Novel biochemical markers in the diagnosis and management of early pregnancy problems

Submitted to the University of Newcastle upon Tyne, Faculty of Medical Sciences as a thesis for the Degree of Doctor of Medicine

Maya Chetty MBBS MRCOG

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Declaration

I hereby certify that the contents of this thesis are my own work and the contributions of others have been acknowledged. No part of this work has been previously submitted for a degree or other qualification to this or any other University.

Maya Chetty

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ABSTRACT

Background and purpose

Early pregnancy problems, including miscarriage, ectopic pregnancy and pregnancy of unknown location, occur commonly and have significant medical, psychological and economic consequences. Biochemical markers are increasingly being used as an adjunct to ultrasonography and this thesis describes three studies exploring the use of novel biochemical markers in the diagnosis and management of early pregnancy problems.

Materials and methods

These are observational studies of women in early pregnancy recruited at Sunderland Royal Hospital and King's College Hospital. Serum samples were taken from women in early pregnancy and lectin affinity chromatography used to characterise the glycosylation of hCG by gestational age and by pregnancy outcome. Women with a diagnosis of a miscarriage, ectopic pregnancy or pregnancy of unknown location had serum levels of hCG, progesterone, inhibin A, IGFBP-1 and inhibin proαC quantified, and statistical analysis was used to see if spontaneous resolution of the pregnancies could be predicted.

Results

Lectin-affinity chromatography reveals five major glyco-isoforms of hCG in early pregnancy, the expression of which changes with gestational age and by pregnancy outcome. The novel markers of the luteo-trophoblastic axis inhibin A, IGFBP-1 and inhibin proαC are found not to be clinically useful in the prediction of spontaneously resolving PULs although when used in the decision trees developed by Elson in 2005, they are useful for predicting spontaneous resolution of miscarriages and failed pregnancies.

Conclusions

Novel biochemical markers have the potential to be a useful addition in the management of early pregnancy problems. Further studies are required to explore the physiological basis of these findings and the clinical applicability of these tools.

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Contribution of Others

The original research questions were devised by Dr J Elson, Dr J Chapman and myself. I designed the studies with significant assistance from Miss J Elson and Dr J Chapman. Under supervision, I set-up the studies and applied for COREC approval. I undertook patient identification and data collection, with assistance from Dr E Sawyer at King's College Hospital. Biochemical assays were performed by Ms G Leadbitter (Sunderland Royal Hospital), Ms T Dew (King's College Hospital), and Dr S Butler (Middlesex University). Dr J Chapman and myself performed the chromatography. I managed the study data and Dr J Elson provided statistical advice and assisted me with the statistical analysis.

List of Abbreviations

ALP	Alkaline phosphatase
CEMACH	Confidential enquiry into maternal and child health
Con-A	Concanavalin A
ELISA	Enzyme linked immunoassay
EPAU	Early pregnancy assessment unit
FSH	Follicle stimulating hormone
GlcNAc	N-acetyl-glucosamine
hCG	Human chorionic gonadotrophin
H-hCG	Hyperglycosylated human chorionic gonadotrophin
ITU	Intensive therapy unit
IUGR	Intrauterine growth restriction
IUCD	Intrauterine contraceptive device
IUP	Intrauterine pregnancy
IGF	Insulin-like growth factor

IGFBP-1	Insulin-like binding protein-1
Inhibin pro-αC-RI	Inhibin pro-αC-related immunoactivity
IVF	*In vitro* fertilisation
LCA	*Lens culinaris* agglutinin
LH	Luitinising hormone
NEQAS	National External Quality Assessment Service
NPV	Negative predictive value
NeuNAc	N-acetylneuramic acid
17-OHP	17α-hydroxyprogesterone
PPV	Positive predictive value
PUL	Pregnancy of unknown location
RCOG	Royal College of Obstetricians and Gynaecologists
ROC	Receiver operating characteristics
SD	Standard deviation
TVS	Transvaginal scan
WGA	Wheat germ agglutinin

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CHAPTER 1. INTRODUCTION

Early pregnancy loss is perhaps the most common medical problem in women of reproductive age. These conditions are a large burden to health services as well as to the physical and psychological wellbeing of women and their partners. In recent times there have been major changes in our approach to the management of early pregnancy problems. With the introduction of early pregnancy assessment units and transvaginal ultrasonography we have moved away from an in-patient and surgical approach, to outpatient services and a more conservative approach to management.

Transvaginal ultrasound is now used routinely in the assessment of early pregnancy problems. This allows a more detailed assessment at earlier gestations than was previous possible. These findings are often used in combination with biochemical markers to give a more complete evaluation or 'profile' of the pregnancy. Serum levels of human chorionic gonadotrophin (hCG) and progesterone are the most commonly used biochemical markers, the optimum way to utilize them in the diagnosis and management of early pregnancy problems remains contentious.

The move away from surgical treatment for early pregnancy problems has economic and clinical advantages. Problems occur however when medical or expectant management is unsuccessful and surgical intervention is required at a later date, often in an 'emergency' situation. The lack of well-defined criteria to differentiate between pregnancies that will spontaneously resolve and those that will not is an ongoing problem for expectant management and is certainly off-putting for patients and clinicians. Expectant management often takes weeks to complete and success rates vary widely. Failure of expectant management after prolonged follow-up is particularly disappointing for women and reduces overall benefits of the management strategy.

A number of novel biochemical markers of the luteal-trophoblastic axis have emerged in the last decade. The aim of this thesis was to investigate these novel biochemical markers in the diagnosis and management of early pregnancy complications. In particular I have tried to identify parameters, which can reliably predict the success of expectant management in women with failing pregnancies. The ability to do so would not only reduce the need for follow-up

but also decrease the need for surgery for both diagnostic and therapeutic indications. The ability to estimate the likelihood of final outcome would allow us to counsel patients appropriately and is likely to increase the uptake of expectant management by both clinicians and patients. This would improve the overall care of women with early pregnancy problems.

CHAPTER 2. LITERATURE REVIEW

2.1 NORMAL EARLY PREGNANCY

2.1.1 Fertilisation, implantation and early development

Following ovulation the ovum is taken up by the fimbrial end of the Fallopian tube and is wafted medially by the rhythmical action of the cilia. Fertilisation of the ovum by spermatozoa occurs either in the peritoneal cavity or within the Fallopian tube. During the following 48 hours the conceptus travels along the Fallopian tube and into the uterine cavity. Within 30 hours of fertilisation the first cell division occurs, in which the fertilised ovum splits into two separate cells. On the fifth day after conception, an additional round of division causes the 32-celled morula to reach the blastocyst stage. This hollow ball is composed of an inner cell mass, eventually giving rise to the fetal and embryonic tissues, and an attached outer shell of cells known as the trophoblast, which will ultimately give rise to the chorion. By the tenth day, the invading trophoblast forms two distinct layers – the cytotrophoblast and the syncytiotrophoblast. The cytotrophoblast (the inner layer) is composed of individual, well defined and rapidly proliferating cells. The outer and thicker layer, the syncytiotrophoblast, comprises of multinucleated cells with indistinct cell borders (Anin *et al.,* 2004).

The conceptus attaches to the secretory endometrium of the uterus by 5 days post ovulation but it is not until day 12 that the blastocyst has burrowed into the endometrium to such an extent that it is completely embedded. There is initial decidualisation at the implantation site, which eventually extends to the whole endometrium. The trophoblast cells produce a proteolytic enzyme which allows invasion into the endometrium. The trophoblastic cells will form the extra-embryonic tissue. The trophoblast differentiates in two ways – the villous and the extravillous trophoblast. The villous trophoblast remains attached to the villous membrane, this is responsible for maternal-fetal gas and nutrient exchange, and hormone secretion. The cells of the extravillous trophoblast proliferate from the tips of anchoring chorionic villi and migrate through the maternal tissues towards decidual arterial walls (interstitial invasion) (Lyall, 2002), or infiltrate the lumens and walls of arteries to cause endovascular

invasion (Kaufmann *et al.,* 2003). Endometrial arteries do not communicate with the intervillous space before 12 weeks gestation because aggregates of trophoblast cells derived from the cytotrophoblast shell plug their distal segments. This protects the conceptus from high oxygen levels during this critical stage of development (Burton *et al.,* 1999). The definitive structure of the placenta is apparent as early as day 21 post ovulation however the uteroplacental circulation is not fully functional until the end of the first trimester (Hustin & Jauniaux, 2000). Placentation, along with other early gestational processes such as implantation, is one of the most important determinants of pregnancy outcome.

2.1.2   Biochemistry in early pregnancy

The syncytiotrophoblast of the developing placenta plays a key part in hormone, protein and growth factor production in early pregnancy. Human chorionic gonadotropin (hCG) is secreted by the syncytiotrophoblast almost immediately after implantation. This maintains the function of the corpus luteum which in turn secretes hormones and growth factors which are essential for the maintenance of early pregnancy. This activity of the corpus luteum decreases after the seventh week of pregnancy at which time the trophoblast and decidua take over as the main hormone-producing unit.

Human chorionic gonadotrophin

The glycoprotein hormone human chorionic gonadotrophin (hCG) has a molecular weight of 36,700d (Midgley & Pierce, 1962) and was first identified in 1927 (Ascheim & Zondek, 1927). It consists of two dissimilar subunits, α and β, which are glycosylated and non-covalently bound. The hCG-α subunit is virtually identical to that of the human pituitary glycoprotein hormones, luteinising hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) (Bahl 1969a; Canfield *et al.,* 1971). The characteristic biological and immunological identities of these hormones are conferred by their specific β-subunits (Pierce and Parsons, 1981). Synthesis of hCG occurs predominantly within trophoblast cells of the blastocyst, the α and β

4

subunits being coded for separately on chromosomes 6 and 19 respectively (Fiddes & Goodman, 1981; Boorstem *et al,* 1982). Post-translational glycosylation comprises the addition of two N-linked oligosaccharides to each subunit; on amino acids 52 and 78 (α subunit) and 13 and 30 (β subunit). In addition to N-linked oligosaccharides, 4 O-linked oligosaccharides are located within the hCG-β–COOH terminus (Bahl, 1969b). HCG is metabolised by the kidney, it is desialated and then excreted into urine (Birken *et al.,* 1996).

HCG secretion begins no later than day 7 in the blastocyst stage (Lopata & Hay, 1989). It has been shown that its level in maternal serum doubles over 1.4-1.6 days from the time of first detection to the thirty-fifth day of pregnancy, and then doubles over 2.0-2.7 days from the thirty-fifth to the forty-second day (Pittaway *et al.,* 1985). The pattern of hCG concentration throughout pregnancy is shown in Figure 1. The half-life of hCG is 32 to 37 hours and the levels of hCG are approximately 1000 IU/L at around 4 weeks of pregnancy, the time of initial visualisation of a gestational sac on transvaginal ultrasound scan. HCG secretion increases with advancing gestational age, reaching a maximal level of 50,000 to 100,000 IU/L at 10 weeks gestation. HCG levels decrease to around 10,000 to 20,000 IU/L by 20 weeks and this plateau is maintained for the rest of pregnancy. Most commercially available monoclonal antibody-based urine pregnancy tests can detect the presence of hCG at a level above 25 IU/L, which corresponds to days 24 to 25 of a regular 28-day cycle. Many different laboratory assay kits exist which are calibrated against different reference preparations of hCG. βhCG may exist in the blood as part of the intact hCG molecule (α and β subunits) or as free βhCG. Assay kits measure either intact hCG, βhCG or total hCG (intact plus βhCG). These need to be taken into account when comparing results from different investigators and appropriate values need to be developed for individual medical centres.

In primate studies it is the exponential increase in hCG levels produced by the implanting embryo and syncytiotrophoblast that appears to prolong the functional lifespan of the corpus luteum (Zeleznik, 1998). In response to hCG, the corpus luteum produces increasing concentrations of progesterone, 17 α-hydroxyprogesterone (17-OHP) and oestradiol, and stimulates the secretion of relaxin. hCG maintains the steroidogenesis of the corpus luteum until the ninth to tenth week of pregnancy by which time placental steroidogenesis is

established and the role is entirely taken over by the placenta. It is also thought that hCG produced by the placenta has a number of autocrine and paracrine actions. These include involvement with the autoregulation of placental steriodogenesis (Menon & Jaffe, 1973), and increasing syncytium formation (Yang *et al.,* 2003). There is also evidence that hCG modulates trophoblast invasion by interfering with endometrial matrix metalloproteinases (MMPs) and their tissue inhibitors (Licht *et al.,* 2001) and influences endometrial differentiation by modulating prolactin and insulin-like growth factor binding protein-1 (IGFBP-1) at the implantation site (Fluhr *et al.,* 2006).

Serum hCG (IU/L)
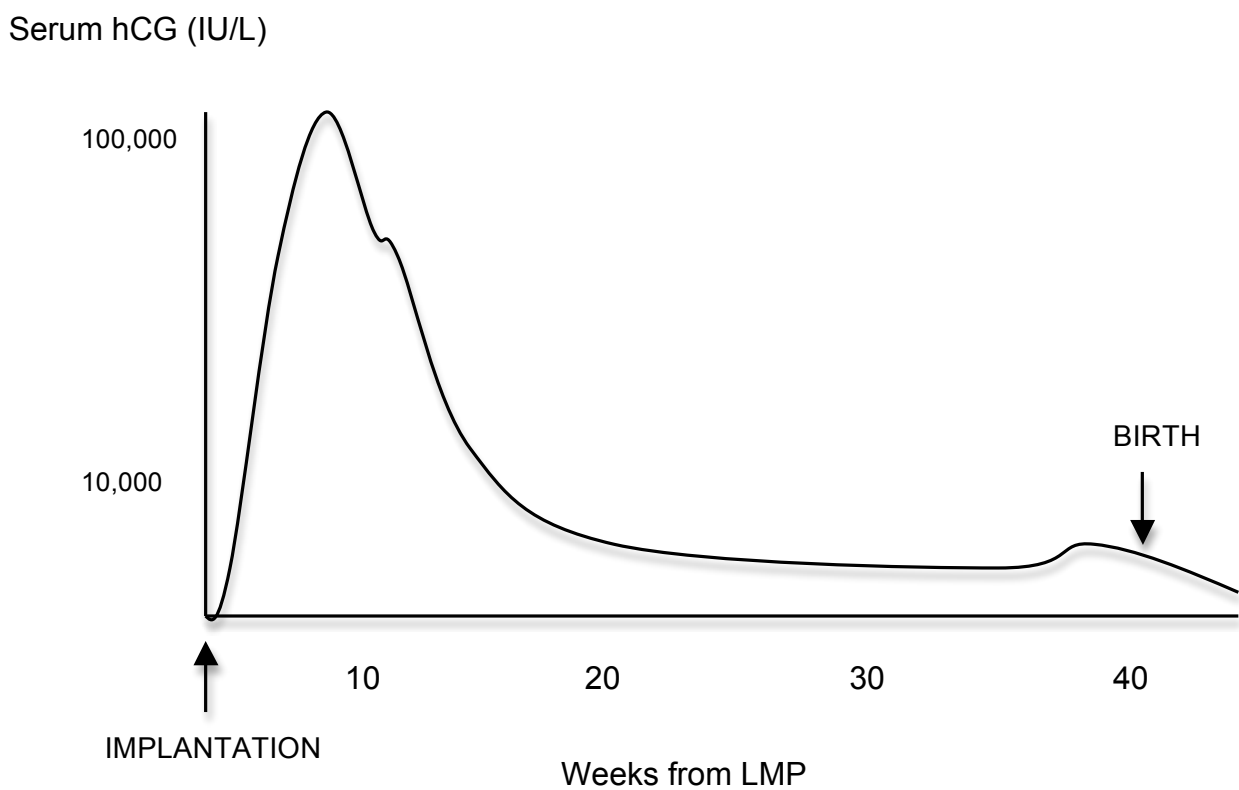
Figure 1. Schematic representation of concentration of human chorionic gonadotrophin (hCG) throughout gestation.

Using antibodies to a linear epitope on the β-hCG-COOH terminus, a hyperglycosylated (H-hCG) form of standard or regular hCG has been identified as the predominant structure in early pregnancy (Sasaki *et al.,* 2008). H-hCG differs from hCG in both the branching and sialic acid content of N- and O-linked oligosaccharides (Elliott *et al.*, 1997; Cole *et al.,* 2003). H-hCG is a product of stem cytotrophoblast cells whereas hCG is produced by syncytiotrophoblasts (Kovalevskaya *et al.,* 2002a; Cole *et al.,* 2006). The expression of H-hCG as a proportion of total hCG declines rapidly in early pregnancy from 92% of total hCG at 3 weeks gestation to <2% total hCG by the second trimester (Cole *et al.,* 2003). The relative proportion of H-hCG to total hCG has been shown to be clinically significant. In particular implantation has been shown to depend upon H-hCG. Low H-hCG levels are predictive of early pregnancy loss (Sutton-Riley *et al.,* 2006) and high concentrations are seen in choriocarcinoma (Khanlian *et al.,* 2003).

