the reagent holder and then push the reagent holder back firmly until you hear the pack lock in place.

3.2.5 Close the primary reagent door.

3.2.6 The system barcode scanner will rescan the reagent barcodes and acknowledge any reagent changes.

3.3 Loading Ancillary Reagents

3.3.1 Mix the ancillary reagent pack by hand.

3.3.2 Load the pack in the ancillary reagent entry with the barcoded side facing outward.

3.3.3 An LED next to the ancillary reagent entry turns green to indicate that the pack is loading.

3.3.4 The system barcode scanner will rescan the reagent barcodes and acknowledge any reagent changes

4 Loading Sample Racks

BIOHAZARD

All products or objects that come in contact with human or animal body fluids should be handled, before and after cleaning, as if capable of transmitting infectious diseases. PPE is provided by Fertility North for your protection.

4.1 Loading Routine Samples (Patient, QC and Calibrators)

4.1.1 If you are using sample cups, label them with the appropriate barcode labels and dispense the sample into the labeled sample cups (see reference to using secondary tubes for patient samples, 4.7 BC-S-787)

4.1.2 Place the sample tubes or cups in the rack. Do so at the workbench with caution. The racks are weighted so as to be quite stable, but once uncapped do not move the rack until you place it onto the queue.

CAUTION: Do not load capped sample tubes. Ensure that all caps are removed from the sample tubes before loading the rack onto the system. Leaving the caps on the tubes can cause mechanical damage to the system.

Operating the Biochemistry Analyser (Siemens Advia Centaur XP)

4.1.3 Ensure that the barcode labels are clearly visible between the slots in the rack.

4.1.4 Load the rack in the sample entry queue. Ensure that the notch in the rack rests on the raised area of the sample entry queue.

CAUTION: Do not manually push a rack into the inprocess queue and do not manually place sample racks near the entrance to the inprocess queue without using the raised area of the sample entry queue as a positioning guide.

4.1.5 If the sample entry queue is in standby, press the sample start button to activate the sample entry queue. The sample entry queue moves the sample racks one at a time to the inprocess queue.

4.2 Loading STAT Samples

4.2.1 STAT samples can be placed in a rack and loaded in the Stat entry. The system assigns STAT priority to these samples and moves the rack to the inprocess queue as soon as space is available.

4.2.2 Samples can also be scheduled as STAT samples at the Worklist – Schedule window and loaded in the sample entry queue. STAT samples loaded in the sample entry queue are processed as soon as the system scans the barcode label and loads the sample rack into the inprocess queue.

4.2.3 If the sample entry queue is in standby, press the sample start button to activate the sample entry queue. The sample entry queue moves the sample racks one at a time to the inprocess queue

5 Scheduling Tests

5.1 Scheduling a Run / Entering a Manual (operator-Initiated) Worklist

5.1.1 You can schedule a run through a laboratory information system (LIS) or you can manually create a worklist. Fertility North currently schedules all work manually. For detailed information about scheduling a run, refer to the ADVIA Centaur and ADVIA Centaur XP systems operating instructions Quick Guide or via the Help Menu on the XP.

5.1.2 *Scheduling Tests for an Individual Patient Specimen*

1. At the workspace, select **Worklist**.
2. Select **Schedule**.
3. Select **Patient**.
4. Select **Schedule by SID** or **Schedule by Rack ID** to indicate how you want the system to identify the sample.
5. Enter the SID or the Rack ID and then press **Enter.** Alternatively, select **Scan Data** to scan the SID using the hand-held barcode reader.
6. If you want to process the sample before routine samples, select **Stat**.

7. Select or enter the tests or the profiles for the sample.
8. If known dilutions are required select **Dilutions**, add values then press **continue**
9. Select **Save**.
10. Load the sample cups in a rack. *See Loading Sample Racks.*
11. When testing is complete, the sample rack will arrive in the exit queue and may be removed for storage.
12. Cap patient tubes before removing from the rack and return to the refrigerator for storage (BC-S-787)

5.1.3 *Scheduling Batches*

Groups of samples requiring identical tests can be scheduled as a batch. The system performs the requested tests on all samples loaded in the starting rack, ending rack, and all racks in between. It is not necessary to nominate the samples position on the rack, merely that everything on that particular rack/s (whether there are two samples on 1 rack or 12 samples over 3 racks) requires the same tests.

1. At the workspace, select **Worklist**.
2. Select **Schedule**.
3. Select **Batch**.
4. Enter the rack number of the starting rack in **Starting Rack** (for example 0003).
5. Enter the rack number of the ending rack in **Ending Rack** (for example, this may also be 0003 if only 2-5 samples are being performed)
6. Select or enter the tests or profiles.
7. Select **Save**.
8. Load the sample cups in a rack. *See Loading Sample Racks.*

5.2 Scheduling Quality Control (QC) Samples

5.2.1 For detailed QC procedural information, refer to the system operating instructions or to the online help system.

5.2.2 This SOP is designed to equip the user to process QC samples only. Further information about Fertility North's QC procedures and run acceptance criteria can be found in BC-S-1048

5.2.3 Siemens recommends the use of commercially available quality control materials with at least 2 levels (low and high). A satisfactory level of performance is achieved when the analyte values obtained are within the Acceptable Control Range for the system or within Fertility North's range, as determined by our internal laboratory quality control scheme. Fertility North runs 3 levels of assay quality control material (low, medium and high). Treat all quality control samples the same as patient samples.

5.2.4 *QC Frequency*

5.2.4.1 Analyse all 3 levels of quality control material on each day that samples are analyzed.

Operating the Biochemistry Analyser (Siemens Advia Centaur XP)

5.2.4.2 Analyse all 3 levels of quality control material each time a two-point calibration is performed.

5.2.4.3 Analyse all 3 levels of quality control material after major service or breakdown of the XP

5.2.5 *QC Operation*

1. At the workspace, select Worklist.
2. Select **Schedule**.
3. Select **Control**.
4. Select or enter a test. The controls previously defined for the test are automatically displayed.
5. Check the box for each level/lot you require
6. Select **Save**.
7. Label three sample cups with QC barcode labels: one for each level.
8. Gently mix the controls and dispense at least 0.6 mL of each control into the labeled sample cups. NOTE: The required volume depends on how many assays are being tested.
9. Load the sample cups in a rack. *See Loading Sample Racks*.

5.3 Scheduling Calibrators

5.3.1 Assays are calibrated when:

5.3.1.1 the calibration interval expires (use the Calibration – Summary window to view calibration status)

5.3.1.2 a calibration is invalid

5.3.1.3 a new lot of assay reagents is used

5.3.1.4 controls are repeatedly out of range

5.3.2 Enter the calibrator information provided on the ADVIA Centaur *Calibrator Assigned Value* card (this is required once when a new lot of calibrator is loaded on to the analyser. The user will be prompted to do so at this time).

5.3.3 Enter the Master Curve information from the *Master Curve* card provided with the primary reagent packs (this is required once when a new lot of reagent is loaded on to the analyser. The user will be prompted to do so at this time).

5.3.4 Ensure the reagent lot you want to calibrate is loaded on the analyser.

1. At the workspace, select **Worklist**.
2. Select **Schedule**.
3. Select **Calibrator**.

4. Select Schedule by SID or Schedule by Rack ID to indicate how you want the system to identify the calibrator.
5. Select or enter a test. The calibrators previously defined for the test are automatically displayed.

   NOTE: Calibrators do not display in the following situations:

   - there are no calibrators defined
   - there are no Master Curves defined
   - there is no reagent onboard
   - the calibration material for defined lots is expired

6. Select a **calibrator.**
7. Select a **reagent lot**.
8. If you schedule by rack, enter the Rack ID in Rack.
9. Select **Save**.
10. Repeat steps 7 through 12 to schedule additional tests for a calibrator.
11. Label two sample cups with calibrator barcode labels: one for the low and another for the high.
12. Gently mix the calibrators and dispense at least 1.0 mL of Low and High Calibrator into the labeled sample cups. NOTE: The required calibrator volume depends on how many assays are being calibrated using these calibrators and the number of calibrator replicates.
13. Load the sample cups in a rack. See *Loading Sample Racks*.

6 Manual Dilution of eE2, P4 and ThCG

6.1 If a Sample is Known to be Beyond the Assay's Dilution Point

6.1.1 The Advia Centaur XP is capable of automatically diluting these 3 assays to the following values

eE2	55000pmol/L
P4	1500nmol/L
ThCG	200000mIU/mL

6.1.2 The corresponding diluents used for each of these assays are

eE2	eE2 Diluent
P4	Multi Diluent 3 (MDil3)
ThCG	ThCG Diluent

Operating the Biochemistry Analyser (Siemens Advia Centaur XP)

6.1.3 Annotate the biochemistry section of the pathology request form in the following way

MD E 1:1 for eE2

MD P 1:1 for P4

MD Q 1:1 for ThCG (Manual Dilution ratio of 1 part diluent to 1 part serum).

6.1.4 Identify, prepare and centrifuge the sample as per normal.

6.1.5 Include a Siemens secondary sample cup in the preparation (as would normally be done for a primary tube that does not have enough serum to run on analyser) Details on secondary cup should include

i) SID Barcode

ii) Patient's full name

iii) DOB

iii) Date of sample and

iv) Details of the manual dilution as described on the request form, ie MD E 1:1

6.1.6 To prepare dilution, add equal proportions of the appropriate diluent and patient serum, measured with Eppendorf pipette (using a fresh disposable tip for each measure).

For example: 250 microlitres of diluent and 250 microlitres of patient serum.

6.1.7 Run the patient sample requesting the highest dilution factor available, being

eE2 5

P4 10

ThCG 200

6.1.8 When calculating the result, you must manually **DOUBLE THE FIGURE** reported from the analyser, annotate and sign the hard copy.

6.2 If the Sample is Not Known to Have an Elevated Level Prior to Testing

6.2.1 You will only discover this when the result is reported as >dilution point.

6.2.2 Proceed following the same preparation as 6.2, with the additional cautions:

i) As the analyser has already reported a result for the SID number, you will have two disparate readings for the one sample.

ii) Clearly annotate what you have done by hand and sign on the XP printout to indicate the second result is a manual dilution.

7 Troubleshooting

7.1 The Advia Centaur XP provides features to help identify and correct system problems. This information is found online (on the software system of the machine) and in Section 5 of the Quick Reference Guide, available in the biochemistry laboratory.

7.2 Viewing System Status

System Status provides a visual indicator that a component or subsystem has a warning or critical condition. The large light on the top cover of the analyser can be seen from both rooms in the laboratory. This warns the operator to check the screen for visual indicators that require attention.

7.3 Using the Event Log

Use the System – Event Log window to identify errors and access online information about possible causes and corrective action procedures. Any warning or critical condition that is captures in the event log causes a change in the background colour of the Event Log Status button:

Yellow indicates that a warning condition exists.

Red indicates that a critical condition exists that may cause the system to stop aspirating or processing samples.

7.4 Accessing Help for Event Codes

Access the Event Log window at the workspace. Follow the detailed instructions available in the Quick Reference Guide (Section 5) to isolate information that offers possible causes and corrective action procedures for a specific error.

7.5 Diagnostic Tools

CAUTION: Do not use the diagnostic tools or perform troubleshooting without observing all safety rules. System components move and can cause injury. Biochemistry staff should only operate in this mode under instruction or guidance from Siemens Technical Support Staff.

7.5.1 Diagnostic Tools allows you to prime the system, test and move system components when you perfrom corrective action procedures.

7.5.2 The System – Diagnostic Tools window put the system in Diagnostic Mode.

7.5.3 You must wait until the system is not processing samples to use diagnostic tools.

Operating the Biochemistry Analyser (Siemens Advia Centaur XP)

CAUTION: The system stops mixing reagents while in Diagnostics Mode. If reagent mixing is turned off for longer than 2 hours, mix all primary reagent packs before resuming operation.

7.6 Technical Assistance

7.6.1 For technical assistance, call Siemens Healthcare on 1800 310 300, quoting the XP's System ID: 114552

7.6.2 You will be connected to a technical support staff member. Be prepared to quote the Event Log Error code and a brief description of the problem you are experiencing.

7.6.3 This process will ''log'' a Job Number in the Siemens system, which will generate contact with a local Technical Staff member. If a resolution cannot be achieved over the telephone, they will attend Fertility North to fix the problem.

8 Back Up

8.1 Fertility North requires a rapid turn-around time for biochemistry tests as the results are part of daily clinical decisions regarding the progression of each patients treatment. External laboratories are not able to prioritise our 5 hormone assays in a manner that allows us to guarantee they will be received the same day.

