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Biomarkers of disease : concentrations in the serum of women during natural and stimulated ovarian cycles and during early pregnancy

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"Biomarkers of disease: Concentrations in the serum of women during natural and stimulated ovarian cycles and during early pregnancy"

A thesis submitted in partial fulfilment

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By

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Abstract

Molecular biomarkers are chemical signatures that all cell types possess. They are used in medicine to evaluate both normal biological events and pathogenic processes. A series of biomarkers associated with cancer of the breast, ovaries and other parts of the female reproductive tract and the monitoring of pregnancy were measured in disease-free women. The biomarkers measured were prostate specific antigen (PSA), CA125, CA15-3, CA72-4, and pregnancy associated plasma protein-a (PAPP-A). The patterns of change during natural and stimulated ovarian cycles and early pregnancy were investigated to determine if these biomarkers could reflect normal events relating to ovulation and implantation/placentation. In addition, the study was able to investigate the possible erroneous crossing of clinical cut-off values associated with disease due to other biological processes rather than the disease itself.

A total of 73 blood samples (10 women) taken throughout the natural menstrual cycle, 64 blood samples (11 women) during stimulated ovarian cycles and 86 blood samples (14 women) during early pregnancy monitoring were collected and all samples were analysed by batch analysis on the Roche Cobas e411. Concentrations of CA125, tPSA, CA15-3 and CA72-4 showed no significant difference between the natural and stimulated ovarian cycle groups ($p \geq 0.5989$). On average the mean PAPP-A of the natural group was 2.41±0.58 mIU/L higher than the stimulated group ($t = 4.10$, $p < 0.001$). CA125 and CA15-3 results were both significantly influenced by the stage of the cycle ($p = <0.0001$), whereas tPSA and PAPP-A concentrations revealed no significant changes ($p \geq 0.65$). In early pregnancy, concentrations of tPSA (in the 9/14 (64%) women with detectable levels) and PAPP-A increased steadily from 4 weeks to 7 weeks ($p<0.01$). There was a significant effect of the stage of gestation ($p=0.0012$) on CA125 concentration with peak levels occurring at 5.5 weeks (57.98±27.45U/mL) before reducing again by week 7. The concentration of CA15-3 reduced as the pregnancy

progressed ($p<0.0001$). The CA72-4 concentration in pregnancy was on average 2.11±0.38U/ml.

In conclusion, batch analysis of all samples from each of the participants was conducted to maximise the possibility that any changes seen in biomarker concentrations were due to biological fluctuations and not because of assay variability. Ovarian stimulation reduced serum PAPP-A levels, whilst CA125 and CA15-3 were unaffected by ovarian stimulation *per se* but showed cyclical changes throughout both natural and stimulated cycles. PAPP-A, CA125, tPSA and CA15-3 all showed consistent changes in early pregnancy, and their combined benefits as markers of different aspects of implantation, embryogenesis and placentation warrants further investigation. Only CA125 in early pregnancy crossed the cut-off associated with disease, ie ovarian cancer, and other gynaecological and inflammatory conditions. Care must therefore be taken when using CA125 determinations to detect disease if the woman is less than 7 weeks pregnant as transient elevations during this time appear normal.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

i. Incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;
ii. Contain any material previously published or written by another person except where due reference is made in the text of this thesis; or
iii. Contain any defamatory material;

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Melissa Jane Stemp

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1 Introduction

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Strimbu & Tavel, 2010). However, many biomarkers of disease are often present in healthy unaffected people too, albeit at low concentrations and so there needs to be certainty that raised levels of such biomarkers do not occur during normal periods of increased cellular growth. A series of biomarkers associated with cancer of the breast, ovaries and other parts of the female reproductive tract were measured in healthy women on the basis that the concentration of these may be more likely to be affected by the reproductive status of the women. The biomarkers measured in the present study were prostate specific antigen (tPSA), CA125, CA15-3, CA72-4 and pregnancy associated plasma protein-a (PAPP-A). The patterns of change throughout natural and stimulated ovarian cycles and in early pregnancy were investigated to determine if these biomarkers could reflect normal events relating to ovulation and implantation/placentation. In addition, the study was able to investigate the possible erroneous crossing of clinical cut-off values associated with disease due to other biological processes rather than the disease itself.

1.1 The Female Reproductive System

1.1.1 The Female Reproductive Cycle

Throughout a female's reproductive years her ovaries and uterus go through monthly cyclical changes which respectively involve developing gametes through oogenesis in the ovaries and preparing the uterus for possible implantation of an embryo (Oktem & Oktay, 2008). The ovaries, the hypothalamus and the anterior pituitary are all involved in secreting hormones which control the phases of the cycle. The term used to describe the formation of gametes in the ovaries is oogenesis. Oogenesis begins early during foetal development, starting when the primordial follicles begin

migrating from the yolk sac into the ovaries where they differentiate into oogonia. Oogonia are diploid with the full 46 chromosomes and undergo mitosis to produce millions of germ cells. Most of the germ cells degenerate through atresia, however, some will form primary oocytes which enter prophase I of meiosis and remain there until puberty (Verlhac & Villeneuve, 2010). The primary oocyte is surrounded by a flattened layer of follicular cells and is now called a primordial follicle. At birth, a female will have anywhere between 200,000 – 2,000,000 primary oocytes; at puberty there are approximately 400,000 remaining. In the average female, only 400 primary oocytes mature and ovulate (Tortora & Derrickson, 2010). At the permanent cessation of the menstrual cycle, a woman is said to have reached menopause, where approximately 1000 follicles are left in each ovary (Oktem & Oktay, 2008).

The onset of menstrual cycling is termed menarche. Young women who have begun menarche will not usually have their first ovulation until around 6 or more months after commencement and the menstrual cycles often do not become regular until years later (Beckman & Feuston, 2003). The proliferative phase of the uterine cycle, in concert with the ovarian cycle, involves building up the stratum functionalis of the endometrium of the uterus in the first two weeks of the cycle (stimulated by oestrogen from the developing follicles in the ovaries). Following the LH surge, progesterone is secreted (at the expense of oestrogen) as the follicle becomes luteinised an in response the endometrium undergoes secretory changes. If fertilization does not occur, progesterone levels (produced by the remnants of the follicle that ovulated, the corpus luteum) drop off which causes the stratum functionalis to slough away in a menstrual bleed (M. Johnson, 2013).

Gonadotrophin-releasing hormone (GnRH) is a hormone secreted by the hypothalamus that stimulates the anterior pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Richards & Pangas,

2010). FSH initiates follicular growth, and LH stimulates the ovarian follicles to keep developing. Just before mid-cycle there are many cumulative GnRH pulses, which stimulate the concentration of LH to surge, initiating ovulation and then the formation of the corpus luteum. From the follicle remnants, the corpus luteum is responsible for producing oestrogen, progesterone, relaxin and inhibin (Tortora & Derrickson, 2010). The oestrogen that is secreted by the ovarian follicles is important for maintaining the female reproductive organs and secondary sex characteristics, amongst other specialised functions. Progesterone is secreted mainly from the corpus luteum and its function is to work with the oestrogen to prepare the endometrium for implantation (and maintain it) as well as preparing the mammary glands for milk production (Tortora & Derrickson, 2010).

1.1.2 Phases of the Female Reproductive Cycle

The typical duration of the female reproductive cycle is between 24 and 36 days, however most texts refer to the cycle as being 28 days, comprising four phases (Tortora & Derrickson, 2010). The menstrual phase is the first 5 days of the cycle and this is where the ovaries, under the influence of FSH, develop several of the primordial follicles into primary and secondary follicles. Development of the follicles can take several months which means that the follicles that have just begun their maturation process won't be ovulated until some months later (Tortora & Derrickson, 2010). During this (menstrual) phase the oestrogen and progesterone levels decrease, stimulating the release of prostaglandins which causes the uterine arterioles to constrict. The constriction of the arterioles deprives the cells of the stratum functionalis of oxygen, killing the cells so that they eventually slough off. The mix of blood, mucus, epithelial cells and tissue fluid from the endometrium flow from the uterus through the cervix and out of the vagina to the exterior as menses (M. H. Johnson & Everitt, 2007).

Next in the cycle is the preovulatory phase. This is the time between the end of the menstrual phase and ovulation. In a 28 day cycle it is recognised as lasting from day 6 to day 13, however in most women this length varies greatly which is the main contributor to the different cycle lengths between women (M. H. Johnson & Everitt, 2007). It is during this phase that some of the secondary follicles start producing oestrogen's (mainly 17β-oestradiol) and inhibin-A. It is also around this time that one of the secondary follicles (the dominant follicle) begins to outgrow the others and produces most of the oestrogens and inhibin-A (M. H. Johnson & Everitt, 2007). The oestrogen's and inhibin down regulate the production of FSH via a negative feedback mechanism operating through the hypothalamus and pituitary gland which causes all of the smaller follicles to stop developing and undergo atresia. It is the dominant follicle that develops into a mature Graafian follicle and it will continue to grow until it reaches between 18-22mm in diameter, forming a bulging cyst on the surface of the ovary (M. H. Johnson & Everitt, 2007). Paralleling the developing follicles in the ovaries, the increase in oestrogen is helping to build the endometrial lining of the uterus by stimulating the cells of the stratum basalis to undergo mitosis and form a new stratum functionalis (Tortora & Derrickson, 2010).

Ovulation in a 28 day cycle happens on day 14. The increase in oestrogen from the follicles stimulates the hypothalamus to release GnRH and the anterior pituitary to secrete LH via a positive feedback mechanism (Tortora & Derrickson, 2010). The surge of LH causes the Graafian follicle to rupture, expelling the secondary oocyte into the pelvic cavity where it is wafted up into the fallopian tube by the fimbriae (M. H. Johnson & Everitt, 2007).

The next phase of the female reproductive cycle is the postovulatory phase. In a 28 day cycle this phase begins on day 15 and lasts until the next menstrual cycle at day 28. Once the Graafian follicle expels its contents the follicle collapses and a clot is formed over the rupture site. Under the

influence of LH, the granulosa and theca interna cells combine and form the corpus luteum (Tortora & Derrickson, 2010). The corpus luteum begins secreting oestrogens, progesterone, inhibin and relaxin. If the oocyte is not fertilized the corpus luteum survives just 2 weeks before degenerating into a corpus albicans (Tortora & Derrickson, 2010). The oestrogens, progesterone, inhibin and relaxin levels decrease and so the levels of LH, FSH and GnRH begin to rise to start a new reproductive cycle. Thus, a woman's fertility is dependent upon these specific endocrine events occurring in the described succinct order (Richards & Pangas, 2010).

1.1.3 Female Hormones and Pregnancy

If the ovulated oocyte is fertilized and the cell starts to divide, the corpus luteum survives past the two weeks with the aid of the hormone human chorionic gonadotrophin (hCG). This hormone is specifically produced by the chorion of the developing embryo from 8 days post-fertilization (Tortora & Derrickson, 2010). The hCG acts similarly to LH as it stimulates the secretory actions of the corpus luteum. The progesterone and oestrogen secreted by the corpus luteum encourages the coiling and growth of endometrial glands along with thickening and vascularisation of the endometrium (Tortora & Derrickson, 2010). The body prepares for the implantation of the embryo, however if this does not occur, the body will undergo menstruation because the progesterone and oestrogen levels decrease as the corpus luteum undergoes luteolysis due to absence of HCG which acts as a sustaining hormone (M. H. Johnson & Everitt, 2007).

If the embryo implants into the endometrium of the uterus, the corpus luteum and the ovaries will continue to secrete progesterone and oestrogens during the first three to four months of pregnancy (M. Johnson, 2013). The hormone levels in this period aren't much greater than at ovulation; however from the third month onwards the placenta starts producing the oestrogens and progesterone at higher levels which maintains the pregnancy (M.

Johnson, 2013; O'Leary, Boyne, Flett, Beilby, & James, 1991). The level of hCG peaks around week 9 of pregnancy and thereafter it declines until birth (Tortora & Derrickson, 2010).

1.2 Infertility in Humans

Infertility in humans is defined as the inability to achieve pregnancy within one year after having regular, unprotected sexual intercourse (Evers, 2002). Ogawa et al (2011) states that infertility affects approximately 10% of all couples in developed countries. Hull et al. (1985) describe reasons for infertility along with the approximate frequency of each, which shows that around 15% of couples will have more than one cause of infertility. Dysfunction in the production or function of sperm accounts for around 30% of infertility cases, which is the largest scoring of the categories. Ovulation failure and unexplained infertility both contribute 25% of infertility cases, tubal infective damage accounts for 20%, endometriosis and coital failure or infrequency account for 5% each, cervical mucus defect is 3% and uterine abnormalities <1% (Mitwally & Casper, 2003). A World Health Organization report stated that in 2003 there were more than 186 million couples in developing countries that were affected by infertility (World Health Organization, 2003). It was highlighted that most of this was attributable to the high prevalence of infectious diseases such as chlamydia and gonorrhoea which are linked with infertility (World Health Organization, 2003). Assisted reproductive technology (ART) can help treat infertile couples unable to conceive naturally. ART has reasonably high pregnancy rates however, it is also associated with the risk of multiple pregnancies and ovarian hyperstimulation syndrome (Van Voorhis, 2006). Advances in medical research have broadened our understanding of why couples are infertile. This has led to the development of a range of diagnostic tools and thus treatment options for the infertile couple. However, Cahill et al (2002), states that in approximately 25% of couples, after a full investigation, the cause for their infertility will not be found.

1.3 Assisted Reproductive Technology (ART)

1.3.1 Introduction

Assisted reproductive technology (ART) is the term used worldwide to describe all treatments or procedures that involve the *in vitro* handling of human oocytes, sperm and human embryos with the intention of achieving a pregnancy (Zegers-Hochshild et al., 2009). The goal of assisted reproductive technology is to assist subfertile women in becoming pregnant if they have been unsuccessful in achieving pregnancy naturally. The procedures outlined in the International Committee for Monitoring Assisted Reproductive Technology (ICMART) glossary that came under the heading of assisted reproductive technologies were; in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo transfer (ET), zygote intrafallopian transfer (ZIFT), gamete intrafallopian transfer (GIFT), gamete and embryo cryopreservation, tubal embryo transfer, gestational surrogacy along with sperm, oocyte and embryo donation (Zegers-Hochshild et al., 2009).

Anderson et al. (2008) described that in Europe, more than 367,000 assisted reproductive technology cycles were performed in more than 1000 clinics over 29 countries in the year 2004. Annually Europe alone generates over 85,000 clinical pregnancies from ART treatments with a pregnancy rate of 28% per transfer in 2004 (Andersen et al., 2008). In the United States of America, over 100,000 assisted reproductive technology cycles are performed each year which results in around 35,000 pregnancies (Society for Assisted Reproductive Technology & American Society for Reproductive Medicine, 2007).

1.3.2 Controlled Ovarian Hyperstimulation

Controlled ovarian hyperstimulation is a treatment where the ovaries are stimulated to produce multiple mature follicles for use with ART treatments such as IVF or ICSI (Mitwally & Casper, 2003). This form of controlled ovarian hyperstimulation is different to ovulation induction treatment because the

aim of this treatment is to produce multiple large follicles, compared with ovulation induction where multiple follicles are not the desired outcome. The two most frequently used medications in ovarian stimulation are; Clomiphene citrate and an injectable form of gonadotrophin such as follicle stimulating hormone (FSH) (Guzick, Carson, & Coutifaris, 1999). Daily doses of FSH ranging from 150-225IU result in a greater yield of follicles and therefore a greater number of oocytes for collection (Fauser & Van Heusden, 1997). The main guideline for ovarian stimulation is to maximise the beneficial outcome of the treatment; high oocyte yield, whilst minimizing the risk of over stimulating the ovaries or creating a multiple pregnancy. For these reasons close monitoring and adjustments are required based on individual outcomes (Fauser, 2008).

Signs of a diminishing ovarian reserve in females include; an elevated basal FSH level, being over the age of 40 years and having and antral follicle count (AFC) of fewer than five follicles sized between 2-10mm prior to treatment and a reduced anti-mullerian hormone (AMH) (Klinkert, Broekmans, Looman, Habbema, & Te Velde, 2005; Klinkert, Broekmans, Looman, & Te Velde, 2004). Klinkert et al. (2004) also explains that it would be unusual for a patient with normal signs of ovarian reserve to have a poor response to gonadotrophin stimulation.

1.3.3 IVF/ICSI

In vitro fertilization (IVF) is a procedure that involves extracting an oocyte from un-ovulated follicle in the ovary and fertilizing it with spermatozoa, outside of the body in the laboratory (Zegers-Hochshild et al., 2009). IVF can be performed on oocytes collected after the natural maturation of follicles in the ovary or after the controlled ovarian hyperstimulation mentioned previously. The difference in outcome is the yield of follicles collected for fertilization. The biological age of a woman will affect her chances of conceiving with IVF treatment. A study conducted in the UK showed that the

pregnancy rate for single embryo transfers in natural IVF cycles was 17% for women aged <30years, 13% for women aged 31-35 years and 11% for women aged 36-40 years (Healy, Trounson, & Andersen, 1994).

Spermatozoa can fail to fertilize an egg, even when they are placed in close proximity to an egg in settings such as IVF. This can occur because of deficiencies in sperm quality or quantity (Gardner, Weissman, Howles, & Shoham, 2009). Intracytoplasmic sperm injection (ICSI) is a procedure which involves injecting a single spermatozoon into the cytoplasm of an oocyte, directly bypassing the zona pellucida and oolemma. This procedure has allowed for greater fertilization rates because men with deficient numbers of progressively motile sperm and men who are classed as azoospermic can have the sperm in the testis and epididymis extracted and injected directly into the egg (Gardner et al., 2009).