Inhibins

Inhibins are heterodimeric glycoprotein hormones consisting of disulfide-linked alpha and beta subunits. Inhibin A has a molecular weight of 32kd and is composed of inhibin α and β_A subunits. It is produced by the corpus luteum during the luteal phase of the ovarian cycle (Muttukrishna *et al.,* 1994) and in early pregnancy (Illingworth *et al.,* 1996) and is also produced by the syncytiotrophoblast in early pregnancy (Birdsall *et al.,* 1997). There is conflicting evidence about which is the major source of inhibin production in early pregnancy. Santoro *et al.,* (1992) looked at inhibin levels in women with premature ovarian failure and donor in vitro fertilisation (IVF) pregnancies i.e. aluteal women. They showed that there was no early rise in inhibin as seen in normal pregnancies, although the levels did reach normal levels towards the end of the first trimester. Lockwood *et al.,* (1997) examined this further by comparing blood samples of women who became pregnant following IVF with fresh embryo transfer i.e. luteal women, with those who became pregnant following IVF with frozen embryo transfer i.e. aluteal women. They concluded that there was no difference in luteal and aluteal pregnancies and that therefore the fetoplacental unit must be the major source of inhibin A in early pregnancy.

Treetampinich *et al.*, (2000) however found that in IVF cycles inhibin A levels were significantly lower in the absence of functioning ovaries and in natural cycles compared with concentrations after ovarian stimulation. They also found that inhibin A concentrations were not significantly different between singleton and multiple pregnancies and therefore concluded that the corpus luteum is the major source of circulating inhibin A in early pregnancy.

In 2002 Muttukrishna *et al.*, found decreased maternal serum levels of inhibin A in pregnancies that went on to miscarry and in 2006 Hwang *et al.*, showed a significant association between the number of fetuses and maternal inhibin A levels in a study of singleton and multiple pregnancies following IVF and embryo transfer. These also confirm that the trophoblast is the major source of inhibin A after the luteo-placental shift in early pregnancy.

Circulating inhibin A levels are at detectable levels by 4 weeks gestation (Lockwood *et al.*, 1997) and climb to a peak at 8-10 weeks of gestation (Tovanabutra *et al.*, 1993; Illingworth *et al.*, 1996, Phupong, Hanprasertpong & Honsawek, 2008). Levels then fall slightly at 16 weeks and thereafter increase progressively to maximal concentrations in week 36 (Fowler *et al.*, 1998). The clearance of inhibin A is fast with a short half-life of around 45 minutes (Muttukrishna *et al.*, 1997). Inhibin A is thought to be involved in regulating placental hCG production by inducing changes in gonadotrophin-releasing hormone (GnRH) secretion (Petraglia *et al.*, 1987). It is also thought to play a part in the cell signalling and therefore possibly trophoblast invasion (Debieve *et al.*, 2000). Animal studies have also suggested a role of inhibin A in maintaining luteal progesterone output (Webley *et al.*, 1994). Its function in humans however, remains unclear. It has a shorter half-life than either hCG or progesterone and therefore may be more sensitive at reflecting changes in the trophoblast. Lower levels have already been demonstrated in women with biochemical pregnancies and missed miscarriages (Glennon Phipps *et al.*, 2000; Muttukrishna *et al.*, 2002). In women with induced pregnancy termination, inhibin A levels have been shown to fall after the administration of misoprostol, which interrupts trophoblastic blood flow and leads to expulsion of the pregnancy (Lahiri *et al.*, 2003). Illingworth *et al.*, (1996) examined inhibin A levels in ectopic pregnancies, complete and incomplete miscarriages, and ongoing pregnancies, and found no significant differences between the groups.

Phipps *et al.,* (2000) found that among dual biomarker combinations for differentiating viable from nonviable pregnancies, the addition of inhibin A to progesterone improved the specificity but not sensitivity of the test. A more recent pilot study (Johns *et al.,* 2007) however, using logistic regression analysis, found that inhibin A alone is the best predictor of first trimester miscarriage. Kirk *et al.* (2009) found that serum inhibin A levels may be of some use in predicting failing PULs and IUPs in the PUL population.

Inhibin pro-αC, a pre-cursor protein of the inhibin α subunit, circulates as a functionally inactive monomer and as part of high molecular weight functional dimers. Inhibin pro-αC-related immunoreactivity (inhibin pro-αC-RI) is a cumulative measurement of monomeric pro-αC subunit and pro-α containing inhibins (Illingworth *et al.,* 1996). Inhibin pro-αC-RI has been found to peak at around day 16 after conception and then fall to a nadir at 16 weeks of gestation, before increasing to a second peak at 36 weeks (Illingworth *et al*., 1996; Fowler *et al.,* 1998). Despite the secondary rise from the early second trimester, absolute levels are at their highest in very early pregnancy (Fowler *et al.,* 1998). In their study of 334 women undergoing IVF, Tong *et al.* (2004) found that this early peak in circulating inhibin pro-αC in very early pregnancy was a consistent and specific feature of clinical pregnancy. Illingworth *et al.* (1996) also found that inhibin pro-αC-RI concentration was an indicator of continuing pregnancy viability, better than either hCG or inhibin A.

In their study of aluteal and luteal pregnancies Lockwood *et al.* (1997) compared serial levels of pro-αC-RI in early pregnancy in these two groups. They found that pro-αC-RI was significantly higher in those pregnancies with multiple corpora lutea compared to those with single corpora lutea and significantly lower in those women with conceptions from frozen embryos i.e. aluteal than those with fresh embryos i.e. luteal. They therefore concluded that the corpus luteum must be the major source in early pregnancy. Fowler *et al.,* (1998) identified a small rise in inhibin A levels and a peak of hCG coinciding with the fall in maternal venous pro-αC concentrations after week 9. These are likely to reflect the luteal to placental shift in support for the pregnancy. Inhibin pro-αC is secreted by the placenta into the fetal circulation at term, and is thought to play a role as a paracrine and endocrine regulator of placental

function (Riley *et al.,* 2000). IVF protocols involving complete ovarian suppression (and therefore absence of luteal tissue) are compatible with successful pregnancy (Lockwood *et al.,* 1998), demonstrating that Inhibin pro-αC is not, however, essential for successful pregnancy.

The early increase in inhibin pro-αC-RI in human pregnancy (Illingworth *et al.,* 1996, Lockwood *et al.,* 1997) supports findings from animal models. Webley *et al.,* (1994) found that in the marmoset monkey circulating inhibin pro-αC-RI were significantly elevated above 'normal' luteal phase concentrations as early as 5 days after ovulation. Measurements of pro-αC-RI concentrations could thus serve as a useful marker of luteal sufficiency during the establishment of a pregnancy.

Steroids

Progesterone

Progesterone is a C-21 steroid hormone derived from cholesterol. It is one of the primary products of the corpus luteum and plays a pivotal role in the establishment and maintenance of pregnancy (Spencer & Bazer, 2004). Progesterone acts on the uterus to stimulate and maintain uterine functions that are permissive to early embryonic development, implantation, placentation and successful fetal and placental development to term. These include endometrial secretory changes, endometrial decidualization, myometrial quiescence, production of a number of endometrial proteins (such has PAPP and PP14), and suppression of matrix metalloproteinases (Schindler, 2004). Recent evidence suggests that the action of progesterone in the endometrium is complex, acting directly (endocrine) and indirectly (paracrine) through both the induction and inhibition of gene expression (Lessey, 2003).

Although progesterone originates almost entirely from the corpus luteum before 7 weeks of gestation, its production shifts more to the placenta after the seventh week, and placental progesterone is sufficient to maintain the pregnancy from this point onwards. From 9 weeks gestation the trophoblast is the dominant source of progesterone (Csapo *et al.,* 1973). Progesterone concentrations in

the maternal blood are less than 2 nmol/L during the follicular phase of the normal menstrual cycle (Abraham *et al.,* 1972; Lindberg *et al.,* 1974) rising to 2-4nmol/L on the day of the LH surge and rising to a plateau of 20-70 nmol/L over the subsequent 7 days. Concentrations rise until 7 weeks of gestation and then remain at a plateau until 10 weeks of gestation, from when concentrations gradually increase to term (see Figure 2). At term, progesterone concentrations can range from 200 to 600 nmol/L (Tulchinsky *et al.,* 1972). Most of the progesterone in the maternal circulation is metabolised to pregnanediol and is excreted in the urine as glucuronide.

The ability of antiprogesterone agents to induce abortion confirms progesterone's crucial role in the maintenance of pregnancy (Baulieu, 1989). It is thought that it may act by inhibiting T-lymphocytic cell-mediated responses involved in tissue rejection. Studies of human trophoblast implanted into rodents showed that progesterone promoted survival, and thus suggested that it is progesterone that blocks the cellular response to foreign antigens (Siiteri *et al.,* 1977). Progesterone is known to be a potent inhibitor of leukaemia inhibitory factor (LIF) which itself plays an important role in trophoblastic invasion (Sunder & Lenton, 2000). Synthetic progestagens have been shown to upregulate nitric oxide synthase in the endometrium and nitric oxide appears to play a role in the maintenance of uterine quiescence (Cameron & Campbell, 1998).

Progesterone production in early pregnancy reflects the dynamics of the corpus luteal-trophoblast axis and the status of the trophoblastic tissue. As progesterone has a shorter half-life than hCG, the progesterone level will reflect any change in the dynamics of the pregnancy earlier. In their prospective cohort study Plante *et al.,* (2008) found that of the single biomarkers, progesterone had the greatest diagnostic accuracy in predicting pregnancy viability.

Serum Progesterone
(nmol/L)

BIRTH

200

100

10   20   30   40

IMPLANTATION

Weeks from LMP
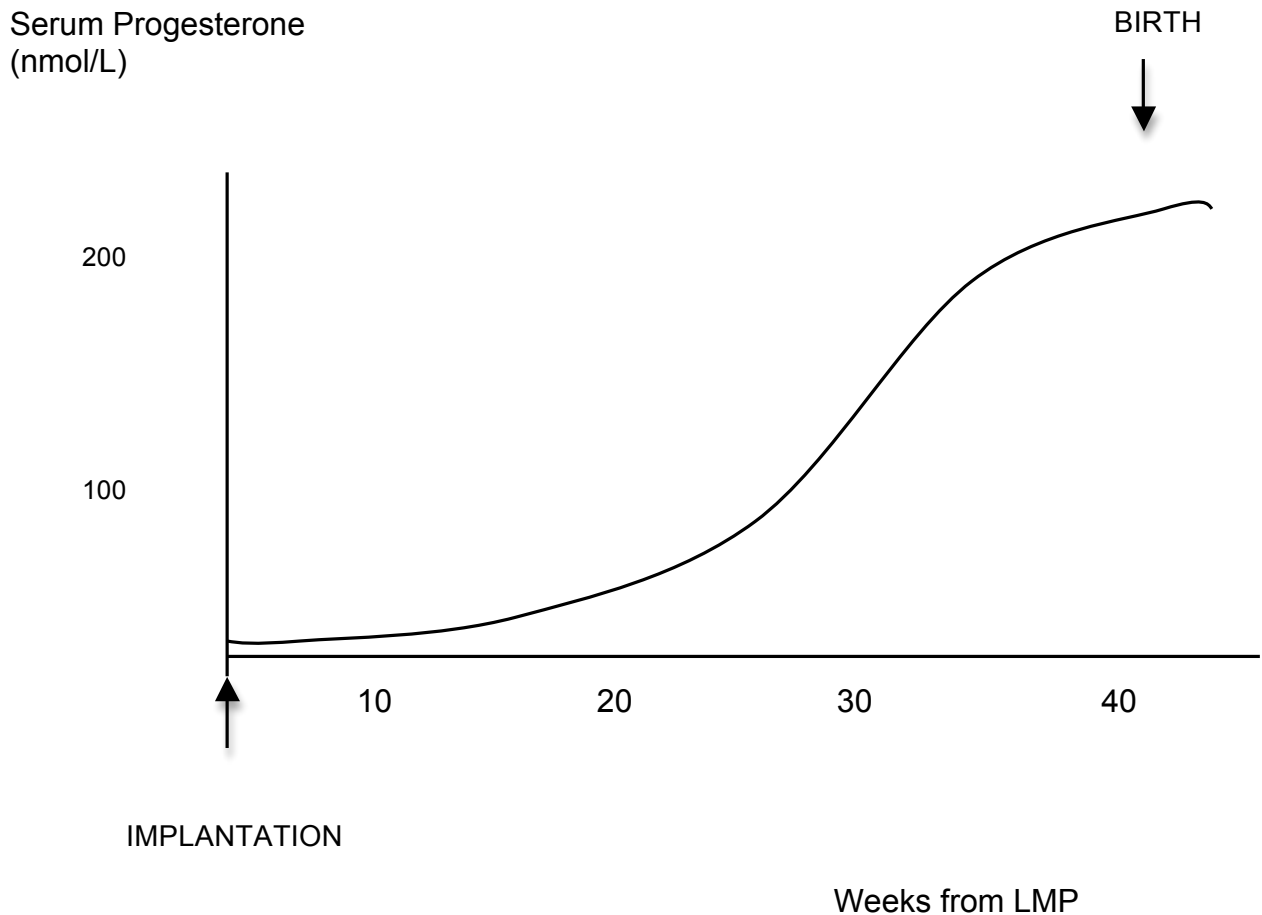
Figure 2. Schematic representations of concentrations of progesterone during the course of human pregnancy.

17 α-hydroxyprogesterone

17 α-hydroxyprogesterone (17-OHP) is a steroid secreted in parallel to progesterone from the corpus luteum (Tulchinsky *et al.,* 1972). The plasma concentration of 17-OHP rises steeply following conception to levels of 2.6 ng/mL in the third week of pregnancy to 5.8ng/mL at the fifth week and then declines to reach a nadir in the 13th week. 17-OHP values reflect corpus luteum function, since the placenta does not have 17 α-hydroxylase to participate in the production of this metabolite (Schindler, 2004). The importance of the corpus luteum in early pregnancy has been known for a long time, supported by the clinical finding that removal of the corpus luteum in early pregnancy (before the 8th week of gestation) is followed by miscarriage (Marthy *et al.,* 1970). Little is known of the functional role of 17-OHP but levels have been shown to be lower

in nonviable intrauterine pregnancies and ectopic pregnancies in a small number of studies (Check *et al.,* 1990; Choe *et al,* 1992; Hubinont, Thomas & Schwers, 1987).

Placental Growth Factors

Insulin-like growth factor binding protein 1 (IGFBP-1) is one of six proteins that specifically binds insulin like growth factors (IGFs) in body fluids and tissues (Shimasaki & Ling, 1992). Insulin-like growth factors (IGF-I and IGF-II), their receptors (IGF-Rs) and IGF-binding proteins (IGFBPs) have vital roles in the regulation of proliferation, differentiation, migration, survival and specific functions of many cell types (Jones & Clemmons, 1995). IGFBPs prolong the half-life of IGFs and modulate IGF activities and bioavailabilities. IGFBP-1 contains 234 amino acids and has a molecular mass of 25kd. The human IGFBP-1 gene is located on chromosome 7 and IGFBP-1 (also known as placental protein 12) is synthesised in large amounts by the decidua of early pregnancy (Rutanen, 1992). It is the predominant IGFBP in amniotic fluid and a major insulin-like growth factor (IGF) binding species in fetal plasma (Drop *et al.,* 1984). It is one of the most important decidual secretory products, with important roles at the embryo-maternal interface in the regulation of placental development, embryo implantation and fetal growth.

The concentration of IGFBP-1 in the maternal circulation increases during pregnancy. There is a rapid rise in the first trimester reaching a peak at 12-16 weeks and levels then decrease after 33 weeks. (Bell, 1988; Crossey *et al.,* 2002). Concentrations are particularly high in decidualized stromal cells and IGFBP-1 is one of the best markers of decidualization (Dunn *et al.,* 2003). In the fetus IGFBP-1 is produced in the liver and pancreas. The exact function of this protein is unclear but it may be present to modulate the potent growth induction by IGF-I, an agent known to be up-regulated by oestrogen (Murphy *et al.,* 1987). It has also been suggested that IGFBP-1 modulates trophoblast invasiveness, acting as a barrier to trophoblast migration (Irwin & Giudice, 1998). Any disruption to this interface may therefore theoretically be reflected in the levels of IGFBP-1. IGFBP-1 is a local modulator of IGF action in fetal

growth (Ben Lagha *et al.,* 2006) and it is also able to mediate progesterone-induced decidualization (Matsumoto *et al.,* 2008).

Two hypotheses exist for the action of IGFBP-1 in early placentation. One is that higher levels of IGFBP-1 inhibit binding of trophoblast to the decidual cells (Irwin & Giudice, 1998). The second is that there is over-production of IGFBP-1 by the deciduum in response to defective implantation. In their study investigating the biochemical markers in the prediction of successful expectant management of miscarriage, Elson *et al.,* (2005a) found that the presence of a raised level of IGFBP-1 was associated with an increased chance of successful expectant management. This suggests that the high level reflect a defect in attachment of the trophoblast to the decidua thus resulting in an increased chance of the retained products being expelled spontaneously. Salim *et al.,* (2004) have subsequently found that women at high risk of miscarriage have higher level of IGFBP-1 in uterine flushings from peri-implantation endometrium, suggesting that abnormal trophoblast invasion may be a mechanism for pregnancy loss in these women.

IGFBP-1 is also being investigated as a marker of obstetric complications. Low levels of IGFBP-1 in cervical secretions are associated with a low-risk of pre-term delivery (Balic *et al.,* 2008) and IGFBP-1 genes have been found to be upregulated in IUGR placentas (Okamoto *et al.,* 2006).

2.1.3   *Ultrasound in early pregnancy*

High resolution transvaginal ultrasonography (TVS) is now the method of choice when assessing early pregnancy and has been shown to be superior to transabdominal ultrasound in diagnostic accuracy (Cacciatore *et al.,* 1989) and the use of TVS has revolutionised the management of early pregnancy problems. Recent RCOG guidelines recommend that all early pregnancy assessment units (EPAUs) should have access to TVS with staff appropriately trained in its use (RCOG, 2006).