8.2 Most machine breakdowns can be resolved within 24 hours. In most instances, it will be more time effective to hold our samples and process them when the analyser is repaired than to send them for processing elsewhere. Any samples already collected will be held and tested retrospectively. Clinical decisions will be made accordingly by Gynaecologists in the absence of results.

8.3 If the machine is expected to be inoperable for greater than 24 hours (for example, machine parts are required that are not available in WA), a decision will be made by the Scientific Director to send patients externally to have their blood collected and processed. These results will be recorded in the usual manner as External Results (BC-S-786). Patients will be individually contacted by nursing staff and advised accordingly. Western Diagnostic Pathology have a collection centre on-site (ground floor below Fertility North) and process their hormones on the same analyser (Advia Centaur XP).

END OF DOCUMENT

APPENDIX H - Assay Protocol for Siemens Advia Centaur XP

Document Number: BC-S-1016 Effective Date: **29-Apr-15**

ASSAY PROTOCOL FOR SIEMENS ADVIA CENTAUR XP eE2, LH, P4, FSH AND ThCG

Purpose:
This procedure outlines the use and application of the Siemens products eE2 (enhanced estradiol), LH (luteinizing hormone), P4 (progesterone), FSH (follicle stimulating hormone) and Total hCG (Human Chorionic gonadotropin) on the Advia Centaur XP biochemistry analyser. It includes storage, preparation (where required), dilution and calibration requirements and reporting conventions.

Associated Documents:	
Siemens Immunoassay Systems Package Inserts	Available via log in to http://www.siemens.com/corp/apps/search/en/index.php and are also stored in biochemistry's MSDS File
Material Safety Data Sheets	Available via Siemens website http://www.siemens.com/corp/apps/search/en/index.php and are also stored in biochemistry's MSDS File
Immunoassay Systems Assay Chart	Prepared and Supplied by Siemens. ref 078D0929-01 Rev.A-ANZ
LB-S-790	Material Safety Data Sheets Maintenance
Advia Centaur Quick Reference Guide	Written Manual available for trained operators. For more information refer to the Operators Guide on board the Centaur XP
Advia Centaur XP Operator's Guide	Available on board the Centaur XP via the help menu.
BC-S-785	Operating the Biochemistry Analyser (Siemens Advia Centaur XP)
BC-S-1044	Ordering and Recording Supplies for the Advia Centaur XP
LB-M-272	FN Manual Part 2 Laboratory
LB-M-328	FN Manual Part 9 Measurement of Uncertainty
BC-F-400	MU report - Biochemistry

Definitions:	
E2	Estradiol
LH	Luteinizing Hormone
P4	Progesterone
FSH	Follicle Stimulating Hormone
hCG	Human Chorionic Gonadotropin
XP or "the analyser"	Advia Centaur XP – Biochemistry Analyser
n/a	Not applicable

Appendices	
Appendix 1	Reporting of Hormone Assay Results

Assay Protocol for Siemens Advia Centaur eE2, LH, P4, FSH AND ThCG

Responsibilities:

Role or Department	Responsible for:
Laboratory Scientist - Biochemistry	• To understand the application, treatment and reporting of assays used to test hormone levels on the Advia Centaur XP.

Table of Contents:

Assay Protocol for Siemens Advia Centaur eE2, LH, P4, FSH AND ThCG

1 Introduction

1.1 Fertility North uses the Siemens Advia Centaur XP Immunoassay System to monitor 5 hormone levels on patients undergoing various forms of fertility treatment. The Advia Centaur XP system provides online help and Information about the use of each of the assays and is available to view and or print during most routine operations. To access the online documentation

1.1.1 Enter through the ***QC*** Symbol at the workspace.

1.1.2 At the application view workspace, select the ***Instructions for Use*** button

1.1.3 Choose the ***Operator's Guide***

1.1.4 Choose ***Assays***

1.1.5 Choose ***Reproductive Endocrinology***

1.1.6 Choose specific Assay to view.

1.2 The Intended use, Summary and Explanation, Principles of the Procedure and all other relevant details are contained herein. These are also provided in paper format along with the appropriate MSDS sheets in the MSDS File.

2 Preparation and Storage

2.1 Records for all Siemens Advia Centaur XP supplies are kept in the Reagent Diary, recording the order number and date ordered, date arrived, batch number [same as previous or new], expiry date, opened date, and if/when QC reported (*see BC-S-1044 Ordering and Recording Supplies for the Advia Centaur XP)*

2.2 All reagents are stored and/or prepared as per the manufacturer's instructions, as listed on the Package Insert information, which can be found with the corresponding assay's MSDS, kept in the Material Safety Data Sheet register folder. The MSDS file is audited annually to maintain the most current copies are stored (LB-S-790)

2.3 Where reconstitution of samples is necessary (for example calibrators), use the pipette pump with a glass volumetric pipette to deliver Reagent Water (filtered in Biochemistry through the Ibis system) from a sterile specimen container.

2.4 A Siemens supplied laminated chart titled, "Immunoassay Systems Assay Chart" (ref 078D0929-01 Rev.A-ANZ) is available in the machine room of the laboratory as a quick guide to various information about the preparation,

calibration and testing of each Assay. This is checked with Siemens Staff and/or online to validate its accuracy and version status annually.

Table 1- Siemens Reagents Identification and Stabilities

Product	BAN No	On Board / Recon Stability	Storage Temp (ºC)	Volume to Add
eE2	10491445	672 hrs	2-8	n/a
FSH	10309974	672 hrs	2-8	n/a
LH	10309972	672 hrs	2-8	n/a
PRGE	10315522	672 hrs	2-8	n/a
ThCG	10308985	672 hrs	2-8	n/a
Multi-diluent (dil) 3	10309944	672 hrs	2-8	n/a
eE2 diluent (dil)	10491878	672 hrs	2-8	n/a
ThCG diluent (dil)	5609230	672 hrs	2-8	n/a
Calibrator (Cal) 30	10379810	28 days	2-8	2ml
Calibrator (Cal) B	10308986	28 days	2-8	5ml
Calibrator (Cal) E	10309079	14 days	2-8	2ml
Cleaning Solution	10310041	n/a	2-25	n/a
Wash 1 Solution	10319550	n/a	2-25	n/a
XP Acid/Base	103110026	n/a	2-25	n/a

3 Dilution

3.1 Estradiol

3.1.1 Samples >11000 pmol/L will be automatically diluted by the analyser.

3.1.2 If high or unpredictable levels are expected samples can be pre-programmed for selected dilutions when assigning the test. (*See BC-S-785 Operating the Biochemistry Analyser)*.

3.1.3 If a value exceeds 55 000pmol/L a manual dilution will be necessary (*See BC-S-785)*

3.2 Progesterone

3.2.1 Samples >150 nmol/L will be automatically diluted by the analyser.

3.2.2 Some luteal phase and pregnant patients may have higher levels and if high or unpredictable levels are expected samples can be pre-programmed for selected dilutions when assigning the test (*See BC-S-785).*

3.2.3 If a value exceeds 1500nmol/L a manual dilution will be necessary (*See BC-S-785)*

3.2.4 As a guide, levels remain fairly constant throughout early pregnancy but vary between patients. Assume level will be similar to the last sample. If borderline, leave to run neat as the test is a short one and the repeat does not result in excessive delays.

3.3 LH (Luteinizing Hormone)

3.3.1 Samples >150 IU/L do not need to be diluted and should just be quoted as such.

3.4 FSH (Follicle Stimulating Hormone)

3.4.1 Samples >110 IU/L do not need to be diluted and should just be quoted as such.

3.5 ThCG (Total Human Chorionic Gonadotrophin)

3.5.1 Samples >1000 IU/L will be automatically diluted by the analyser.

3.5.2 Only samples of patients who are pregnant will have continually escalating levels and will need to be diluted. Samples can be pre-programmed for selected dilutions when assigning the test (*See BC-S-785).*

3.5.3 If a value exceeds 200 000 IU/L a manual dilution will be necessary (*See BC-S-785)*

3.5.4 As a guide, if the previous result was <500 IU/L, then run neat; if >500 IU/L, then run at 1/100; and if >50000 IU/L, then run at 1/200.

4 Calibration

4.1 Prior to calibration check assay and calibration lot numbers: Scan new assay master curve barcodes if required. Try and ensure that the calibrator has been reconstituted at least a day prior to use and at the other extreme, that it is not expired. Reconstitute according to Manufacturer's instructions (to be found on the package insert, kept with MSDS information) The bottles specify the amount of water to add and this is underscored to annotate that the volume has been checked. To reconstitute samples, use the pipette pump with a glass volumetric pipette to deliver Reagent Water (filtered in Biochemistry through Ibis system) from a sterile specimen container, recording on the label the date of reconstitution and the date of expiry. Perform any calibrations due prior to daily assay runs (*See BC-S-785)*

4.2 Frequency of Calibration

4.2.1 LH, Progesterone, ThCG and FSH – 28 days

4.2.2 Estradiol – 21 days

4.2.3 When new reagent and calibration lots are introduced.

4.2.4 Upon poor QC performance

4.3 Application of Calibrators

4.3.1 LH, FSH, ThCG – Calibrator B

4.3.2 Progesterone – Calibrator E

4.3.3 Estradiol – Calibrator 30

5 Reporting Results – Hormone Assays

5.1 In any measurement there is an uncertainty associated with that measurement and biochemical testing is no exception. Please refer to *LB-M-328 Fertility North Manual 9 – Measurement of Uncertainty* for details regarding MU.

5.2 Reference ranges used by Fertility North (i.e. – follicular, midcycle, luteal, peri menopausal and pregnant) are those published by Western Diagnostic Pathology.

END OF DOCUMENT

Assay Protocol for Siemens Advia Centaur eE2, LH, P4, FSH AND ThCG

APPENDIX 1

REPORTING OF HORMONE ASSAY RESULTS

E2 [Estradiol]
a] If less than 150 report as <150
b] All other values reported as whole numbers

LH [Luteinising Hormone]
a] If less than 1 report as <1.0
b] Values between 1 and 150 report to 1 decimal place.
c] If greater than 150 report as >150

P4 [Progesterone]
a] If less than 1 report as <1.0
b] All other values report to 1 decimal place.
A check range applies between 4.0 and 6.0 **

** A clinical threshold of 4.5 nmol/L is currently used in the management of IVF/ICSI cycles as the upper limit of normality on the day of ovulatory trigger. Values in excess of this suggest a disturbed hormonal milieu causing asynchrony between the embryo and endometrium, and so all embryos are cryopreserved for transfer in a subsequent cycle. Unfortunately, commercial progesterone assays do not operate optimally at this level resulting in a relatively high coefficient of variation both within- and between-assay (Fleming, 2008). In an attempt to increase reliability of the results, Fertility North has elected to identify a check range of 4.0-6.0 nmol/L such that any results falling within this range will be automatically repeated. The reported result will then be the mean of the two duplicates rather than relying upon the singlicate result, removing the need for clinical staff to order repeats.

Fleming R. (2008). Progesterone elevation on the day of hCG: methodological issues. *Human Reproduction Update*; 14, 391-392.

hCG [human Chorionic Gonadotropin]
a] If less than 2 report as <2
b] All other values reported as whole numbers.

FSH [Follicle Stimulating Hormone]
a] If less than 1 report as <1.0
b] Values between 1 and 150 report to 1 decimal place
c] If greater than 110 report as >110

APPENDIX I – Control and Storage of Biochemistry Reagents and Consumables

Document Number: BC-S-1044 Effective Date: **03-Mar-15**

Control and Storage of Biochemistry Reagents and Consumables

Purpose:
The purpose of this document is to describe the ordering, receipt and storage of laboratory reagents and consumables for Biochemistry. To ensure adequate stock levels are maintained and delivery notes are matched with invoices for payment. To maintain traceability of reagent use and Batch/Lots. This SOP must be used for training biochemistry staff and contains all the information necessary to complete regular competencies.

Associated Documents:	
QC-M-525	Quality Manual
	Q:\Approved Supplier List
	Q:\Approved Supplier List\Approved Consumables Supplier List.xls
	Biochemistry Order Book/Reagent Diary
BC-S-785	Operation of the Biochemistry Analyser (Siemens Centaur XP)
BC-S-1016	Assay Protocol for Siemens Advia Centaur XP
BC-S-1043	Cleaning and Routine Maintenance of the Biochemistry Laboratory
BC-S-1048	Biochemistry Quality Control

Definitions:	
FN	Fertility North

Responsibilities:

Role or Department	Responsible for:
Biochemistry	• Ordering stock and ensuring adequate stock levels of reagent and consumables are maintained • Receipting stock • Ensuring items are not used outside expiry dates • Authorising payment of invoices once goods are received • Use the Reagent Diary to monitor and trace stock use and Batch/Lot numbers.