1.4 Biomarkers

A biomarker is defined as "a characteristic that is measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (Atkinson et al., 2001) and, as such, biomarkers can include a range of measures from images obtained by X-rays to molecular biomarkers that can be measured in blood. Biomarkers are used in many areas of medicine, for example oncology (Bhatt, Mathur, Farooque, Verma, & Dwarakanath, 2010), but there is an increased awareness of the potential benefits of biomarkers when applied to reproductive medicine (Palmer & Barnhart, 2013).

1.4.1 Tumour markers

Tumour markers are commonly used in the detection and monitoring of cancer. Cancer is an umbrella term that describes many different diseases, all of which involve somatic or germinal cells that have alterations in different

genes, allowing the cells to resist natural cell death processes, so they proliferate uncontrollably and indefinitely (Cho, 2007). The American Cancer Society projected that in 2012 there would be 1,638,910 new cases of cancer and 577,190 deaths as a results of cancer in the United States (Siegel, Naishadham, & Jemal, 2012). Using biomarkers to detect diseases such as cancer plays an important role in medicine, and it is important to define the exact relationship that the biomarkers have with the actual pathology. Whilst tumour markers are often the normal endogenous products found in the plasma and serum of healthy individuals in very small concentrations (Trape, Molina, & Sant, 2004), these molecules are produced by tumour cells in greater amounts which can be an indicator of cancer presence (Malati, 2007). Tumour markers can be expressed in the body's tissues, present as intracellular substances, or they might be released into the circulation and present in the serum (Bates & Longo, 1987; Harnden, 1985; Virji, Mercer, & Herberman, 1988). The majority of tumour markers are proteins, glycoproteins, proteoglycans, carbohydrates, glycolipids, enzymes or hormones (Sarandakou, Protonotariou, & Rizos, 2007; Trape et al., 2011).

When a cell becomes cancerous, the processes of differentiation can result in the excess synthesis and release of molecules, or the induction of other cells to synthesize these molecules (Trape et al., 2011). The products that the cells produce are called tumour markers, and detecting any elevations of tumour markers is used frequently for the diagnosis of tumours, the prognosis for the patient, identifying the stage of disease and in monitoring the growth of the tumour (Malati, 2007). The criteria for the "ideal" tumour marker requires that it should be sensitive and have low false negatives, be highly specific with low false positives, the predictive values should be highly positive or negative and it should have an accuracy of 100% in diagnosing and differentiating between healthy patients and patients with tumours (Malati, 2007). The ideal tumour marker would show a positive correlation between the tumour marker and tumour size, predict any early recurrence of tumour cells, have prognostic ability, and be able to detect early tumours (Bates,

1991). The ideal tumour marker should be either highly specific to one type of tumour or a known universal marker for many tumour types, as well as being straightforward to run as an assay, indicating any changes in the cancers presence. The reality is that no one tumour marker is specific to any one malignancy; they are usually only specific to an organ or group of malignancies (Malati, 2007). The efficacy of a tumour marker to effectively diagnose disease relies on its positive and negative predictive value. The positive predictive value shows the probability that the disease the marker is looking for is actually present if the test shows a positive result. The converse applies for the negative predictive value, where the test result should be negative if there is no disease present. The sensitivity of a marker is if a positive result occurs there will positively be a tumour present and the specificity of the tumour marker shows how many healthy individuals test negative for the marker (Malati, 2007).

Tumour markers can in some cases increase in concentration in patients that are healthy and have no neoplasia, which results in a false positive test result (Trape et al., 2004). The pathophysiological processes that are responsible for this increase include cell necrosis or inflammation which may lead to the release and therefore increase in concentrations of cell products in the serum, or a dysfunction in the organ that is responsible for eliminating the molecule resulting in a reduction in the molecule's catabolism (Trape et al., 2011). Although there has been a lot of research into cancer biomarkers and other biomarkers of disease (Palmer & Barnhart, 2013), most of the research is related to the specificity and sensitivity to the diseases the markers are associated with, the ability to monitor treatment therapies using biomarkers, and the strength of biomarkers to give a prognosis for disease (Bates, 1991). Some of this research has highlighted that these antigens are also present in varying amounts in the tissues, serum and other biological fluids of healthy individuals which could be the result of the variation in reproductive hormones releasing many growth factors and peptides that are used as tumour markers. This has led to the questioning of what the normal

fluctuations of these tumour markers are in healthy women throughout the natural menstrual cycle, stimulated ovarian cycle and early pregnancy (Palmer & Barnhart, 2013). An understanding of the changes in serum concentrations of the markers in healthy patients would allow for a more accurate interpretation of results by clinicians. The tumour markers that have been explored in this research project include the carbohydrate antigens CA125, CA15-3, CA72-4, and the enzyme prostate specific antigen (tPSA).

1.4.1.1 Serum CA125

CA125 is a tumour associated antigen which belongs to the family of hybridoma-defined tumour markers which is used in the monitoring of epithelial ovarian carcinoma. CA125 can be detected in the serum and is found in a large proportion of non-mucinous ovarian tumours that are of epithelial origin (McLemore et al., 2012; Schlageter, Cassinat, Toubert, Borschneck, & Rain, 1998; Toki, Kubota, Lu, & Nakayama, 2000). CA125 is not present on the surface epithelium of normal foetal and adult ovaries; however it has been demonstrated in the epithelium of the endometrium, endocervix and oviduct (Kabawat, Bast, Welch, Knapp, & Colvin, 1983). CA125 has also been found in amniotic fluid and coelomic epithelium which are both tissues of foetal origin. Elevated levels of CA125 have also been associated with benign gynaecological diseases such as endometriosis, cervitis, ovarian cysts, ovarian metaplasia, adenomyosis and myomatous uterus (Toki et al., 2000). Based on a study which used 888 blood donors and 56 healthy participants, the normal value of CA125 in the identification of carcinoma was set at an upper limit of 35U/ml (Klug, Bast, Niloff, Knapp, & Zurawski, 1984). The highest levels of CA125 are said to occur in patients suffering from ovarian carcinoma, however research has shown that asymptomatic healthy women can have elevated levels of CA125 without the presence of disease (Toki et al., 2000). One study found that in healthy women the mean CA125 concentration is positively correlated with age where 12.7% of women aged 40-55 years,

16% of women aged 56-70 and 32.3% of women aged over 70 years had serum concentrations of CA125 over the normal limits (Dehaghani, Ghiam, Hosseini, Mansouri, & Ghaderi, 2007).

Various authors have suggested that the circulatory CA125 concentration increases during the menstruation phase of the natural cycle. In a study by Bon et al. (1999), the CA125 concentration was measured in healthy women during spontaneous ovulatory cycles. This study confirmed earlier reports that there was a phase dependent increase in CA125 concentration during menstruation in women and that there is a negative correlation between oestradiol and CA125 levels in spontaneous ovulatory cycles (Haga, Sakamoto, Hiroshi, Yoshimura, & Akagi, 1986; Jager, Meier, Wildt, Sauerbrei, & Lang, 1988; Pittaway & Fayez, 1987; Touitou, Darbois, Bogdan, Auzeby, & Keusseoglou, 1989). CA125 was again examined during the menstrual cycle in apparently healthy women in a more recent study where the concentration of CA125 over the menstrual cycle was compared between two different automated analysers, with the conclusion that CA125 is highest during menses however the changes are not clinically significant in healthy women (McLemore, 2012). The endometrium has been shown to be responsible for the cyclical changes of CA125 in the menstrual cycle however the exact mechanisms that allow the release of CA125 into the circulation has not yet been explained (Kafali, Artunc, & Erdem, 2007; Ziemet, Muller-Holzner, Marth, Daxenbichler, & Dapunt, 1993).

CA125 has been measured throughout ovarian stimulation cycles for ART treatment showing levels are significantly higher in the luteal phase rather than during the stimulation phase or at oocyte collection (Zweers, De Boever, Serreyn, & Vandekerckhove, 1990). It has been postulated that CA125 may well be a good predictor of pregnancy success for ART treatments because concentrations on the day of oocyte retrieval

>10IU/ml had an accuracy of 86.6% for pregnancy, with CA125 concentration being a measure of endometrial receptivity and therefore predictor of pregnancy success (Tavmergen, Sendag, Goker, & Levi, 2001). Significant differences in CA125 levels have also been shown between viable pregnancies and both anembryonic and ectopic pregnancies, however due to overlaps in the ranges of values for each group the findings were not of clinical significance (Jacobs et al., 1990).

Many authors have published reports showing that CA125 plasma concentrations are increased during pregnancy (Aslam, Ong, Woelfer, Nicolaides, & Jurkovic, 2000; Halila, Stenman, & Seppala, 1986; Kobayashi, Takashima, ASagawa, Mori, & Fujii, 1993; Niloff, Klug, Schaetzl, Reynolds, & Bast, 1984). Early reports from Niloff et al. (1984) and Touitou et al. (1989) showed that 16% and 12.5% respectively of pregnant women in the first trimester had elevated levels of CA125 and other publications then advanced this and concluded that 20-24% of pregnant women, regardless of gestation, had elevated levels of CA125 (Halila et al., 1986; Pittaway & Fayez, 1987). The CA125 antigen was then shown to be expressed during embryonic development where levels of CA125 are significantly higher in the first and third trimesters of pregnancy (Bon et al., 2001; Hardardottir et al., 1990). Other studies have shown maternal CA125 levels to reach an average of 85U/mL in the first trimester and then drop to the average non-pregnant levels during the second and third trimester (Haga et al., 1986; Seki, Kikuchi, & Kato, 1986). This was supported by a later study which showed peak CA125 levels occurring between weeks 6-7 of gestation in naturally conceived pregnancies and further findings where pregnancies achieved via assisted conception had levels of at least twice those found in non-pregnant women (Jacobs et al., 1990). Research by Ocer, Bese, Saridogan, Aydinli, and Atasii (1992) suggested that maternal serum CA125 >65U/ml in the first trimester presented a risk of spontaneous abortion of 83.3% and that in those pregnancies with bleeding lasting 3 or more days, with elevated CA125

levels, this risk rose to 100%. A later study found that all of the successful pregnancies in the study group had CA125 levels <93U/ml between weeks 7-12 gestation and those that aborted had levels >125U/ml (Azogui, Yaronovski, Zohar, & Ben-Shlomo, 1996). Furthermore, one study found that tubal pregnancies with no uterine bleeding had conversely much lower CA125 concentrations than intrauterine pregnancies whereby levels were within the normal non-pregnant range of <35U/ml (Kobayashi et al., 1993).

Some scientists hypothesize that the first trimester increase in CA125 is the result of decidual CA125 having access to maternal circulation via tubal reflux because after the fusion of the decidua capsularis and decidua parietalis which obliterate the endometrium around 10-12 weeks, tubal closure follows and then the maternal CA125 levels decrease (Kobayashi et al., 1993). Other reports suggested that it is the invasion of the trophoblast in the decidua during placentation that causes the increase in CA125 in the first trimester and an increase in the third trimester is the result of the subsequent deplacentation during which decidual CA125 can gain access to the maternal circulation (Kobayashi, Sagawa, & Nakamura, 1989). When indicated as a predictor of spontaneous abortion, authors suggest that the elevated CA125 level is an indicator of the decidual destruction which is associated with pregnancy outcome (Ocer et al., 1992).

Although it may be argued that CA125 has been investigated extensively in women, it was important that this project included CA125 so that the results from this study can be compared to previous research to confirm that the method being used to measure the compounds is robust and reproducible for all of the other analytes. This research aims to add to the current literature using the latest in automated immunoassay technology and serial sampling of individual women.

1.4.1.2 Prostate Specific Antigen (PSA)

Prostate specific antigen (PSA) is a serine protease which is produced by both normal and neoplastic epithelial cells of the prostate. PSA is regulated by androgens and has been extensively studied as a biomarker for prostate cancer (Bhatt et al., 2010; Chu, 1997; Luke & Coffery, 1994). It was first discovered in 1971, and then isolated in 1979 from an extract of human prostate, however, it wasn't until 1980 when Papsidero and his colleagues began measuring PSA quantitatively that its properties for clinical use as a biomarker for identifying prostate cancer were identified (Bhatt et al., 2010; Papsidero, Wang, Valenzuela, Murphy, & Chu, 1980; M. Wang, Valenzuela, Murphy, & Chu, 1979). The epithelium of the prostate synthesizes PSA and plays the role of effectively preventing the release of the protease into the circulatory system (Malati, 2007). PSA can leave the prostate and is often present, normally in small amounts, in the serum of healthy individuals. The protease can form complexes with many protease inhibitors in the serum and seminal fluid, giving rise to multiple molecular forms which circulate through the blood. The major immunoreactive form of PSA is the 100KD complex PSA-ACT, which constitutes up to 90% of total PSA in the serum. There are other complexes, such as PSA-AT and PSA-PCI, which occur at significantly lower concentrations in the serum i.e. less than 1% of PSA. Finally, PSA can form a complex with alpha-2 macroglobulin (α_2 M) which encapsulates the PSA molecule, masking the epitopes on the surface of the antigen further preventing its immunodetection in enzyme immunoassays (Malati, 2007).

PSA levels in the serum are often elevated in men with prostate cancer which has made it the most clinically significant tumour marker for the disease (Papsidero et al., 1980). This led to the American Urological Association (AUA) and the Food and Drug Administration (FDA) in the United States, originally promoting recommendations that all men over the age of 50 who have no family history of prostate cancer, and those

with a family history who are over the age of 40, have annual digital rectal examinations for prostate abnormalities as well as serum PSA level checks (Malati, 2007). However, prostate cancer can be present in the body even when the serum level for PSA is not elevated (Thompson et al., 2004). Due to this, there have been many calls to stop using PSA as a routine screening test in men and now it is no longer used in the USA (Friedrich, 2011).

Prostate-specific antigen has certainly been incorrectly named since advances in the sensitivity of technology have shown PSA to be expressed in females (Aksoy, Akcay, Umudum, Yildirim, & Memisogullari, 2002; Giai et al., 1995). PSA was found to be expressed by normal, hyperplastic and neoplastic female breast tissue where it is present in 30%-40% of breast tumours in females (H. Yu, Diamandis, & Sutherland, 1994; H. Yu et al., 1995). The PSA concentration does not correlate with the reproductive hormones LH, FSH and oestradiol, however, it is said to follow the progesterone concentration peak (N. Zarghami, L. Grass, E. Sauter, & E. Diamandis, 1997). Steroid hormone receptor-positive breast carcinoma cell line T-47D was used in a tissue culture system which reproduces the process of PSA production by breast cells to show that only upon stimulation by steroid hormones does the cell line produce PSA in a dose-dependent manner (N. Zarghami et al., 1997). According to one study investigating women over the age of 50 years, PSA levels are not significantly different between healthy women and those with breast cancer (Giai et al., 1995).

Further studies have shown that PSA is produced in a cyclical manner during the natural menstrual cycle where the peak concentration is during the mid to late follicular phase and lowest during the mid to late luteal phase of the cycle (N. Zarghami et al., 1997). These findings have been confirmed by a later study which showed PSA concentrations in the saliva of women were increased in the follicular phase and around mid-cycle

compared to the luteal and early follicular phases (Aksoy, Akcay, et al., 2002). Prostate specific antigen concentrations are very low in female serum and it is not known why some women have measurable levels of PSA and others do not. Zarghami et al. (1997) suggest that it is because of an inability of PSA to diffuse out of the breast tissue into the blood circulation.

Prostate specific antigen was identified in amniotic fluid during pregnancy where the concentration increased from 11 weeks until 21 weeks of gestation before it decreased until birth (H. Yu et al., 1995). This study also found that PSA is in greater concentrations in the serum of pregnant women compared to non-pregnant women (H. Yu et al., 1995). The same group also detected PSA in the breast milk of lactating women, although concentrations were variable between women and its biological role in milk is still unknown (H. Yu & Diamandis, 1995). As such, this led the authors to suggest that as well as serving as a growth factor regulator in cancer, PSA may play a role in normal foetal development throughout pregnancy. There are conflicting opinions about PSA and its role in pre-natal screening for Down Syndrome. One study conducted in 1998 concluded that the concentration of PSA in Down Syndrome pregnancies was significantly higher than in normal pregnancies (Lambert-Messerlian, Canick, Melegos, & Diamandis, 1998). However, another study from the same year could not confirm that there was any variation in maternal PSA concentrations with gestational age nor could they confirm that maternal PSA levels were increased in pregnancies affected by Down Syndrome (Spencer & Carpenter, 1998). Another study found PSA in the endometrium of the uterus and that it may play a role in uterine regulation (Mannello et al., 1996). Although the physiological role and biological significance of PSA is still not completely understood it has been hypothesised as a growth factor regulator which is produced by cells exhibiting steroid hormone receptors (E. Diamandis & H. Yu, 1995).

1.4.1.3 Serum CA15-3

CA15-3 is a mucin-like glycoprotein encoded by the gene MUC1 which was first identified by two monoclonal antibodies 115D8 and DF3 (Kamal et al., 2011; Schlageter et al., 1998). Mucins provide a protective layer on the surface of epithelial cells by providing lubrication, preventing dehydration and protecting against proteolysis due to its high degree of glycosylation (Al-Azemi, Refaat, Aplin, & Ledger, 2009). MUC1 is heterogeneously expressed on the surface of epithelial cells, including those in the breast and upper reproductive tract and is thought to prevent embryo implantation (Al-Azemi et al., 2009). Meseguer et al. (2001), suggesting that the human blastocyst can produce certain factors that remove MUC1 from a site to facilitate implantation in the endometrium. Expression of MUC1 has been shown to be progesterone dependent and is up regulated in endometrial epithelial cells in the luteal phase of the menstrual cycle (Aplin, Hey, & Graham, 1998; Hey, Graham, Seif, & Aplin, 1994).