The gestational sac is the first pregnancy structure that can be detected by ultrasound. It is usually visualised from 4^{+3} weeks gestation onwards when it

measures 2-3 mm in diameter (Yeh *et al.,* 1986). The gestational sac includes the chorionic cavity, a rim of invading chorionic villi and the underlying decidual reaction. It is usually located eccentrically in the upper part of the uterine cavity and grows approximately 1 mm in diameter per day changing its shape from being round (up to 1 cm in size) to more elliptical thereafter. The early gestational sac contains two separate fluid filled compartments: the amniotic and exocoelomic (chorionic) cavity. In very early pregnancy the exocoelomic cavity predominates. From 8 weeks the amniotic cavity expands rapidly and soon occupies most of the gestational sac. By the end of the first trimester the amniotic and chorionic membranes fuse resulting in the complete obliteration of the exocoelomic cavity. Normal ranges for gestational and amniotic sac size in early pregnancy have been established.

The yolk sac is first seen during the fifth week as a circular, well-defined, echo-free area within the gestational sac measuring 3-4 mm in diameter. The yolk sac grows slowly until it reaches a maximum diameter of 6 mm at 10 weeks gestation (Jauniaux *et al.,* 1991). It is the yolk sac that plays a major role in the nutrition of the early embryo. The yolk sac floats within the exocoelomic cavity and has been shown to have absorptive epithelia. It is thought that nutrients of maternal origin absorbed by the trophoblast pass into the coelomic fluid and are absorbed by the yolk sac (Burton, Hempstock & Jauniaux*,* 2001).
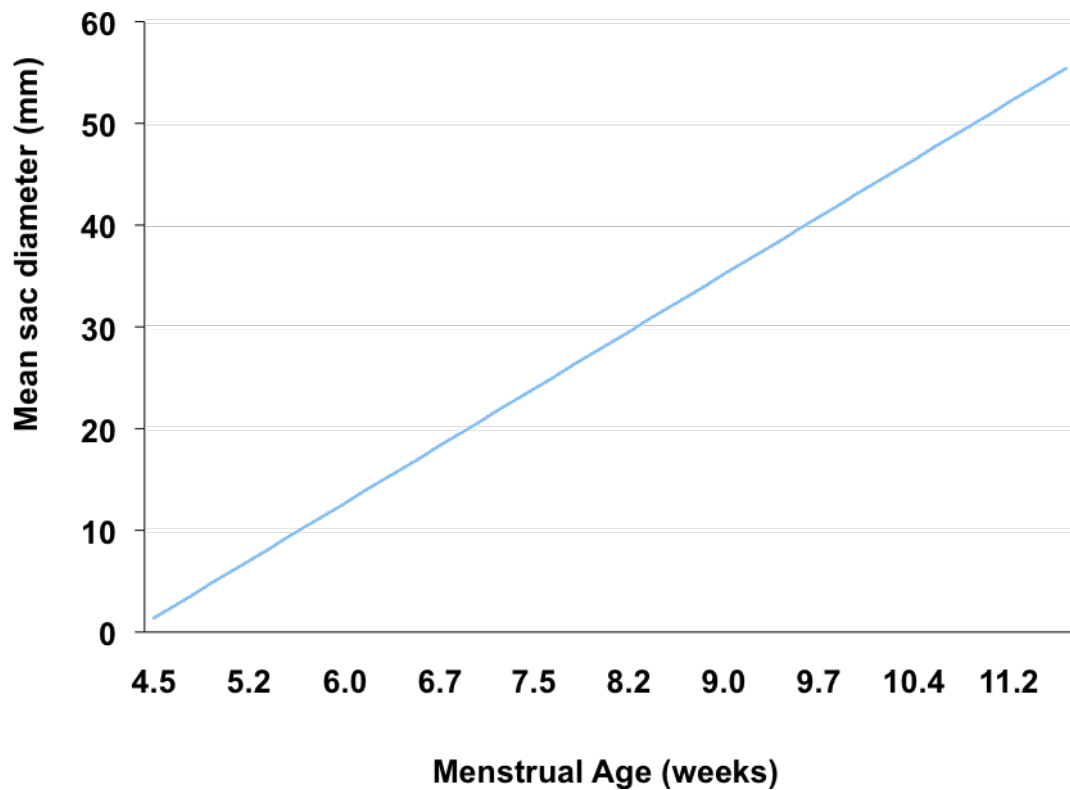
Figure 3. Gestational sac size correlated with menstrual age during the first 12 weeks (Nyberg *et al.,* 1987).

The embryo can first be demonstrated on TVS at the beginning of the sixth week, when it measures around 2 mm. It is first seen as a straight echogenic line, adjacent to the yolk sac and close to the connecting stalk. The cardiac activity begins at approximately day 37 (5^{+2} weeks) menstrual age (Bree *et al.,* 1989). This corresponds to the crown-rump length of 1.5-3 mm. When the embryo reaches 5 mm in length it can consistently be seen separate from the yolk sac and all embryos of that size should have visible cardiac activity. This corresponds to 6^{+3} weeks gestational age and the sac diameter should measure 15-20 mm. The embryo grows at around 1 mm per day and the average crown-rump length is 12 mm at 7^{+3} weeks and 20 mm at 9 weeks.

Gestation	Key landmarks
5 weeks	Empty gestational sac (mean diameter 10 mm)
5 ½ weeks	Gestational sac with yolk sac visible
6 weeks	Gestational sac (mean diameter 16 mm) and yolk sac with adjacent heart beat but small embryo (3 mm)
6 ½ weeks	Embryo with crown-rump length of 6 mm with heart beat visible (rate 125 bpm)
7 weeks	Embryo with crown-rump length of 10 mm with heart beat visible (rate 150 bpm)
8 weeks	Embryo with crown-rump of 16 mm with separate amniotic sac and coelomic cavity with yolk sac. Fetal body movements visible, heart rate 175 bpm.

Table 1. Key chronological landmarks in the development of the embryo, as seen on transvaginal ultrasound examination (Hately *et al.,* 1995).

2.2 EARLY PREGNANCY PROBLEMS

2.2.1   *Initial assessment*

Early pregnancy failure is a large burden on health services because of its high incidence and complex clinical management, which often requires the use of multiple diagnostic tests and in-patient surgical treatment. Assessment of early pregnancy is indicated in women with clinical symptoms suggestive of miscarriage or ectopic pregnancy and in asymptomatic women who have experienced miscarriage in the past and those at high risk of ectopic pregnancy (Ankum *et al.,* 1996). Assessment tools include ultrasonography and measurement of biochemical markers (see Figure 4).

Figure 4. Basic diagnostic algorithm for early pregnancy loss (RCOG 2006)

USS – TAS / TVS

'Pregnancy of Known Location'

'Pregnancy of Unknown Location'

Intrauterine pregnancy

Ectopic pregnancy

Viable IUP

Diagnostic algorithm for 'PUL'

IUP 'Uncertain viability'

Rescan in 7-10 days

Resolved PUL

Non-viable IUP

KEY
PUL- Pregnancy of unknown location
USS- Ultrasound scan
TAS- Transabdominal scan
IUP- Intrauterine pregnancy
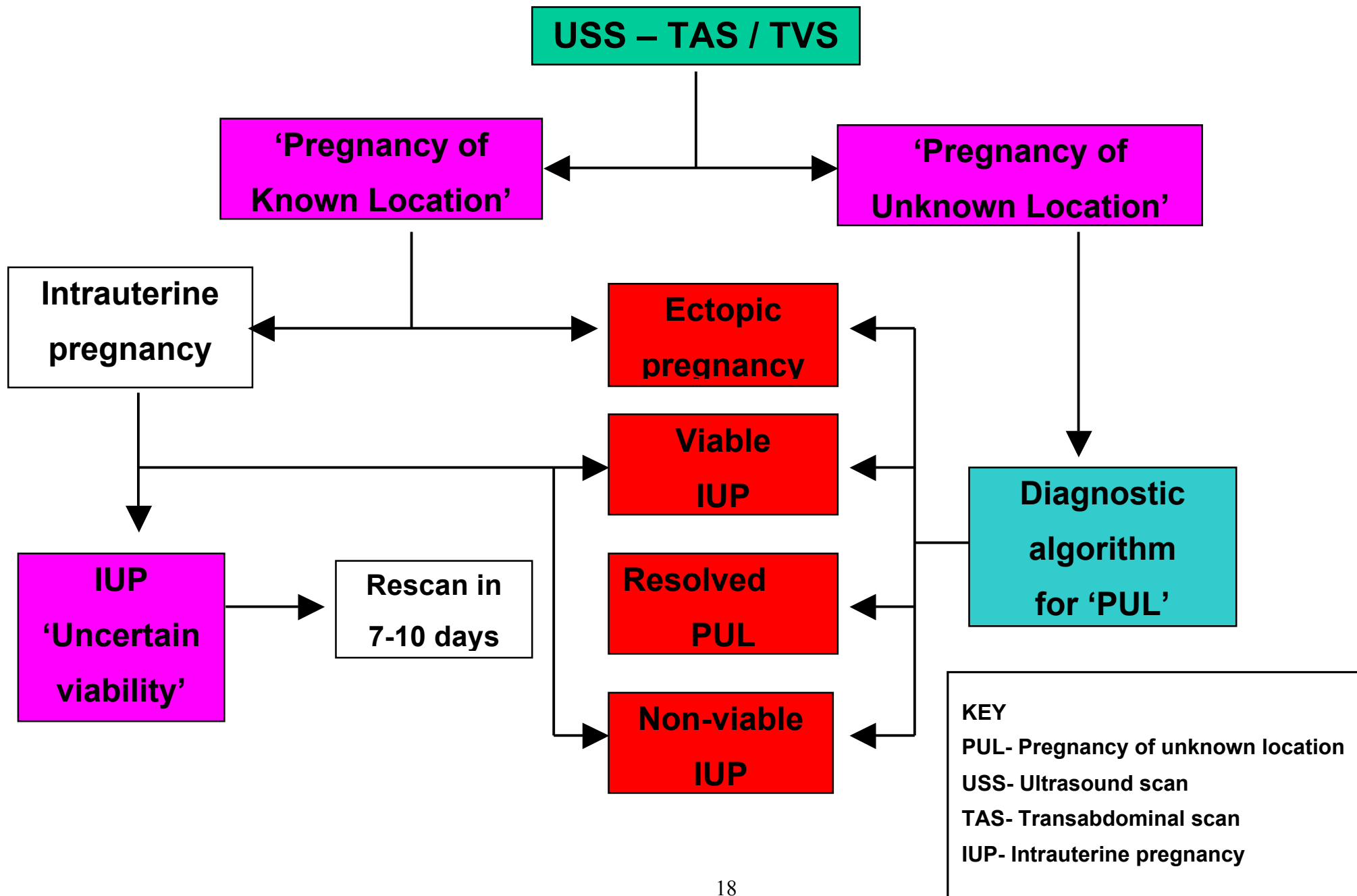
2.2.2 Pregnancy of Unknown Location

Epidemiology and aetiology

In 5-31% of patients presenting to an EPAU it may not be possible to confirm the pregnancy site by TVS at first visit (Banerjee *et al.,* 2001; Hahlin *et al.,* 1995; Banerjee *et al.,* 1999; Cacciatore *et al.,* 1988). A recent consensus statement (Barnhart *et al.,* 2011) has emphasised the importance of consistency in the terminology used for the classification of these initial ultrasound findings. The use of five categories is suggested: definite ectopic pregnancy, probable ectopic pregnancy, PUL, probable intrauterine pregnancy, and definite intrauterine pregnancy.

Pregnancy of unknown location (PUL) is a descriptive term rather than a pathological diagnosis which describes when there is a positive pregnancy test but no evidence of an intrauterine or extrauterine pregnancy on TVS. There are three possible diagnoses: a viable intrauterine pregnancy that is too small to see on TVS, a complete miscarriage where the pregnancy has already been lost, and an ectopic pregnancy. Failing PULs (or trophoblast in regression) account for 44-69% of the PUL population (Hajenius *et al.,* 1995; Banerjee *et al.,* 2001) and are never visualised using transvaginal ultrasonography. An indeterminate proportion of these represent complete miscarriages and self-limiting forms of ectopic pregnancies (Condous *et al.,* 2006).

	Number of cases	Failing PULs (%)	Normal IUPs (%)	Miscarriage (%)	Ectopic Pregnancy (%)
Hajenius *et al*., 1995	265	44	31		26
Banerjee *et al.,* 1999	127	50	27	9	14
Banerjee *et al.,* 2001	104	69	22	2	7
Condous *et al.,* 2005	527	57	34		
Facey *et al.,* 2006	74	69	9.5	9.5	12

Table 2. Comparison of PUL final outcomes

Biochemical markers

Serial measurements of serum hCG are often used in the assessment of pregnancies of unknown location and a large number of women are subjected to invasive diagnostic procedures. An ectopic pregnancy is "diagnosed" if the hCG does not double in 2-3 days as seen in normal intrauterine pregnancies. This approach is based on the assumption that all ectopic pregnancies follow the same abnormal development pattern, however as many as 10% of ectopics develop in the same way as normal intrauterine pregnancies. In addition, a large proportion of ectopics are failing pregnancies, similar to the miscarriage of an intrauterine pregnancy. An abnormal doubling time is therefore neither a sensitive nor specific method for diagnosing ectopic pregnancies (Shepherd *et al.,* 1990). Another approach used to diagnose ectopic pregnancy is the use of a serum cut-off level above 1000 IU/L of hCG at which point a normal,

intrauterine pregnancy should be seen by transvaginal sonography (Cacciatore *et al.,* 1990).

Lower levels of hCG in pregnancies that are destined to fail have been well documented. As hCG production is directly related to the amount of trophoblast present it has been suggested that suboptimal serial changes in hCG may be a more accurate marker of trophoblast viability.

Recently a number of diagnostic models for prediction of resolution of pregnancies of unknown location have been suggested. In 1995, Hahlin *et al.* used a combination of serum progesterone and serial hCG measurements to predict safe expectant management. If the serum hCG change was less than 5% and the progesterone less than 20 nmol/L then the sensitivity of the model was 73% and the specificity 97%. In 1999, Banerjee *et al.,* developed a logistic regression model using the biochemical markers hCG and progesterone along with endometrial thickness and the presence of bleeding. This model had a sensitivity and specificity of 92% in identifying failed pregnancies undergoing spontaneous resolution. Prospective evaluation of both models by Banerjee *et al.,* in 2001 however, showed that using a serum progesterone of less than 20 nmol/L was as accurate as either model (see Figure 5).

The use of hCG ratios (hCG 48 hours/hCG 0 hour) in the prediction of spontaneous resolution of pregnancies of unknown location (PULs) has recently been assessed in a prospective cohort study (Condous *et al.,* 2006). An hCG ratio of <0.87 was found to outperform absolute serum hCG levels with a sensitivity of 92.7% and a specificity of 96.7%. Unfortunately there has not yet been any valid comparison of hCG ratios versus the use of initial hCG with progesterone to suggest which model is more efficacious and cost effective. A recent prospective observational study has looked at hCG ratio versus progesterone in women with PUL (Bignardi *et al*., 2010). This study looked at prediction of viability once an IUP was detected at follow-up scan, and not PUL outcome. HCG ratio was found to perform only slightly better than a single serum progesterone (area under ROC curves 0.756 and 0.678 respectively).

Expectant management of PULs has been shown to be safe, reduce the need for unnecessary surgical intervention and is not associated with serious adverse outcomes (Condous, Okaro & Bourne, 2003). Nevertheless, 9-29% of women

will require surgical intervention due to a worsening clinical condition or non-declining hCG (Hahlin, Thorburn & Bryman, 1995; Banerjee *et al.,* 1999).
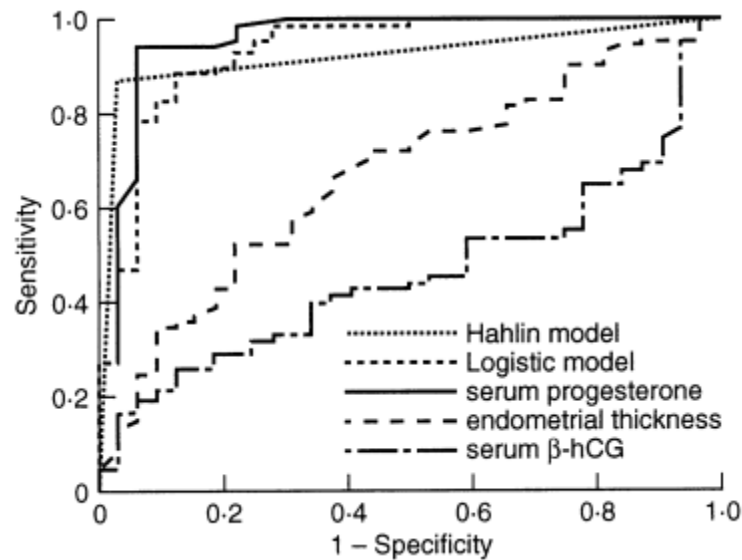
Figure 5. Receiver Operating Characteristics curves demonstrating the performance of Hahlin's and the logistic regression model, serum progesterone, serum β-hCG and endometrial thickness in their ability in predicting correctly which pregnancies will resolve without the need for any intervention (Banerjee *et al*., 2001).