Control and Storage of Biochemistry Reagents and Consumables

Table of Contents:

1 Introduction

1.1 Biochemistry staff are responsible for ensuring that there are adequate stores on site of equipment, reagents, disposables, and other laboratory supplies. The duty will be given specifically to one or more staff members on a rotational basis.

1.2 The Approved Supplier List located Q:\Approved Supplier List provides a comprehensive list of suppliers that the biochemistry department use, as outlined in Section 9.4 of *QC-M-525 Quality Manual.*

1.3 If an order is placed and supplies have not arrived within a reasonable time frame, the order must be followed up with the supplier.

2 Placing an Order

2.1 Stock levels are checked on a weekly basis (BC-S-1043 Weekly Maintenance) and items running low are ordered accordingly. To perform a full stock take, print Laboratory Consumables Supplier List located Q:\Approved Supplier List\Approved Consumables Supplier List.xls. This document contains up to date information regarding items ordered, catalogue numbers, costs, delivery costs, supplier contact details including how to order from individual companies, and minimum stock levels.

2.2 Standing Orders: The biochemistry department has a standing order with BioRad for the Current Lot of Quality Control Material (BC-S-1048) and it will arrive during the first week of every month. For details see Q:\Approved Supplier List\Approved Consumables Supplier List.xls

2.3 Physically identity the quantity of each item held in the refrigerator (reagents) or in the laboratory and record the number required for order.

2.4 Take note of delivery costs for the required items; if other items can be ordered from the same company (to save on freight costs) do so.

Control and Storage of Biochemistry Reagents and Consumables

2.5 Using the *Order Book/Reagent Diary* record the order, assigning the next chronological order number FNxxx , e.g. FN860. The details required are the supplier, the date (ordered) and the items required. Each separate reagent pack will require its own line in the diary, whether it is a different reagent type or a multiple of the same e.g. Ordering 3 x eE2 reagent boxes requires them to be eE2 A, eE2 B, eE2 C so they may be registered and identified individually.

2.6 All of the reagents and consumables used on the Advia Centaur XP are ordered from Siemens. Orders are placed via email using the order form available in the Biochemistry (B:) drive and sent to diagorders.au@siemens.com. A confirmation will be received by return email. Check the goods contained in the confirmation match the goods ordered. File the confirmation and a copy of the emailed order in the Siemens Order File.

2.7 Orders being placed containing refrigerated items (i.e. reagents) must be placed no later than Wednesday afternoon to allow adequate time for them to arrive delivered from the Eastern States to Fertility North before the weekend.

2.8 Advia Centaur XP reagent is expensive with a limited shelf life, so purchasing must not be overestimated. However, delivery becomes costly if orders are placed too frequently therefore emergency orders should be avoided. Before deciding upon numbers for purchasing, consider an item's rate of use and package volume. Check the expiry dates on current batches to check items are still ok to use in the upcoming weeks.

3 Receipt of Items

3.1 Consumables

3.1.1 When receiving goods check the Delivery Note corresponds with the items received and also the hand written *Order Book/Diary Entry*.

3.1.2 If all the items correspond sign and date the Delivery Note and fill in the corresponding columns in the *Reagent Diary* (see 3.2.2) including the date the goods were received and a signature of the staff member accepting them.

3.1.3 If the order is Siemens reagents/consumables, attach the delivery docket to the copy of the email order form in the Siemens Order File to await the invoice for authorisation and matching.

3.1.4 For partially received orders, indicate in the *Order Book* which items were received, and await the rest of the order.

3.1.5 If the receiving goods are incorrect or not fit for purpose, contact the company from whom the goods were ordered to immediately to arrange a return or exchange.

3.1.6 If the items are not fit for purpose e.g. damaged, delivered un-refrigerated, sterility compromised or date of expiry exceeded, quarantine the items 'Out of Use' until the return of items can be arranged as outlined in Section 16.4 of *QC-M-525 Quality Manual*.

Control and Storage of Biochemistry Reagents and Consumables

3.2 Siemens Reagents (Primary and Ancilliary) Calibrators, Wash, Acid/Base and Cleaning Solution

3.2.1 The *Order book* is simultaneously referred to as the *Reagent Diary*, as it performs both functions. The Biochemistry department orders almost exclusively product for the Biochemistry analyser. This is sourced from the 1 supplier prescribed by the machine in operation, currently Siemens Healthcare.

3.2.2 For the receipt of these items, follow steps in 3.1 ensuring the delivery is unpacked and cold products placed in the refrigerator as quickly as possible to ensure they do not warm to room temperature.

3.2.3 The *Reagent Diary* is set up across two A4 pages. It includes columns for the record of

a) Order Number
b) Goods Ordered
c) Company
d) Date Ordered
e) Lot Number of product
f) NEW (Indication of New/Changed Reagent Lot)
g) Date Order Received
h) Signature of staff member responsible for accepting, checking and writing in order
i) Date Lot Expires (if applicable)
j) Date Product is Opened
k) Signature of staff member opening product

3.2.4 Products are entered in to the diary upon receipt.

3.2.5 The outside of each product is labelled with

a) The **Order Number** (eg FN860) If there are multiples of one type they are each given a separate identification (egFN860A, FN860B) and a separate entry in the Reagent diary to record their specifics.
b) **SAME** or **NEW** to signify whether the lot is the same as the one currently in use and/or already held in stock or new and in this case will require scanning and calibration before use (BC-S-785 and BC-S-1016).
c) "**DATE OPENED**:" The date the product is opened will be recorded here
d) A series of **DOTS** across the front forward facing panel of the reagent box (where applicable) to represent the individual number of reagent containers held within. Each dot is struck through with a line as the corresponding cartridge is used, offering a visual representation of stock through the glass door of the refrigerator without having to open the box or fridge.

Control and Storage of Biochemistry Reagents and Consumables

3.2.6 As each product is opened, the remaining columns in the reagent diary are filled in. When opening a new box or packet of consumables, use in the order of Date Received (from oldest to newest) to ensure a continuous turnover of stock and avoid 'older' stock being left in storage.

3.2.7 Once supplies are added to the XP, they are recorded and tracked for on-board stability and expiration. The XP records the Lot and Calibration details for every test performed on the analyser.

3.3 Biochemistry Invoice Authorisation

3.3.1 The Accounts Co-Ordinator at FN will deliver all biochemistry invoices to the Biochemistry department office area. Only authorised staff members can sign invoices to indicate payment can be made.

3.3.2 Invoices are matched up with the appropriate Delivery Note, and in the case of Siemens orders, the copy of the emailed order.

3.3.3 All items on the invoices are carefully checked before authorisation. The authorised staff member must sign their name beside the amount payable. Discrepancies must be raised with the supplier, Accounts Co-Ordinator and Biochemistry Manager. Authorisation for payment is withheld until issues are resolved.

3.3.4 Signed invoices are stapled with the supporting documents and passed to the Accounts Co-Ordinator for processing.

END OF DOCUMENT

APPENDIX J – Siemens Immunoassay Reagents and Calibrators

						Calibrator			
Hormone Assay	**Onboard Stability (days)**	**Wash Fluids**	**Probe Wash Reagents**	**Ancillary Reagents**	**Diluent**	**ID**	**Volume (ml)**	**Reconstitution Stability (days)**	**Calibration Interval (days)**
E2	42	Wash 1	-	eE2R	eE2 dil	30	2	28	21
P4	28	Water	APW1	P4 Releasing Agent	Multi-dil 3	E	2	14	28
QhCG	21	Water	-	-	ThCG dil	B	5	28	28
TSH	60	Wash 1	-	-	Multi-dil 1	B	5	28	28
fT3	28	Water	-	-	-	A	5	28	28

APPENDIX K - Recording Blood Test Results that are Collected and Analysed Internally

FertilityNorth
Fertility & Endometriosis Treatment Centre

SOP

Document Number: BC-S-691 Effective Date: **03-Mar-15**

Recording Blood Test Results that are Collected and Analysed Internally

Purpose:
This document provides explanation and instruction on creating a Daily Worksheet to report Biochemistry's test results. This is done using Fertility North's reporting software via data entry and a validation step, where results are then available to clinicians and nursing staff on the Daysheet Report. This SOP must be used for training biochemistry staff and contains all the information necessary to complete regular competencies.

Associated Documents:	
BC-S-784	Producing Sample Identification Barcodes
BC-S-785	Operating the Biochemistry Analyser (Siemens Advia Centaur XP)
BC-S-1016	Assay Protocol for Siemens Advia Centaur XP

Definitions:	
LIS	Laboratory Interface System
XP, analyser	The Siemens Advia Centaur XP biochemistry analyser

Responsibilities:

Role or Department	Responsible for:
Laboratory Scientist-Biochemistry	• To accurately retrieve and transcribe the daily test results obtained on the Advia Centaur XP into the reporting software and validate them so they are available for comment by clinicians and nursing staff.

Table of Contents:

Recording Blood Test Results that are Collected and Analysed Internally

1 General Information

1.1 Fertility North does not currently use an LIS (Laboratory Interface System) to link the Biochemistry Analyser and Reporting Computer Software. This is performed manually via data entry by the Biochemistry Scientist on Duty.

1.2 A number of checking steps have been incorporated into this process to minimise error.

1.2.1 Samples are barcoded (BC-S- 784) not only by a unique number but with a test profile, supplying another level of identification. This allows for the tests to be ‘’Batched” (BC-S-785), eliminating the need to enter any other data by hand to request a test on the analyser.

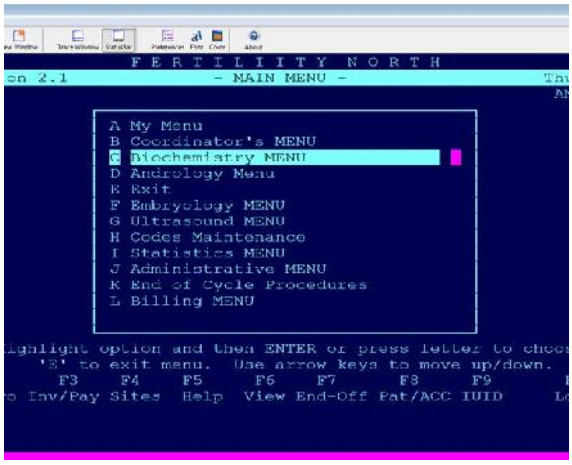
1.2.2 A worksheet for results is set up numerically in the same order as the daily barcoded samples. This is identical to the printout received from the analyser, allowing direct continuity for the Scientist performing the data entry.

1.2.3 All data entry of the results is performed in duplicate. A value must be entered ***identically twice*** to be accepted into the worksheet.

1.2.4 Nothing can be seen by other users of the software programme during production of results. They are only visualised on The Daysheet Report and in a patient’s procedure record after validation.

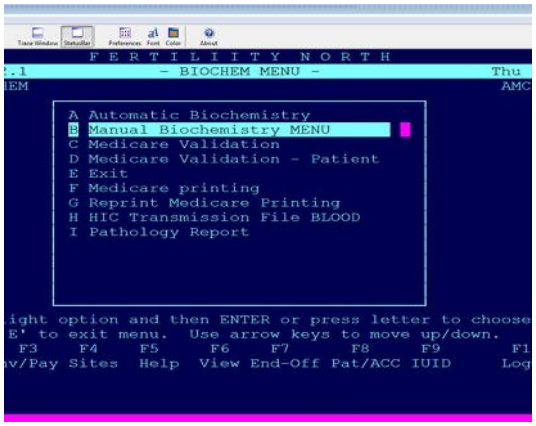
2 To Prepare a Daily Worksheet

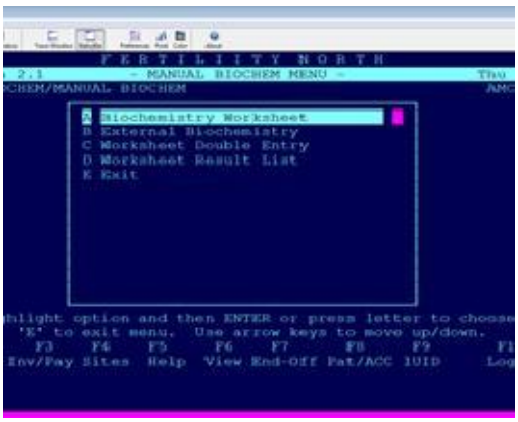
2.1 Access the computer software using your identifying log in and select the Biochemistry menu (C).