Serum levels of CA15-3 are considered normal if they are under the upper limit of 25 U/ml (Bhatt et al., 2010). Elevated levels of CA15-3 in women with breast cancer were identified as being involved in the cancer's metastasis (Duffy, Shering, Sherry, McDermott, & O'Higgins, 2000). Of the many different tumour markers that have been examined in breast cancer, CA15-3 is used most extensively as its expression in most breast cancers is greatly increased (Clinton et al., 2003; O'Brien, Gough, Skehill, Grimes, & Given, 1994). In women with breast cancer, where the disease status is operable, around 11% of cases have elevated levels of CA15-3, and in those with metastatic breast cancer, 60% have elevated levels (Park, Oh, Kim, & Kim, 2008). Post-operative prognosis for patients with high CA15-3 concentrations is much worse than for those with low concentrations, although it has been found that patients with high serum CA15-3 levels

have a good response to more aggressive treatments (Park et al., 2008; Shering, Sherry, McDermott, Higgins, & Duffy, 1998).

However, the sensitivity of CA15-3 for marking breast cancer has been reported as being low due to high levels in benign breast tissue diseases, ovarian, lung, pancreatic, stomach, uterine and colorectal cancers and hepatic dysfunction (Duffy et al., 2000; Nalini, Delphine, Silvia, Makhija, & Uthappa, 2005; Sakaguchi, 2004). Elevated concentrations of CA15-3 have been found in patient serum where there is an absence of neoplasia (Trape et al., 2011). In a study conducted on post-menopausal women who were all asymptomatic and healthy, 5.9% had serum concentrations of CA15-3 higher than the cut-off 25 U/ml (Dehaghani et al., 2007). CA15-3 has been found in the serum of patients with cobalamin deficiency and macrocytosis in concentrations that are ten times the upper reference limit for healthy people (Symeonidis et al., 2004). Amoura (2005) found that 22% of patients suffering different inflammatory myopathies had serum concentrations of CA15-3 over the upper reference limit, suggesting that there may be other factors influencing serum CA15-3 concentrations.

Research into other tumour markers such as CA125, as mentioned previously, has shown that the fluctuations in some tumour marker concentrations are phase dependent in the menstrual cycle of healthy women. Bon et al. (1999) described the differences in CA15-3 concentrations in different phases of the spontaneous ovulatory cycle as being not of statistical significance. However, another report found conflicting results whereby they showed significant phase dependent differences in CA15-3 concentrations throughout the menstrual cycle (Erbagci, Yilmaz, & Kutlar, 1999). Dehaghani et al. (2007) showed that a group of women with a history of using the oral contraceptive pill for greater than one year had a significantly larger number of subjects within the normal range for CA15-3, with only 1 out of the 76 women above the

normal upper limit compared to 11 out of 127 women in the group of women who did not use oral contraceptives.

Different studies have found that CA15-3 concentrations in pregnancy vary between slightly elevated, moderately elevated and significantly elevated (Cheli et al., 1999; Panidis, Vlassis, Matalliotakis, Skiadopoulos, & Kalogeropoulos, 1988). When comparing levels from each trimester, although levels were shown to increase with gestation they do not cross clinical thresholds (Bon et al., 2001; Correale et al., 1993; Schlageter et al., 1998; Touitou et al., 1989). The rise in maternal serum concentrations of CA15-3 during the latter stages of pregnancy has been suggested to be due to the proliferation of the breast epithelium during this phase or that it might be an artefact of the immunoassay as it was developed against milk-fat globule membranes (Bon et al., 2001; Hilkens et al., 1984). Other studies have found that concentrations of CA15-3 do not change significantly with pregnancy and that it can still be used as a biomarker for breast cancer if gestational related reference ranges are put in place (Botsis et al., 1999; Touitou et al., 1989).

1.4.1.4 Serum CA72-4

CA72-4 is a tumour associated antigen that is defined through its reactivity with the two murine monoclonal antibodies CC-49 and B72-3 (Hareyama, Sakuragi, Makinoda, & Fujimoto, 1996). CA72-4 was once described as a useful tumour marker for all epithelial derived tumours and gastric carcinomas (Ohuchi, Takahashi, & Matoba, 1989). This research showed that the sensitivity of CA72-4 for gastric carcinoma was 38%, which is greater than the tumour markers CA19-9 which is 33%, CEA at 31% and CA125 at 21% (Ohuchi et al., 1989; Villena, Lopez Encuentra, & Ecvhave Sustaet, 1996). CA72-4 was postulated as a complimentary biomarker to CA125 in the screening of ovarian cancer, where one study found that when combining the biomarkers the sensitivity for detecting early stage

disease increased from 45% to 70% (Skates et al., 2004). With advances in technology in recent years, immunoassays are now much more sensitive so any evidence from this time is somewhat limited. These antibodies recognise the tumour associated glycoprotein (TAG-72) in the serum of patients with various adenocarcinomas as well as in foetal epithelium (Ohuchi et al., 1989; Villena et al., 1996).

1.4.2 Reproductive Markers

1.4.2.1 Reproductive Hormones

Reproductive hormones such as oestradiol, progesterone, luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) are measured and evaluated as indicators of normal biological processes and events that occur throughout the natural menstrual cycle and in pregnancy (O'Leary et al., 1991; Sherman & Korenman, 1975). These hormones are also useful tools in assisted reproductive medicine as they are measured throughout exaggerated biological processes like those during hyperstimulated ovarian cycles for IVF/ICSI to monitor follicle growth and endometrial development (Wikland & Hillensjo, 2004).

During the menstrual cycle, oestradiol is produced in increasing amounts by the granulosa cells of the developing follicle and when the follicle is mature it will trigger luteinizing hormone to surge to induce ovulation. The follicular phase is variable between women as shown by Cole, Ladner, and Byrn (2009) where the timing of the LH peak was between 10-20 days. The phase after ovulation is called the luteal phase which has been shown to be consistent in length between women (McIntosh, Matthews, Crocker, Broom, & Cox, 1980), where oestradiol and progesterone prepare the endometrium for implantation and serum concentrations of progesterone will start to increase from ovulation and are maintained by the corpus luteum if pregnancy is achieved. Human chorionic gonadotrophin (hCG) is a hormone produced by the chorion of the

developing embryo from around 8 days post fertilisation where maternal serum concentrations double every 2-3 days. These reproductive markers were measured in all women throughout this study to ensure accurate timing of biological events throughout all three groups of cycles.

1.4.2.2 Pregnancy-associated plasma protein-A (PAPP-A)

Pregnancy-associated plasma protein-A (PAPP-A) was identified as one of four proteins found in the serum of pregnant women in 1974 and was later documented as being produced by the syncytiotrophoblastic cells of the placenta where it is released in increasing amounts into maternal circulation with increasing gestation (Folkersen, Grudzinskas, Hindersson, Teisner, & Westergaard, 1981; Lin, Galbert, Kiefer, Spellacy, & Gall, 1974). The concentration of maternally circulating PAPP-A is reduced in the first trimester of pregnancies that are affected by trisomy 18, trisomy 21, blighted ova and is also associated with with IUGR (intra-uterine growth retardation) causing low birth weight, premature birth and pre-eclampsia (Brizot et al., 1996; D'Antonio et al., 2013; Dane, Dane, Batmaz, Ates, & Dansuk, 2013; Poon, Maiz, Valencia, Plasencia, & Nicolaides, 2009; Smith et al., 2002; NJ Wald, Watt, & Hackshaw, 1999; Yovich, Willcox, Grudzinskas, Chapman, & Bolton, 1986). Early reports suggested that reduced PAPP-A concentrations in maternal serum are due to altered post-translational events which affect protein transport across the placenta or its secretion, rendering PAPP-A screening tests useful as a marker of placental function (Brizot et al., 1996). First trimester combined screening tests measure PAPP-A and free β-hCG at 9-12 weeks as well as nuchal translucency by ultrasound at 11-13 weeks. This is combined with the gestational age, maternal age and weight to give an estimate of the risk of the foetus having trisomy 18 or 21 (bAmor et al., 2009).

Children born as a result of ART in 2008 accounted for 3.3% of all live births in Australia (Y. A. Wang, Chambers, & Sullivan, 2010). There have

been comparisons made between serum biomarker concentrations for second trimester screening in naturally conceived pregnancies and IVF conceived pregnancies. In some studies PAPP-A levels were shown to be reduced in pregnancies conceived via ART (Anckaert, Schiettecatte, Sleurs, Devroey, & Smitz, 2008; bAmor et al., 2009; Bersinger et al., 2004; Hui et al., 2005; Liao, Heath, Kametas, Spencer, & Nicolaides, 2001; Matilainen et al., 2011; Maymon & Shulman, 2002a, 2004; Orlandi et al., 2002; Tul & Novak-Antolic, 2006), however in others there was no significant difference (Bellver et al., 2005; Ghisoni et al., 2003; Lambert-Messerlian et al., 2006; Niemimaa et al., 2001; Wojdemann et al., 2001). Tul and Novak-Antolic (2006) found a correlation between the number of oocytes retrieved with PAPP-A concentrations, whereby higher oocyte numbers results in a lower PAPP-A, leading to the suggestion that the difference may be due to the multiple corpora lutea present. bAmor et al. (2009) found that PAPP-A levels were reduced in both fresh and frozen-thawed embryo transfers when compared to naturally conceived pregnancies, however, fresh transfers did have significantly lower PAPP-A levels than frozen-thawed transfers. They also showed that there was no significant difference in PAPP-A levels between IVF, ICSI and GIFT subtypes of ART pregnancies.

Increases in false-positive rate (FPR) in prenatal screening tests for Down syndrome have been reported for pregnancies conceived through assisted reproduction (bAmor et al., 2009; Barkai et al., 1996; Frishman et al., 1997; Lambert-Messerlian et al., 2006; Orlandi et al., 2002; Ribbert et al., 1996; N. Wald, White, Morris, Huttly, & Canick, 1999). Having a lower PAPP-A level at first trimester screening and therefore receiving a false-positive result (FPR) might subsequently lead to unnecessary amniocentesis procedures exposing these pregnancies to undue risk. However, after adjustment for maternal age many other studies conclude that the FPR is not increased in ART pregnancies (Bellver et al., 2005; Liao et al., 2001; Matilainen et al., 2011; Maymon & Shulman, 2002b;

Wojdemann et al., 2001). Contradictory results such as these suggest that there may be many confounding factors on these prenatal screening tests such as exogenous hormone treatment regimens which have been suggested as the reason for lower PAPP-A levels in these pregnancies (bAmor et al., 2009; Bersinger et al., 2004; Hui et al., 2005; Tul & Novak-Antolic, 2006).

1.5 Aims of the Study

The study combined the measurement of several biomarkers using the latest generation of immunoassays, with the serial monitoring of individual women in three different reproductive situations namely natural and stimulated ovarian cycles as well as early pregnancy. A better understanding of the behaviour of such biomarkers in women may help us monitor reproductive events more clearly, as well as assist in the clinical interpretation of these markers should they be elevated above clinical threshold for disease. The aims of the present study were therefore:

1. To use the current generation of immunoassays to measure selected serum biomarkers (CA125, Prostate specific antigen [PSA], CA15-3, CA72-4 and PAPP-A) in women of reproductive age, and to describe the assay performance characteristics at these levels.

2. Measure these compounds in disease-free women during the natural menstrual cycle, stimulated ovarian cycle and early pregnancy.

3. Identify changes in these biomarkers that may give additional information about ovarian function, implantation and placentation.

4. Determine if these markers exceed the clinical thresholds associated with disease, and identify any possible need to revise reference ranges in the different reproductive phases.

2 Materials and Methods

2.1 Participants

2.1.1 Ethics Approval

Ethics approval was granted by the Joondalup Health Campus Research Ethics Committee [JHCREC, Project 1216] and the Edith Cowan University Human Research Ethics Committee [ECUHREC, Project 7281], see appendices 1 and 2.

2.1.2 Selection of Participants

Participants recruited for this research project were women attending Fertility North for fertility treatment. None of the women had evidence of cancer or endometriosis. On each given day that the clinic was open, women on current treatment plans came to the clinic in the morning between 7:00am and 8:30am to have their blood taken as part of their management. To identify which patients were due for blood collection on each day, we used an electronic diary (Genie; Genie Solution Pty Ltd, Indooroopilly, Queensland) which lists all women attending the clinic on that day. The program permits all of the patient's medical information and history to be retrieved at any given time. On each day during the recruitment period, the list of patients was screened to determine which women were attending for their first blood sample of their cycle that day and what type of treatment they were starting. If they were day 2 of a natural cycle or a fresh IVF or ICSI cycle, these women were approached that morning and recruited using the information letter and consent forms approved by the two governing ethics committees. If there were any women starting a pregnancy monitoring cycle of blood sampling then these women were also approached in the same manner.

2.1.3 Study Cycles

2.1.3.1 Natural Menstrual Cycle

Participants in this study group included women who were attending Fertility North for a tracking cycle which takes place in the initial investigation prior to commencing fertility treatment. These women are on no medications that affect the natural reproductive cycle e.g. the oral contraceptive pill or hormone replacement therapy. Blood was taken on day 2-3 of the natural cycle, where the first day of menses is called day 1. Oestradiol, progesterone, LH and FSH are measured from this first sample. The next blood sample was taken 5-7 days later when the oestradiol, progesterone and luteinizing hormone concentrations were measured. These three hormones were measured until ovulation. Ovulation was defined as being when there was a serum LH surge and a rise in progesterone concentration from a baseline level. This was then confirmed by ovarian ultrasound when the leading follicle could be seen. Seven days after ovulation the woman's oestradiol and progesterone concentration were measured as an indication of luteal function, and then at fourteen days after ovulation a pregnancy test was performed by measuring oestradiol and progesterone as well as human chorionic gonadotrophin (hCG).

2.1.3.2 Stimulated Ovarian Cycle

Participants in this study group included women starting an IVF or ICSI cycle at Fertility North. These women had their blood taken on day 2 of their cycle where the oestradiol, progesterone, luteinizing hormone and follicle stimulating hormone concentrations were measured. The women embarked on a stimulatory regimen using a protocol including either a GnRH agonist in a short down regulation (flare) cycle (Frydman et al, 1988; Garcia et al, 1990; Acharya et al, 1992), long down regulation cycle (Porter et al, 1984; Shaw et al, 1985) or a GnRH antagonist in an antagonist cycle (Diedrich et al, 1994). After 6 days of receiving FSH

injections the women returned for another blood test to measure their oestradiol and follicle stimulating hormone levels to gauge their response to stimulation. The oestradiol levels were measured approximately every 2 days during stimulation to further monitor the ovarian response and identify whether an increased dose of FSH was required due to a low response, or if the woman was at risk of ovarian hyperstimulation syndrome (OHSS) because of an inordinately high response. Seven days after the patient was triggered to ovulate, another blood test was performed to measure the oestradiol and progesterone levels. At fourteen days after ovulation, the patient had a pregnancy test indicating oestradiol, progesterone and human chorionic gonadotrophin (hCG) concentrations. If the woman was pregnant she had a blood test every two or three days to measure her progesterone and hCG levels until she had a visible foetal heart beat identified by ultrasound around seven weeks gestation.

2.1.3.3 Early Pregnancy

Participants in this group included women who had undergone fertility treatment at Fertility North and had become pregnant as a result of this treatment. At fourteen days after ovulation women had a pregnancy test which measured their oestradiol, progesterone and human chorionic gonadotrophin (hCG) levels. A hCG concentration of >25 IU/mL is regarded as a positive pregnancy test and the hCG concentration should approximately double every two to three days. Therefore, blood samples were taken every two to three days during an early pregnancy monitoring cycle, until the woman had an ultrasound scan at around 6-7 weeks of gestation. Women in this study group take one 200mg Progesterone BD pessary (Orion, Balcatta WA) which helps to support the pregnancy.

2.2 Serum Collection & Storage

All blood samples were collected by Fertility North phlebotomists on site. Blood was collected using syringes and transferred into 5ml Vacutainer SST™ tubes

(Becton Dickinson, UK), labelled and bagged, before delivery to the laboratory with a request form. Receipt of the sample by the laboratory was then confirmed by adding the patient and requested tests to the "blood" screen in Genie for that day, and selecting the patient as per the daily blood samples. The sample was then allocated a laboratory number, with copies of the bar code being placed on the blood tube and on the pathology referral form. The blood was allowed to clot at room temperature and then centrifuged at 4000rpm for 4 minutes. The sample was then analysed for the requested reproductive hormones according to the patient's treatment and then stored at -80˚C for retrospective analysis of tumour markers at a later date. Once each patient had finished having blood samples collected, all of the blood samples from that completed treatment cycle were thawed at room temperature and then analysed for biomarker concentrations in one batch analysis on the same day that they were thawed. Once the sample was analysed, any excess serum was replaced into the -80˚C freezer for storage. The research detailed in this thesis was carried out between February 2012 and December 2013.

2.3 Determination of Serum Hormone Concentration

The Siemens Centaur CP Automated Analyser (Siemens, Bayswater, Victoria 3053, Australia) was used to measure oestradiol, luteinising hormone (LH), progesterone and human chorionic gonadotrophin (hCG).