2.2.3   *Miscarriage*

Epidemiology and aetiology

A miscarriage is an intrauterine pregnancy that ends spontaneously before the fetus has reached viability. This is currently defined by the World Health Organisation as the spontaneous expulsion from its mother of a fetus weighing less than 500 g or before 24 weeks of gestation. A missed miscarriage is defined as an anembryonic pregnancy or where there is early fetal demise but

the gestational sac remains *in utero*. An incomplete miscarriage is one where part but not all of the products of conception have been passed from the uterus. A complete miscarriage is one where all of the products of conception have been passed and the uterus is now empty.

Miscarriage is the most common complication of early pregnancy. It has been estimated that the overall miscarriage rate is around 40% (Wang *et al.,* 2003). The majority of these losses occur before the missed menstrual period but bleeding complicates 21% of clinically detected pregnancies and 12-15% are lost (Kline *et al.,* 1989; Nybo Anderson *et al.,* 2000). Miscarriage accounts for 50000 inpatient admissions to hospitals in the UK annually (Bradley & Hamilton-Fairley, 1998).

Failure of spiral artery transformation and inadequate placentation is important in spontaneous miscarriage (Haxton & Bell 1983; Gaillard *et al.,* 1993). Khong *et al.* (1987) and Hustin, Jauniaux & Schaaps (1990) both showed that by examining the histological specimens of spontaneous miscarriages, defective transformation of the spiral arteries, and a reduced trophoblastic infiltration of the decidua could be seen. Colour Doppler studies have shown that at 7-12 weeks of gestation a high resistance to flow in decidual arteries is associated with up to a 43% risk of subsequent miscarriage (Jaffe & Warsof, 1992). Jauniaux *et al.* (1994) found that in missed miscarriages the trophoblastic shell was thinner and discontinuous, and that the intervillous space and endometrium were massively infiltrated with blood. They suggest that this is due to reduction or absence of vascular plugs allowing free access of blood to the intervillous space with subsequent arrest of embryoplacental flow, or retroplacental haemorrhage and abruption. The mechanical cause of most miscarriages is thought to be this premature entry of maternal blood into the intervillous space. The low oxygen state of the early placenta would appear to be necessary for the differentiation of the trophoblast, angiogenesis and protein synthesis and increasing the oxygen flow to the intervillous space would appear to be a factor in early pregnancy failure (Jauniaux *et al.,* 2000).

A woman may present at various stages of the process of miscarriage. Ultrasound classification of miscarriages has superseded clinical classification, which was based on the amount of bleeding and the state of the internal cervical os on examination (see Figure 6).
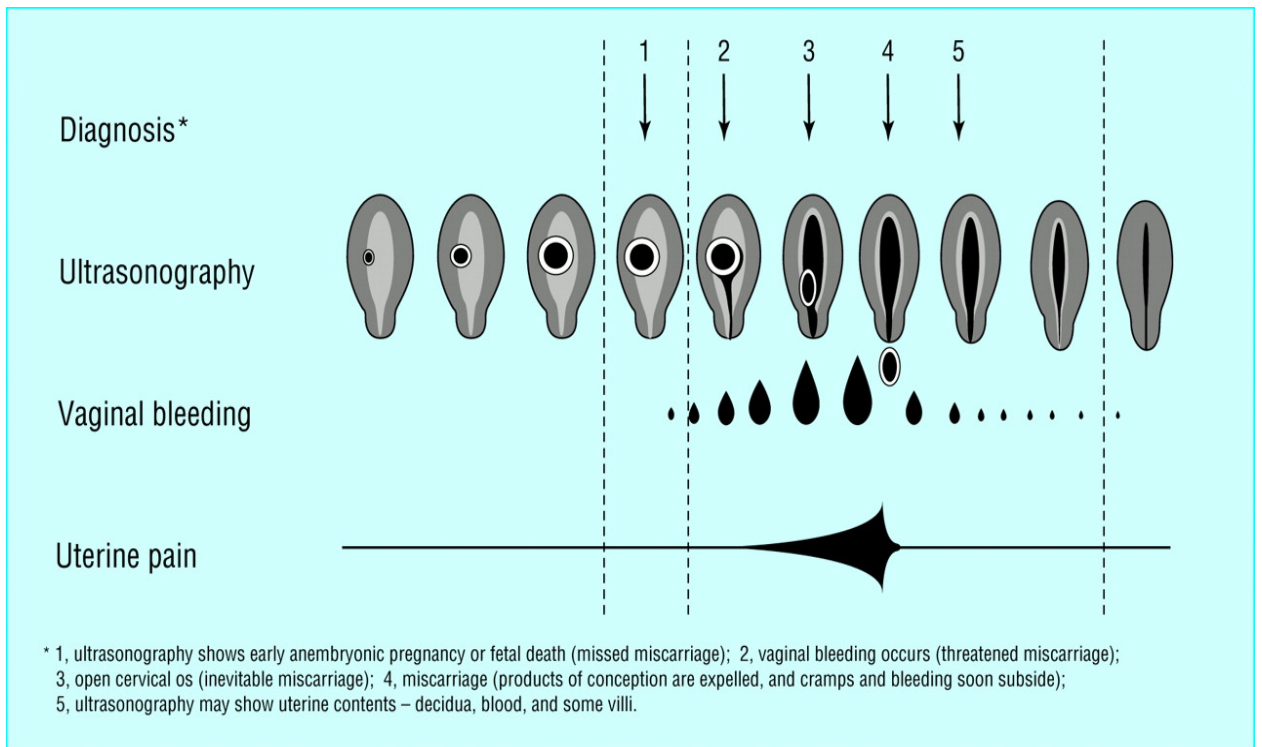
Figure 6. Natural course of miscarriage, with opportunities for intervention as illustrated by Ankum *et al.,* (2001).

Ultrasound findings on initial scan	Action required
Gestational sac of mean diameter >20 mm with no evidence of an embryo or yolk sac	Repeat scan in one week
Crown-rump length >10 mm with no heart action	Repeat scan in one week
Gestational sac <15 mm or crown-rump length <10 mm	Repeat scan in two weeks

Table 3. Guidelines for establishing the death of an embryo by ultrasound (Hately *et al.,* 1995)

Biochemical markers

Biochemical markers have been used to predict early pregnancy outcome. A number of small studies have also shown low levels of progesterone in nonviable pregnancies (see Table 4) and a progesterone level of less than 25 nmol/L in an anembryonic pregnancy has been shown to be diagnostic of nonviability (see Table 5).

Authors	Diagnosis	Cut-off level of progesterone for viability (nmol/L)	Specificity
Hahlin, Sjoblom & Lindblom (1991)	Miscarriage/ectopic	30 nmol/L	77%
Cunningham *et al.,* (1993)	Asymptomatic Symptomatic	45 nmol/L 30 nmol/L	91% 94%
Stewart, Nazar-Stewart & Toivola (1995)	Miscarriage/ectopic	24 nmol/L	88%
Aksoy *et al.,* (1996)	Miscarriage	36 nmol/L	90%

Table 4. Comparison of the cut-off levels of progesterone used in the diagnosis of early pregnancy failure

Authors	Cut-off level of Progesterone for viability (nmol/L)	Specificity	Sensitivity
Riss *et al.,* (1989)	48 nmol/L	83%	87%
Elson *et al.,* (2003)	25 nmol/L	40%	100%

Table 5. Comparison of the cut-off levels of progesterone used in the diagnosis of viability in anembryonic pregnancy

Studies have demonstrated the variable expression of a single glyco-isoform of hCG in early successful and failed pregnancies. These studies use specific antibodies to C5 hCG (monoclonal antibody B152) which is a hyperglycosylated structure related to choriocarcinoma hCG. Relatively high levels of this isoform hyperglycosylated hCG (H-hCG) have been described in the first 5-6 weeks gestation, with a subsequent decline in expression as pregnancy progresses but which persists in failing pregnancy (Sutton Riley *et al.,* 2006). Whether over expression of H-hCG is an isolated phenomenon or is associated with variable expression of other isoforms is not known.

Management

Diagnosis of miscarriage has traditionally been followed by surgical curettage, on the assumption that this decreases the risk of subsequent gynaecological infection. Surgical management is not, however, without complications (Chung *et al.,* 1999) and a recent randomised control trial has shown that the incidence of gynaecological infection after first trimester miscarriage is low (2-3%) and does not differ by the method of management (Trinder *et al.,* 2006). Surgical evacuation does remain the treatment of choice if endometrial thickness is >50 mm, bleeding is excessive, vital signs are unstable or infected tissue is present

in the uterine cavity (in which case surgery must be performed with antibiotic cover) (Sagili & Divers, 2007). Fewer than 10% of women who miscarry fall into these categories (Ballagh *et al.,* 1998).

Alternative management options include medical management and expectant management. Medical management uses drugs to aid expulsion of retained products. It is the preferred choice of women over surgical management (Demetroulis *et al.,* 2001), and there are cost savings (Petrou *et al.,* 2006). Trials have reported widely varying success rates, from 13% to 93% (De Jonge *et al.,* 1995; Sahin *et al.,* 2001). Comparisons are limited by the use of different drug regimens and different measures of success. No statistical difference in efficacy has been shown between surgical and medical management in incomplete miscarriage (Muffley *et al.,* 2002) and in women with incomplete miscarriages medical treatment has not been shown to offer any benefits over expectant management (Nielsen*,* Hahlin & Platz-Christensen, 1999).

Expectant management follows the natural history of the condition, allowing the spontaneous passage of retained products of conception. It avoids the iatrogenic problems associated with both medical and surgical treatment and is the most cost-effective (Petrou *et al.,* 2006). In incomplete miscarriage expectant management has been shown to be very effective. Luise *et al.,* (2002) showed a 91% success rate and Nielsen & Hahlin (1995) found that expectant management resulted in complete uterine evacuation over three days in 79% of cases of incomplete miscarriage. Efficacy was reduced to 37% after seven days when expectant management was used to treat women with miscarriage mostly diagnosed as early fetal demise (Wieringa-de Waard *et al.,* 2002). A recent prospective observational study has shown that after just 2 weeks of expectant management, spontaneous resolution had occurred in 71% of incomplete miscarriages, 53% of "empty sacs" and 35% of missed miscarriages (Casikar *et al.,* 2010).

Attempts have been made to determine parameters which predict the success of expectant management. Schwarzler *et al.* (1999) examined 108 patients with missed miscarriages, of which 78 underwent expectant management. They used colour Doppler to examine the intervillous blood flow and found that those with intervillous blood flow were more likely to undergo spontaneous

miscarriage than those without. By examining the patients' characteristics with logistic regression analysis, they showed that both hCG and progesterone were able to predict spontaneous miscarriages. However only progesterone was statistically significant. Nielsen, Hahlin & Oden*, (1996) designed a logistic regression model to identify women with first trimester spontaneous miscarriages suitable for expectant management. They used data from 103 women undergoing expectant management of incomplete miscarriages of which 81 completed spontaneously and 22 went on to have surgical management. Those patients who miscarried completely were more likely to have lower serum progesterone, 17-OHP, and hCG levels. They also had a significantly smaller intrauterine diameter of retained products of conception. They created a logistic regression model involving progesterone, hCG and intrauterine diameter measured by ultrasound scan. This model had a 98% positive predictive value (PPV) and 44% negative predictive value (NPV) for the probability that no more than 2% of women would undergo surgical management, and a 90% PPV and 67% NPV that 80% of the women with the highest probability of complete miscarriage were managed expectantly. The time limit allowed for expectant management was only three days. A recent study of 54 women (Elson *et al.,* 2005a) has shown that by using some of these markers (including IGFBP-1, inhibin A, and inhibin pro αC) they could predict the appropriate management for 81% of women with miscarriages (see Figure 7). These data need to be confirmed to ensure that it is appropriate for general clinical use.

Eventual Outcome for Miscarriages
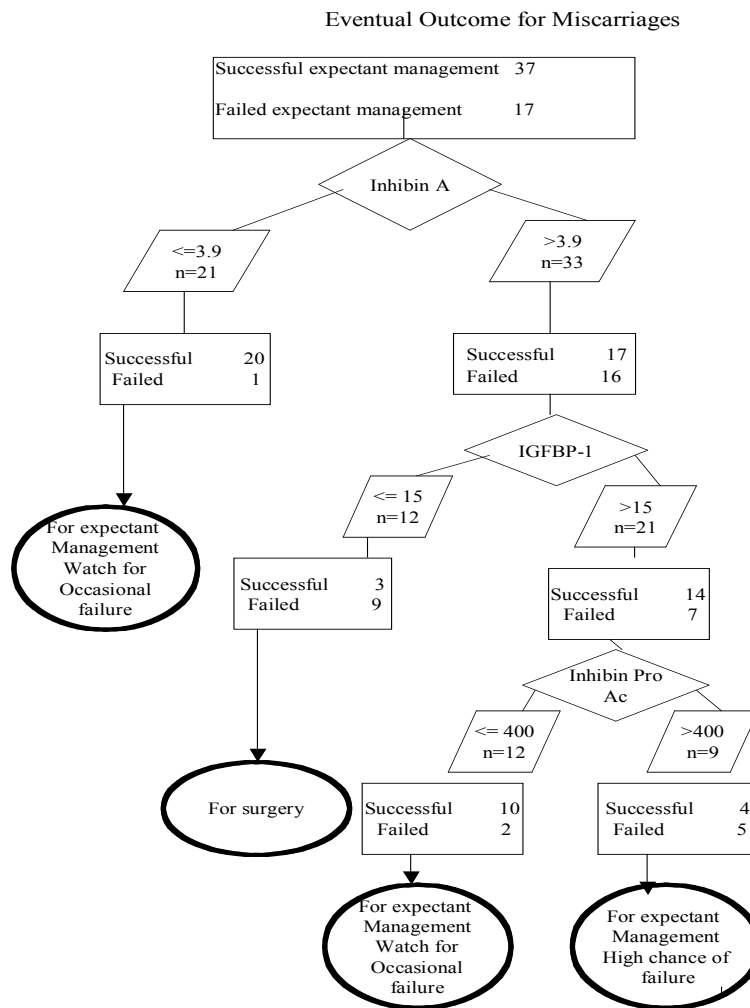
Figure 7. Decision tree analysis for expectant management of miscarriage (Elson *et al.,* 2005a)

2.2.4 Ectopic pregnancy

Epidemiology and aetiology

An ectopic pregnancy is defined as implantation of the fertilised ovum outside the uterine cavity. In the UK during the period 2006-2008 more than 35000 ectopic pregnancies were estimated to have occurred and there were 6 maternal deaths resulting from ectopic pregnancy (CMACE, 2011). This rate has declined since the last triennial report and is the lowest since figures were first estimated in 1988. Whether this is a trend or an anomaly remains to be seen.

The majority of ectopic pregnancies (95%) are tubal in origin while a small proportion may be interstitial, primary abdominal, ovarian or cervical. 80% of tubal ectopics are located in the ampullary segment of the tube (Wong & Clark, 1968). The clinical presentation is very variable. Diagnosis of all women at risk for ectopic pregnancy should be prompt but it is not always an emergency. A haemodynamically stable woman should be diagnosed before rupture, a goal that can usually be accomplished without laparoscopy. For women who present in shock, immediate surgery is both diagnostic and therapeutic (ASRM, 2006).

Kemp *et al.,* (1999) suggested that the placentation could be the key factor in the difference between viable and nonviable tubal pregnancies. They compared the histology in these two groups and found that the viable ectopic pregnancies were more likely to be implanted on the mesosalpingial side of the tube, that they had deeper trophoblastic invasion and that they had increased villous vascularisation.

Factors with a proven role in increasing the risk of ectopic pregnancies include previous ectopic pregnancy and previous tubal surgery, tubal pathology, *in utero* diethylstilboestrol exposure, previous pelvic inflammatory disease, pregnancy after sterilisation and with intrauterine contraceptive device (IUCD) in situ, and smoking (Marchbanks *et al.,* 1988; Ankum *et al* 1996; Bouyer *et al.,* 2003). In theory an abnormal conceptus could be predisposed to ectopic implantation due to delayed migration but studies have not confirmed an important role for chromosomal abnormalities in the aetiology of ectopic pregnancies (Goddijn *et al.,* 1996).

Biochemical markers

Biochemical markers have been used both to diagnose ectopic pregnancy and in its management. Abnormal implantation leads to reduced levels of βhCG being seen in ectopic pregnancies. Traditionally an ectopic pregnancy is diagnosed if the hCG does not double in 2-3 days as seen in normal intrauterine pregnancies. However, this may also be the case in a failing intrauterine pregnancy. An abnormal doubling time is neither sensitive nor specific method to diagnose ectopic pregnancy (Shepherd *et al.,* 1990). Another approach used to diagnose ectopic pregnancy is a serum cut-off level above 1000-1500 IU/L of βhCG at which point an intrauterine pregnancy should be seen by transvaginal sonography. However this fails to take into account the time to return to normal of serum βhCG levels following miscarriage or the diagnostic accuracy of ultrasound in the presence of uterine anomalies such as fibroids (Barnhart *et al.,* 1999). The serum level of hCG would appear to be higher in those women with deeper trophoblastic invasion into the tubal wall than in those where the ectopic trophoblast is limited to the lumen or tubal mucosa (Natale *et al.,* 2003).