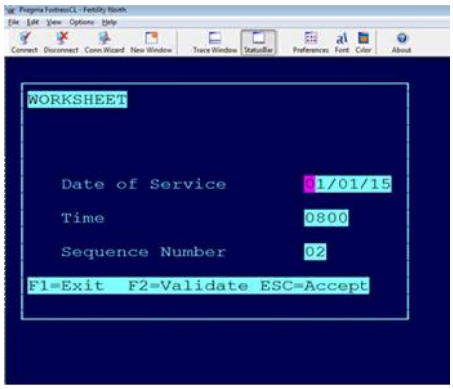
Recording Blood Test Results that are Collected and Analysed Internally

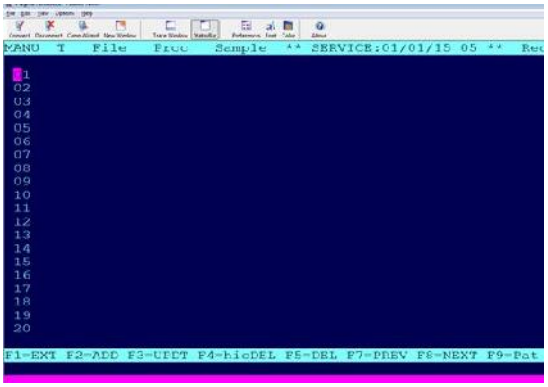
2.2 Select option (B) Manual Biochemistry and then (A) Biochemistry Worksheet





2.3 Worksheet Sequence No. 02 is the first in the manual series and will automatically appear. Choose ESC to accept.





2.4 When a worksheet is bought up you have the following options:

```
F1=EXT F2=ADD F3=UPDT F4=hicDEL F5=DEL F6=RPEAT F7=PREV F8=NEXT F9=Pat F10=INS
```

2.5 Select F2 which allows you to add a new entry. Enter the patient's date of birth (as this is the most unique identifier) and select the matching patient. If there are multiple people with the same date of birth, a list of patients will be bought up. Scroll though the list and press ESC when the highlighted name is correct.

2.6 This will bring you into a new screen which allows you to add a record of the patient's tests. You must make sure there is a procedure number in the PROCEDURE # field and there must be a sample number in the SAMPLE field. Assign the sample number using the barcode reader unless it is unavailable in which case the number can be entered manually.

Recording Blood Test Results that are Collected and Analysed Internally

2.7 Select F3 and, using arrows to find the tests your are performing, Select F4 to assign. Unless samples are going into patient history rather than a cycle [assigned by nurses] the daily reference must accompany the sample. [F5 is used to add or change the daily status if required]. This reference will automatically appear if the patient has a PROCEDURE # displayed.

2.8 Once all the correct data is filled in press ESC to accept the data or F1 to exit back to the worksheet to add another patient.

2.9 Continue this process, adding all patients to the worksheet. Once all samples are entered, select F1 to go back to the menu. The Biochemistry worksheet is saved.

3 To Enter Patient Test Results Into the Daily Worksheet

3.1 Results are manually entered from the Advia Centaur XP output. To print results

3.1.1 Select **Print** from the Workspace

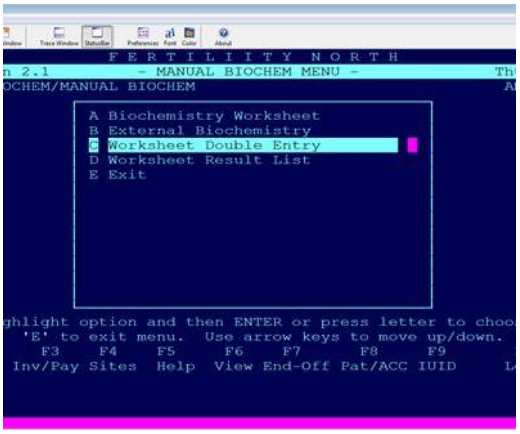
3.1.2 Select **Report Options**

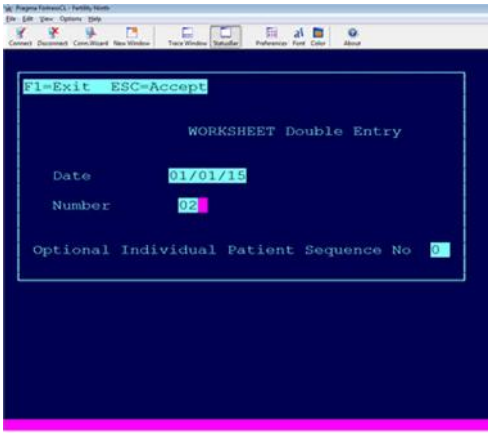
3.1.3 Edit the **Starting Date**/Time and **Ending Date**/Time to include all the results you need to print

3.1.4 Check that **View**: Results, **Sort**: SID and **Format**: Uncompressed

3.1.5 Select **Print Report**

3.2 Results are transferred into the computer software by selecting (C) Worksheet Double Entry.





Recording Blood Test Results that are Collected and Analysed Internally

3.3 The Date will default to the current day, the worksheet number will default to the current Manual Biochemistry Sheet. Each result is repeated (twice) to ensure the correct value is entered. All values are entered exactly as they appear on the Biochemistry analyser's printout (see BC-S-1016)

3.4 Once complete, the worksheet is printed by choosing (D) Worksheet Result List and cross-checked against the Advia Centaur XP Printout.

4 To Validate a Daily Worksheet

4.1 When the Biochemistry Scientist on duty has completed and checked results, they must validate the worksheet, making it available on the Daysheet Report for viewing and comment by nursing staff and clinicians.

NOTE: No biochemistry test selections or results are visible in the database until the Biochemistry Worksheet has been validated. The Biochemistry Worksheet cannot be validated until it is complete.

4.2 To validate the worksheet choose (A) Biochemistry Worksheet.

4.3 Select the correctly numbered worksheet (there may be more than one) in the display that appears next and press F2 to validate results.

4.4 You will be asked to confirm your selection by pressing "Y".

4.5 A validation sheet will automatically print. The Scientist responsible for entering the results into the worksheet must sign the validation form and file it with the complete set of day's paperwork including all result printouts from the XP and the completed reporting software worksheets.

END OF DOCUMENT

APPENDIX L – Biochemistry Quality Control

Document Number: BC-S-1048 Effective Date: **03-Mar-15**

Biochemistry Quality Control

Purpose:
To monitor laboratory performance in order to (i) help maintain high level of precision and (ii) estimate accuracy. This SOP must be used for training biochemistry staff and contains all the information necessary to complete regular competencies.

Associated Documents:	
NATA Technical Note 17	Guidelines for the validation and verification of quantitative and qualitative test methods.
NATA Technical Note 28	In-house calibrations and measurement uncertainty.
BC-F-400	Measurement uncertainty associated with the measurement of reproductive hormones at Fertility North
LB-M-328	FN Manual Part 9 Measurement of Uncertainty
BC-T-601	EQA Schedules Deadlines.
BC-S-785	Operation of the Biochemistry Analyser (Siemens Advia Centaur XP)
BioRad LyphoCheck Immunoassay Plus Control	http://www.myeinserts.com/40280
ADVIA QC Application	ADVIA QC Application Quick Reference Guide and ADVIA QC Online
BC-S-1044	Control and Storage of Biochemistry Reagents and Consumables

Definitions:	
Accuracy	Describes how close the result is to its true value.
EQA	External quality assurance
IQC	Internal quality control
Measurement uncertainty	Characterisation of the dispersion of results that can be attributed to a test on a sample.
Precision	Describes the spread of repeated measurements, and relates to the analysis "getting the same measurement each time".
Reproducibility	Precision estimate when series of measurements made under different conditions, eg on different days using routine conditions.

Responsibilities:

Role or Department	Responsible for:
Scientific Director	Overseeing the Biochemistry Laboratory performance
Biochemistry Manager	Ensuring Quality Control is monitored and recorded.
Biochemistry Staff	Acceptance, validation and reporting of daily QC results. Monitoring of QC material stock. Scheduling and testing External QAP samples when due.

Biochemistry Quality Control

Table of Contents:

1 Introduction

The monitoring of assay performance is to be done using two tools, namely:

1.1 Internal quality control. This involves the analysis of the same set of samples, each selected to represent a different part of the calibration curve, on each day the assay is run. Confirmation that the results fall within set limits ensures that drift (systematic error) or rogue values (random errors) are minimised. Data generated can also be used to estimate precision in the form of reproducibility, and ultimately measurement uncertainty.

1.2 External quality assurance. This involves the analysis of samples distributed to a number of laboratories. The results obtained by each laboratory can be then be compared to those of the others, and against a target value (consensus of the laboratories or a known true value).

2 Internal Quality Control

2.1 *General Information*

2.1.1 Lyphocheck Immunoassay Plus Control is intended as an assayed quality control serum to monitor the precision of the testing procedures for the analytes run at Fertility North. This product is to be used according to the manufacturer's (Bio-Rad) instructions, which are found on the package insert (available with each kit of 4 sets of vials) and also at http://www.myeinserts.com/40280 (the number representing the specific Lot).

2.1.2 The control material is on a standing order (BC-S-1044), stored by Bio-Rad Laboratories in the United States, not in Australia where the order is placed. It is therefore recommended to maintain at least 2 weeks supply in advance of requirements onsite. One package of 12 x 5ml bottles (4 sets of the tri-levels) arrives at the beginning of each calendar month until the current lot expires or the stock is exhausted. A few months before

this time, a new lot must be trialled, evaluated and deemed suitable before the order of another lot is placed. The reservation of a specific lot of controls typically lasts between 1 and 2 years.

2.2 Selection and Trial of New Lots

2.2.1 There is considerable variation between lots of control material, and some may be better suited to the particular tests and reference ranges used at Fertility North.

2.2.2 Maintaining one lot for as long as possible is cost effective, eliminating price rises, wasted reagents and staff's time evaluating new batches.

2.2.3 Liaising with local Bio-Rad staff, one set of an available lot of control material will be sent for Fertility North to trial. These are to be prepared (as per instructions below) and processed in a single run on the biochemistry analyser (see BC-S-785). This minimizes other machine variables from the outcome. This may be achieved by either aliqotting in to sample cups and running a series of many tubes, or by running just one sample cup of each of the three levels with many replicates of each of test.

2.2.4 The Scientific Director will evaluate the results and if suitable, the Lot will be reserved for its available duration.

2.2.5 Establishing the Mean

When the quality control lot number changes, a new established mean is required. Manufacturer's (BioRad) recommended limits are adopted. This mean is then reviewed and adjusted further if necessary. Outliers (<3SD>) are excluded before calculations are done.

2.3 Preparation of BioRad Ligands

2.3.1 Biorad controls are lyophilised and delivered at ambient temperature, but are stored in the biochemistry refrigerator upon arrival.

2.3.2 Select the next unopened set of the box in use (1 set contains 3 vials with Black, Grey and White coloured lids labelled 1, 2 and 3).

2.3.3 Underscore the 5ml notation on the bottle's label (indicating a self-check of the quantity required) and add a ''reconstituted" date to the label (and the rubber stopper if you wish).For example, "recon 05/07"

2.3.4 Add exactly 5ml reagent water obtained from the Biochemistry water filter to each vial, using a glass volumetric pipette as the measuring device.

2.3.5 Gently rotate on the spinner for a minimum of 20 minutes to ensure homogeneity before aliqotting for use. It is preferred that this is done on the day before the current sets expire, but this is not a requirement of use. Once reconstituted, the product is stable for seven days whilst kept refrigerated.

2.3.6 Before sampling, allow the product to reach room temperature.

2.3.7 Quality Control samples are processed on the biochemistry analyser (BC-S-785)

2.4 Run Acceptance and Performance Assessment

2.4.1 All QC results are stored by the Advia Centaur XP automatically via the Advia QC Application (See Advia QC online and Advia QC Quick Reference Guide). Advia QC provides both real time and long term evaluation of the analyser and method performance. It includes:

a) Collection of control results
b) Calculation and display of statistical data
c) Assessing data for errors
d) Notification of QC violations
e) Tools for reviewing and managing control results
f) Tools for creating printed reports
g) Identification and reporting of events such as lot changes and calibration date.