2.3.1 Basic Assay Procedure

The automated analyser was run daily according to the manufacturer's instructions and the standard operating procedure of Fertility North [Appendix 3]. All reagents were stored and used as per the manufacturer's instructions as shown in Table 1 and Appendix 5 [Chapter 3]. To reconstitute calibrators and control material, the pipette pump with a glass volumetric pipette was used to deliver deionised water (Ibis Technology, Mt Hawthorn). A record of the sample, the date of reconstitution and the date of expiry were written on the side of the reagent bottles.

2.3.2 Internal and External Quality Control

Internal quality control for each assay was performed using the BioRad Immunoassay Plus 1, 2, 3 (Bio-Rad Laboratories, Irvine, CA) in each run. Ligands were reconstituted to 5 mls and 7 x 600µl lots aliquotted for the next 7 days. All results were stored by the Advia Centaur CP automatically and transferred to a computer on a monthly basis into QC reporter 2. A repeat patient sample from the previous day was also run and results recorded daily. All of the reproductive hormone assays were also assessed using two external quality assurance schemes named RCPA Endocrine scheme (RCPA, New South Wales) and EQASRM (EQASRM, Western Australia). These schemes were used as a means to compare the results generated from the method being used to the results of other laboratories in the country that are also using the same method.

2.4 Determination of Serum Biomarker Concentration

The Roche Cobas e411 Automated Analyser (Roche Diagnostics, Germany) was used to measure the biomarkers; PAPP-A, CA125, CA15-3, CA72-4 and total PSA (tPSA). This was done as a batch analysis to minimise variability attributable to the reagent stability.

2.4.1 Basic Assay Procedure

The automated analyser was run daily according to the manufacturer's instructions, and the standard operating procedure of Fertility North [Appendix 4]. All reagents were stored and used as per the manufacturer's instructions as shown in Table 2 and Appendix 6 [Chapter 3]. The lyophilized calibrators and control material were reconstituted with deionised water (Ibis Technology, Mt Hawthorn) as shown in Appendix 7. A record of the sample the date of reconstitution and the date of expiry was written on the side of the reagent bottles. The reagent kits were stored in the biochemistry

refrigerator at 2-8˚C until use. After opening, they were stable for 12 weeks at 2-8˚C or 3-8 weeks on-board the analyser. When in use reagent packs were removed from the fridge and placed on the reagent rotor with the lids unclipped after coming to room temperature. The temperature of the rotor was controlled at 20˚C. Reagents were left to warm to this temperature before use. The rotor lid was replaced and locked once all reagent packs were loaded. After use, the lids on the reagent bottles were snapped closed and the reagent is placed back into the refrigerator for storage until its next use.

2.4.2 Calibrations

The reagent kits were calibrated within 24hours after the initial opening. Thereafter, renewed calibrations were required every 7 days per reagent kit until all tests were used. The Roche CalSet specific to each of the assays were used to calibrate the reagent kits. The calibrators come in liquid and lyophilized form and were all stored at 2-8˚C on receipt and these were stable until the stated expiry date. The lyophilized calibrators were reconstituted as per the manufacturer's instructions and once reconstituted the calibrators were stored at 2-8˚C where they were stable for 6 weeks. Aliquots of reconstituted calibrator were frozen at -20˚C where they were stable for 12 weeks until thawed for use. The calibrators were left to warm to room temperature before use and the reconstituted aliquots were used only once.

2.4.3 Internal and External Quality Control

Internal quality control was assessed using the Elecsys PreciControl Tumor Marker (Roche Diagnostics, Germany) with two levels, the BioRad Lyphochek Tumor Marker Plus Control (Bio-Rad Laboratories, Irvine, CA) and the Elecsys PreciControl Maternal Care (Roche Diagnostics, Germany) with three levels, which were run each day that an assay was performed and after every calibration. The CA125 assay was enrolled in an external quality assurance scheme (RCPA, Endocrine scheme) under Fertility North however there were no external quality assurance schemes available for the other biomarker

assays. Reconstitution details and stability of the quality control material are given in Appendix 7. The assay's performance characteristics are described in Table 1 [Chapter 3].

2.5 Statistical Analysis

Measurement of quality control samples was expressed as the mean and standard deviation, to allow the calculation of coefficients of variation. However, the results of clinical samples are expressed as means and standard error of the mean (sem) as an estimate of how close to the population mean the sample mean is likely to be.

For the ovarian cycle analyses, a linear mixed effects model was used to test the mean differences in marker concentration between women in stimulated and natural cycles across the cycle phases. For each model, the response variable (CA125, tPSA, CA153 and PAPP-A) was log transformed before analysis as the data appeared non-normal and thus the error distribution might affect the calculation of standard errors and the p-values required for null hypothesis testing. Group (IVF and 'natural') and Phase were included as fixed effect factors and 'Subject' and 'Time' were modelled as random effects (as in some subjects there were multiple time points measured within phases). The interaction between Group and Phase was also modelled. The analysis was done using the R version 3.0.0 computing software (R Core Team, 2013). Models were built using the 'nlme' (Pinheiro, Bates, DebRoy, Sarkar, & R Development Core Team, 2012). Small sample size and gaps in IVF sampling due to the nature of IVF need to be taken into account in cautiously interpreting this data.

For the early pregnancy analyses the response variable (tPSA, CA125, CA15-3, PAPP-A and hCG) was log transformed before analysis as the data appeared non-normal and thus the error distribution might affect the calculation of standard errors and the p-values required for null hypothesis testing. Gestation

was included as fixed effect factors and 'Subject' and 'Time' were modelled as random effects (as in some subjects there were multiple time points measured within the phase). The interaction between Group and Gestation was also modelled. The analysis was done using the R version 3.0.0 computing software (R Core Team, 2013). Models were built using the 'nlme' (Pinheiro et al., 2012) mixed models package and graphics were produced using the 'ggplot2' package (Wickham, 2009).

2.6 Day and Phase of Cycle

The menstrual cycle, as described earlier, is considerably variable in length from day one of the cycle to ovulation. In order to normalise the follicular phase for statistical analysis a formula was applied to calculate an adjusted day (Hadlow et al., 2013).

$$\text{Adjusted day} = \text{Actual day} \times (14/\text{Actual day of ovulation})$$

The menstrual cycle was further divided into seven phases using the day of the cycle (Hehenkamp et al., 2006) relative to ovulation as;

(1) Early Follicular (EF) - 10 days or more before ovulation

(2) Mid Follicular (MF) - 6 to 9 days before ovulation

(3) Late Follicular (LF) - 2 to 5 days before ovulation

(4) Periovulatory (PO) - 1 day before ovulation

(5) Ovulation (O) - the day of ovulation

(6) Mid-Luteal (ML) – 5 to 8 days after ovulation

(7) Late Luteal (LL) – 9 to 14 days after ovulation

3.0 Results

3.1 Performance characteristics of Roche automated immunoassays used to measure CA125, tPSA, CA15-3, CA72-4 and PAPP-A.

Results obtained with the Biorad Lyphochek Tumor Marker Plus trilevel quality control, Roche Elecsys PreciControl Tumor Marker QC bi-level quality control material and Roche Elecsys PreciControl Maternal Care QC tri-level quality control material are shown in Table's 1 and 2 . The reproducibility of results from each assay was described by calculating the coefficient of variation (CV). Good reproducibility was shown in the measurement of CA125 (CV <6.2%) and CA15-3 (CV <7.5%). PAPP-A and tPSA were more variable (CV <11.4% and <13.4% respectively) and CA72-4 was more so (CV <20.4%). The BioRad QC material appeared to give greater reproducibility compared to the Roche QC such that all of the CV's were lower for the BioRad QC for 6 of the 8 QCs.

The variability associated with the measurement of serum is shown in Table 3 where pooled serum from women recruited in the study was analysed daily. Similar patterns were seen with the CA125 (CV <4.7%) and CA15-3 (CV <6.5%). However, the low concentrations of CA72-4, PAPP-A and tPSA resulted in higher variability with CVs of <24.1%, <27.0% and <38.5% respectively. Estimates of within-run variability seen with clinical samples are shown in Table 4, with PAPP-A, CA125, CA15-3 and CA72-4 all being <5.0%. tPSA was more variable with a CV of 12.94%.

Table 1. Between-run variability assessed using commercially available quality control material (CA125, CA15-3, CA72-4 and tPSA).

Marker	Level	Biorad Lyphochek Tumor Marker Plus		Elecsys PreciControl Tumor Marker	
		μ ± sd	CV (%)	μ ± sd	CV (%)
CA125	Low	28.45 ± 1.46	5.12	28.52 ± 1.75	6.13
U/ml	Medium	70.67 ± 4.02	5.69	-	-
(n=43)	High	194.66 ± 6.74	3.46	87.30 ± 4.40	5.04
CA15-3	Low	22.25 ± 1.41	6.32	18.52 ± 1.39	7.49
U/ml	Medium	57.12 ± 3.97	6.95	-	-
(n=42)	High	111.48 ± 7.55	6.77	84.58 ± 5.85	6.92
CA72-4	Low	4.54 ± 0.87	19.15	4.94 ± 1.01	20.44
U/ml	Medium	11.98 ± 1.72	14.33	-	-
(n=33)	High	44.84 ± 3.25	7.26	44.75 ± 5.19	11.61
tPSA	Low	0.072 ± 0.01	13.39	-	-
ng/ml	Medium	3.30 ± 0.29	8.84	3.90 ± 0.35	9.04
(n=45)	High	15.55 ± 1.45	9.31	39.33 ± 3.63	9.23

Table 2. Between-run variability assessed using commercially available quality control material (PAPP-A).

Marker	Level	Elecsys PreciControl Maternal Care	
		μ ± sd	CV (%)
PAPP-A	Level 1	5535.63 ± 629.03	11.36
mIU/L	Level 2	2761.58 ± 256.88	9.30
(n=38)	Level 3	271.42 ± 20.65	7.61

Table 3. Pooled human serum showing between-run variability.

Marker	Pool A		Pool B		Pool C	
	μ ± sd	CV (%)	μ ± sd	CV (%)	μ ± sd	CV (%)
CA125 U/ml (n=23)	27.18± 0.98	3.59	36.58± 1.53	4.18	40.36± 1.88	4.67
CA15-3 U/ml (n=21)	15.51± 0.94	6.09	15.68± 1.02	6.49	16.45± 0.99	6.00
CA72-4 U/ml (n=15)	2.37± 0.46	19.45	1.28± 0.31	24.05	1.66± 0.38	22.86
tPSA ng/ml (n=22)	0.007± 0.001	16.88	0.003± 0.001	38.46	0.004± 0.001	28.87
PAPP-A mIU/L (n =22)	9.04± 1.31	14.47	6.19± 1.67	26.99	37.15± 1.69	4.54

Table 4. Pooled human serum showing within-run variability.

Marker	Pooled Serum	
	μ ± sd	CV (%)
CA125 U/ml (n=25)	36.20 ± 0.90	2.48
CA15-3 U/ml (n=25)	15.70 ± 0.33	2.07
CA72-4 U/ml (n=25)	1.28 ± 0.06	4.68
tPSA ng/ml (n=25)	0.006 ± 0.001	12.94
PAPP-A mIU/L (n = 25)	9.25 ± 0.21	2.25

3.2 Natural and Stimulated Ovarian Cycles

3.2.1 Length of Follicular Phase

The day of ovulation (as defined in 2.1.3.1) for the 10 natural cycles is shown in Figure 1 (a). It was extremely variable, ranging from day 10 to day 25. The day of ovulation in the 11 cycles stimulated with exogenous gonadotrophin is shown in Figure 1 (b). This was less variable than the natural cycles, ranging between day 12 and day 15 of the cycle.

3.2.2 Reproductive Hormones

The reproductive hormone concentrations in natural and stimulated ovarian cycles are shown in Table 5. In the early follicular phase the oestradiol concentrations are similar between natural (165.6±11.8pmol/L) and stimulated cycles (156.0±6.6pmol/L). Large differences in oestradiol are seen by the mid-follicular phase where natural cycle concentrations increase by a factor of ~1.6 times to an average of 265.0±43.0pmol/L compared to an increase by a factor of ~7.3 times to an average of 1133.7±241.1pmol/L in stimulated cycles. The oestradiol concentrations in the late follicular phase in natural cycles on average doubled to 528.0±51.2pmol/L compared to an increase by a factor of ~3.5 to an average of 3980.0±435.8pmol/L in the stimulated cycles. Seven days after ovulation the oestradiol concentrations in both cycles decreased slightly to 484.8±68.4pmol/L in natural cycles and 2187.0±301.1pmol/L in stimulated cycles.

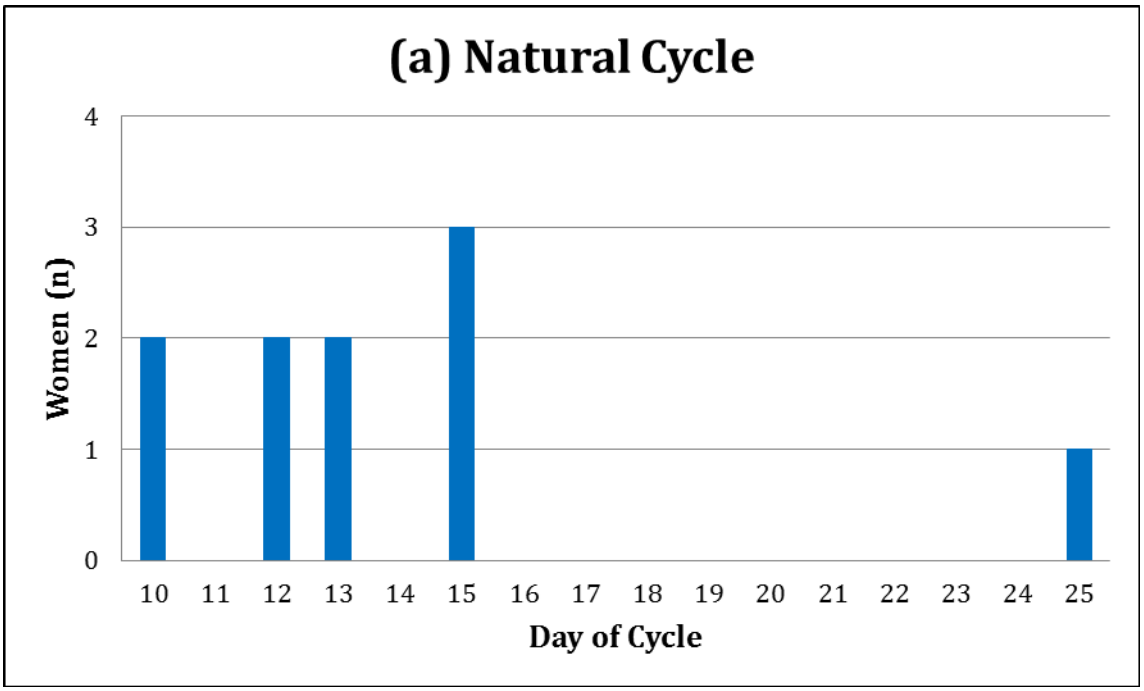
Progesterone remained relatively constant throughout the early follicular, mid-follicular and late follicular phases in natural cycles where concentrations ranged from 1.3±0.2-1.9±0.3nmol/L. It wasn't until after ovulation during the mid-luteal phase that progesterone was seen to increase in natural cycles (36.5±4.6nmol/L). Progesterone was only measured in the early follicular and mid-luteal phase during stimulated cycles. The progesterone concentration in stimulated cycles (2.3±0.6nmol/L) was similar

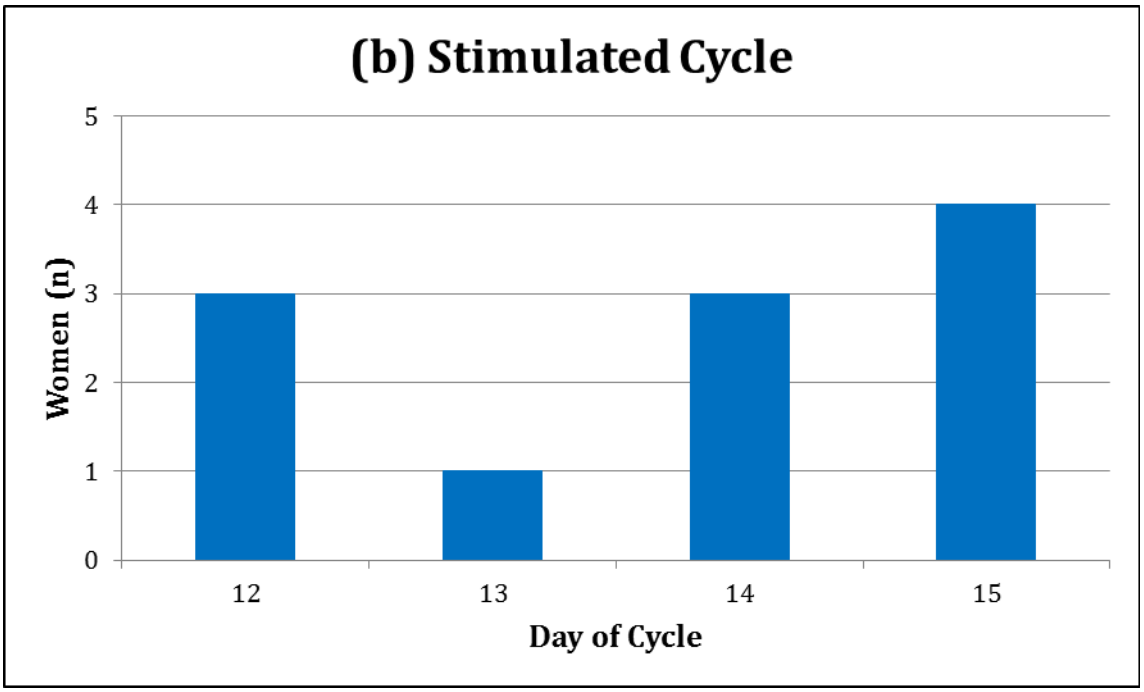
to natural cycles (1.9±0.3 nmol/L) in the early follicular phase however concentrations were much greater in the mid-luteal phase of stimulated cycles (197.2±30.0) compared to the mid-luteal concentrations in natural cycles (36.5±4.6).