Several studies have demonstrated reduced progesterone and 17-OHP levels in ectopic pregnancies (Hahlin *et al.,* 1991; Choe *et al.,* 1992; Stewart *et al.,* 1995). This is thought to be due to abnormal implantation thus affecting the luteal-placental axis (Sauer *et al.,* 1988). Progesterone and βhCG may therefore be used together with ultrasound for the diagnosis and management of pregnancies of unknown location as these pregnancies are likely to resolve spontaneously regardless of location (Banerjee *et al.,* 1999).

Only one small study has assessed the value of more novel biochemical markers in the diagnosis of ectopic pregnancy. Illingworth *et al.,* (1996) examined eight women with ectopic pregnancies and compared their inhibin A and pro-αC-RI levels with levels of eight women with ongoing intrauterine pregnancies. There was no significant difference between the two groups.

Ultrasound

Traditionally the findings of a positive pregnancy test and an empty uterus seen at the time of ultrasound scan have been synonymous with the presence of an

ectopic pregnancy. However with the use of transvaginal ultrasound, approximately 85% of ectopic pregnancies can be visualised directly (Ofili-Yebovi *et al.,* 2003) and so transvaginal ultrasound has become the single diagnostic tool of choice for ectopic pregnancy. Like laparoscopy, ultrasound does not confer 100% sensitivity for the diagnosis of tubal ectopic pregnancy, however it is safe, inexpensive and non-invasive, is acceptable by women and, in trained hands, is highly reproducible (Condous, 2007).

The following transvaginal ultrasonagraphic criteria are used for the diagnosis of ectopic pregnancy: (1) an inhomogenous adnexal mass ("blob sign") (Condous *et al.,* 2005); (2) an empty extrauterine sac with a hyperechoic ring ("bagel-sign") (Goldstein & Timor-Tritsch, 1995); and (3) a yolk sac of fetal pole with or without cardiac activity in an extrauterine sac. Recent studies using high-resolution sonography have shown the most common morphology to be a solid ectopic pregnancy (Elson *et al.,* 2000) and in a meta-analysis of ten studies, a non-cystic adnexal mass or an inhomogenous mass was diagnostic of an ectopic pregnancy with a sensitivity and specificity of 84.4% and 98.9% respectively (Brown & Doubilet, 1994).

Management

There are three main methods of currently managing an ectopic pregnancy: surgical, medical and expectant. Surgical management is indicated in all haemodynamically unstable patients and in selected other cases. Surgical options include salpingectomy or salpingostomy, done either as an open procedure or laparoscopically. Three randomised trials have shown that laparoscopy is superior to laparotomy in haemodynamically stable patients (Vermesh *et al.,* 1989, Lundroff *et al.,* 1991, Koninckx *et al.,* 1991).

The folic acid antagonist methotrexate has been widely used for the medical treatment of ectopic pregnancies since the late 1980s. Methotrexate inhibits dihydrofolate reductase and so prevents the reduction of folic acid to tetrahydrofolate, a key step in the synthesis of DNA and RNA precursors. Methotrexate therefore leads to interference with DNA synthesis and cell multiplication in the conceptus. It can be given either intramuscularly or by

direct injection into the ectopic pregnancy, either laparoscopically or under ultrasound guidance. In the UK methotrexate is most commonly given as a single intramuscular dose of 50 mg/m^2. Single dose regimens have reported success rates varying from 64-94% (Stovall & Ling, 1993, Stika *et al.,* 1996). Methotrexate treatment is more likely to be successful if the initial hCG level is low. Ransom *et al.,* (1994) also showed that ectopic pregnancies with serum progesterone <10 nmol/L are more likely to be successfully treated by systemic methotrexate injection.

It is now well recognised that not all ectopic pregnancies require treatment as some will resolve spontaneously. Expectant management is becoming increasingly important as the ability to detect small ectopic pregnancies and tubal miscarriages increases. It is important that the ectopic pregnancy is actually visualised to avoid mistakenly managing expectantly live or large ectopic pregnancies where the risk of failure is high.

Large multicentre randomised controlled trials are currently underway in the UK and Netherlands (The METEX study, van Mello *et al.,* 2008) to compare methotrexate and expectant management in haemodynamically stable patients with an ectopic pregnancy and low serum hCG or PUL with low but plateauing hCG concentrations. Results and guidance from these ongoing studies are eagerly awaited.

Studies show that around a quarter of ectopic pregnancies will be suitable for expectant management (Ylostalo *et al.,* 1992; Elson *et al.,* 2004). The selection criteria for expectant management varies but those ectopic pregnancies with a viable fetus or the presence of haemoperitoneum would be considered unsuitable for all but surgical management. In expectant management once the ectopic pregnancy is diagnosed management varies but consists of follow-up with a combination of serial ultrasound scans, hCG and progesterone measurements. The hCG levels are monitored until they drop below 20 IU/L indicating spontaneous resorption of the pregnancy. An increase in the size of the ectopic pregnancy or a rise in the serum hCG levels would be an indication to consider surgery.

Success rates for expectant management vary between 50-100% (Lund, 1955; Sauer *et al.,* 1987). Several attempts have been made to examine the clinical,

ultrasound and biochemical parameters that can predict the success of expectant management. Fernandez *et al.,* (1988) looked at 14 patients with ectopic pregnancies confirmed by laparoscopy. 64% of these resolved spontaneously. This study found that a serum hCG below 1,000 IU/L appeared to be the best marker for successful expectant management. Garcia *et al.,* (1987) reported on 13 women with ectopic pregnancies of less than 4cm in size diagnosed at laparoscopy. Only one case required surgical intervention. They found that serum βhCG, progesterone and oestradiol levels were all below the ranges expected for normal pregnancies but did not describe any threshold levels. Shalev *et al.,* (1995) examined 60 women with laparoscopically diagnosed ectopic pregnancies. They found that the presenting level of hCG, the rate of fall of hCG and the size of the ectopic pregnancy at laparoscopy were significant factors in predicting successful expectant management. They suggested that using a presenting level of hCG of <2,000 IU/L allowed a 60% success rate.

Ylostalo *et al.,* (1992) examined 83 patients, which represented 26% of all their ectopic pregnancies over a 2-year period. They found that around 69% of the 83 cases, or 18% of all ectopic pregnancies, resolved spontaneously. They also used 4 cm as a cut-off for the size of the ectopic pregnancy and included those ectopic pregnancies with a fetal pole and no fetal heartbeat. Whilst they also found that the hCG levels were significantly higher in the group with failed expectant management than those who finally resolved spontaneously, there were cases with high initial values with successful expectant management. No attempt to define whether the morphology of the ectopic pregnancies contributed to the final outcome was examined.

Sauer *et al.,* (1987) compared the biochemical profiles of spontaneously resolving ectopics, viable ectopics and normal intrauterine pregnancies. They found that hCG, progesterone, 17-OHP, and oestradiol were all significantly lower in ectopic pregnancies. By using a low threshold for progesterone of 4 nmol/L, they found that the ectopic pregnancies with progesterone below this level had a shorter time to resolution. There was a high degree of correlation between 17-OHP and progesterone. The fall in progesterone and 17-OHP preceded the fall in hCG levels by 7-29 days.

Cacciatore *et al.,* (1995) examined the sonographic findings and hCG levels in expectantly managed ectopic pregnancies. They found that 69% of 71 patients had spontaneously resolving ectopic pregnancies. They concluded that whilst initial hCG and size of the ectopic pregnancy did not differ between the two groups, a decrease in the size of the ectopic pregnancy by day 7 was a significant predictor.

Elson *et al.,* (2004) found significant differences in demographic, ultrasound and biochemical findings between spontaneously resolving ectopics and those requiring treatment. They devised a decision tree that may be used as a guide to estimate the probability of successful expectant management in individual cases (see Figure 8). This could predict outcome with a probability of 88% in five out of the seven subgroups, which accounted for 69% of the study population. As novel biochemical markers have been shown to be more accurate indicators of the luteal-trophoblastic axis (Elson *et al.*, 2005a) it is likely that they may be useful for predicting the spontaneous resolution of ectopic pregnancies more accurately.

Fertility rates after expectant management have been examined and patients treated in this way have good long-term fertility outcomes with spontaneous pregnancy rates of around 80% (Carp *et al.,* 1986). The risk of repeat ectopic pregnancies is low, around 4%.
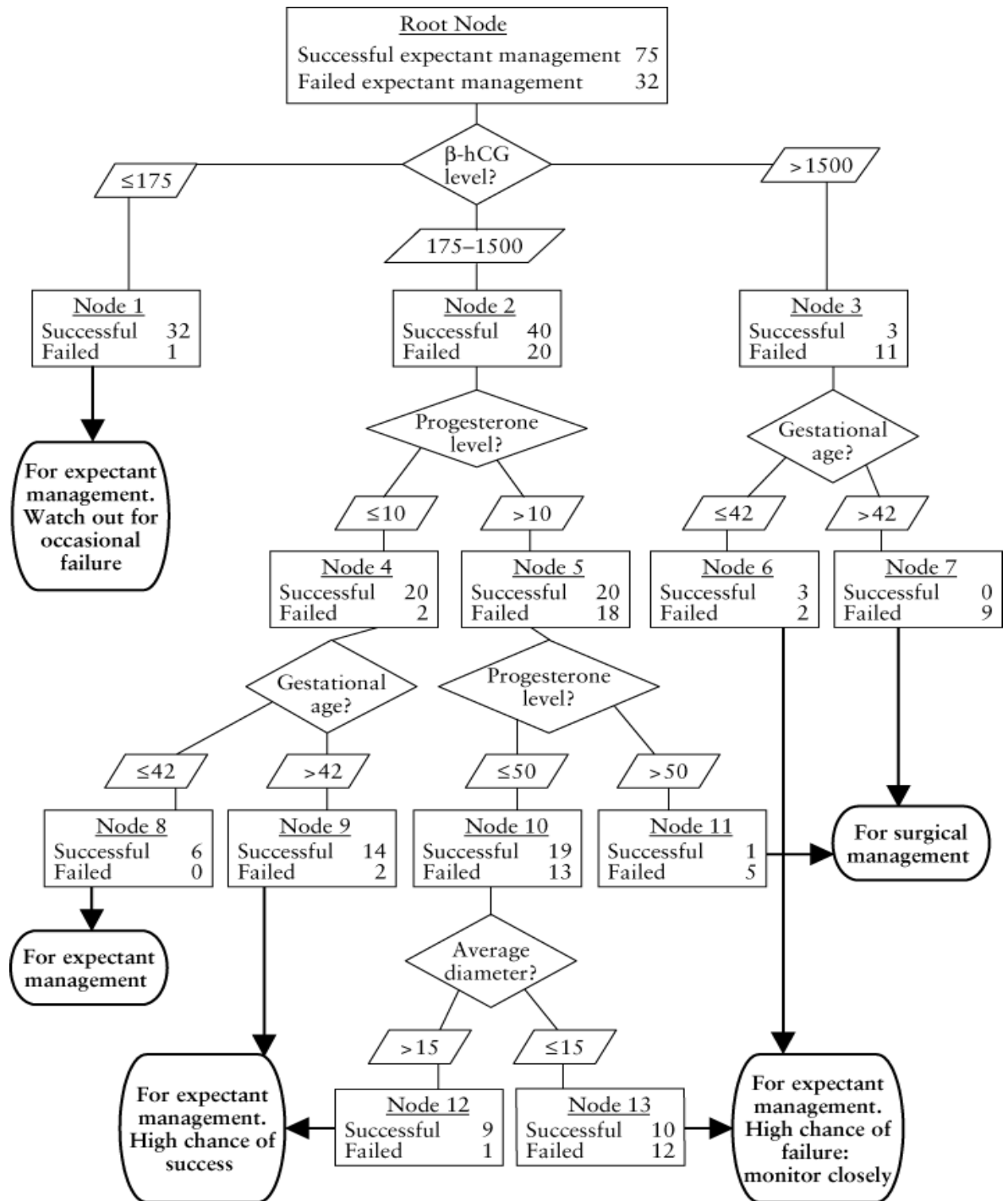
Figure 8. Schematic representation of the decision tree analysis employed in the study for the expectant management of tubal ectopic pregnancy (Elson *et al.,* 2004*).*

2.3 SUMMARY

It is clear from the literature review presented that the management of early pregnancy problems has benefited from the introduction of transvaginal ultrasound scanning and the adjunctive use of biochemical markers. We have also identified a number of areas in need of further research to improve our understanding of the pathophysiology of early pregnancy failure and to improve the care received by patients with early pregnancy problems. Our research questions include is the glycosylation of hCG involved in early pregnancy failure? Can the successful expectant management of miscarriage and failed pregnancies be predicted using novel biochemical markers as described by Elson *et al*., (2005a, 2005b) in our own population? And will these biochemical markers similarly predict the successful expectant management of pregnancies of unknown location?

The original research presented endeavors to answer these questions. The glycosylation of hCG and expectant management studies were conceived separately and are not related apart from in their overarching theme 'the use of novel biochemical markers in the diagnosis and management of early pregnancy problems'.

CHAPTER 3. MATERIALS AND METHODS

3.1 PATIENT RECRUITMENT

Patients for these prospective observational studies were recruited from the Early Pregnancy Assessment Unit of Sunderland Royal Hospital during the period August 2005 and June 2008. The Unit serves a local community of 330,000 residents including both inner city and rural areas with a high level of socio-economic deprivation. This is a secondary referral unit seeing approximately 1300 women per year. Women can be referred by their midwife, general practitioner, accident and emergency department, family planning department or hospital doctor if they have pain or bleeding in early pregnancy. The unit also has an open access policy for women who have had a previous ectopic pregnancy or two previous miscarriages.

Patients were also recruited from the Early Pregnancy Assessment Unit of King's College Hospital, London, for the pregnancy of unknown location and ectopic pregnancy studies, during the period August 2006 and July 2007. The Unit serves a racially mixed inner city population with a high level of socio-economic deprivation. This is a tertiary referral unit seeing approximately 2,500 women per year. The unit has an open access policy and additionally sees women referred by their general practitioner or hospital consultants.

In both units all women are triaged by a nurse and if appropriate undergo a urine pregnancy test *(Clearview HCG II^{TM}, Unipath, Bedford, UK)*. This test is a monoclonal antibody test which according to the manufacturers specifications has a sensitivity of 99% at a urine β-hCG level greater than 25 IU/L. Those women with a positive test then undergo ultrasound scanning and biochemical testing as appropriate.

Clinically stable women with an ultrasound diagnosis of pregnancy of unknown location, missed or incomplete miscarriage, or ectopic pregnancy, who were suitable for and chose expectant management were eligible to take part in the expectant management studies.

Informed written consent was taken from all women prior to inclusion in the studies.

3.2 ETHICS COMMITTEE APPROVAL

Approval for recruitment of women into the glycosylation of hCG study was granted by Sunderland Local Research Ethics Committee (SLREC 704) and for the expectant management studies by Northumberland Regional Ethics Committee (05/Q0902/63).

3.3 ULTRASOUND

3.3.1 Ultrasound Equipment

The equipment used at Sunderland Royal Hospital was a Toshiba Powervision 6000, with a Toshiba IPVM-651VT 6MHz transvaginal probe (Toshiba Medical Systems Ltd, Tokyo, Japan) when a TVS was indicated. The equipment used at King's College Hospital was an Aloka ultrasound system with a 5MHz transvaginal probe. (Aloka SSD-5000, Aloka Co. Ltd, Tokyo, Japan). The mechanical index (MI) was continuously displayed during examination and it was always kept <1.

3.3.2 Ultrasound Method

Ultrasounds were performed by sonographers and verbal consent for the procedure was obtained in all cases. In accordance with departmental guidelines initial scans were performed transabdominally when the estimated gestational age was nine weeks or greater, and transvaginally when less than nine weeks. Transvaginal scans were performed following transabdominal scans when required. The bladder was emptied prior to transvaginal scanning. The probe was introduced gently into the vagina and the cervix and uterus demonstrated in the sagittal plane. The probe was then rotated through 90° and the uterus examined in the coronal plane from fundus to the cervical region. Whilst in the coronal plane the tip of the probe was tilted to the patients right and the right ovary and adnexa examined in the coronal and sagittal planes. The tip of the probe was then tilted to the other side and the left ovary and adnexa then examined in the same way. Finally the pouch of Douglas was inspected for the presence of free fluid.