2.4.2 For each assay, BioRad Immunoassay Plus 1, 2, 3 are run daily, prior to any patient samples being tested.

2.4.3 A repeat patient sample from the previous day is also run daily.

2.4.4 The daily quality control values are assessed and the assay run accepted or rejected according to the criteria indicated in the flow chart (Appendix 1).

2.4.5 Monthly graphs and details of BioRad Immunoassay Plus 1, 2, 3 are printed from the Advia QC programme and submitted to the Scientific Director for review and discussion. Once signed and dated, the paper copies are filed. Changes such as assay limits are adjusted as required.

3 External Quality Assurance

The laboratory is to be enrolled in two schemes for the measurement of the reproductive hormones FSH, oestradiol, LH, progesterone and hCG. The RCPA Chemical Pathology QA programme covers a range of the five hormones associated with women receiving no ovarian stimulation whereas the EQASRM programme specifically uses samples from women undergoing ovarian stimulation in the context of an IVF programme and hence markedly increased oestradiol concentrations.

3.1 RCPA Chemical Pathology QA Programme

3.1.1 This programme operates in six month cycles with twelve samples (six pairs) assayed over this period. Each sample must be assayed in singlicate to mirror patient samples which are also done in singlicate. The due dates for the current cycle are detailed on the EQA Schedules Deadlines (BC-T-601).

3.1.2 The interim reports must be seen by the Scientific Director for evaluation and corrective action initiated for unacceptable results.

3.1.3 The end-of-cycle report gives an indication of accuracy (bias) for our method compared with all users or a pre-determined target value obtained by a reference method. However, direct comparisons with other like-method users can be seen in the interim reports or online in the data analysis option. Estimates of imprecision are also given based on the limited data set from the six pairs. These end-of-cycle reports are reviewed six monthly by the Scientific Director and corrective action initiated for unacceptable results.

3.1.4 Sample Preparation

a) Samples are refrigerated until use.

b) They are lyophilised and require reconstitution. Add exactly 5ml reagent water obtained from the biochemistry water filter to each vial, using a glass volumetric pipette as the measuring device. Gently rotate on the spinner for a minimum of 20 minutes to ensure homogeneity before use.

c) Samples are to be analysed as soon as possible on the day of reconstitution and tested in an identical manner to patients (BC-S-785)

3.1.5 Contact

Details of the programme can be seen on www.rcpaqap.com.au . To contact the scheme organiser, use the contact form on the website by logging in to the My QAP portal at www.myqap.com.au and log a case in there for prompt response. Telephone support is at 1300 782920.

3.1.6 Data entry online

a) Go to www.rcpaqap.com.au
b) Hover above "Participant Login" tab and then select Chemical Pathology.
c) Login with the laboratory user name and password.
d) Choose "Data entry" from the left hand menu and wait until the programme is loaded fully.
e) Select "data entry" tab.
f) Select "Endocrine Programme" tab and wait till fully loaded.
g) Enter data and save.

3.1.7 Viewing summaries and reports

a) This is only for the Scientific Director or nominated deputy.
b) Go to www.rcpaqap.com.au
c) Hover above "Participant Login" tab and then select "myQAP".
d) Login with the e-mail and and password.
e) Select "Reports" tab.
f) Complete survey report filter definitions to find the survey of interest.
g) Click on the green "Filter Records" icon to locate the surveys and reports from that period.

3.2 EQASRM

3.2.1 This programme operates by monitoring unknown oestradiol samples, which are sent to the clinic from the External Quality Assurance Scheme for Reproductive Medicine (EQASRM). Samples are sent quarterly and are representative of values typically obtained in an IVF program. The due dates for the current cycle are detailed on the EQA Schedules Deadlines (BC-T-601).

3.2.2 Each sample should be analysed in singlicate to mirror patient samples and results forwarded to EQASRM by the due date.

3.2.3 Reports with values and individual methods of participating laboratories are compiled and forwarded to the laboratory. These are reviewed upon receipt by the Scientific Director and corrective action initiated for unacceptable results.

3.2.4 Sample Preparation

a) Samples are refrigerated until use.

b) Samples are serum and do not require any preparation. They are analysed in an identical manner to patients (BC-S-785)

c) Samples are run neat but may require dilution, The XP will automatically repeat and dilute if necessary.

3.2.5 Contact

Details of the programme can be seen on www.eqasrm.com.au and e-mail contact made at info@eqasrm.com.au . The postal address is External Quality Assurance Schemes for Reproductive Medicine, PO Box 162, Northlands, Western Australia 6905.

3.2.6 Data entry online

a) Go to www.eqasrm.com.au

b) Logon with the laboratory user name and password.

c) Go to the member's section and click on the "submit results" tab.

d) Click on the scheme you wish to enter results for and follow the prompts.

e) Check your entries thoroughly as once the confirmation page has been submitted, results cannot be changed.

f) Logout once you have entered the results.

3.2.7 Viewing summaries

a) Go to www.eqasrm.com.au

b) Logon with the laboratory user name and password.

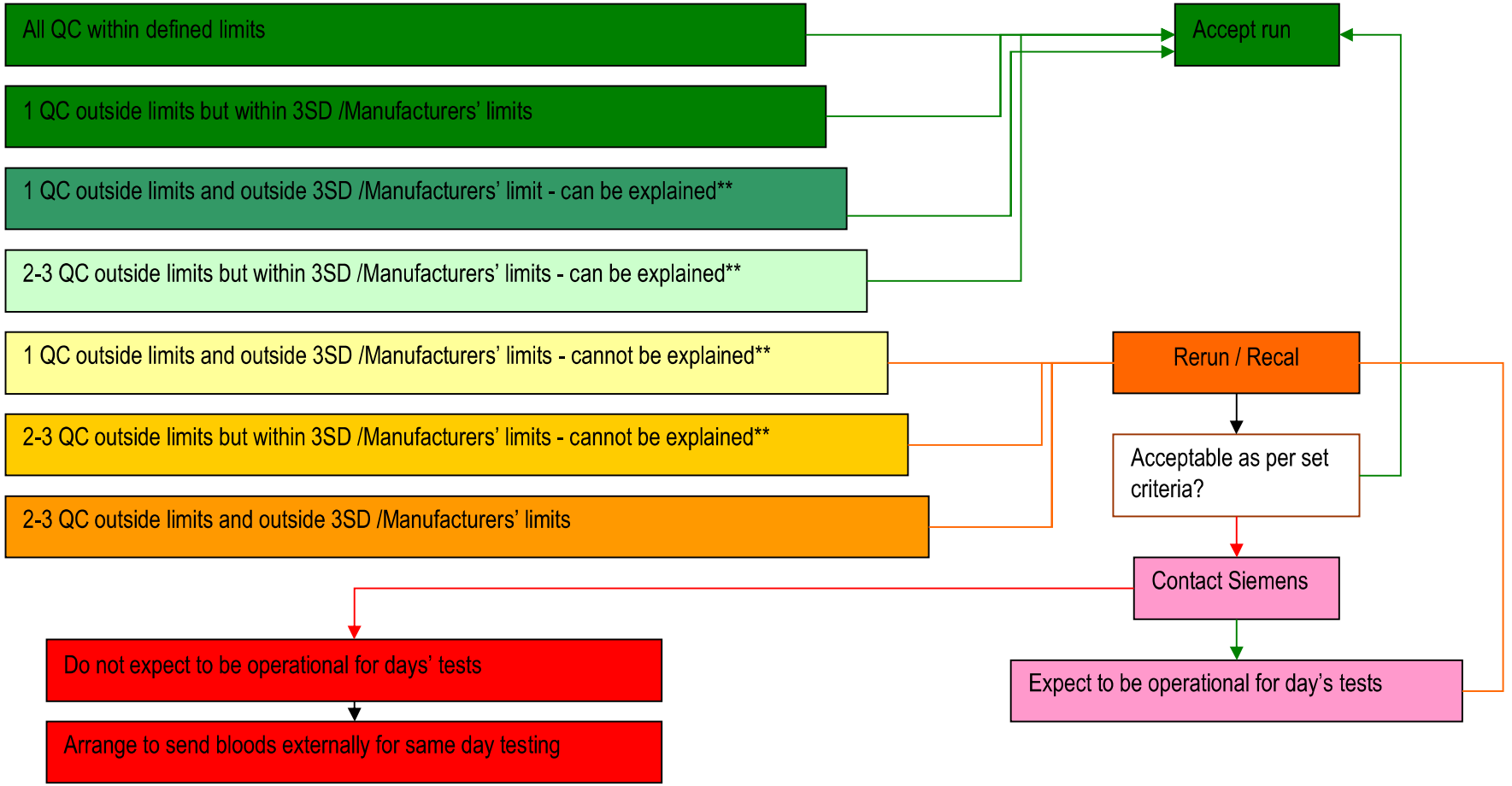
c) Go to the member's section and click on the "distribution summaries" tab.

d) Select which scheme and distribution you require and click on the "view" button.

e) Data for all methods will be shown but only graphs pertaining to your user group will be displayed. A graph of the bias is also available.

f) Printouts are of data only, not graphs.

Biochemistry Quality Control



**** Some Possible Explanations - 1] Trend suggesting target shift possible? 2] Suspected dilution error?***

END OF DOCUMENT

APPENDIX M – Hormone Measurements of Uncertainty

FertilityNorth
Fertility & Endometriosis Treatment Centre

MEASUREMENT UNCERTAINTY ASSOCIATED WITH THE MEASUREMENT OF REPRODUCTIVE HORMONES AT FERTILITY NORTH

Introduction

Every test is subject to some level of error, and estimates of measurement uncertainty (MU) provide information about how large this error might be. At Fertility North we calculate the MU using the variability of the daily internal quality control samples. The MU can be used to derive an interval such that one can be 95% confident that a reported result includes the true value.

MU and hormone assays

The MU for the five reproductive hormones (oestradiol, progesterone, LH, FSH and hCG) are shown in the table below. The main points are:

a) The hormones are measured on the Siemens Centaur XP automated analyser.
b) The daily QC samples are purchased from BioRad and represent three levels, nominally low, medium and high controls.
c) The MU is expressed as a percentage.
d) The 95% confidence limits of a result (X) can be calculated with a coverage factor of 2 as X ± (X*MU).
e) The MU gives an indication of the magnitude of change in a result that must be observed to give 95% confidence that is not due to the inherent errors of the method.

Hormone	QC	Mean	MU	95% confidence interval
Oestradiol (pmol/l)	I	311	15.1%	264 – 358
	II	1076	9.9%	969 – 1183
	III	2589	13.3%	2251 – 2945
Progesterone (nmol/l)	I	2.7	24.8%	2.0 – 3.4
	II	22.0	19.6%	17.7 – 26.3
	III	61.0	14.5%	52.2 – 69.8
LH (IU/L)	I	1.3	13.5%	1.1 – 1.5
	II	20.8	13.3%	18.0 – 23.5
	III	71.5	14.5%	61.2 – 81.9
FSH (IU/L)	I	8.0	12.7%	7.0 – 9.0
	II	27.5	11.3%	24.4 – 30.6
	III	58.0	9.7%	52.4 – 63.6
hCG (IU/L)	I	6.8	25.6%	5.1 – 8.6
	II	23.3	16.2%	19.5 – 27.1
	III	171.2	13.0%	149.0 – 193.4

SUMMARY

1. **Despite a highly sophisticated automated analyser, there is a degree of variability in the hormone concentrations measured on different occasions.**
2. **Care should be taken when interpreting the results and allocating or adjusting treatment.**

APPENDIX N - AMH Gen II ELISA Manufacturer's Guide

FOR PROFESSIONAL USE ONLY

AMH Gen II ELISA

REF A79765

CAUTION

Not for sale in U.S.A.

INTENDED USE

The Anti-Mullerian Hormone (AMH) Gen II enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of AMH in human serum and lithium heparin plasma. This assay is intended for *in vitro* diagnostic use.

SUMMARY AND EXPLANATION

AMH is a glycoprotein dimer composed of two 72 kDa monomers linked by disulfide bridges.[1,2,3,4,5,6,7,8] It belongs to the transforming growth factor-β family. AMH performs various physiological functions. In males, AMH is secreted by the Sertoli cells. During embryonic development, AMH is responsible for Mullerian duct regression. AMH continues to be produced by the testicles until puberty and then decreases slowly to residual post-puberty values. In females, AMH is produced in small amounts by ovarian granulosa cells after birth until menopause, and then becomes undetectable.