Table 5. Reproductive hormone concentrations (mean±sem) in natural and stimulated ovarian cycles. The stage of the cycle was classified as early follicular (EF), mid-follicular (MF), late follicular (LF) and mid-luteal (ML).

Stage	Oestradiol (pmol/L)		Progesterone (nmol/L)	
	Natural	Stimulated	Natural	Stimulated
EF	165.6±11.8	156±6.6	1.9±0.3	2.3±0.6
MF	265.0±43.0	1133.7±241.1	1.4±0.2	-
LF	528.0±51.2	3980.0±435.8	1.3±0.2	-
ML	484.8±68.4	2187.0±301.1	36.5±4.6	197.2±30.0

Figure 1. Day of ovulation for women during (a) Natural cycles, and (b) Stimulated cycles.





3.2.3 Changes in serum CA125, CA15-3, CA72-4, tPSA and PAPP-A concentrations.

Serum concentrations of CA125, CA15-3, CA72-4, tPSA and PAPP-A during natural and stimulated cycles are shown in Tables 6 and 7. When comparing results from natural versus stimulated ovarian cycles, CA125 concentrations showed no significant difference (p=0.5989) [Table 8]. Concentrations of CA125 were however significantly influenced by the stage of the ovarian cycle (p=<0.0001). In natural menstrual cycles, the concentration of CA125 was highest during the early follicular phase of the cycle, which is concurrent with menstruation (25.92±4.45 U/ml) and lowest in the late-follicular phase prior to ovulation (16.76±2.39 U/ml) [Table 6 & Appendix 8]. In stimulated ovarian cycles, concentrations of CA125 were highest during the late luteal phase just prior to menstruation (23.57±3.91 U/ml) and lowest at the mid-follicular phase (13.41±1.90 U/ml) [Table 7 & Appendix 8].

All samples in the ovarian cycles had detectable levels of CA15-3 [Tables 6 and 7]. One individuals sample from the natural cycle group (29.54 U/ml) and one from the stimulated group (29.51 U/ml) measured above the clinical cut-off value for CA15-3 of 25 U/mL, however all other samples from both individuals were below the clinical cut-off. There was no significant difference in the CA15-3 concentration between natural and stimulated ovarian cycles (p=0.8694) as shown in Table 9. However, there were significant differences in concentrations seen between the phases of the cycle (<0.0001) [Table 9]. In natural menstrual cycles the concentration of CA15-3 was highest in the mid-follicular phase (17.52±2.31 U/ml) and lowest in peri-ovulation (13.67±1.51 U/ml) [Table 6].

Total prostate specific antigen (tPSA) was detectable in 6/10 (60%) of women at some point in the natural menstrual cycle and 10/11 (91%) of women in stimulated cycles. Concentrations of tPSA were low during natural and stimulated cycles and there was no significant difference in tPSA

concentrations between natural cycles and stimulated cycles (p=0.9193), or between different stages of the cycle (p=0.8769) [Table 10]. The concentration of tPSA was highest in the early follicular and late luteal phase which coincides with menstruation, however this did not reach statistical significance [Table 6].

There were 6/10 (60%) of the individuals in natural menstrual cycles and 6/11 (54.5%) of individuals in the stimulated cycles that had detectable levels of CA72-4 for at least one of the samples. CA72-4 concentrations were overall on average 1.47±0.31 U/ml in natural cycles and 1.58±0.35 U/ml in stimulated ovarian cycles. There was a larger degree of variation between the individuals than there was at different phases of the cycles [Table 6]. The results are very unstable and inconsistencies are greatest for CA72-4 compared to the other biomarkers.

All samples had detectable levels of PAPP-A in natural and stimulated cycles. There was no evidence of any significant changes in the mean concentration of PAPP-A during natural and stimulated ovarian cycles (p=0.4859) and there was no evidence for an interaction effect between PAPP-A concentrations and the phase of the cycle (p=0.8491)[Table 11]. There was however a significant difference in PAPP-A concentrations in the natural cycles compared to the stimulated cycles whereby on average the mean PAPP-A of the natural group was 2.41±0.58 mIU/L higher than the stimulated group (p< 0.0001)[Table 11].

Table 6. Biomarker concentrations (mean ± sem) during the natural menstrual cycle.

Phase	CA125 (U/ml)	CA15-3 (U/ml)	CA72-4 (U/ml)	tPSA (ng/ml)	PAPP-A (mIU/L)
Early Follicular	25.92±4.45	15.59±1.67	1.86±0.92	0.012±0.009	8.97±0.79
Mid-Follicular	21.36±3.21	17.52±2.31	1.21±0.50	0.007±0.004	8.91±1.04
Late-Follicular	16.76±2.38	15.91±1.69	1.10±0.33	0.009±0.006	8.59±0.64
Peri-Ovulatory	17.91±2.64	13.67±1.51	2.46±1.47	0.009±0.006	9.28±0.90
Ovulation	17.49±2.80	15.26±1.91	1.65±0.80	0.008±0.004	8.8±0.99
Mid-Luteal	20.39±1.83	16.85±1.94	1.04±0.31	0.004±0.002	8.40±0.81
Late Luteal	19.77±2.51	15.03±1.95	1.52±0.96	0.012±0.006	8.30±0.89

Table 7. Biomarker concentrations (mean ± sem) during stimulated ovarian cycles.

Phase	CA125 (U/ml)	CA15-3 (U/ml)	CA72-4 (U/ml)	tPSA (ng/ml)	PAPP-A (mIU/L)
Early Follicular	17.06±2.37	15.10±2.00	2.33±1.12	0.0036±0.004	6.24±0.54
Mid-Follicular	13.41±1.90	14.18±2.09	1.13±0.39	0.0085±0.002	6.73±0.59
Late-Follicular	13.62±1.64	14.60±1.90	0.98±0.27	0.0036±0.003	6.57±0.35
Mid-Luteal	22.89±13.45	16.99±2.15	1.72±0.88	0.0151±0.001	6.19±0.53
Late Luteal	23.57±3.91	16.84±2.23	1.53±1.01	0.0053±0.004	5.97±0.49

Table 8. ANOVA table for the analysis of the concentration of CA125 in natural and stimulated cycles across phases.

CA125	numDF	denDF	F-value	p-value
(intercept)	1	90	926.1185	<0.0001
Group	1	19	0.2862	0.5989
Phase	1	90	6.2613	0.0141
Group:phase	1	90	21.5411	<0.0001

Table 9. ANOVA table for the analysis of the concentration of CA15-3 in natural and stimulated ovarian cycles across phases.

CA15-3	numDF	denDF	F-value	p-value
(intercept)	1	91	756.9088	<0.0001
Group	1	19	0.0278	0.8694
Phase	1	91	35.4565	<0.0001
Group:phase	1	91	23.0392	<0.001

Table 10. ANOVA table for the analysis of the concentration of Prostate Specific Antigen (tPSA) in natural and stimulated ovarian cycles across phases.

PSA	numDF	denDF	F-value	p-value
(intercept)	1	67	734.6268	<0.0001
Group	1	14	0.0107	0.9193
Phase	1	67	0.0157	0.9006
Group:phase	1	67	0.0242	0.8769

Table 11. ANOVA table for the analysis of the concentration of Pregnancy Association Plasma Protein-A (PAPP-A) in natural and stimulated ovarian cycles across phases.

	numDF	denDF	F-value	p-value
(Intercept)	1	96	768.6488	<.0001
Group	1	25	56.8412	<.0001
Phase	1	96	0.4893	0.4859
Group:Phase	1	25	0.8491	0.8491

3.3 Early Pregnancy

3.3.1 Reproductive hormones

The serum hCG concentrations increased from 296.8±70.9 at 4 weeks to 52191.3±11310.7 at 7 weeks [Table 12]. This increase in hCG during early pregnancy is highly significant (p=<0.0001) [Table 13]. Progesterone concentrations were steady ranging from 179.2±42.1 at week 4 to 287.8±78.8 at week 7 of gestation [Table 12].

3.3.2 Serum PAPP-A, CA125, CA15-3, CA72-4 and tPSA.

The PAPP-A, CA125, CA15-3, CA72-4 and tPSA biomarker concentrations are shown in Table 17. The concentration of PAPP-A increased steadily from 6.51±0.56mIU/L at 4 weeks to 125.38±54.2mIU/L at 7 weeks. The changes seen in early pregnancy were highly significant (p=<0.0001) for PAPP-A [Table 14] and all pregnancies were viable and resulted in a live birth.

The concentrations of CA125 in early pregnancy are shown in Table 18. There was a significant effect of the stage of gestation on CA125 concentration (p=0.0012) [Table 15 & Appendix 9]. However, there was considerable variability in the timing of peak CA125 [Figure 2]. When the values are normalised to the time of peak value there are clear patterns of change in CA125 concentration [Figure 3].

Figure 2. A frequency distribution of the time of peak CA125 concentration for 14 pregnant women.

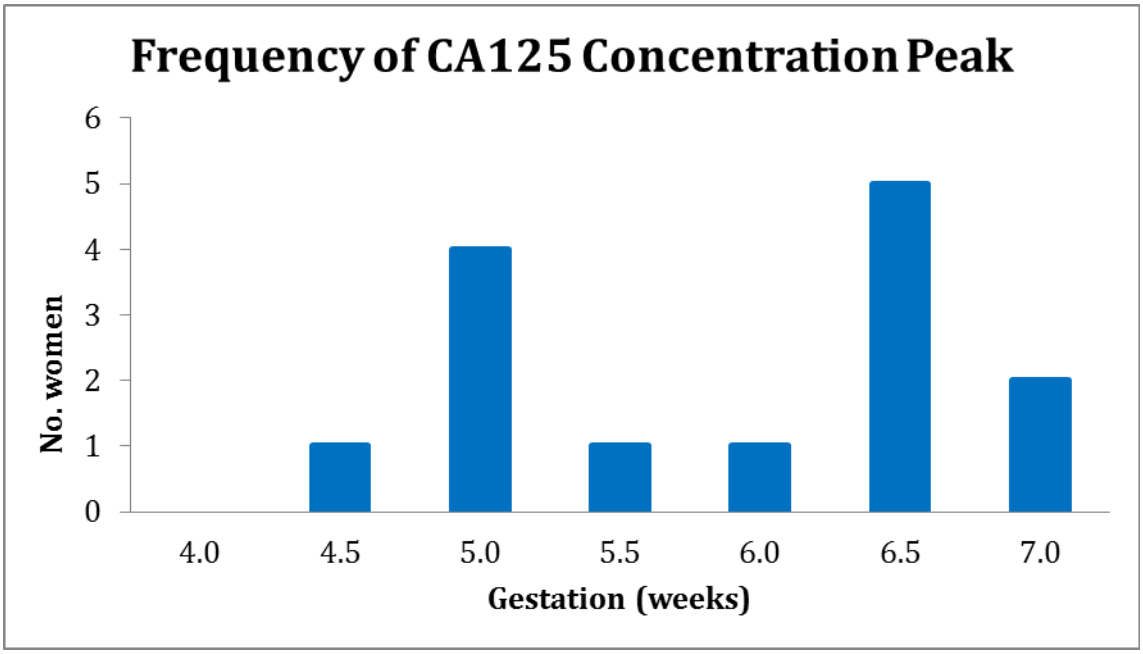
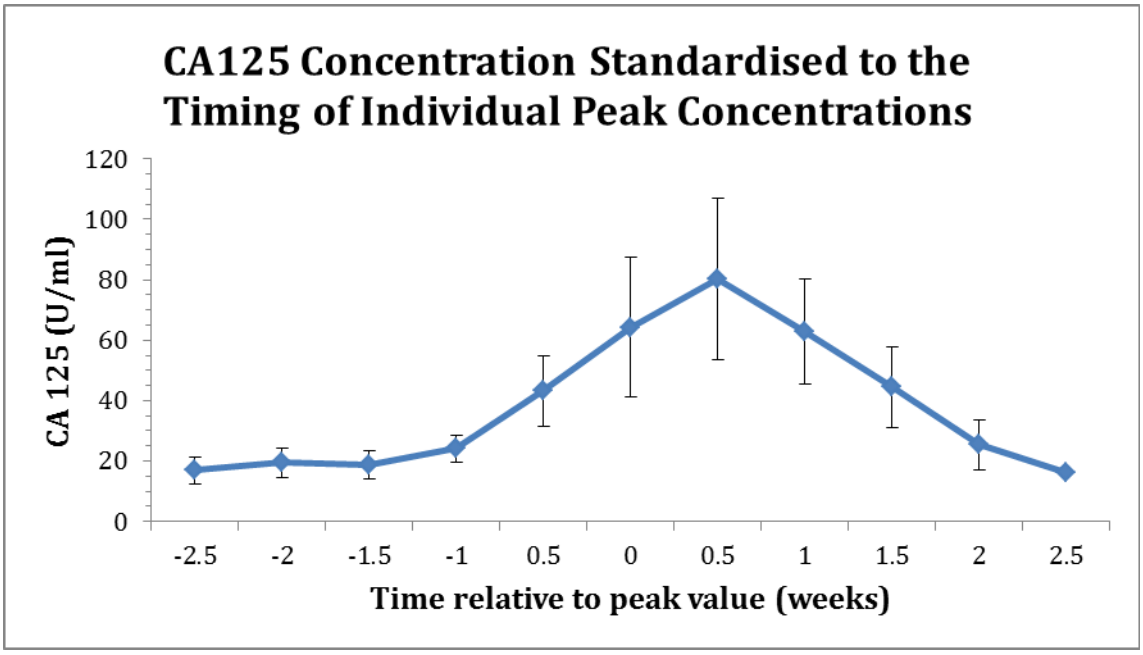


Figure 3. The serum CA125 concentration (U/ml) relative to the time of the peak concentration for 14 pregnant women.



There were 9/14 (64%) of the pregnant women that had detectable levels of tPSA in at least one sample during early pregnancy. The concentration of PSA increased steadily from 0.005±0.001 at 4 weeks to 0.01 ± 0.003 at 7 weeks [Table 18 & Appendix 9], with an average increase of 0.0015 ± 0.0004ng/ml for every one unit increase in gestation time (p=0.000) [Table 16]. In effect, the concentration of tPSA in maternal serum on average doubled from week four to seven of gestation.

All participants had detectable levels of CA15-3 throughout early pregnancy where there was a significant effect of the stage of gestation on the CA15-3 concentration (p=0.000) [Table 17 & Appendix 9]. The average concentration of CA15-3 was 16.44±2.22U/ml at 4 weeks gestation and over the time period reduced to an average of 12.97±2.35U/ml [Table 17]. These concentrations were all below the clinical cut-off value of 25U/ml.

Of the participants, only 57% of women in early pregnancy had detectable levels of CA72-4 for at least one of their samples. The clinical cut-off values for CA72-4 are variable and transferability of expected values to individual laboratories can be considerably different. The CA72-4 concentration in pregnancy was on average 2.14±3.5U/ml, however, because of the variability of results both within-individuals and between-individuals a statistical analysis was not performed as the distribution did not fit the linear mixed effects model.

Table 12. Reproductive hormone concentrations (mean±sem) in early pregnancy according to the gestational age.

Gestation	Hormone Concentrations		
	Oestradiol (pmol/L)	Progesterone (nmol/L)	hCG (IU/L)
4 weeks	1797.5±394.3	179.2±42.1	296.8±70.9
4.5 weeks	-	179.7±43.5	1773.0±262.0
5 weeks	-	179.3±37.2	5359.6±1212.8
5.5 weeks	-	196.8±43.4	15085.9±2666.5
6 weeks	-	169.3±34.9	31725.0±5367.8
6.5 weeks	-	167.9±42.5	48306.0±7371.0
7 weeks	-	287.8±78.8	52191.3±11310.7

Table 13. ANOVA table for the linear mixed effects model for hCG levels during pregnancy.

hCG	numDF	denDF	F-value	p-value
(Intercept)	1	69	5634.490	<0.0001
Gestation	1	69	1153.922	<0.0001

Table 14. ANOVA table for the linear mixed effects model for PAPP-A levels during pregnancy.

PAPP-A	numDF	denDF	F-value	p-value
(Intercept)	1	69	1094.4500	<0.0001
Gestation	1	69	549.0459	<0.0001

Table 15. ANOVA table for the linear mixed effects model for CA125 levels during pregnancy.

CA125	numDF	denDF	F-value	p-value
(Intercept)	1	69	207.11741	<0.0001
Gestation	1	69	11.38667	0.0012

Table 16. ANOVA table for the linear mixed effects model for Prostate Specific Antigen (tPSA) levels during pregnancy.

PSA	numDF	denDF	F-value	p-value
(Intercept)	1	45	2967.6833	<0.0001
Gestation	1	45	15.6762	0.000

Table 17. ANOVA table for the linear mixed effects model for CA15-3 levels during pregnancy.

CA15-3	numDF	denDF	F-value	p-value
(Intercept)	1	69	528.8371	<0.0001
Gestation	1	69	15.7576	0.000

Table.18 Biomarker concentrations (mean ± sem) during early pregnancy.