3.3.3 Ultrasound measurements

All measurements were done on a frozen ultrasound image with callipers. The endometrial thickness was measured from a longitudinal image through the thickest area of the endometrium, from the outermost border of the endometrium on one side to that on the other side.
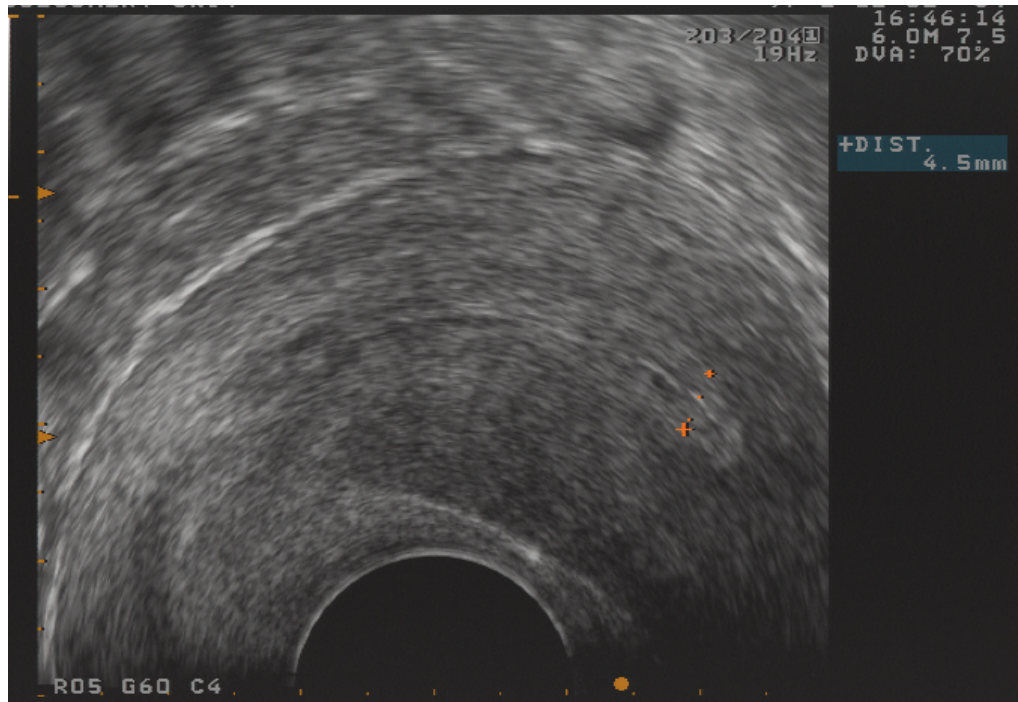
Figure 9. Longitudinal ultrasound image of the uterus, with the measurement of endometrial thickness from outermost border of the endometrium on one side to the other *(image courtesy of J. Elson)*

In the case of an incomplete miscarriage, the intrauterine diameter of the retained products of conception was determined by taking two further measurements in the coronal plane at the thickest area and calculating the diameter as endometrial thickness x diameter 2 x diameter 3 divided by 3.
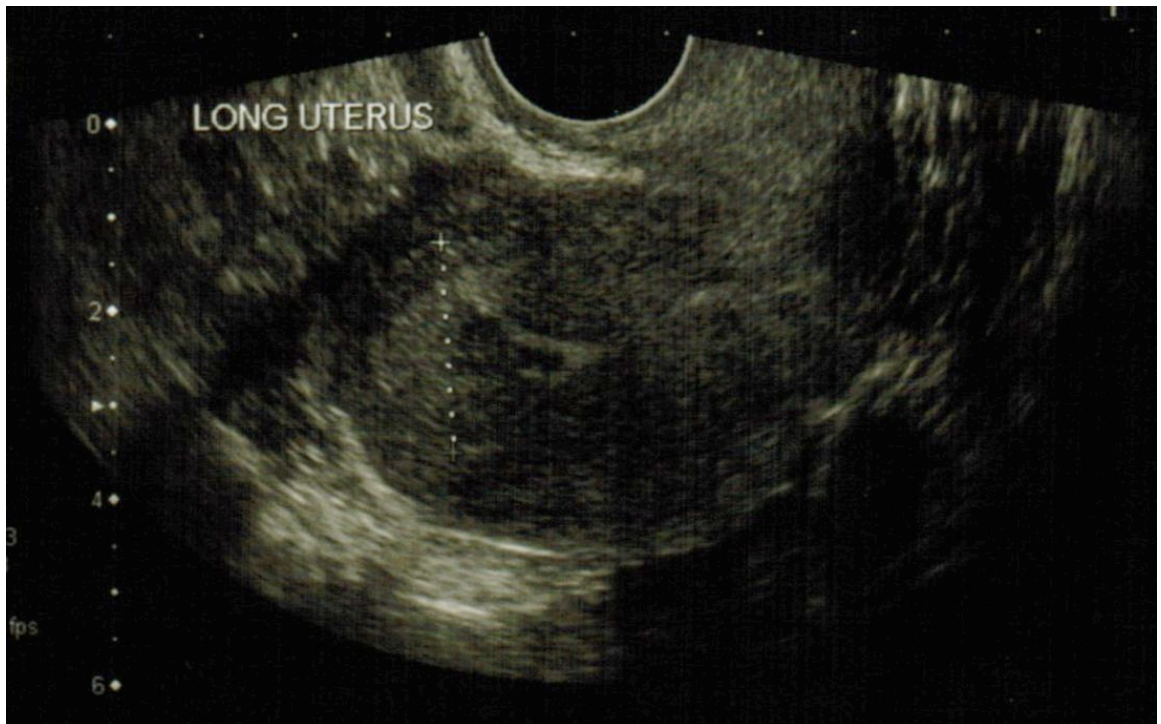
Figure 10. Ultrasound measurement of retained products of conception – longitudinal section (Endometrial thickness 22.5 mm)

Tubal ectopic pregnancies were diagnosed only when there was an adnexal mass with morphological characteristics of an ectopic separate to the ovary and corpus luteum. For ectopic pregnancies the average diameter of the ectopic pregnancy was calculated by measuring the ectopic pregnancy in three dimensions.

The morphology of the ectopic pregnancy was classified into four categories: gestational sac with an embryo, gestational sac with a yolk sac, gestational sac with no detectable embryonic structures and homogenous or solid tubal mass.
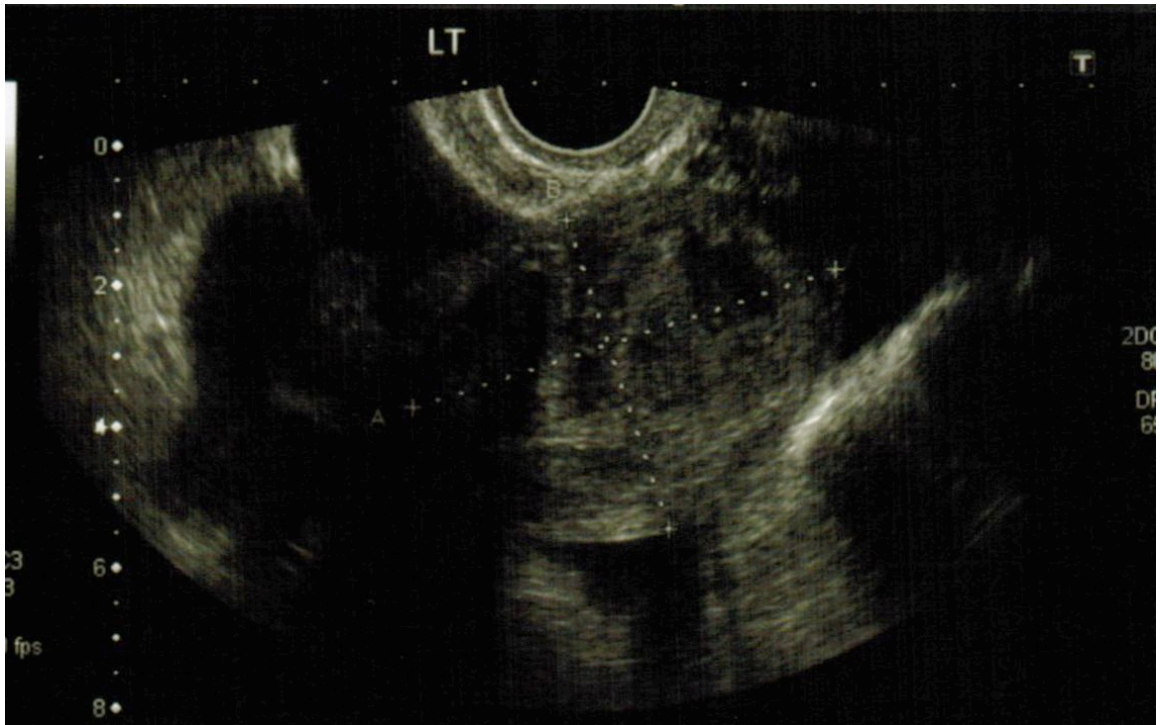
Figure 11. Ultrasound measurements of an ectopic pregnancy showing a solid left sided tubal mass

Measurements of intrauterine contents in missed miscarriages or normal pregnancies are demonstrated in Figure 12.

Gestational (chorionic) sac - Measurements should be
performed from the inner edges of trophoblast in three
planes. The diameters measured correspond to those of the
chorionic cavity. The maximum and mean diameters
should be recorded. The volume is calculated
using formula for ellipsoid
V=AxBxCx 0.523.

Amniotic sac - The three perpendicular diameters should
be measured and the mean diameter calculated. As the
amnion is very thin the measurements should be taken
from the centre of the membrane.

Yolk sac - Three diameters are measured from the outer
wall of the yolk sac.

 Crown- rump length - In early pregnancy this is the
greatest length of the embryo as the crown and rump
cannot be distinguished. From 7 weeks onwards the
 measurement should be taken in the sagittal section, with
care taken not to include the yolk sac.
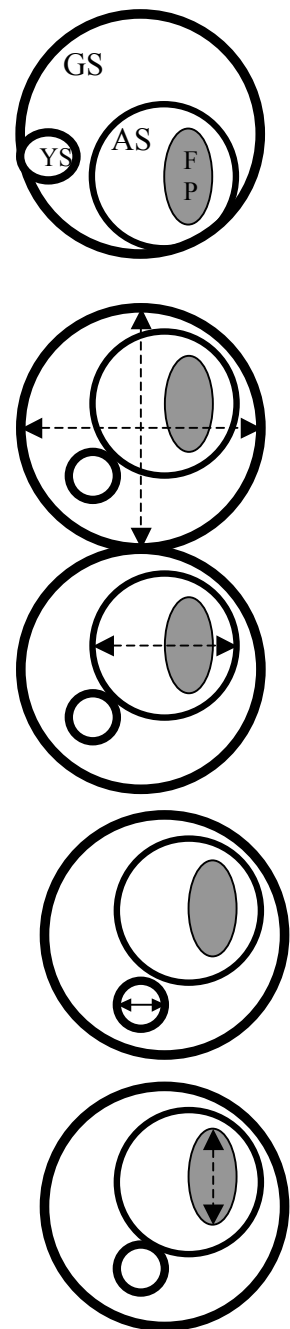
**Figure 12. Schematic representation of measurement of ultrasound
images of early pregnancy showing gestational sac (GS), yolk sac (YS),
amniotic sac (AS) and fetal pole (FP)** *(diagram courtesy of J. Elson)*

Diagnosis of miscarriages were made in accordance with departmental guidelines as outlined in Table 6.

The diagnosis of miscarriage can be made if ultrasound demonstrates one of the following:
• Retained products of conception (>15 mm AP diameter)   • Gestation sac >20 mm (mean sac diameter) with no contents or fetal pole   • Fetal pole >10 mm with no fetal heart pulsation   • No change in 2 scans with intrauterine pregnancy over 10 days

Table 6. Diagnostic criteria for diagnosis of miscarriage from CHS trust protocol 'Management of Miscarriage'

Figure 13. Ultrasound picture of a missed miscarriage with no change in two scans over 10 days

3.4 BIOCHEMISTRY

Blood samples were collected in plain tubes. All blood samples were centrifuged for 10 minutes at 1,000 RPM at room temperature and the serum extracted and frozen at -20°C. The hCG and progesterone assays for those patients with either pregnancies of unknown location or ectopic pregnancies were measured immediately. All others samples were frozen for later analysis in batches.

The panel of novel biochemical markers was chosen to reflect all aspects of the luteo-trophoblastic unit. Progesterone, inhibin pro-αC and 17-OHP are products of and reflect the function of the corpus luteum. hCG is the major product trophoblast and IGFBP-1 is secreted by the decidua. Inhibin A is produced both by the trophoblast and the corpus luteum.

3.4.1 hCG assay

Serum hCG concentrations (intact hCG plus the hCG β-subunit) were quantified using an automated immunoassay technique and expressed in IU/L using the World Health Organisation Third International Reference 75/537. For the glycosylation of hCG study the analysis was carried out using the Immulite 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA). For the expectant management studies the analysis was carried out using the Roche E170 (Roche Diagnostics, Mannheim, Germany) analyser at Sunderland Royal Hospital and the Bayer Immuno1^{TM} (Bayer Diagnostics, Basingstoke, UK) was used at King's College Hospital.

Immulite

Immulite hCG is a solid-phase, two site, chemiluminescent enzyme immunometric assay designed for the quantitative measurement of hCG in serum.

- o 5 μL of sample, reagent (alkaline phosphatase conjugated to polyclonal ovine anti-hCG in buffer) and beads are added together and incubated for 30 minutes.
- o Reagent is removed from beads by spinning the reaction tube at high speed along its vertical axis.
- o Beads are washed then chemiluminescent substrate is added.
- o The light emitted is detected at 477 nm by a photomultiplier tube.

The inter and intra assay coefficients of variation are less than 10%.

Roche E170

 The test principle is a competitive immunoassay using two incubations.

- o 1st incubation: 30 μL of sample, biotinylated monoclonal hCG-specific antibodies, and a monoclonal hCG specific antibody labelled with a ruthenium complex react to form a sandwich complex.

- o 2nd incubation: after addition of streptavadin-coated microparticles the complex becomes bound to the solid phase via interaction of biotin and streptavadin.

- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via a reagent bar code.

Bayer Immuno1

The immunoassay technique used was a heterogenous sandwich magnetic separation assay (MSA).

- The hCG antibody conjugate (R1) and the hCG antibody conjugate 2 (R2) are reacted with the patient sample and incubated at 37 ^{0}C.

- The monoclonal Immunomagnetic Particle (mIMP) reagent is added and a second incubation period occurs during which the antibody complex is bound. The mIMP/antibody complex is then washed and the para-nitrophenyl phosphate (pNPP) substrate is added. The alkaline phosphatase (ALP) in the antibody conjugate reacts with the pNPP to form para-nitrophenoxide and phosphate. Increasing absorbance due to formation of para-nitrophenoxide is monitored at 405 nm and 450 nm.

- The dose/response curve will be directly proportional to the hCG concentration in the sample.

The inter and intra assay coefficients of variation are less than 10%.

A test series of 4 samples analysed at both sites (Sunderland Royal Hospital and King's College Hospital) shows an 11% between-method variation (range 0-17%).

3.4.2 Progesterone assay

Progesterone levels were quantified using an automated immunoassay and expressed in nmol/L. At Sunderland Royal Hospital the analysis was carried

out using the Roche E170 (Roche Diagnostics, Mannheim, Germany) analyser and at King's College Hospital the Bayer Immuno1™ (Bayer Diagnostics, Basingstoke, UK) was used.

Roche E170

The test principle is a competitive immunoassay using two incubations.

- o 1st incubation: 30μL sample – in the presence of a biotinylated monoclonal progesterone-specific antibody and a progesterone derivative labelled with ruthenium complex – are incubated with Danazol to release progesterone. Progesterone from the sample competes with the labelled progesterone derivative for the antibody binding site.

- o 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The amount of the labelled progesterone derivative bound to the solid phase is inversely proportional to the progesterone content of the sample.

- o The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

- o Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Bayer Immuno1

The immunoassay method used is a heterogenous competitive immunoassay.

- o Anti-progesterone antibody (R1) is reacted with the patient sample and incubated at 37°C.

- o Progesterone enzyme conjugate (R2), which competes with the progesterone in the sample for binding sites on the antibody is then added followed by the mIMP. A second incubation occurs during which the antibody/hapten complex is washed and the pNPP substrate is then added. The alkaline phosphatase in the antibody conjugate reacts with the pNPP to form para-nitrophenoxide and phosphate. Increasing absorbance due to formation of para-nitrophenoxide is monitored at 405 nm and 450 nm.

- o The colour production in the reaction is inversely proportional to the progesterone concentration.

The inter and intra assay coefficients of variation are less than 10%.

Our test series of 4 samples comparing the two methods confirms a 2-3 nmol between-method variation, as quoted by UK National External Quality Assessment Service (NEQAS). This is taken into account during statistical analysis.

3.4.3 17 - α-OH progesterone assay

This was quantified using an enzyme linked immunoassay DSL-10-6800 ACTIVE (Diagnostic Systems Laboratories, USA) and expressed as ng/mL. This ELISA uses the competitive binding enzyme immunoassay format. In the assay, standards, controls and unknowns containing 17α-OH progesterone (17-OHP) are incubated with biotin-labeled 17-OHP and rabbit anti-17-OHP antiserum in microtitration wells coated with goat anti-rabbit gamma globulin where the unlabeled and biotin-labeled antigens compete for a limited number of anti-17α-OH progesterone binding sites. After incubation and washing, the wells are incubated with streptavidin-horseradish peroxidase (HRPO), which binds to the biotinylated 17α-OH progesterone. The unbound streptavidin-HRPO is washed away, followed by incubation with the substrate

tetramethylbenzidine (TMB). An acidic stopping solution is then added to stop the competition reaction, and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm. The intra-assay coefficients of variation were 5.1% at 0.49 ng/mL and 4.9% at 6.13 ng/mL, the inter-assay coefficients of variation were 6.76% at 0.49 ng/mL and 5.38% at 6.63 ng/mL.

Assay Procedure

1. Pipet 50 µL of the standards, controls and unknowns into the microtiter wells.

2. Add 50 µL of the 17-OHP biotin conjugate solution to each well.

3. Add 100 µL 17-OHP antiserum to each well.

4. Incubate the wells shaking at a fast speed (500-700 rpm) at room temperature (~25°C) for 1 hour.

5. Aspirate and wash each well 5 times with the wash solution and

blot dry.

6. Add 200 µL of streptavidin-enzyme conjugate solution to each well.

7. Incubate the wells shaking at a fast speed (500-700 rpm) on an orbital microplate shaker, for 30 minutes at room temperature (~25°C).