PRINCIPLE OF THE TEST

The AMH Gen II ELISA is an enzymatically amplified two-site immunoassay. In the assay, calibrators, controls and samples are incubated in microtitration wells which have been coated with anti-AMH antibody. After incubation and washing, anti-AMH detection antibody labeled with biotin is added to each well. After a second incubation and washing step, streptavidin-horseradish peroxidase (HRP) is added to the wells. After a third incubation and washing step, the substrate tetramethylbenzidine (TMB) is added to the wells. Lastly, an acidic stopping solution is added. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm and between 600 and 630 nm. The absorbance measured is directly proportional to the concentration of AMH in the samples. A set of AMH calibrators is used to plot a calibration curve of absorbance versus AMH concentration. The AMH concentrations in the samples can then be calculated from this calibration curve.

MATERIALS SUPPLIED

Ab PLATE **Anti-AMH Gen II Antibody Coated Microtitration strips:**
A56025

- One stripholder, containing 96 polystyrene microtitration wells with mouse monoclonal anti-AMH IgG immobilized to the inside wall of each well.
- Store at 2 to 8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

AMH Gen II Sample Diluent:
A56026

- One bottle, 13 mL, containing buffer with bovine serum albumin (BSA), < 0.5% ProClin* 300 and sodium azide.
- Store unopened at 2 to 8°C until the expiration date.

BIO CONJ RTU **AMH Gen II Antibody-Biotin Conjugate:**
A56023

- Provided ready to use.
- One bottle, 13 mL, containing biotinylated anti-AMH antibody in buffer with protein (bovine, mouse), < 0.3% ProClin 300 and sodium azide.
- Store at 2 to 8°C until expiration date.

STREP CONJ RTU **Streptavidin-Enzyme Conjugate:**
A56024

- Provided ready to use.
- One bottle, 13 mL, containing streptavidin-HRP in buffer with protein (mouse, fish) and < 10% methanol.
- Store at 2 to 8°C until expiration date.

ASSAY BUFFER **AMH Gen II Assay Buffer:**
A56021

- Two bottles, 13 mL, containing buffer with BSA, protein (bovine, mouse), < 0.3% ProClin 300 and sodium azide.
- Store at 2 to 8°C until expiration date.

TMB SOLN **TMB Chromogen Solution:**
DSL-10-9755

- One bottle, 11 mL, containing a solution of TMB in citrate buffer with hydrogen peroxide.
- Store at 2 to 8°C until expiration date.

WASHCONC I **Wash Concentrate I:**
DSL-10-4030

- One bottle, 100 mL, containing buffered saline with a nonionic detergent.
- Store at 2 to 8°C or room temperature (~25°C) until expiration date.
- Dilute 10-fold with deionized water prior to use.

STOP SOLN A **Stopping Solution A:**
DSL-10-9780

- One bottle, 11 mL, containing 0.2 M sulfuric acid.
- Store at 2 to 8°C or room temperature (~25°C) until expiration date.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. **AMH Gen II Calibrators and Controls A79766**
2. Appropriate size tube (for sample premix)
3. Microtitration plate reader capable of absorbance measurement at 450/405 nm and preferentially capable of dual wavelength (reference filter) at 600 to 630 nm
4. Deionized water
5. Precision pipette(s) to deliver 10–1000 µL
6. Microtitration plate shaker capable of 600–800 orbital revolutions per minute (rpm)
7. Microtitration plate washer
8. Vortex mixer
9. Absorbent materials for blotting the strips
10. Graph paper for manual data reduction

WARNINGS AND PRECAUTIONS

- **For *in vitro* diagnostic use.**
- Use good laboratory practices.[9]
- Samples and blood-derived products may be routinely processed with minimum risk using the procedure described. However, handle these products as potentially infectious according to universal precautions and good clinical laboratory practices, regardless of their origin, treatment or prior certification.[10] Use an appropriate disinfectant for decontamination. Store and dispose of these materials and their containers in accordance with local regulations and guidelines.

⚠ Caution

- Sodium azide preservative may form explosive compounds in metal drain lines. See NIOSH Bulletin: Explosive Azide Hazard (8/16/76).
  To avoid the possible build-up of azide compounds, flush wastepipes with water after the disposal of undiluted reagent. Sodium azide disposal must be in accordance with appropriate local regulations.

- AMH Gen II Assay Buffer

WARNING
H317 May cause an allergic skin reaction.
P280 Wear protective gloves, protective clothing and eye/face protection.
P333+P313 If skin irritation or rash occurs: Get medical advice/attention.
P362+P364 Take off contaminated clothing and wash it before use.
reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC# 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC# 220-239-6](3:1) < 0.05%

- AMH Gen II Biotin Conjugate

WARNING
H317 May cause an allergic skin reaction.
P280 Wear protective gloves, protective clothing and eye/face protection.
P333+P313 If skin irritation or rash occurs: Get medical advice/attention.
P362+P364 Take off contaminated clothing and wash it before use.
reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC# 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC# 220-239-6](3:1) < 0.05%

- AMH Gen II Streptavidin Conjugate

DANGER
H226 Flammable liquid and vapour.
H302 Harmful if swallowed.
H313 May be harmful In contact with skin
H370 Causes damage to organs.

P210 Keep away from heat, hot surfaces, and sparks. No smoking.
P280 Wear protective gloves, protective clothing and eye/face protection.

P308+P311 If exposed or concerned: Call a doctor/physician.
P312 Call a POISON CENTER or doctor/physician if you feel unwell.
Methanol 1 - 9%

- AMH Gen II Sample Diluent

WARNING
H317 May cause an allergic skin reaction.
P280 Wear protective gloves, protective clothing and eye/face protection.
P333+P313 If skin irritation or rash occurs: Get medical advice/attention.
P362+P364 Take off contaminated clothing and wash it before use.
reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC# 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC# 220-239-6](3:1) < 0.05%

- Stopping Solution A

DANGER
H314 Causes severe skin burns and eye damage.
P280 Wear protective gloves, protective clothing and eye/face protection.
P301+P330+P331 IF SWALLOWED: rinse mouth. Do NOT induce vomiting.
P303+P361+P353 IF ON SKIN (or hair): Rinse skin with water.
P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310 Immediately call a POISON CENTER or doctor/physician.
Sulfuric Acid 1 - 3%

- Xi: Irritant: < 0.5% ProClin 300.

R 43: May cause sensitization by skin contact.
S 37: Wear suitable gloves.
S 28: After contact with skin, wash immediately with plenty of soap and water.

- Xn: Harmful: < 10% Methanol.

R 20/21/22: Harmful by inhalation, in contact with skin and if swallowed.
R 68/20/21/22: Harmful: possible risk of irreversible effects through inhalation, in contact with skin and if swallowed.
S 36/37: Wear suitable protective clothing and gloves.
S 45: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

- SDS Safety Data Sheet is available at techdocs.beckmancoulter.com

SAMPLE COLLECTION AND PREPARATION

- Serum and lithium heparin plasma are the recommended samples.
- Observe the following recommendations for handling, processing and storing blood samples:[11]
  a.) Collect all blood samples observing routine precautions for venipuncture.
  b.) Allow serum samples to clot completely before centrifugation.
  c.) Keep tubes stoppered at all times.
  d.) Within two hours after centrifugation, transfer at least 500 µL of cell-free sample to a storage tube. Tightly stopper the tube immediately.
  e.) Serum and lithium heparin plasma may be stored at 2 to 8°C for 48 hours.
  f.) If the assay will not be completed within 48 hours, or for shipment of samples, freeze at -20°C.
- Use the following guidelines when preparing samples:
  a.) Ensure residual fibrin and cellular matter have been removed prior to analysis.
  b.) Follow blood collection tube manufacturer's recommendations for centrifugation.
- Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products. Variations in these products may exist between manufacturers and, at times, from lot-to-lot.
- Avoid repeated freezing and thawing of samples.
- Avoid assaying lipemic or hemolyzed samples.

PROCEDURAL NOTES

- A thorough understanding of this package insert is necessary for successful use of the AMH Gen II ELISA.
- It is the responsibility of the customer to validate the assay for their use.
- Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert.
- A calibration curve must be included with each assay.
- Bring all kit reagents to room temperature (~25ºC) before use.
- Thoroughly mix the reagents before use by gentle inversion.
- Do not mix various lots of any kit component within an individual assay.
- Do not use any component beyond the expiration date shown on its label.
- Incomplete washing will adversely affect the outcome and assay precision.
- To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stopping solution into the wells in the same order and speed used to add the TMB chromogen solution.
- Avoid microbial contamination of reagents, especially of the conjugate and the assay buffer.
- Avoid contamination of the TMB chromogen solution with the conjugates.
- Use a clean disposable pipette tip for each reagent, calibrator, control or sample.
- For dispensing sulfuric acid and TMB chromogen solution, avoid pipettes with metal parts.

- The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies.
- Use deionized water.
- Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.

TEST PROCEDURE

Preparation of Reagents

1. ***Wash Solution:*** Dilute 1 part Wash Concentrate I with 9 parts deionized water. The resulting working strength wash solution is stable for one month at room temperature (~25°C) when stored in a tightly sealed bottle.
2. ***Microtitration Wells:*** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

Assay Procedure

Allow all samples and reagents to reach room temperature (~25°C). Mix reagents thoroughly by gentle inversion before use. After reconstitution of reagents, mix thoroughly, avoiding foam. Calibrators, controls and samples should be assayed in duplicate.

NOTE: All samples reading higher than the highest calibrator should be thoroughly mixed and diluted in the AMH Gen II Sample Diluent prior to assay. For pediatric male samples: Dilute 1 part sample with 9 parts Sample Diluent before testing.

1. Before adding sample to the AMH Gen II ELISA microplate, you must prepare all calibrators, controls, and samples with the **AMH Gen II Assay Buffer** (REF A56021).
   In a sample tube, prepare 1 part of each calibrator, control, or test sample respectively (including diluted pediatric male samples) with 5 parts AMH Gen II Assay Buffer (for example, 60 µL calibrator, control, or sample + 300 µL AMH Gen II Assay Buffer). Mix thoroughly.
   NOTE: This is a preparation of the AMH calibrators, controls and test samples with the AMH Gen II Assay Buffer. No dilution factor is required.
2. Mark the microtitration strips to be used.
3. Within 1 hour, pipet 120 µL of the premixed calibrators, controls and samples to the appropriate wells using a precision pipette.
4. Incubate the wells, shaking at 600–800 rpm on an orbital microplate shaker, for one hour at room temperature (~25°C).
5. Prepare the wash solution as described under the "Preparation of Reagents" section of this package insert.
6. Aspirate and wash each well five times with the wash solution using an automatic microplate washer or manually using a precision pipette. Blot and dry by inverting plate on absorbent material.
   NOTE: Use of an automatic microplate washer is strongly recommended. Incomplete washing will adversely affect assay precision. If a microplate washer is not available, follow these steps to wash the plate manually:
   (a) Completely aspirate the liquid from each well
   (b) Dispense 400 µL of the wash solution into each well using a precision pipette
   (c) Aspirate the liquid again
   (d) Repeat steps (b) and (c) four times
7. Add 100 µL of the antibody-biotin conjugate solution to each well using a precision pipette.
8. Incubate the wells, shaking at 600–800 rpm on an orbital microplate shaker, for one hour at room temperature (~25°C).
9. Aspirate and wash each well five times with the wash solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
10. Add 100 µL of the streptavidin-enzyme conjugate to each well using a precision pipette.
11. Incubate the wells, shaking at 600–800 rpm on an orbital microplate shaker, for 30 minutes at room temperature (~25°C).
12. Aspirate and wash each well five times with the wash solution using an automatic microplate washer. Blot dry by inverting plate on absorbent material.
13. Add 100 µL of the TMB chromogen solution to each well using a precision pipette.
    Avoid exposure to direct sunlight.
14. Incubate the wells, shaking at 600–800 rpm on an orbital microplate shaker, for 8–12 minutes at room temperature (~25°C).
    NOTE: Be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Visually monitor the color development to optimize the incubation time.
15. Add 100 µL of the stopping solution to each well using a precision pipette.
16. Read the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450 nm.
    NOTE: 1) While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "Blank".
    2) If wavelength correction is available, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set between 600 and 630 nm.