Gestation (Weeks)	CA125 (U/ml)	CA15-3 (U/ml)	CA72-4 (U/ml)	tPSA (ng/ml)	PAPP-A (mIU/ml)
4 (n=7-9)	22.79 ±3.73	16.44 ±2.22	2.15 ±1.08	0.005 ±0.001	6.51 ±0.56
4.5 (n=9-14)	30.40 ±6.02	15.19 ±1.56	2.19 ±1.05	0.005 ±0.001	6.86 ±0.40
5 (n=9-14)	47.65 ±15.12	14.59 ±1.60	2.42 ±1.24	0.006 ±0.001	8.29 ±0.66
5.5 (n=9-14)	57.98 ±27.45	14.30 ±1.58	2.15 ±1.08	0.007 ±0.001	14.58 ±1.47
6 (n=9-14)	48.15 ±19.13	14.28 ±1.54	1.68 ±0.66	0.008 ±0.001	34.41 ±4.45
6.5 (n=8-13)	42.59 ±10.56	14.04 ±1.53	1.79 ±0.74	0.009 ±0.001	59.37 ±7.09
7 (n=4-6)	29.92 ±6.44	12.97 ±2.35	2.60 ±1.40	0.010 ±0.003	125.38 ±22.11

4.0 Discussion

4.1 Assay Characteristics

The use of immunoassays allows precise quantitative measurements to be made when measuring analytes. However, different assays often have different characteristics due to the choice of reagent or their calibration, resulting in different numerical values. This is important when comparing work from various laboratories or over a range of time frames. For example, the expression of PAPP-A results in mIU/ml in the present study compared to µg/L elsewhere (Sinosich, Porter, Sloss, Bonifacio, & Saunders, 1984; Yovich et al., 1986) reflects the change in methodology and the move to a different standardisation. Between-assay differences have been reported when measuring CA15-3 with commercial kits from different companies, resulting from differences in the antibodies produced against different sections of the antigen as well as calibration rather than specificity (Klee & Schreiber, 2004). This is perhaps not too surprising as many of the companies used similar capture and signal antibodies in their sandwich immunometric assays. Different CA125 assays have used different reference preparations, such that results are then expressed in different units. Whilst the current study used an assay with results in U/ml, Tavmergen et al. (2001) used a method reporting in IU/ml. The performance of the CA125 assays can also show between-supplier variability, and large differences have been reported between assays supplied by Siemens and Panomics (McLemore et al., 2012).

Secondary to the variation seen between the assays used in different research projects, there is also some variation associated with using the same assay in different runs. The between-batch assay variation differs amongst analytes and so it is important to assess the between-batch variation associated with the method being used to ensure that any changes seen are the result of biological fluctuations and not because of the assay variation. In this present study the between-batch variation was further compared with the within-batch variation

to ensure that the method used only had the smallest assay variation associated with the results. The assessment of the between-batch variation was done by measuring artificial quality control (QC) material from both the company that manufactures the assay and an external source on up to 45 different occasions. Along with the manufactured QC samples, pooled human serum was also analysed to assess the assays at levels found in our study population.

The results for the between-batch variation measured using the commercial QC material showed good reproducibility for the CA125 and CA15-3 assays however the PAPP-A, tPSA and CA72-4 assays showed considerably greater variability. The results obtained using the pooled human serum showed similar patterns for CA125 and CA15-3 however again the lower concentrations of the tPSA, CA72-4 and PAPP-A in the pooled human serum resulted in a larger degree of variability. The results for the within-batch variation measured using the pooled human serum show good reproducibility whereby the within-batch variation for CA125, CA15-3, CA72-4 and PAPP-A all show CVs of <5.0%. The within-batch results for tPSA were more variable than the other assays however the serum concentrations for tPSA are lower in females and thus near the assays lower limit of detection. The difference in the between-batch variation and within-batch variation shows that the analysis of longitudinal samples from individual women should be done in one batch analysis where possible. This ensures that any changes seen in the concentrations of the biomarkers are due to biological fluctuations and it reduces the interference that might be the result of the assay itself.

4.2 Ovarian Cycles

The lengths of the follicular phase in the natural cycles included in this present study were variable ranging between 10-25 days. This is similar to previous work performed by Cole et al. (2009) which showed the distribution of the LH peak as being between 10-20 days, and Lenton, Landgren, and Sexton (1984) who reported a distribution of 8.2-21 days. The range of lengths of the follicular

phase in stimulated cycles was less, being between 12-15 days as they were all controlled ovarian cycles and the differences in days is attributable to the individual response to the stimulation protocol. The hormone levels in all cycles followed the classical patterns of change, confirming that the modelling and expression of results according to the stage of cycle is appropriate.

There were phase dependent changes seen in CA125 concentrations for both natural and stimulated ovarian cycles. This study showed that there was no effect of ovarian hyperstimulation on CA125 levels and that both natural and stimulated ovarian cycles showed similar changing patterns. The results from the natural group agreed with the literature in that the highest CA125 levels were found during menstruation (Bon et al., 1999; Haga et al., 1986; Jager et al., 1988; McLemore et al., 2012; Pittaway & Fayez, 1987; Touitou et al., 1989).The stimulated group results also agreed with the literature where the highest CA125 levels were found in the luteal phase of the cycle (Zweers et al., 1990). It was suggested that CA125 levels were negatively correlated to oestradiol levels however the lack of difference between stimulated and natural cycles suggests that even with grossly elevated oestradiol levels, there is no significant difference in CA125 concentrations. This would also confirm reports that it is in fact the endometrium that is responsible for the cyclical changes in CA125 concentrations and it is the disruption of the endometrium during menses that allows increased amounts of CA125 to enter the blood stream (Bon et al., 1999; Kafali et al., 2007; Weintraub, Bischof, Tseng, Redard, & Vassilakos, 1990). It was also proposed that pregnancy outcomes following ART treatment could be predicted by measuring CA125 on the day of oocyte retrieval and that levels >10IU/ml were correlated with an 86.6% positive pregnancy rate based on a prospective study of 75 ART cycles (Tavmergen et al., 2001). Of the 8 participants in this study that had a CA125 of >10U/ml before oocyte retrieval, only 3 of those became pregnant (37.5%) which is markedly lower than the literature had suggested. There was also one participant who had a CA125 level <10U/ml that did become pregnant. Although these results are interesting they

are limited by the relatively small sample size but may warrant further investigation.

Serum concentrations of CA15-3 in natural menstrual cycles were not statistically different to those found in stimulated cycles suggesting that ovarian stimulation *per* se for the purposes of IVF and ICSI procedures does not affect circulating serum CA15-3 levels. The CA15-3 concentration did however show some interaction with the phases of the cycles which is in agreement with the literature (Erbagci, Yilmaz, & Kutlar, 1999). The MUC1 gene, which encodes the CA15-3 glycoprotein, is expressed in the upper female reproductive tract and its function has been suggested to be to prevent ectopic embryo implantation (Al-Azemi et al., 2009). Other studies have shown that MUC1 expression is progesterone dependent whereby it is up regulated in the luteal phase of the menstrual cycle (Aplin, Hey, & Graham, 1998; Hey, Graham, Seif, & Aplin, 1994). These findings are fitting because if MUC1 is up regulated by progesterone in the luteal phase, it would suggest that it is indeed a part of the body's mechanisms to avoid ectopic pregnancy. This current study has shown that serum concentrations of CA15-3 are at their highest in the mid-follicular phase of the natural menstrual cycle, which is at a time that progesterone is at its lowest levels, suggesting that although CA15-3 is encoded by the MUC1 gene, it does not appear to be progesterone dependent. This being said, the previous research on MUC1 expression was carried out on tissue samples whereas this is an analysis of serum concentrations so the 'lag' in peak CA15-3 concentrations may not reflect activity at a local level.

Prostate specific antigen (PSA) was thought to be produced exclusively by prostatic tissue and was therefore used to monitor prostate cancer (Armbruster, 1993). However, it has been associated with a number of tissues and biological events in women (EP Diamandis & H Yu, 1995) such as in the breast (He Yu et al., 1996) and during the ovarian cycle (Aksoy, Akçay, Umudum, Yildirim, & Memisogullari, 2002; N. Zarghami, L. Grass, E. R. Sauter, & E. P.

Diamandis, 1997). Total prostate-specific antigen was detectable in 60% of women in natural cycles and 91% of women during stimulated ovarian cycles. The range of mean results from each phase was between 0.004ng/ml – 0.012ng/ml for both natural and stimulated ovarian cycles. There was no significant relationship between tPSA concentration and phase of the cycle, nor was there any significant difference between tPSA concentrations in natural and stimulated cycles. Zarghami et al., (1997) indicated that tPSA in the menstrual cycle followed the progesterone concentration peak with a 10-12 day delay which was suggestive that tPSA changed in a cyclical manner. This present study shows that tPSA concentrations were highest in the early follicular and late luteal phase which is relative to menstruation although this did not reach statistical significance. Total prostate specific antigen concentrations are very low in female serum and it is not known why some women have measurable levels of tPSA and others do not. The physiological function of tPSA in females is yet to be determined.

Ohuchi et al. (1989) described CA72-4 as a useful tumour marker for all tumours derived from epithelial cells highlighting the tumour markers increased sensitivity to gastric carcinoma compared to other tumour markers such as CA19-9, CEA and CA125. It was also proposed that when CA72-4 is used as a complimentary biomarker to CA125, the sensitivity for detecting early stage ovarian cancer increased from 45% to 70% (Skates et al., 2004). The CA72-4 assay used in this research failed to register any results above the assays lower limit of detection in 40-45.5% of individuals from both natural and stimulated ovarian cycles. Of those individuals that did have detectable levels of CA72-4, the results showed an extremely high degree of variability both within each individual (whereby each sample from the same cycle was vastly different to the others) and between patients where the difference was so large that there were no obvious patterns of change. It was for these reasons that statistical analysis was not carried out and it was concluded that the assay was too unreliable for use as a diagnostic measure in the clinical setting.

Similar to the misnomer with PSA, the name pregnancy-associated plasma protein-A (PAPP-A) does not necessarily mean this protein is associated only with pregnancy. Although most of the research surrounding PAPP-A has been performed during pregnancy (D'Antonio et al., 2013; Dane et al., 2013; Folkersen et al., 1981; Jacobs et al., 1990; Lin et al., 1974; NJ Wald, Hackshaw, Diamandis, & Melegos, 1999; Yovich et al., 1986), there are recent studies which indicate differences in PAPP-A concentrations during ART treatment cycles (bAmor et al., 2009; Maymon & Shulman, 2002b; Orlandi et al., 2002; Tul & Novak-Antolic, 2006). This present study found a significant difference in the mean concentration of PAPP-A between natural and stimulated ovarian cycles, where stimulated ovarian cycles were on average 2.41±0.58mIU/L lower than natural cycles. The present study also showed that throughout each of the two types of cycles there were no significant changes in PAPP-A levels. Findings of lower serum PAPP-A concentrations in women during stimulated ovarian cycles confirms previous work where it was shown that women had lower serum PAPP-A levels with higher oocyte number after oocyte retrieval, leading to the proposal that differences in PAPP-A concentrations may be due to the presence of multiple corpora lutea in the ovaries (Tul & Novak-Antolic, 2006). Amor, et al. (2009) found that PAPP-A levels were reduced in both fresh and frozen-thawed embryo transfers when compared to naturally conceived pregnancies, however, fresh transfers did have significantly lower PAPP-A levels than frozen-thawed transfers. This again provides evidence for the multiple corpora lutea theory where the ovaries in frozen-thawed embryo transfer cycles are not hyperstimulated to create multiple follicle development like those of fresh cycles. Similar to the literature which states that PAPP-A concentrations are similar between ART subtypes, this present study also found that there was no significant difference between the PAPP-A concentrations in IVF versus ICSI cycles.

4.3 Early Pregnancy

All fourteen of the pregnancies included in this study resulted in a live birth with no complications. The reproductive hormone levels in all individuals

followed the classical pattern of change throughout early pregnancy, confirming that the modelling used to express the results according to the timing of gestation was appropriate. The progesterone levels were maintained at a constant level via the administration of progesterone pessaries which aid in the support of the pregnancy.

There have been many publications (Aslam et al., 2000; Halila et al., 1986; Kobayashi et al., 1993; Niloff et al., 1984; Pittaway & Fayez, 1987) where CA125 has been measured and found to increase during pregnancy; however most of these are reports from cross sectional studies that compare single CA125 measurements from each of the gestational trimesters. This study was different in that serial blood samples were collected from the same women from 4 weeks gestation through until seven weeks gestation. As the women in this study all fell pregnant following ART treatment, important events such as ovulation were known and implantation could be inferred from this timing. Pregnancy tests are done fourteen days after ovulation is confirmed, which is also referred to in obstetrics as 4 weeks gestation relative to the last menstrual period. This study found that in early pregnancy CA125 levels were generally lower than the clinical cut-off value at week 4, however they did increase significantly on average at week 5.5 before reducing again back to the normal range by week 7. It was observed that although the statistical mean for peak concentrations was week 5.5, there was a degree of variation on the time of gestation that these peak levels did actually occur. It showed that there were two time points, namely week 5 and 6.5 that had more individuals have their peak CA125 concentration. This being said, although different women had different timing for elevated CA125 concentrations, when the data was standardised so that all concentration peaks were labelled as t=0 and the other samples were timed accordingly, the same significant changes in CA125 concentrations were seen. This elevation of CA125 is concurrent with placentation events occurring in the endometrium and along with previous suggestions that circulating CA125 is attributable to endometrium disruption during menses, it would suggest again that it is the disruption of the endometrium during placentation that may be

responsible for this elevation. There have been other studies suggesting that pregnancies with elevated CA125 (>65U/ml) in the first trimester were at an increased risk of spontaneous abortion however in successful pregnancies the CA125 reduced to <93U/ml between weeks 7-12 gestation, although those pregnancies which did abort still had levels >120U/ml (Azogui et al., 1996; Ocer et al., 1992). The current study had four pregnant participants that had peak CA125 levels between 94-341U/ml where all pregnancies resulted in a live birth. Of these four pregnancies with elevated CA125, all but one reduced to <93U/ml by week 7, however the one participant whose CA125 did not reach <93U/ml had her last sample collected at week 6.5 gestation so it is not known if this would have reduced further or not. The results in this study do seem to follow the trends highlighted in previous work and would agree with the argument that as a predictor of spontaneous abortion, an elevated CA125 level is an indicator of the decidual destruction which is associated with pregnancy outcome (Ocer et al., 1992).

During early pregnancy there was a significant effect of the stage of gestation on CA15-3 concentrations, where the CA15-3 concentrations declined as the pregnancy progressed to week 7. Other studies reported in the literature have used cross-sectional samples collected later in pregnancy, and have shown CA15-3 to increase towards delivery, being higher in the third trimester than first and second trimesters using (Bon et al., 2001; Correale et al., 1993). Research by Meseguer et al. (2001) found that the MUC1 gene which encodes CA15-3 is an anti-adhesive molecule that is expressed on endometrial epithelial cells and the human blastocyst. The study also found that there was an increase in the MUC1 expression during the apposition phase of implantation followed by a localised down regulation of MUC1 during adhesion at the site of implantation. In the present study, serum concentrations of CA15-3 decrease from week four to week seven of gestation which is suggestive that the declining serum concentrations of CA15-3 might be a potential biomarker of certain implantation events and warrants further investigation.

Prostate specific antigen was detectable in 9/14 (64%) of the pregnant women in this study. In these women, PSA on average increased steadily from week 4 through week 7 of gestation where concentrations effectively doubled. There have not been any studies that indicate the behaviour of PSA this early in pregnancy however other literature does confirm that PSA in amniotic fluid increased from weeks 11 to 21 and thereafter declined until birth (H. Yu et al., 1995). Prostate specific antigen is expressed by normal, hyperplastic and neoplastic female breast tissue so it was of interest to measure PSA in women during early pregnancy when breast changes can occur. The PSA was seen to increase significantly in some women whilst the breast tumour marker CA15-3 when measured in the same women decreased. It would then appear that the PSA circulating in the serum is not of the same origin as CA15-3, and the role of PSA as a marker of normal embryo implantation and development warrants further study.

With increasing numbers of pregnancies being achieved through ART and much research into the risks associated with a pregnancy achieved from ART, there have been many reports that PAPP-A levels at the first trimester screening are reduced compared to those conceived naturally. As PAPP-A is measured as part of the Down syndrome screening tests, an increase in the false positive rate would mean an increase in the number of unnecessary amniocentesis procedures being carried out on these already precious pregnancies. The present study measured PAPP-A levels during the first vital weeks of pregnancy until week seven of gestation with the intention of giving insight into the behaviour of PAPP-A during this time period. It was found that PAPP-A concentrations increased steadily from 6.51±0.56mIU/ml to125.38±22.11mIU/ml by week 7, where levels at week four were similar to those found throughout the stimulated ovarian cycles. These pregnancies were all conceived following ART treatment which would explain the similar PAPP-A levels at week four of gestation.

Although described as a useful biomarker of various different conditions (Ohuchi et al., 1989), CA72-4 concentrations in this population of women were extremely variable. The results in the group of pregnant women showed a high degree of variability within each of the individuals however there were no obvious patterns of change. Close to half (43%) of the women in this study did not have detectable levels of CA72-4, where the lower limit of detection is <0.2U/ml. It was for this reason that statistical analysis was not carried out and the assay was deemed too unreliable for use in the clinical setting.