8. Aspirate and wash each well 5 times with the wash solution and blot dry.

9. Add 100 µL of the TMB chromogen solution to each well.

10. Incubate the wells shaking at a fast speed (500-700 rpm) at room temperature (~25°C) for 30 minutes.   Avoid exposure to direct sunlight.

11. Add 100 µL of the stopping solution to each well.

12. Read the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450 nm.

3.4.4 Inhibin A assay

Inhibin A was quantified using an enzymatically amplified "two-step" sandwich-type immunoassay DSL-10-28100 ACTIVE (Diagnostic Systems Laboratory, USA) and expressed as pg/mL. In the assay, duplicates of standards, controls and unknown serum samples are incubated in microtitration wells that have been coated with anti-inhibin βA subunit antibody. After incubation and washing, the wells are treated with another anti-inhibin alpha subunit detection antibody labelled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells are incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured is directly proportional to the concentration of inhibin A present. A set of inhibin A standards were used to plot a standard curve of absorbance versus inhibin A concentration from which the inhibin A concentrations in the unknowns can be calculated.

Assay Procedure

1. Pipet 50 μL of the standards, controls, and unknowns to the wells of the microtitre plate.

2. Add 50 μL of Inhibin A sample buffer A to each well.

3. Add 50 μL of Inhibin A sample buffer B to each well.

4. Incubate the wells, shaking at 500-700 rpm on an orbital microplate shaker, for 3 hours at room temperature.

5. Aspirate and wash each well 6 times with the Wash Solution and blot dry.

6. Add 100 μL of the Inhibin A antibody-enzyme conjugate solution to each well.

7. Incubate the wells on an orbital microplate shaker set at 500-700 rpm for 1 hour at room temperature.

8. Aspirate and wash each well 6 times with the wash solution and blot dry

9. Add 100 μL of the TMB chromogen solution to each well

10. Incubate the wells on an orbital microplate shaker set at 500-700 rpm for 15

minutes at room temperature. Avoid exposure to direct sunlight.

11. Add 100 µL of the stopping solution to each well using a semi-automatic dispenser.

12. Read the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450 nm.

3.4.5 Inhibin proαC-RI assay

Inhibin pro-αC was quantified using a solid phase sandwich ELISA (Oxford Bio-Innovation MCA 1254KZZ), and expressed as pg/mL. The wells of a microtitre plate come dry-coated with a monoclonal antibody specific for the pro region of the alpha subunit of inhibin. Samples are incubated in the wells so that the antigen binds to the 'capture' or immobilised antibody via its pro region of the alpha subunit. Following washing of the plate a 'second' or detection antibody is added. This is the Fab fragment of a monoclonal antibody specific to the alpha subunit of inhibin coupled to alkaline phosphatase. Any unreacted material is then removed by washing before the detection of alkaline phosphatase using a sensitive amplified substrate reaction. This results in a red reaction product with a colour intensity which is directly proportional to the concentration of inhibin-pro-αC related materials present in the original sample. The assay has less than 0.1% cross reactivity with inhibin A, inhibin B, activin A, activin B and follistatin.

Assay Procedure

1. Add 50 μL of each sample and standard dilution in duplicate, to the wells of the microtitre plate.

2. Add 50 μL of assay diluent in duplicate wells as a zero analyte sample. Cover the plate with a plate sealer and incubate overnight at 4°C.

3. To 1 vial of MCA1254A alkaline phosphatase conjugated Fab mouse anti human inhibin alpha subunit, add 1 mL of pro-αC assay diluent. Replace the top and mix.   Remove the contents and add to a further 5 mL of assay diluent.

4. Wash the wells of the microtitre plate by filling each well to the top with Pro-αC washing buffer allowing to stand for about 15 seconds and then decanting or aspirating each well thoroughly.   Repeat this step a further 3 times. Invert the plate to drain on absorbent paper.

5. Add 50 µL MCA1254A prepared in step 3 to each well of the microtitre plate.

6. Cover and seal the plate and incubate at room temperature for 1 hour.

7. Wash as in step 4 but with 8 cycles and ending with the wells filled with buffer.   Leave the plate to soak for 15 minutes at room temperature whilst preparing the substrate.

8. Prepare the substrate by adding the substrate diluent to the lyophilised substrate. Mix for 5 minutes.

9. Remove the buffer from the plate wells and further wash the plate for 2-3 cycles.   Drain the plate dry by inversion on absorbent paper.

10. To each well of the plate add 50 µL of substrate solution.

11. Cover and seal the plate and incubate at room temperature for 2 hours.

12. Prepare the amplifier by adding the amplifier diluent to the lyophilised amplifier.   Mix for 5 minutes.

13. To each well of the plate add 50 µL of amplifier solution. Agitate gently to mix.

14. Cover the plate and incubate at room temperature. Colour will appear quite rapidly. Read the absorbance values, at 5 minute intervals, of each well at 490 nm. Preferably referencing at 620 nm.

15. Stop the reaction by the addition of 50 µL of STOP solution to each well when the 200 pg/mL standard has reached an absorbance of 2.0 at 490 nm (approximately 10-20 minutes depending on ambient temperature).

3.4.6 IGFBP-1 assay

IGFBP-1 levels were quantified using The ACTIVE Total IGFBP-1 ELISA (Diagnostic Systems Laboratories, USA), an enzymatically amplified two-step "sandwich" assay, and expressed as µg/L. In the assay, standards, controls and samples are incubated in microtitration wells which have been coated with anti-IGFBP-1 antibody. After incubation and washing, anti-IGFBP-1 detection antibody labeled with enzyme- horseradish peroxidase (HRP) is added to each well. After a second incubation and washing step, the substrate tetramethylbenzidine (TMB) is added to the wells. The reaction is then terminated by adding an acidic stopping solution. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm and between 600 and 630 nm. The absorbance measured is directly proportional to the concentration of IGFBP-1 in the samples. A set of IGFBP-1 standards were used to plot a standard curve of absorbance versus IGFBP-1 concentration. The total IGFBP-1 concentrations in the samples were then calculated from this standard curve. The inter- and intra-assay coefficients of variation are less than 10%.

Assay Procedure

1. Pipette 25 µL of the standards, controls and samples assay into the appropriate microtiter wells.

2. Add 50 µL of the assay buffer to each well.

3. Incubate the wells, shaking at 500-700 rpm on an orbital microplate shaker, for one hour at room temperature.

4. Aspirate and wash the wells 5 times with the wash solution and blot dry.

5. Pipette 100 µL of the antibody-enzyme conjugate into the wells.

6. Incubate the wells, shaking at 500-700 rpm on an orbital microplate shaker for 30 minutes at room temperature.

7. Wash the wells 5 times with the wash solution and blot dry.

8. Add 100µL of TMB chromogen solution into each well.

9. Incubate the wells, shaking at 500-700 rpm on an orbital microplate shaker for 10 minutes at room temperature.

10. Stop the reaction by adding 100 μL of stopping solution into each well.

11. Measure the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450 nm.

3.4.7 H-hCG assay

A specific monoclonal antibody to hCG-H (antibody B152) was generated against the hCG with 100% hexasaccharide O-linked structures (100% H-hCG) produced by a single patient with choriocarcinoma by Cole *et al.,* (1999). Using this monoclonal antibody a microtiter plate two antibody (B152 plus anti-β tracer) assay was established for detecting intact H-hCG. H-hCG was quantified using this two-step sandwich-type ELISA, and expressed as μg/L. The inter-assay variance is 8.9%. The microtiter plate assay recognizes H-hCG (100% immunoreactivity), sialic acid deficient H-hCG (100%), H-hCG free β-subunit (60%), pure CHO-cell recombinant hCG (<1%), and pure CHO (Chinese Hamster ovary) -cell recombinant hCG free β-subunit (<1%) (Cole *et al.*, 1999).

Assay procedure

1. Microtiter plates are coated by incubation 16-24 hours at $4^{o}C$ with capture antibody (0.2 mL per well of a solution containing 2.5 mg/L antibody B152 in 0.25 mol/L $NaHCO_3$ and 0.1 mol/L NaCl).

2. Plates are washed three times with water and blotted dry

3. Wells are blocked with phosphate-buffered saline, pH 7.4, containing 10 g/L bovine serum albumin and 0.4 g/L sodium azide.

4. Incubate for 1 hour at room temperature, then plates are again washed three times with water and blotted dry.

5. C5 hCG at concentrations of 0, 60, 12 and 2.4 μg/L is added to quadruplicate wells of the plate. Buffer is added, and the plates are incubated for 4 hours at room temperature on an orbital plate shaker.

6. Plates are again washed 3 times with water and blotted dry.

7. Tracer antibody is added to each well. After an additional 2 hours incubation at room temperature on the plate shaker, plates are again washed three times with water and blotted dry.

8. 0.2 mL of substrate is added to each well.

9. Incubate for 15 minutes at room temperature

10. The reaction is stopped by the addition of 0.05 mL of HCl.

11. The plates are read on a microtiter plate reader at 450 nm.

3.4.8 Chromatography methods

Partially purified hCG was prepared from 1 mL maternal serum by ion-exchange chromatography. Fractions containing hCG were pooled and chromatographed on Superdex 75 pg 16/60 or immobilised lectins, equilibrated in 20 mmol/L Tris HCl, 500 mmol/L NaCl; pH 7.4. For lectin chromatography, samples were either run on individual columns of concanavalin-A (Con-A), *Lens culinaris* agglutinin (LCA), and wheat germ agglutinin (WGA), or Con-A and WGA columns in series.

A. Ion-exchange chromatography

- o Ion exchange columns, 5 mL Sepharose-Q, are generated by washing in 20mmol/L Tris, pH 7.4, 1 M/L NaCl, followed by 20 mmol/L Tris pH 7.4
- o Maternal serum, 1 mL, is desalted on 5 mL Sephadex (Hi-Trap – desalting) column in 20 mmol/L Tris, pH 7.4
- o Desalted serum sample is applied to ion-exchange column
- o Protein is eluted from ion-exchange column in a 0 – 1 M/L NaCl gradient
- o Fractions containing hCG immunoactivity are pooled and retained for either gel-filtration or lectin affinity chromatography.

B. Gel filtration chromatography

- o Gel chromatography columns, Sephadex 75 pg 16/60, are equilibrated in 20 mmol/L Tris pH 7.4, 0.5 M NaCl/L and calibrated with dextran blue, albumin, 63.7 kDa, ovalbumin, 48.6 kDa, chymotrypsinogen A, 20 kDa and ribonuclease A, 15.7 kDa (low molecular weight gel filtration calibration kit; Amersham Pharmacia Biotech).
- o Protein is eluted with 20 mmol/L Tris pH 7.4, 0.5 M NaCl/L at 1 mL/min.
- o Fractions are assayed for hCG immunoactivity.

C. Lectin affinity chromatography

- o Columns of 1 mL. immobilised lectin (ConA, WGA, LCA – Sepharose) are generated by washing in 20 mL 20 mmol/L Tris, pH 8.5, 1 M/L NaCl followed by 20 mL 20 mmol/L Tris, pH 7.4, 0.5 M NaCl/L, 1 mmol/ $CaCl_2$, $MnCl_2$, $MgCl_2$.
- o Immunoactive hCG is run on to the column, washed with 20 mmol/L Tris, pH 7.4, 0.5 M NaCl
- o Bound immunoactive hCG is eluted from lectin by appropriate sugar.
- o Fractions are assayed for hCG immunoactivity.

Gel filtration chromatography

Partially purified samples as described above were chromatographed on superdex pg 75. Partition coefficients (Kav) were derived from calibration using dextran blue, albumin, 63.7 kDa, ovalbumin, 48.6 kDa, chymotrypsinogen A, 20 kDa and ribonuclease A, 15.7 kDa (low molecular weight gel filtration calibration kit; Amersham Pharmacia Biotech).

Lectin affinity chromatography

(i) Lectin columns were washed at 1 mL/min and unbound protein collected in 1 ml fractions for 20 column volumes. Bound protein was eluted from lectin columns in either 500 mmol/L mannose (Con-A and LCA) or 500 mmol/L N-acetyl-glucosamine (GlcNAc) (WGA), in 20 mmol/L Tris HCl, 500mmol/L NaCl pH 7.4, at 1 mL/min over 10 column volumes. 1 mL fractions were collected. Fractions containing bound and unbound protein were assayed for hCG immunoactivity.

(ii) Pooled fractions containing hCG immunoactivity from ion-exchange chromatography were applied to Con A columns as above and washed with 20 column volumes of Tris HCl, 500 mmol/L NaCl pH 7.4. The eluent containing unbound protein was discarded. A column containing WGA, 1 mL, was then connected in series after the Con A column. Protein which had bound to the Con A column was eluted with 500 mmol/L mannose, the eluent passing through the WGA column, to a total of 15 column volumes. Protein which bound to WGA after being displaced from Con A by 500 mmol/L mannose, was finally eluted from the WGA column by 40 column bed volumes 500 mmol/L GlcNAc.

Resolution of hCG isoforms

Binding of serum hCG to WGA was further assessed on unfractionated serum samples. Serum, 50 mcL, was made up to 2.5 mL final volume in Tris HCl, 500

mmol/L NaCl, pH 7.4 and applied directly to 1 mL. WGA equilibrated in the same buffer at 0.2 mL/min the initial ion-exchange step having been omitted. After washing with 10 column volumes, a gradient of 3-15 mmol/L GlcNAc was run through the column and 50 fractions (volume 2 mL) were collected at 0.2 mL/min. Fractions were assayed for hCG content (Immulite 2000).

3.5 STATISTICAL ANALYSIS

All data collected were stored in Microsoft Excel spreadsheets. The Statistical Package for Social Sciences, version 16.0 (SPSS, Statistical Analysis Systems, Chicago, Illinois, USA) was used for all calculations.

3.5.1 Decision tree analysis

The Classification and Regression Trees (C&RT) tree-building algorithm, within SPSS version 16.0, was used to 'grow' the trees. This is a recursive partitioning method for predicting continuous dependent variables (regression) and categorical predictor variables (classification) (StatSoft, 2011). It determines a set of *if-then* logical (split) conditions through sequential analysis of variables. The algorithm finds the split at each node that will generate the greatest improvement in predictive accuracy and so permits accurate prediction or classification of cases. The resulting 'trees' have the advantages of mimicking clinical decision-making and, as no presumptions are made about relationships between variables, they are particularly useful when there is no *a priori* knowledge.

The stopping rules for the iterative process were set as the following; the tree should have a maximum of five levels, a minimum of five cases were to be present for a split to be calculated, and any given split should not generate a group with less than two cases. Limiting the number of levels to five avoids 'overfitting', which is particularly important with a small data set. V-fold cross-validation repeats the analysis many times over with different randomly drawn samples from the data. This was used to validate our decision-trees and is a powerful and essential step for generating useful trees.

Decision trees attempt to find a strong relationship between input values and target values in a group of observations that form a data set. When a set of input values is identified as having a strong relationship to a target value all of these values are grouped, and that becomes a branch on the decision tree. Decision trees are a simple but powerful form of multiple variable analysis and the resulting trees present the data and relationships in a form that is readily understandable and applicable. They can be used as the basis for 'clinical decision rules' that help to translate findings from original research studies into clinical practice (Ebell, 2010).

CHAPTER 4. GLYCOSYLATION OF MATERNAL SERUM HCG IN EARLY PREGNANCY

4.1 INTRODUCTION

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone produced by trophoblast that was first identified in 1927 (Ascheim & Zondek, 1927). hCG is a heterodimer composed of α- and β- subunits which are non-covalently bound. The α-subunit is shared with the other glycoprotein hormones, luteinizing hormone, follicular stimulating hormone, and thyroid stimulating hormone, whereas the β- subunit is specific for each hormone. By the 1970s the amino acid sequences of the hCG subunits had been determined and it was found that hCG contained 4 N-linked and 4 O-linked oligosaccharides of variable structure (Bahl 1969b; Bahl *et al.,* 1972; Morgan *et al.,* 1975; Kennedy & Chaplin, 1976; Kessler *et al.,* 1979; Green *et al.,* 1986). These carbohydrates make up about one-third of the molecular weight of hCG and the glycosylation is of structural and functional importance, affecting the signal transduction and the half-life of the hormone (Lustbader *et al.,* 1998).

HCG, like the other glycoprotein hormones, exists as a family of 'glycoforms' which differ in their oligosaccharide structures (Lambert *et al.,* 1998). The hormone hCG has a high degree of structural heterogeneity associated with these differences in carbohydrate moieties, in particular the terminal sialic acid residues. These are the major determinant of the charge of the hCG glyco-isoforms (Abushoufa *et al*., 2000). These glyco-isoforms of hCG have been demonstrated within normal pregnancy, failing pregnancy and trisomy 21 pregnancy (Elliott *et al.,* 1997; O'Connor *et al.,* 1998; Abushoufa *et al.,* 2000).

One of the roles of hCG is the promotion of progesterone synthesis through early pregnancy. In early pregnancy hCG promotes progesterone production by the corpus luteal cells, this is essential to maintaining pregnancy (Csapo *et al.,* 1973). The corpus luteum replaces pituitary LH in controlling progesterone production from implantation to around 7-9 weeks of gestation, from when the syncytiotrophoblast cells make progesterone independent of hCG stimulation until term. This is known as the luteal-placental shift (Csapo *et al.,* 1973). The need for production of hCG throughout pregnancy has recently been explained

as hCG has been found to have critical functions in trophoblast differentiation and in fetal nutrition through myometrial spiral artery angiogenesis (Cole, 2009).