RESULTS

1. Calculate the mean absorbance for each calibrator, control or sample.
2. Subtract the mean absorbance of Calibrator A (blank) from the mean absorbance of calibrators B-G, control or sample. Plot the log of the mean blank-subtracted absorbance readings for calibrators B-G along the y-axis versus log of the AMH concentrations in ng/mL along the x-axis, using a cubic regression curve-fit. Use of curve fits other than recommended may cause results to vary.
3. Determine the AMH concentrations of the controls and samples from the calibration curve by matching their mean absorbance readings with the corresponding AMH concentrations.
4. Any sample reading higher than the highest calibrator should be appropriately diluted using sample diluent and reassayed. For pediatric male samples: Dilute 1 part sample with 9 parts Sample Diluent before testing.
5. Any sample reading lower than the analytical sensitivity should be reported as such.
6. Multiply the value by a dilution factor, if required.
   NOTE: If the absorbance readings exceed the limitations of the plate reader, a second reading at 405 nm is needed (reference filter between 600 and 630 nm if available). In this case, proceed to construct a second calibration curve as above with the absorbance readings of all calibrators at 405 nm. The concentration of the off-scale samples at 450 nm is then read from the new calibration curve. The readings at 405 nm should not replace the on-scale readings at 450 nm.

LIMITATIONS

- The reagents supplied in this kit are optimized to measure AMH levels in serum and lithium heparin plasma.
- For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the sample. Samples from individuals which have been regularly exposed to animals or have received immunotherapy or diagnostic procedures utilizing immunoglobulins or immunoglobulin fragments may produce antibodies, e.g. HAMA, that interfere with immunoassays. Additionally, other heterophile antibodies such as human anti-goat antibodies may be present in samples.[12,13]
- Other potential interferences from endogenous binding proteins and drugs in the patient sample could be present and may cause erroneous results in immunoassays. Carefully evaluate the results of patients suspected of having

these types of interferences.[14,15]

- If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial.

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- AMH Gen II ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for AMH Gen II ELISA controls are provided with the AMH Gen II Calibrator and Control kit.
- A full calibration curve, plus low and high level controls, should be included in each assay.
- The TMB chromogen solution should be colorless to very light yellow. Development of a blue color may indicate reagent contamination or instability.

EXPECTED VALUES

1. Each laboratory should establish its own reference ranges to assure proper representation of specific populations.

SAMPLES	MEDIAN AGE (yrs)	MEDIAN IN (ng/mL)	2.5–97.5TH PERCENTILE IN (ng/mL)
Random Males (N=136)	38	5.7	1.3-14.8
Random Females (N=95)	30	2.4	ND-12.6
Males fertility clinic (N=100)	37	5.3	0.8-14.6
Females 3rd day of cycle (N=106)	-	1.5	ND-10.6
Post Menopausal Females (N=45)†	71	ND	ND
Boys (N=36)†	4.8	56.3	3.8-159.8
Girls (N=36)†	5.0	1.3	ND-8.9

†Non parametric reference at 90% limit.
ND = Non-Detectable

TYPICAL CALIBRATION CURVE

WELL NO.	WELL CONTENTS	MEAN ABSORBANCE	CONC. (ng/mL)
	CALIBRATORS		
A1, A2	A	(Blank)	0
B1, B2	B	0.019	0.16
C1, C2	C	0.053	0.4
D1, D2	D	0.17	1.2
E1, E2	E	0.57	4.0
F1, F2	F	1.47	10.0
G1, G2	G	3.11	22.5

CAUTION: The above data must not be employed in lieu of data obtained by the user in the laboratory.

SPECIFIC PERFORMANCE CHARACTERISTICS

All analytical characteristics are stated in ng/mL. To convert to SI units (International System of Units):

1 ng/mL = 7.14 pM

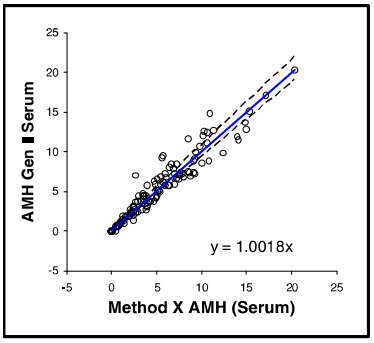
Method Comparison

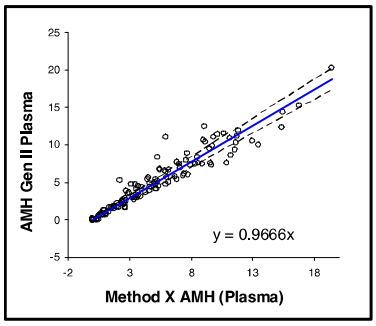
The AMH Gen II ELISA has been compared to another commercially available AMH kit (Method X). One hundred nineteen male and female serum and lithium heparin plasma samples, ranging in age from 20-50 years were assayed and linear regression analysis of the results yielded the following:

Regression:

A. AMH SERUM =1.0 (Method X)
(r = 0.98; 97.5% CI = 0.95-0.98, P <0.0001)

B. AMH PLASMA =0.967 (Method X)
(r = 0.98; 97.5% CI = 0.95-0.98, P <0.0001)





Dilution Recovery (Linearity)

Multiple dilutions of four samples containing various AMH levels with AMH Gen II sample diluent (zero) resulted in the following data.[16]

SAMPLE	DILUTION FACTOR (1:X)	EXPECTED VALUE IN (ng/mL)	OBSERVED VALUE IN (ng/mL)	% RECOVERY
1	Neat value	4.94	N/A	N/A
	2	2.47	2.66	108
	4	1.24	1.37	110
	8	0.62	0.63	102
	16	0.31	0.26	84
2	Neat value	6.47	N/A	N/A
	2	3.24	3.46	107
	4	1.62	1.77	109
	8	0.81	0.88	109
	16	0.41	0.37	90
3	Neat value	10.34	N/A	N/A
	2	5.17	5.23	101
	4	2.58	2.59	100
	8	1.29	1.34	104
	16	0.65	0.59	91
4	Neat value	12.86	N/A	N/A
	2	6.43	6.36	99
	4	3.22	3.11	97
	8	1.61	1.58	98
	16	0.80	0.68	85

Spiking Recovery

Addition of three different levels of AMH to four patient samples with low AMH resulted in the following data:

SAMPLE	ENDOGENOUS CONC. (ng/mL)	EXPECTED CONC. (ng/mL)	OBSERVED CONC. (ng/mL)	% RECOVERY
1	0.67	1.97	1.96	99
		3.16	3.20	101
		4.24	4.45	105
2	1.16	2.44	2.53	104
		3.60	3.81	106
		4.66	5.01	107
3	2.21	3.44	3.86	112
		4.55	4.48	98
		5.57	5.69	102
4	1.47	2.73	2.77	101
		3.88	3.87	100
		4.93	5.07	103

Imprecision:

Reproducibility of the AMH Gen II assay was determined in a study using two in-house serum pool based controls (Q1, Q2) and two kit controls (C1, C2) with two lots of reagents. The study included a total of 40 assays, four replicates per assay.[17]

SAMPLE	MEAN CONC.	WITHIN RUN	BETWEEN RUN	TOTAL
	(ng/mL)	% CV	% CV	% CV
Q1	4.42	5.4	5.6	7.7
Q2	14.03	3.6	4.5	5.8
C1	3.82	3.7	4.4	5.7
C2	16.45	3.4	4.0	5.3

Analytical Specificity

The antibodies used in the assay bind to the mature region of AMH, which is more stable against proteolysis compared to pro-hormone region. This highly characterized dual monoclonal antibody pair is specific to AMH and does not detect inhibin A, activin A, FSH and LH at 2 times their physiological concentrations.

Interference

When potential interferents (hemoglobin, triglycerides and bilirubin), were added at least at two times their physiological concentration, AMH concentrations were within ±10% of the control as represented in the following table.[18]

INTERFERENTS	ANALYTE CONC.	UNSPIKED SAMPLE (ng/mL)	SPIKED SAMPLE (ng/mL)	% DIFFERENCE TO REFERENCE
HEMOGLOBIN	2 mg/mL	2.85	2.96	3.9
TRIGLYCERIDES	20 mg/mL	2.48	2.31	-6.9
BILIRUBIN	0.6 mg/mL	2.5	2.51	0.4

Limit of Detection (LoD):

The lowest amount of AMH in a sample that can be detected with a 95% probability is 0.08 ng/mL. The value was determined by processing a complete seven point calibration curve, controls and five serum samples in the range of zero to 1.5 ng/mL.[19] Two assay runs per day were performed over 10 days with all samples run in duplicate per run.

Limit of Quantitation (LoQ):

The estimated minimum dose achieved at 20% total imprecision is 0.17 ng/mL. The value was determined by processing a complete seven point calibration curve, controls and seven human serum samples with at least two samples that were less than the median of normal and minimum of three samples that were greater than the median of normal over 20 runs and 10 days in duplicates.[19]

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APPENDIX O – AMH calibrators

FOR PROFESSIONAL USE ONLY

AMH Gen II CALIBRATORS AND CONTROLS REF A79766

CAUTION

Not for sale in U.S.A.

INTENDED USE

The Anti-Mullerian Hormone (AMH) Gen II Calibrators are intended to calibrate the AMH Gen II ELISA assay (A79765) for the quantitative measurement of AMH in human serum and lithium heparin plasma. AMH Gen II Controls are intended for use as quality controls to monitor the precision of laboratory testing procedures for the AMH Gen II ELISA. This assay is intended for *in vitro* diagnostic use.

SUMMARY AND EXPLANATION

Quantitative assay calibration is the process by which samples with known analyte concentration (i.e. assay calibrators) are tested in the same manner as patient samples to measure the response. The mathematical relationship between the measured response and the known analyte concentrations establishes the calibration curve. This mathematical relationship, or calibration curve, is used to convert absorbance measurements of patient samples to specific quantitative analyte concentration. Quality control materials simulate the characteristics of patient samples and are essential for monitoring the system performance of the AMH ELISA assay. In addition, they are an integral part of good laboratory practices.[1,2,3,4,5,6] When performing the AMH assay, include quality control materials to validate the integrity of the assay. The assayed values should fall within the acceptable range if the test system is working properly.

PRINCIPLE OF THE TEST

Two levels of controls are available to allow performance monitoring of the AMH Gen II ELISA. In the A79765 assay, calibrators, controls and samples are incubated in microtitration wells which have been coated with anti-AMH antibody. After incubation and washing, the wells are treated with anti-AMH detection antibody labeled with biotin. After a second incubation and washing step, the wells are incubated with streptavidin-horseradish peroxidase (HRP). After a third incubation and washing step, the wells are incubated with the substrate tetramethylbenzidine (TMB). Lastly, an acidic stopping solution is added. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as primary reference filter. The absorbance measured is directly proportional to the concentration of AMH in the samples. A set of AMH Gen II calibrators is used to plot a calibration curve of absorbance versus AMH concentration. The AMH concentrations in the samples can then be calculated.

MATERIALS SUPPLIED

CAL | A **AMH Gen II Calibrator A:**
A56010

- One vial, 0.5 mL, labeled A, containing 0 ng/mL AMH in bovine serum with sodium azide.
- Provided ready to use.
- Freeze upon receipt at -20°C. For multiple use, aliquot and freeze at -20°C.
- Stable until the expiration date stated on the label when stored at -20°C.
- Mix contents thoroughly by gentle inversion before use.
- After initial use, the thawed vials are stable at 2 to 8°C for seven days. Label the vials with the date of thaw.
- Return calibrator to 2 to 8°C after each use.
- Thaw calibrator no more than two times.
- Signs of possible deterioration are control values out of range.

CAL | B-G **AMH Gen II Calibrator B-G:**
A56011, A56012, A56013, A56014, A56015, A56016

- Six vials, 0.5 mL, labeled B-G, containing concentrations of approximately 0.16, 0.4, 1.2, 4.0, 10.0 and 22.5 ng/mL bovine AMH in bovine serum with sodium azide.
- Provided ready to use.
- Freeze upon receipt at -20°C. For multiple use, aliquot and freeze at -20°C.
- Stable until the expiration date stated on the label when stored at -20°C.
- Mix contents thoroughly by gentle inversion before use.
- After initial use, the thawed vials are stable at 2 to 8°C for seven days. Label the vials with the date of thaw.
- Return calibrators to 2 to 8°C after each use.
- Thaw calibrators no more than two times.
- Signs of possible deterioration are control values out of range.
- Refer to Calibrator/Control Value card for exact concentration.

CONTROL | I-II **AMH Gen II Controls:**
A56017, A56018

- One set of controls, 0.5 mL each, labeled I and II, containing low and high concentrations of bovine AMH in bovine serum with sodium azide.
- Provided ready to use.
- Freeze upon receipt at -20°C. For multiple use, aliquot and freeze at -20°C.
- Stable until the expiration date stated on the label when stored at -20°C.
- Mix contents thoroughly by gentle inversion before use.
- After initial use, the thawed vials are stable at 2 to 8°C for seven days. Label the vials with the date of thaw.
- Return controls to 2 to 8°C after each use.
- Thaw controls no more than two times.
- Signs of possible deterioration are control values out of range.
- Refer to Calibrator/Control Value card for exact concentration.