4.4 Crossing of Clinical Thresholds

Whilst many biomarkers may be associated with the underlying process of a disease, there can be many ways in which the use of the biomarker may have limited clinical utility (Palmer & Barnhart, 2013). This can be seen with biomarkers that were once used routinely in diagnostic medicine and originally thought to be specific to particular diseases, but have subsequently been shown to either (a) have a limited response in some individuals despite the presence of the disease or (b) be associated with other biological processes and possibly cloud the interpretation. A good example of the former is tPSA and prostate cancer where the measurement of PSA was once recommended as a screening test for the disease, but the recommendation has been withdrawn after studies later highlighted that the disease can be present even when serum concentrations of tPSA are not elevated (Thompson et al., 2004). The current study measured a variety of biomarkers in disease-free women during different stages of reproductive activity, and was then able to comment upon the possibility that the biomarkers may cross erroneously clinical thresholds, because of other biological processes rather than the presence of the disease itself. Although there were changes in concentrations for most of the biomarkers, it was only CA125 during early pregnancy that consistently crossed the cut-off associated with disease and may have significant clinical implications. The clinical cut-off for CA125 as recommended by the assay manufacturer is 35U/ml where it is said that concentrations greater than 35U/ml are associated with ovarian cancer and other gynaecological and

inflammatory conditions. This study showed that CA125 concentrations in early pregnancy peak between 5 and 6.5 weeks gestation (Appendix 11) and that the maximum concentrations often exceed the clinical cut-off for many women (Appendix 12). These pregnancies all resulted in a live birth so it can be inferred that a transient elevation in CA125 is not associated with adverse pregnancy outcomes.

4.5 Summary

4.5.1 Findings

This study made evident the importance of conducting a batch analysis of all samples from each of the participants to make certain that any changes seen in biomarker concentrations was due to biological fluctuation and not because of the increased batch to batch variation that is associated with some assays. This study provided useful information about the precision of the assays and highlighted weaknesses in some such as CA72-4 and tPSA, particularly at low concentrations.

Ovarian stimulation appeared to affect serum PAPP-A levels which may extent into pregnancy, with the IVF/ICSI cycles having significantly lower concentrations, whilst CA125 and CA15-3 were unaffected by ovarian stimulation *per se* but showed cyclical changes throughout both the natural and stimulated cycles. tPSA and CA72-4 showed no consistent changes in either natural or stimulated cycles. PAPP-A, CA125, tPSA and CA15-3 (but not CA72-4) all showed consistent but different patterns of change in early pregnancy.

4.5.2 Implications

The biomarkers studied provided additional information over and above the reproductive hormones in monitoring the biological events of ovarian cycles and early pregnancy. In pregnancy, PAPP-A, CA125, PSA and CA15-3 are

useful in combination for assessing different aspects of implantation, embryogenesis and placentation. Care should be taken when interpreting CA125 as a screen for gynaecological disease during early pregnancy as transient elevations during this time are normal.

4.5.3 Limitations

Although participant numbers were not large (n=35) the number of serum samples (n=223) were adequate to provide statistically significant data at all levels of the study and to reveal consistent patterns. A limited number of biomarkers were measured, and the additional value of others cannot be excluded. All patients showed normal responses in terms of ovulation and implantation, and the value of these biomarkers in disordered ovulation or pregnancy failure cannot be commented upon.

4.5.4 Future Research

The value of additional biomarkers that can be measured using automated assays should be investigated. The biomarkers of the current study should also be applied to patients showing disordered ovulation or having pregnancies with non-viable outcomes such as ectopic implantation, blighted ovum and spontaneous abortion.

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6.0 Appendices

Appendix 1 – Information letter and consent forms

RESEARCH PROJECT

INFORMATION

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

The title for this student research project is:

Tumour markers: concentrations in the serum of healthy women during natural and stimulated ovarian cycles, and during early pregnancy

Every healthy person carries low levels of what are known as disease markers. When diseases occur, the level of a particular marker may increase and therefore help with diagnosis (and monitoring) of the disease. But we do not know whether and how hormonal changes in various stages of the reproductive cycle may influence the level of these disease markers.

We are inviting you to participate in this study because we are looking for samples from perfectly healthy women.

Aim of the project

a) Measure selected blood tumour markers in women, namely prostate specific antigen (PSA), CA15-3, CA72-4, and CA125.
b) Measure these compounds in healthy women during the natural menstrual cycle, stimulated ovarian cycle and early pregnancy.
c) Determine if the markers fluctuate or are stable, and identify possible changes in these compounds that are a normal part of ovulation and pregnancy.
d) Measure other markers if they become available during the course of the present study, for the same reasons.

How can you help?

Once your blood samples have had hormones measured in the normal way, we would like to use the remainder of the samples in the project rather than it being discarded.

How will the sample be used?

The laboratory at Fertility North will measure levels of the various tumour markers in your blood.

Will these tests tell me if I have cancer?

No, you have been selected because you are healthy. Results can only be interpreted accurately for women at risk of the disease if we understand what happens to these markers in normal women.

Will I get to see the results?

If you agree for your samples to be used then we shall send you a copy of the final report of the project.

Will the study help me?

Not directly as the results will not affect your treatment or management. However, it will hopefully enable us to understand the dynamics of these tumour markers, which will help others.

Who will see the results of my sample?

The sample will be coded so that your identity will only be known to the staff at Fertility North.

What if I do not want my sample to be used?

You are free to refuse for your samples to be used. This will not prejudice your treatment in any way.

Who should I ask if there are any questions?

Dr Phillip Matson (Scientific Director), Dr Vince Chapple (Medical Director), Melissa Stemp (Biochemist) and Dr Angus Stewart (senior Lecturer ECU) are the people involved in the project. These, 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.

FNC 22. CONSENT FOR THE USE OF BLOOD IN THE RESEARCH PROJECT ENTITLED

Tumour markers: concentrations in the serum of healthy women during natural and stimulated ovarian cycles, and during early pregnancy.

Name: ……..

Address: ……..

DOB: ……..

I agree for my blood samples taken in this cycle to be used in this project

☐ only for the markers listed in the Research Project Information document,

☐ for the markers listed in the Research Project Information document and any other markers as additional tumour marker assays become available during the course of this study

I also understand that:

a) Refusal would not have prejudiced my treatment in any way.
b) I am not required to have any extra blood samples taken other than those ordered by my doctor for my own management.
c) I will be sent a copy of the final report of the study so that I may see how the blood samples were used, and the conclusions made.
d) I am free to ask questions of the investigators during the study.
e) I will be given a copy of this consent form.

SIGNATURES

Volunteer: …….. 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 2 – Ethics approval letters

Phill Matson

From: Research Ethics <research.ethics@ecu.edu.au>
Sent: Wednesday, 5 September 2012 3:51 PM
To: Melissa STEMP
Cc: Peter ROBERTS; Phill Matson; Research Assessments
Subject: 7281 STEMP ethics approval
Attachments: Conditions of approval MULTICENTRE.pdf

Dear Melissa

Project 7281 STEMP
Tumour markers: concentrations in the serum of healthy women during natural and stimulated ovarian cycles, and during early pregnancy

Student Number: 10184554

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 5 September 2012 to 1 August 2014.

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 **research.ethics@ecu.edu.au** Tel: +61 08 6304 2170 | **Mobile: 0428 035 397** | Fax: +61 08 6304 5044 | CRICOS IPC 00279B

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20 July 2012

Miss M Stemp
Fertility North, Suite 213
Specialist Medical Centre
Joondalup Health Campus

Dear Miss Stemp

RE: Tumour markers: concentrations in the serum of healthy women during natural and stimulated overaisn cyclees, and during early pregnancy (1216)

The Human Research Ethics Committee of Joondalup Health Campus is pleased to notify you that your proposal to undertake research 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

Ann Y Hammer
Executive Officer, JHC HREC

cc Dr P Matson, Co-Investigator, Fertility North

Appendix 3 – Centaur CP SOP

FertilityNorth
Fertility & Endometriosis Treatment Centre

SOP

Document Number: BC-S-590 Effective Date: **09-Aug-13**

Operation and Maintenance of the Siemens Centaur CP Analyser

Purpose:
To provide Fertility North staff guidance in how to operate the Siemens Centaur CP Automated Analyser in a consistent and compliant manner.

Associated Documents:	
BC-F-451	CP Barcode Checklist
BC-F-459	CP Tube Cap Placemat
	Advia Centaur CP Manual Operator's Guide

Definitions:	
FN	Fertility North

Responsibilities:

Role or Department	Responsible for:
Laboratory Technician	• Ensuring that this procedure is adhered to in relation to measuring reproductive hormones.
Scientific Director	• Ensuring that this procedure is followed by all staff in the Biochemistry department. • Ensuring that all results are validated and reported correctly on the appropriate Fertility North documents.

Table of Contents:

Operation and Maintenance of the Siemens Centaur CP Analyser

1 Introduction

1.1 Assay protocol for estradiol, luteinizing hormone, progesterone, human chorionic gonadotrophin and follicle stimulating hormones.

1.2 Assay – Refer to the Advia Centaur CP - Immunoassay System (See Advia Centaur CP Manual Operator's Guide).

2 Reagent Preparation and Storage

2.1 Reagents –For all Siemens Advia Centaur CP supplies a Reagent Diary is kept recording the Order No and date, Arrival date, Batch No [same as previous or new], Expiry date, Date opened, and if/when QC reported. All reagents are stored as per manufacturer's instructions. The Order Number and same/new batch are also written on the box. The date the box is opened is also recorded on the box.

2.2 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 sample the date of reconstitution and the date of expiry.

Product	Cat No	On Board/Recon Stability	Storage Temp	Volume to Add
eE2	10491445	672 hrs	2-8 degrees	Na
FSH	1360521	672 hrs	2-8 degrees	Na
LH	2212941	672 hrs	2-8 degrees	Na
PRGE	1586287	672 hrs	2-8 degrees	Na
ThCG	6031151	672 hrs	2-8 degrees	Na
Multi-dil 3	5389133	672 hrs	2-8 degrees	Na
eE2 dil	10491878	672 hrs	2-8 degrees	Na
ThCG dil	5609230	672 hrs	2-8 degrees	Na
Cal 30	10379810	14 days	2-8 degrees	2ml
Cal B	649625	28 days	2-8 degrees	5ml
Cal E	4634452	14 days	2-8 degrees	2ml
Immunoassay Plus	370	na	2-25 degrees	5ml
Cleaning Soln	9908593	na	2-25 degrees	Na
Wash 1 Soln	1137199	na	2-25 degrees	Na
R1/R2	497043	na	2-25 degrees	Na

Operation and Maintenance of the Siemens Centaur CP Analyser

3 Assay Procedure

3.1 Preparing the Advia Centaur CP for operation

a) Check on board supplies: - Water, Wash 1, R1/R2, primary/secondary reagents and cuvettes. [These should have been replenished at the end of the last run]
b) Check that waste bin is empty. [This should have been done at the end of the last run]
c) Perform the Aspirate Probe Bubble Detector Calibration [Maintenance, Aspirate Probe Bubble Detector Calibration, and Perform]
d) Perform the Automated System Prime [Maintenance, Automated System Prime, and Perform] and Close to initialise to Ready Mode.
e) Load Quality Control Ligands, a Patient Repeat from the previous day and any calibrator tubes required. Ligands are reconstituted to 5 mls and 7 x 600ul lots are aliquotted and available for the next 7 days. If calibrations are due, 500ul from the appropriate reconstituted bottle is put into labelled tubes after ensuring it is within its expiry date.
f) Perform any calibrations due by selecting the reagent tray, selecting reagent due for calibration and selecting calibrate. [See3.9 Advia Centaur CP Manual Operator's Guide]
g) When this is underway the Ligands and a Patient Repeat from the previous day can have their tests assigned. To assign tests, select the sample tray and when the sample is highlighted, add each test. If the sample is urgent it can jump the queue by also activating the stat button which shows as orange. If dilutions are required, the dilutions button is selected and then the test and the dilution/s. Close the window and if no other tests are running you will also need to start CP operation by selecting the start button.
h) Once calibrations and controls have been run and accepted [see 1.01.21] patient samples can be loaded as they become available and tests assigned.

3.2 Preparation and completion of a Daily Worksheet

a) Open PowerTerm, and select D- Biochemistry menu.
b) Select A-New Worksheet, and ESC to accept the worksheet number and date.
c) Select F2, enter the date of birth, and select the matching patient.
d) Assign the sample number using the barcode reader unless it is unavailable in which case the number can be entered manually.
e) Select F3 and using arrows to find test 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 for this].

f) Once the samples are entered, hit F1 to go back to the menu. The Biochemistry worksheet is saved.

g) Results are manually entered from the Advia Centaur CP output - Results, Print, Today's Records, Consolidated Results and OK.

h) Results are transferred into PowerTerm by selecting C-Worksheet Double Entry from the Biochemistry menu and entering each result twice.

i) Once complete, the worksheet is printed by choosing D-Worksheet Result List and cross-checked against the Advia Centaur CP.

j) When Biochemistry staff have completed and checked results, they must validate the worksheet, making it available for viewing and comment by Clinicians and nursing staff. To validate the worksheet choose A-Biochemistry Worksheet, ensuring the correct sheet is chosen by number (there may be more than one) then choose F2 to validate and Y to confirm.

k) Sign validation form and file with complete set of day's paperwork including all result printouts from the CP and completed worksheets from Powerterm.

4 Dilutions of Assays (ADVIA CENAUR CP)

4.1 Estradiol

Samples >11000 pmol/L will be automatically diluted by the Advia Centaur CP. If high or unpredictable levels are expected samples can be pre programmed for selected dilutions when assigning the test. (See 2.42 Advia Centaur CP Operator's Guide).

4.2 Progesterone

Samples >150 nmol/L will be automatically diluted by the Advia Centaur CP. 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 2.42 Advia Centaur CP Operator's Guide)

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.

4.3 LH (Luteinizing Hormone)

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

4.4 FSH (Follicle Stimulating Hormone)

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

4.5 ThCG (Total Human Chorionic Gonadotrophin)

Samples >1000 IU/L will be automatically diluted by the Advia Centaur CP. 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 2.42 Advia Centaur CP Operator's Guide)

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

5 Assay Calibration

5.1 For estradiol, luteinizing hormone, progesterone, human chorionic gonadotrophin and follicle stimulating hormones.

5.2 Frequency of calibration [see 3.9 Advia Centaur CP Operator's Guide)

a) LH, Progesterone and ThCG – Monthly
b) Estradiol and FSH – 14 days
c) When new reagent and calibration lots are introduced.
d) Upon poor QC performance

5.3 Application of Calibrators

a) LH, FSH, ThCG – Cal B
b) Progesterone – Cal E
c) Estradiol – Cal 30

The bottles specify the amount of water to add. When this is done the reconstitution and expiry dates are written on the bottle.

Operation and Maintenance of the Siemens Centaur CP Analyser

5.4 Calibration of Assays (see3.11-17 Advia Centaur CP Operator's Guide)

a) 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.

b) Prior to daily assay runs, perform any calibrations due by selecting reagent tray, selecting reagent due for calibration and selecting calibrate.

END OF DOCUMENT

Appendix 4 – Roche SOP

FertilityNorth
Fertility & Endometriosis Treatment Centre

SOP

Document Number: BC-S-542 Effective Date: **09-Aug-13**

Operation and Maintenance of the Roche COBAS e411 Analyser

Purpose:
To provide Fertility North staff guidance in how to operate the Roche Cobas e411 Automated Analyser in a consistent and compliant manner.

Associated Documents:	
BC-F-410	Screening Tests Schedule
BC-F-411	Screening Results Worksheet
	Roche Cobas e411 analyser operators manual

Definitions:	
FN	Fertility North

Responsibilities:

Role or Department	Responsible for:
Laboratory Technician	• Ensuring that this procedure is adhered to in relation to screening bloods.
Scientific Director	• Ensuring that this procedure is followed by all staff in the Biochemistry department. • Ensuring that all results are validated and reported correctly on the appropriate Fertility North documents.

Table of Contents:

Operation and Maintenance of the Roche COBAS e411 Analyser

1 Start-up / Pre-routine Operation Procedures

1.1 Preparing the Roche Cobas e411 for Operation

1.1.1 Perform a visible inspection of the machine, noting all surfaces are clean and free from debris, probes and microbead mixer paddle are in good condition and not bent, no tubing is pinched or bent, and pipette syringes and associated tubing are free of bubbles and not leaking system water. If these fail inspection, then remedy by cleaning or calling the Roche Support Service.

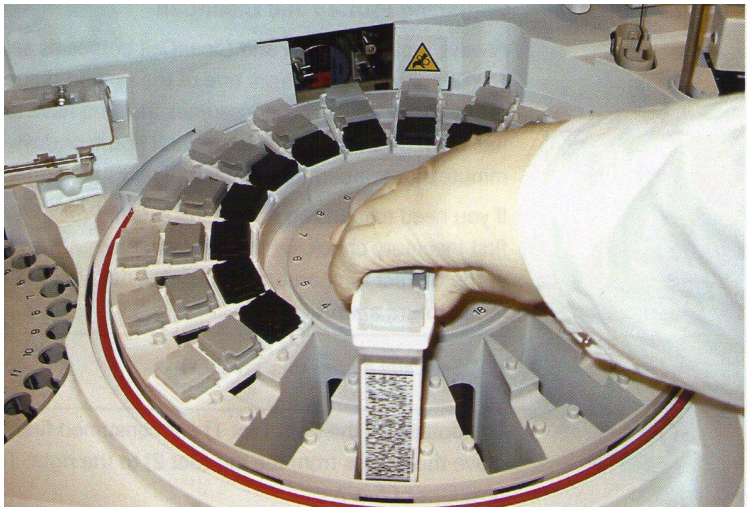
1.1.2 Ensure that printer is switched on and contains paper.