TRACEABILITY

The (analyte) measurand in the AMH Gen II Calibrators is traceable to the manufacturer's working calibrators. The assigned values were established using representative samples from this lot of calibrator and are specific to the assay methodologies of the AMH Gen II reagents. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. **AMH Gen II Reagent A79765**
2. Appropriate size tube (for sample premix)
3. Microtitration plate reader capable of absorbance measurement at 450/405 nm and preferentially capable of dual wavelength (reference filter) at 600 to 630 nm
4. Deionized water
5. Precision pipette(s) to deliver 10–250 µL
6. Microtitration plate shaker capable of 600–800 orbital revolutions per minute (rpm)
7. Microtitration plate washer
8. Vortex mixer
9. Absorbent materials for blotting the strips
10. Graph paper for manual data reduction

QUALITY CONTROL VALUE ASSIGNMENT

The control values were derived by analyzing control replicates run in the A79765 assay, and are lot-specific. Individual laboratory means may vary from the listed values, but should fall within the corresponding acceptable range. Variation between laboratories may be caused by differences in laboratory technique, assay incubation temperature and instrumentation. It is recommended that each laboratory establish its own mean and acceptable range values and use those provided only as guides.

WARNINGS AND PRECAUTIONS

- **For *in vitro* diagnostic use.**
- Use good laboratory practices.[7]
- Samples and blood-derived products may be routinely processed with minimum risk using the procedure described. However, handle these products as potentially infectious according to universal precautions and good clinical laboratory practices, regardless of their origin, treatment or prior certification.[8] Use an appropriate disinfectant for decontamination. Store and dispose of these materials and their containers in accordance with local regulations and guidelines.

⚠ Caution

- Sodium azide preservative may form explosive compounds in metal drain lines. See NIOSH Bulletin: Explosive Azide Hazard (8/16/76).
  To avoid the possible build-up of azide compounds, flush wastepipes with water after the disposal of undiluted reagent. Sodium azide disposal must be in accordance with appropriate local regulations.
- **GHS HAZARD CLASSIFICATION**
  Not classified as hazardous
- SDS The Safety Data Sheet (SDS) is available upon request or at techdocs.beckmancoulter.com

CALIBRATION DETAIL

The AMH Gen II calibrators are provided at seven levels —zero and approximately 0.16, 0.4, 1.2, 4.0, 10.0 and 22.5 ng/mL. Run the AMH Gen II Calibrators A-G and Control Levels I and II in duplicate.

CURVE DETAIL

1. Subtract the mean absorbance of Calibrator A (blank) from the mean absorbance of calibrators B-G, control or sample. Plot the log of the mean blank-subtracted absorbance readings for calibrators B-G along the y-axis versus log of the AMH concentrations in ng/mL along the x-axis, using a cubic regression curve-fit. Use of curve-fits other than recommended may cause results to vary.
2. Determine the AMH concentrations of the controls and samples from the calibration curve by matching their mean absorbance readings with the corresponding AMH concentrations.

LIMITATIONS

If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial.

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Tercüme edilen IFU'lar techdocs.beckmancoulter.com adresinden veya Beckman Coulter satış temsilcinizden alınabilir (B00574).

Переведенные инструкции по применению можно найти на веб-сайте techdocs.beckmancoulter.com или получить от местного представителя компании Beckman Coulter (A92295).

Oversatte bruksanvisninger finnes på techdocs.beckmancoulter.com eller fra din Beckman Coulter-salgsrepresentant (A92287).

Preložený návod na použitie je k dispozícii na webovej stránke techdocs.beckmancoulter.com alebo ho môžete získať od svojho zástupcu spoločnosti Beckman Coulter (B37782).

Przetłumaczone instrukcje obsługi dostępne są na stronie internetowej techdocs.beckmancoulter.com lub u przedstawiciela handlowego firmy Beckman Coulter (B51695).

APPENDIX P – Patient Medications

Medication Key

Common Name	Abbreviation	Chemical name	Units	Administered	Use	Company
Aspirin	ASP	Acetylsalicylic acid	mg	Oral tablet	Anticoagulant	Apotex
Cetrotide	CET	Cetrorelix acetate	µg	Injection	GnRH antagonist	Merck Serono
Clexane	CLX	Enoxaparin sodium	mg	Injection	Prevention of venous thromboembolism	Sanofi-Aventis
Clomid	CLO	Clomiphene citrate	mg	Oral tablet	Synthetic ovulation stimulant	Sanofi-Aventis
Crinone	CRI	Progesterone	mg	Vaginal gel	Pregnancy support	Merck Serono
Doxycycline	ABS	Doxycycline	mg	Oral tablet	Antibiotic	Sandoz
Elonva	ELO	Corifollitropin alfa	mg	Injection	Controlled ovarian hyperstimulation	Merck Sharp & Dohme
Endometrin	EPS	Progesterone	mg	Pessaries	Pregnancy support	Ferring
Gonal-F	GON	Follitropin alfa	IU	Injection	Controlled ovarian hyperstimulation	Merck Serono
Lucrin	LUC	Leuprorelin acetate	mg	Injection	GnRH agonist	AbbVie
Luveris	LUV	lutropin alfa	IU	Injection	LH	Merck Serono
Menopur	MEN	human menopausal gonadotrophin	IU	Injection	Controlled ovarian hyperstimulation	Ferring
Oral Contraceptive Pill	OCP	Ethinyloestradiol/ Drospirenone	µg/ mg	Oral tablet	Regulate cycle and time ovulation	Bayer
Orgalutran	ORG	Ganirelix	mg	Injection	GnRH antagonist	Merck Sharp & Dohme
Oripro	PPS	Progesterone	mg	Pessaries	Pregnancy support	Merck Serono

Ovidrel	OVI	Choriogonadotropin alfa	µg	Injection	Superovulation egg trigger	Merck Serono
Panafcortelone	PRD	Pregnisolone	mg	Oral tablet	Anti-rejection	Aspen Pharmacare Australia
Pregnyl	PRE	Choriogonadotropin alfa	IU	Injection	Final egg release	Merck Sharp & Dohme
Progynova	PRO	Oestradiol valerate	mg	Oral tablet	Hormone replacement therapy	Bayer
Proluton	PTN	Progesterone	mg	Injection	Pregnancy support	Bayer
Provera	PRV	Medroxyprogesterone acetate	mg	Oral tablet	Induce a bleed	Pfizer Australia
Puregon	PUR	Follitropin alfa or beta	IU	Injection	Controlled ovarian hyperstimulation	Merck Sharp & Dohme
Scitropin	SCI	Somatropin	mg	Injection	Recombinant human growth hormone for egg quality	SciGen Australia
Synarel	SYN	Nafarelin	mg	Nasal spray	GnRH agonist	Pfizer Australia

Patient ID	Cycle	Medications taken by each patient								
37	AIH	PUR50	OVI250	OVI60						
52	AIH	GON50	OVI250	OVI60	PPS400					
60	AIH	GON62.5	OVI250	OVI60	PPS400					
76	AIH	GON50	OVI250	OVI60						
78	AIH	GON25	OVI250	OVI60	PPS400					
82	AIH	GON25	OVI250	OVI60	PPS400					
85	AIH	GON25	OVI250	OVI60	PPS200					
4	FET	GON50	OVI250	OVI60	PPS600					
5	FET	PRO16	PPS1200	PTN25	CLX20	ASP100				
7	FET	GON50	OVI250	OVI60	PPS400					
11	FET	GON75	OVI10	ABS100	OVI250	PRD10	PPS600			
16	FET	OCP3/30	PRO16	PPS1200	PTN25					
19	FET	GON37.5	OVI250	PPS400						
21	FET	GON25	OVI250	OVI60						
26	FET	GON37.5	OVI250	PRD10	OVI60	PPS400	CLX40			
27	FET	GON62.5	OVI250	OVI60	PPS400					
28	FET	GON25	OVI250	OVI60						

32	FET	GON37.5	OVI250	OVI60	PPS400					
34	FET	PRO8	PRD10	PPS2400	PTN25	CRI180	CLX40	ASP100		
38	FET	PRO16	PPS800	PTN25	CRI90					
39	FET	GON37.5	OVI250	PRD10	OVI60	PPS400	CLX20	ASP100		
46	FET	GON50	OVI250	PPS400						
51	FET	PUR25	OVI250	OVI60	PPS200					
55	FET	GON37.5	OVI250	OVI60	PPS400					
57	FET	PRO16	ABS100	PPS1200	PTN25	CLX20	ASP100			
58	FET	GON37.5	OVI250	OVI60	PPS400					
61	FET	GON50	ABS100	OVI250	PRD10	PPS400	CLX40			
64	FET	PRO10	PPS800	PTN25						
65	FET	GON50	OVI60	PPS600						
68	FET	GON50	OVI250	OVI60						
69	FET	GON25	OVI250	OVI60	PPS400					
71	FET	GON50	OVI250	OVI60	PPS400					
8	ICSI	SYN400	PUR300	OVI500	PPS400					
15	ICSI	ORG250	GON150	OVI10	OVI500	OVI60	PPS400			
17	ICSI	ORG250	GON125	OVI10	OVI500	PPS400				

22	ICSI	CET250	GON175	LUV75	OVI500	PPS400				
23	ICSI	ORG250	GON150	OVI500	PPS400					
24	ICSI	CET250	GON400	OVI10	SCI40	PRE10000	PRD10	OVI60	PPS600	CLX40
29	ICSI	LUC10	PUR200	OVI500	PPS400					
31	ICSI	ORG250	GON200	OVI10	PRE10000	PPS400				
33	ICSI	SYN400	PUR200	OVI20	OVI500	PPS200				
35	ICSI	OCP3/30	LUC10	ORG250	GON175	OVI10	PRE10000	PPS400		
36	ICSI	ORG250	PUR100	OVI10	PRE10000	PPS400				
41	ICSI	SYN400	PUR175	OVI20	ABS100	PRE10000	PRD10	PPS400	ASP100	
50	ICSI	ORG250	PUR200	OVI500	PPS400					
54	ICSI	ORG250	GON125	OVI10	OVI500	PPS400				
59	ICSI	SYN400	GON200	OVI10	PRE10000	PPS400				
70	ICSI	GON450	OVI10	PRE10000	PRD10	PPS400	CLX20	ASP100		
72	ICSI	SYN400	GON200	OVI500	PPS400					
10	IVF	OCP3/30	LUC10	GON175	LUV75	OVI500	PPS400			
13	IVF	ORG250	GON150	OVI500	PPS400					
14	IVF	SYN400	PUR300	OVI500	PRD10	PPS400				
45	IVF	ORG250	MEN150	OVI500	PRD10	PPS400	CLX20	ASP100		

56	IVF	LUC10	GON150	OVI500	PPS400	PRV5				
66	IVF	ORG500	GON200	PRE10000	PPS400					
67	IVF	LUC10	SYN400	GON300	OVI500	PPS400				
79	IVF	SYN400	PUR250	ABS100	PRE10000	CRI90	CLX20	ASP100		
1	OI	GON25	OVI250	OVI60	CLX20	ASP100				
2	OI	GON100	OVI250	PPS800	CRI90					
6	OI	CLO100	OVI250	OVI60						
18	OI	CLO50	OVI250	OVI60						
20	OI	CLO50	OVI250	OVI60	PPS200					
30	OI	CLO5	OVI250	OVI60	PPS200					
43	OI	GON37.5	OVI250	OVI60	PPS400					
44	OI	GON62.5	OVI250	OVI60	PPS400	CLX20	ASP100			
47	OI	GON50	OVI250	OVI60	PPS200					
48	OI	CLO100	OVI250	OVI60						
49	OI	CLO2.5	OVI250	OVI60						
53	OI	CLO50	OVI250	OVI60						
83	OI	GON50	OVI250	PPS400						
3	TRACK	None								

9	TRACK	None								
12	TRACK	None								
25	TRACK	PPS200								
40	TRACK	None								
42	TRACK	PPS200								
62	TRACK	PPS200								
63	TRACK	PPS200								
73	TRACK	PPS200								
74	TRACK	None								
75	TRACK	PPS200								
77	TRACK	PPS400								
80	TRACK	PPS200	CLX20							
81	TRACK	None								
84	TRACK	None								