1.1.3 With the analyser in Standby mode, top up system water with a 100+1 solution of SysWash in deionised water (10ml: 1L).

1.1.4 Empty liquid waste container and rinse with water, and empty solid waste tray. Ensure safety glasses and gloves are worn when emptying biohazard material.

1.1.5 Allow reagent packs to warm to room temperature and then load on to the reagent rotor the reagent packs for the tests to be run that day. The rotor is temperature controlled to 20°C.

1.1.6 Open lids and check for surface bubbles. If present, aspirate gently with a plastic pipette.

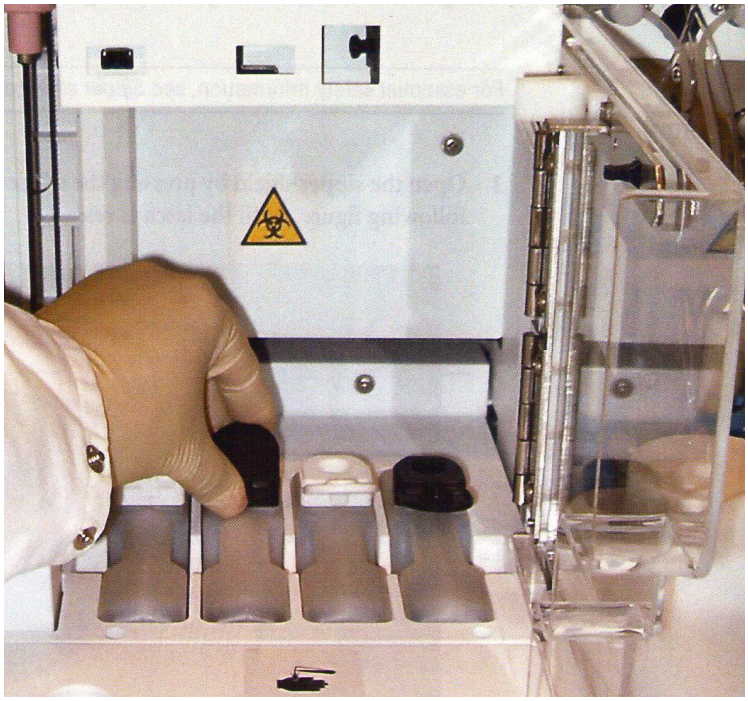


1.1.7 Check the reagents (ProCell and CleanCell bottle sets) and consumables (AssayCup and AssayTip trays), and ensure sufficient are on board. Top up as required, ensuring that new lots of reagents are calibrated, and the

Operation and Maintenance of the Roche COBAS e411 Analyser

ProCell and CleanCell are added in pairs. The chambers holding the ProCell and CleanCell are kept to a temperature of 28.0°C, and so reagents must be allowed 15 minutes to achieve this working temperature.

1.1.8 Open the ProCell and CleanCell lids.



1.1.9 From the home screen select the button to run a Reagent Scan. The reagent scan will check how many AssayTips and AssayCups are onboard as well as the ProCell and CleanCell levels. It also scans the barcodes on any reagent packs that are on board the analyser. The analyser will register any new lots and determine if the new kits requires a calibration before it will allow it to be run.

1.1.10 The analyser is now ready for use.

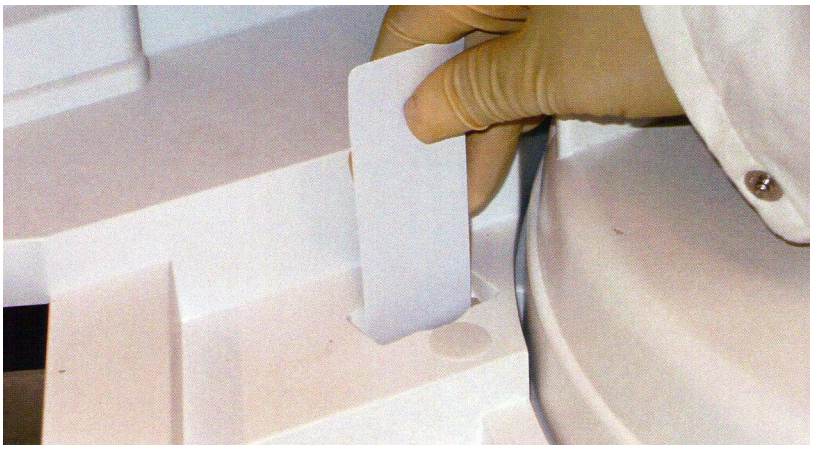
Operation and Maintenance of the Roche COBAS e411 Analyser

2 Routine Operation

1.1 Instrument Calibration / Quality Control

2.1.1 Calibrations should be performed at the frequency summarised in Table 2.1 and indicated below for the individual assays. Expired calibrators should not be used and should be discarded.

2.1.2 Calibrator details are entered using the calibrator barcode card which is inserted in the barcode reader to the left of the reagent rotor.



2.1.3 If using Position Assignment to indicate the Calibrators, then ensure that the barcode on the tube is not visible to the scanner and that the lot number of the Calibrator is assigned to the correct positions. This is done at Workplace>Calibration>Calibrator>Position Assignment. Select the Calibrator lot on the left of the screen, and the rack position on the right of the screen, and choose Assign. If a previous Lot is in the rack positions then these will need to be removed first.

2.1.4 Check if a Calibration or QC is requested by the system (Overview Screen > Calib/QC Load List)

2.1.5 Check if calibration renewal is due on each of the reagent packs. If manual calibrations are required select these from the Calibration menu > Status > Highlight test > Select Full > Save.

2.1.6 Running the Calibrations is simply a case of placing the Calibrators in the correct rack position in the front tray and pressing Start. Ensure that the Calibration was successful before running QCs and samples.

2.1.7 Request non-Roche controls from the QC menu > Status Highlight Control > Select > Save.

Operation and Maintenance of the Roche COBAS e411 Analyser

2.1.8 Prepare calibrator and control materials i.e. reconstitute lyophilized material or thaw frozen material. Always refer to the specific calibrator or control package insert for details.

2.1.9 Load required controls using the designated Control racks. When loading controls, ensure both levels of the same control are next to each other on the rack with level 1 in the position in front of level 2.

2.1.10 Start measuring controls (Start > Start).

2.1.11 Once sampling is complete, remove calibrators and controls from the racks, promptly close the lids and return them to the refrigerator.

2.1.12 Validate Calibration and Control results. Write into Endocrinology or Serology workbook and file the printed copy under Roche Daily QC & Calibration Printouts.

Operation and Maintenance of the Roche COBAS e411 Analyser

1.2 Sample Processing

2.2.1 Remove samples from the fridge and allow them to warm to room temperature.

2.2.2 Using sample racks held on a metal tray, samples are added to each rack with their barcode visible through the rack opening, as listed in the tests schedule (BC-F-410) which defines which position each patient sample is in.

2.2.3 Once all the racks are loaded on the tray, the tray is placed in to the front compartment (A-line) of the analyser, ensuring there is a tray on the back area, C-line, to receive the racks.

2.2.4 Press Start so that the samples pass through bar code reader.

2.2.5 Choose Workplace>Data Review and select the first tube that was read.

2.2.6 Program the patient samples by pressing Workplace > Test Selection.

2.2.7 Select the Routine option from the Sample area on the top left of the screen. A sequence number is assigned automatically.

2.2.8 Select the Normal or Reduced option for Sample Cup.

2.2.9 Select the required dilution, if any.

2.2.10 Select the tests for the sample on the test key matrix. Once selected the test appears white.

2.2.11 Choose Save to complete the test selection.

2.2.12 Once done, bring the tray forward from the C-line back to the A-line, and replace a tray in the C-line.

2.2.13 Press Start > Start to run the samples for sampling and analysis.

2.2.14 Track sample processing by selecting Sample Tracking from the Overview screen.

Operation and Maintenance of the Roche COBAS e411 Analyser

1.3 Result Evaluation

2.3.1 Print patients result reports by choosing Workplace > Data Review. Highlight all patient samples and press Print > Print.

2.3.2 Transcribe individual patient results onto the Screening Results Worksheet document (BC-F-411). This sheet will have the matching patient barcode that is on the sample tube and the Screening Test Schedule (BC-F-410) at the header of the patient's results column.

2.3.3 The assay scientist validates the results written onto the Screening Results Worksheet from the hard copy that has been printed off the Roche Cobas e411 Analyser.

2.3.4 A secondary scientist that did not run the assays then co-validates the results written onto the Screening Results Worksheet from the original hard copy that was printed off the Roche Cobas e411 Analyser.

2.3.5 After the results have been validated by both scientists, the results are transcribed into the appropriate word documents; "Serology Female Master" or "Serology Male Master" or "Endocrinology Female Master" or "Endocrinology Male Master".

2.3.6 A copy of each individual patients report is saved as Surname, Initial and the date of processing.

2.3.7 The report is copied and pasted into a new template in the patients file on Genie. The appropriate template can be found under the Lab Reports heading. Once the template is complete and all patient details are checked and correct the document is saved to the patients file.

2.3.8 A copy of the report is printed from the patients overview screen and is paired with the original results print out from the Roche Cobas e411 Analyser and the Screening Results Worksheet. These are sent to the Scientific Director for final validation.

1.4 Result Final Validation

2.4.1 The Scientific Director, or his nominated deputy, is to give the final validation of all screening results by viewing the patient's final report generated through the Genie program and validating that the result is the same as what was originally printed off the Roche Cobas e411 Analyser.

Operation and Maintenance of the Roche COBAS e411 Analyser

1.5 End of Operation / Daily Maintenance

2.5.1 Once the analyser has gone back into Standby mode you are then able to open the reagent rotor lid and press down on the reagent caps to snap them shut. Remove all reagent packs and store in the refrigerator. Replace rotor lid and lock.

2.5.2 Replace AssayTip and AssayCup trays that are empty.

2.5.3 Empty solid waste container and liquid waste containers ensuring safety glasses and gloves are worn to avoid contact with hazardous material.

2.5.4 Clean the sample/reagent probe with alcohol swabs daily and check for condensation inside compartments.

2.5.5 Close the ProCell and CleanCell lids to prevent evaporation.

2.5.6 Perform any other scheduled maintenance e.g. weekly or monthly maintenance.

- Weekly. Clean the sipper probe to prevent contamination and carryover. In addition, spills on the incubator may cause gripper movement alarms, and so the incubator and aspiration station should be cleaned. Empty the system water container irrespective of the amount contained and rinse with water, then refill with 1+100 diluted SysWash.
- Fortnightly. Clean the rinse stations for the sample/reagent probe and microbead mixer, plus the one for the sipper probe. Liquid flow cleaning should be performed in the correct sequence to keep the sipper liquid flow clean and maintain the integrity of the measuring cell.
- Bimonthly. Replace the pinch valve tubing every two months.
- As needed. Clean the liquid waste container and the microbead mixer. Also, clean the ProCell and CleanCell compartments. Clean the reagent rotor and compartment.
- Ensure the Cobas e411 analyser maintenance log is completed.

END OF DOCUMENT

Appendix 5 – Siemens Reagent Stabilities

Product	Cat No	On Board/Recon Stability	Storage Temp	Volume to Add
eE2	10491445	672 hrs	2-8 degrees	Na
FSH	1360521	672 hrs	2-8 degrees	Na
LH	2212941	672 hrs	2-8 degrees	Na
PRGE	1586287	672 hrs	2-8 degrees	Na
ThCG	6031151	672 hrs	2-8 degrees	Na
Multi-dil 3	5389133	672 hrs	2-8 degrees	Na
eE2 dil	10491878	672 hrs	2-8 degrees	Na
ThCG dil	5609230	672 hrs	2-8 degrees	Na
Cal 30	10379810	14 days	2-8 degrees	2ml
Cal B	649625	28 days	2-8 degrees	5ml
Cal E	4634452	14 days	2-8 degrees	2ml
Immunoassay Plus	370	-	2-25 degrees	5ml
Cleaning Soln	9908593	-	2-25 degrees	Na
Wash 1 Soln	1137199	-	2-25 degrees	Na
R1/R2	497043	-	2-25 degrees	Na

Na = not applicable as product is supplied in liquid form.

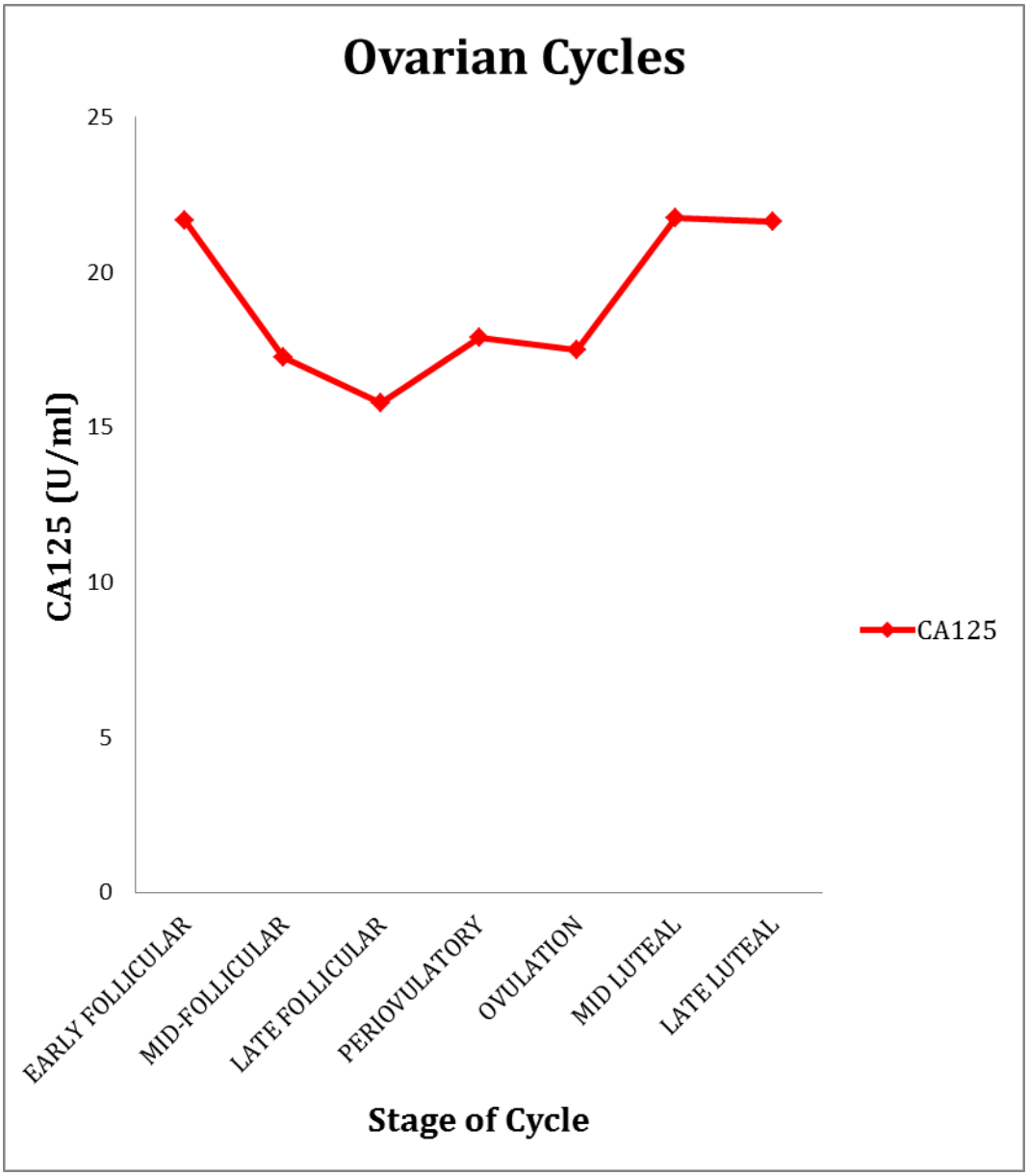
Appendix 6 – Roche Reagent Stabilities

Assay	Test time	Stability			Calibration			Measuring range
		Unopened 2-8°C	Opened 2-8°C	On-board analyser	Initial	On-board analyser	Stored same lot	
CA125 II	18 mins	Exp. Date	12 weeks	6 weeks	Within 24hrs	7 days	1 month	0.6-5,000 U/mL
CA15-3 II	18 mins	Exp. Date	12 weeks	5 weeks	Within 24hrs	7 days	1 month	1.00-300 U/mL
CA72-4	18 mins	Exp. Date	12 weeks	8 weeks	Within 24 hrs	7 days	1 month	0.200-300 U/mL
tPSA	18 mins	Exp. Date	12 weeks	4 weeks	Within 24hrs	7 days	1 month	0.003-100 ng/mL
PAPP-A	18 mins	Exp. Date	4 weeks	3 weeks	Within 24 hrs	7 days	1 month	4-10000 mIU/L

Appendix 7 – Roche QC Material

Assay	QC material	No. Levels	Reconstitution	Storage	Stability
CA125, CA15-3, CA72-4, tPSA	Elecsys PreciControl Tumor marker	2	3 mls water each	-20˚C	1 month
CA125, CA15-3, CA72-4, tPSA	Biorad Lyphochek Tumor Marker Plus	3	2 mls water each	-20˚C	1 month
PAPP-A	Elecsys PreciControl Maternal Care	3	3 mls water each	-20 ˚C	3 months

Appendix 8 – Change in mean concentration of CA125 in ovarian cycles, natural and stimulated combined.



Appendix 9– Change in the mean concentration during early pregnancy of CA125, CA15-3 and tPSA.