Recently, using antibodies against specific epitopes within hCG molecules derived from choriocarcinoma hCG, a hyperglycosylated variant of hCG, known as hyperglycosylated hCG (H-hCG), has been identified. H-hCG is made by extravillous cytotrophoblast cells (Kovalevskaya, 2002a) whereas villous syncytiotrophoblast cells make 'regular' hCG. H-hCG also differs from regular hCG in structure and function (Skarulis *et al.,* 1992; Kovalevskaya *et al.,* 1999; Cole *et al.,* 1997). Regular hCG and H-hCG have the same peptide sequence with structural differences only in their oligosaccharide side chains (Elliott *et al.,* 1997). H-hCG glycosylation differs from regular hCG with a tendency to greater branching of the N-linked oligosaccharides and much larger O-linked oligosaccharides (Elliott *et al.,* 1997; Kobata & Takeuchi, 1999; Valmu *et al.,* 2006).

H-hCG is the predominant form of hCG produced in the first week of pregnancy and this variant is an autocrine (rather than endocrine) factor. H-hCG inhibits apoptosis in extravillous invasive cytotrophoblast cells promoting cell invasion and growth (Cole, 2009) thus it is critical to efficient placentation and therefore for successful pregnancy. At three weeks gestation H-hCG comprises 89% of total hCG immunoactivity, falling to 16% by seven weeks gestation, 5% at 9 weeks and < 1.3% after 14 weeks (Cole, 2009). Total hCG, therefore, undergoes a shift in glycosylation, from predominantly H-hCG forms to regular hCG.

We have hypothesised that the shift from H-hCG to regular hCG is associated with further modifications to hCG glycosylation and that this pattern of glycosylation is important for successful pregnancy. In order to test this hypothesis we have identified glyco-isoforms of total hCG, based upon lectin binding, through late first trimester and into the second trimester. We have compared our findings from successful pregnancies to those seen in failing pregnancies. Samples were also assayed for hCG and H-hCG to investigate the contribution of these to any differences seen.

4.2 STUDY DESIGN

This was a prospective study of 76 women who presented to the early pregnancy assessment unit or the antenatal clinic at Sunderland Royal Hospital. All women underwent an ultrasound scan to determine the viability and gestation of their pregnancy. Gestation was calculated from the date of the last menstrual period and subsequently altered according to scan findings when a discrepancy of seven or more days existed (as per departmental guidelines).

Venous blood samples were taken from all of the women. Samples were allowed to clot at room temperature, centrifuged and serum stored in 1 mL aliquots at -30°C until analysis. Written, informed consent was given by all participants. Final pregnancy outcomes were identified from the hospital patient database and dichotomised into failed (including both failures at initial scan and subsequent failures) and viable pregnancies. Viability at anomaly scan was used as a surrogate measure of viability when delivery had not yet taken place at time of data collection.

The study was approved by Sunderland Local Research Ethics Committee.

4.3 METHODS

4.3.1   *Gross structure and general characteristics of maternal serum hCG*

Partially purified hCG was prepared from 1 mL maternal serum by ion-exchange chromatography. Fractions containing hCG were pooled and chromatographed on Superdex 75 pg 16/60 or immobilised lectins, equilibrated in 20 mmol/L Tris HCl, 500 mmol/L NaCl; pH 7.4.

For lectin chromatography, samples were either run on individual columns of Concanavalin-A Sepharose (Con-A), *Lens culinaris* agglutinin Sepharose (LCA), and wheat germ agglutinin Sepharose (WGA), or Con-A and WGA columns in series.

a. Gel filtration chromatography

Partially purified samples as described above were chromatographed on superdex pg 75. Kav were derived from calibration using dextran blue, albumin, 63.7 kDa, ovalbumin, 48.6 kDa, chymotrypsinogen A, 20 kDa and ribonuclease A, 15.7 kDa (low molecular weight gel filtration calibration kit; Amersham Pharmacia Biotech).

b. *Lectin affinity chromatography*

(i) Lectin columns were washed at 1 mL/min and unbound protein collected in 1 mL fractions for 20 column volumes. Bound protein was eluted from lectin columns in either 500 mmol/L mannose (Con-A and LCA) or 500mmol/L GlcNAc (WGA), in 20 mmol/L Tris HCl, 500 mmol/L NaCl pH 7.4, at 1 mL/min over 10 column volumes. 1 mL fractions were collected. Fractions containing bound and unbound protein were assayed for hCG immunoactivity.

(ii) Pooled fractions containing hCG immunoactivity from ion-exchange chromatography were applied to Con A columns as above and washed with 20 column volumes of Tris HCl, 500 mmol/L NaCl pH 7.4. The eluent containing unbound protein was discarded. A column containing WGA, 1 mL, was then connected in series after the Con A column. Protein which had bound to the Con A column was eluted with 500 mmol/L mannose, the eluent passing through the WGA column, to a total of 15 column volumes. Protein which bound to WGA after being displaced from Con A by 500 mmol/L mannose, was

finally eluted from the WGA column by 40 column bed volumes 500 mmol/L GlcNAc.

4.3.2 Resolution of hCG isoforms

Binding of serum hCG to WGA was further assessed on unfractionated serum samples. Serum, 50 mcL, was made up to 2.5 mL final volume in Tris HCl, 500 mmol/L NaCl, pH 7.4 and applied directly to 1 mL. WGA equilibrated in the same buffer at 0.2 mL/min the initial ion-exchange step having been omitted. After washing with 10 column volumes, a gradient of 3-15 mmol/L GlcNAc was run through the column and 50 fractions (volume 2 mL) were collected at 0. 2 mL/min. Fractions were assayed for hCG content. A selection of samples from the peaks of concentration were assayed for H-hCG.

4.3.3 hCG assay

Measurement of hCG was by commercially available immunoradiometric assay (Vankrieken & De Heotogh, 1995) on Immulite 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA) which is highly sensitive for both hCG and H-hCG (Cole *et al.,* 2004).

4.3.4 H-hCG assay

Peaks of hCG immunoactivity from WGA chromatography were concentrated by ultracentrifugation (Amicon Ultra-4 Centrifuge Filter Units; Millipore UK Ltd, Hertfordshire, UK), reconstituted and assayed for H-hCG using monoclonal antibody B152 microtiter plate assay which recognizes H-hCG with 100% immunoreactivity (Cole *et al.*, 1999).

4.3.5 Statistical analysis

For statistical, graphic and tabulated presentation, chromatography results, measured as hCG U/L /fraction, were expressed as the percentage within each fraction of the total hCG eluted from the column. Mann-Whitney U-test was used to compare the heights of the peak values of discrete hCG isoforms. Results were tabulated as the median value and ranges of the peak values for each isoform.

4.4 RESULTS

4.4.1 Gross structure and general characteristics of maternal serum hCG

The stability of hCG and specificity of hCG affinity for WGA were demonstrated by gel filtration chromatography and lectin affinity chromatography on ConA / WGA in series respectively. Gel filtration chromatography, following ion-exchange chromatography, of 1 mL serum (20-fold greater starting volume than that used for WGA chromatography) revealed a single peak of hCG immunoactivity, Kav= 0.34- 0.36, with no difference between any groups. No hCG immunoactivity was identified eluting with a smaller molecular size which could be considered indicative of free β-hCG. Using a starting volume of 1 ml serum, hCG was eluted from WGA in 500 mmol/L GlcNAC but not 500 mmol/L mannose, indicative of specific WGA-hCG binding and elution.

Of the three lectins tested, 100% hCG binding to ConA and WGA was observed, whereas hCG binding to LCA varied between 23-27%. No significant difference in binding between samples from various lengths of gestation or pregnancy outcome was found. When samples were chromatographed on columns of ConA and WGA in series, hCG from all sources bound to WGA, from which it was eluted specifically by 500 mmol/L GlcNAc but not 500 mmol/L mannose*.*

4.4.2 Resolution of hCG isoforms

Chromatography on WGA of serum hCG identified 5 distinct hCG glyco-
isoforms when eluted by a 3–15 mmol/L GlcNAc gradient. Each of these glyco-
isoforms was present at all stages of gestation (see Figure 14). When samples
from early, <8 weeks gestation, were compared to those from second trimester,
16–20 weeks gestation, highly significant differences in the relative expression
of each isoform were observed (see Figure 14 and Table 7).
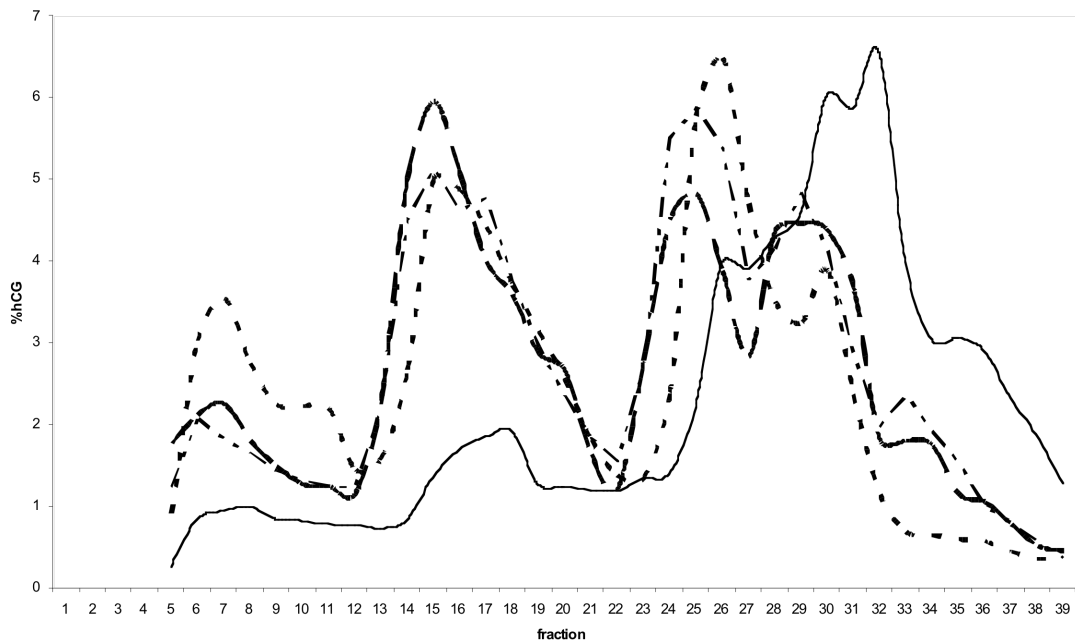
Median values for:

<8 weeks	———————
8-<12 weeks	— — —
12-<16 weeks	— · — · — ·
16-<20 weeks	— — — — ·

Samples, 50mcL maternal serum, eluted in 3-15 mmol/L GlcNAc gradient through 1 mL
immobilised lectin over 80 mL.

Fraction volume = 2 mL.

Results expressed as percentage / fraction of total hCG immunoactivity eluted from
column (y axis) against fraction (x axis).

Figure 14. **WGA lectin affinity chromatography by gestational age**

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
<8weeks gestation					
Median peak value, n=17	1.07	2.58	4.92	6.70	3.97
Range	0.48-3.06	0.59-6.41	2.11-19.25	2.73-14.14	0.62-14.77
>16-20 weeks gestation					
Median peak value, n=20	2.43	5.06	6.76	4.48	0.73
Range	1.16-10.83	3.16–11.64	4.05-11.13	1.59-13.62	0.36-2.77
Mann-Whitney U-test	*P<0.001**	*P=0.0001**	*P=0.0142**	*P=0.003**	*P<0.001**

* significant at $P < 0.05$

Table 7. Comparison of median peak values between samples at <8 weeks (n=17) and >16-20 weeks gestation (n=20)

Comparison of samples from <8, 8-<12, 12-<16 and 16-20 weeks gestation showed that the major shift in hCG glycoform expression occurred between <8 and 8-<12 weeks gestation. Thereafter little difference in hCG glyco-isoform expression occurred (see Figure 14 and Table 8).

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
<8 weeks gestation Median peak value, n=17 Range	1.07 0.48-3.06	2.58 0.59-6.41	4.92 2.11-19.25	6.70 2.73-14.14	3.97 0.62-14.77
8-<12 weeks gestation Median peak value, n=13 Range	2.43 0.78-5.01	5.98 1.96-11.50	5.53 1.51-10.80	5.43 3.14-10.88	2.05 0.83-11.20
Mann – Whitney U-test	*P=0.0009**	*P=0.0013**	*P=0.55*	*P=0.1026*	*P=0.0107**
8-<12 weeks gestation Median peak value, n=13 Range	2.43 0.78-5.01	5.98 1.93-11.50	5.53 1.51-10.80	5.43 3.14-10.88	2.05 0.83-11.20
12-<16 weeks gestation Median peak value, n=8 Range	2.12 1.60-3.85	5.40 4.39-8.22	6.28 4.93-8.18	5.43 3.55 – 6.93	1.93 1.00–3.55
Mann-Whitney U-test	*P=0.5382*	*P=0.9135*	*P=0.685*	*P=0.7999*	*P=0.7445*
12-<16 weeks gestation Median peak value, n=8 Range	2.12 1.60 - 3.85	5.40 4.39 - 8.22	6.28 4.93 - 8.18	5.43 3.55 – 6.93	1.93 1.00 – 3.55
>16-20 weeks gestation Median peak value, n=20 Range	2.43 1.16–10.83	5.06 3.16-11.64	6.76 4.05-11.13	4.48 1.59-13.62	0.73 0.36–2.77
Mann-Whitney U-test	*P=0. 0236**	*P=0. 939*	*P=0. 6655*	*P=0. 2742*	*P=0. 0018**

* significant at *P* < 0. 05

Table 8. Comparison of median peak values between successive stages of early pregnancy.

Significant differences are apparent between samples between <8 (n=17) and 8-<12 (n=13) weeks pregnancy. No difference is a observed between 8-<12 (n=13) and 12-<16 (n=8) weeks gestation. Some difference between 12-<16 (n=8) and >16-20 (n=20) weeks gestation. The greatest difference between successive stages is observed for that between <8 and 8-<12 weeks gestation.

Chromatography on WGA of samples from failing pregnancies at <8 weeks gestation showed significantly different hCG glyco-isoform expression as compared with viable pregnancies at similar gestations (see Figure 15 and Table 9).
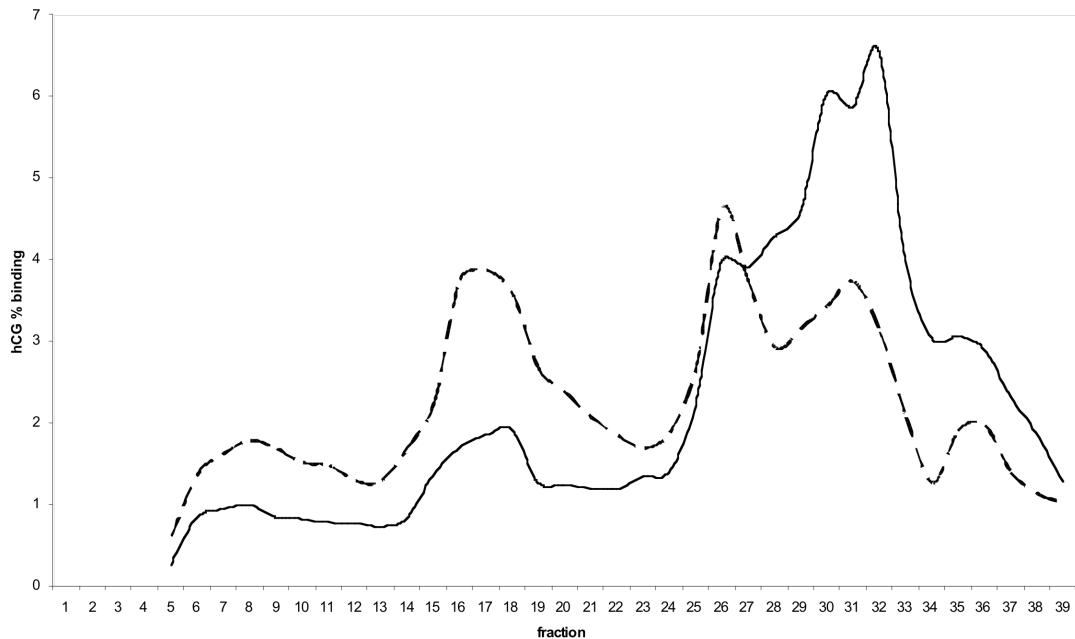
Median values for   viable _____   & failed --------- pregnancies

Samples, 50 mcL maternal serum, eluted in 3-15 mmol/L GlcNAc gradient through 1 mL immoblised lectin over 80 mL.

Fraction volume = 2 mL.

Results expressed as percentage / fraction of total hCG immunoactivity eluted from column (y axis) against fraction (x axis).

Figure 15. **WGA lectin affinity chromatography by pregnancy outcome**

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
<8weeks gestation; viable Median peak value, n=17	1.07	2.58	4.92	6.70	3.97
Range	0.48-3.06	0.59-6.41	2.11-19.25	2.73-14.14	0.62-14.77
<8weeks gestation; failed Median peak value, n=18	2.32	5.31	4.85	4.71	2.71
Range	1.12-5.98	1.091-11.64	1.93-10.79	1.33-15.86	0.30-5.79
Mann Whitney U test	*P=0.0003**	*P=0.0079**	*P=0.986*	*P=0.0259**	*P=0.0333**

*significant at *P* < 0. 05

Table 9. **Comparison of median peak values observed for samples at less than 8 weeks gestation between viable (n=17) and failed (n=18) pregnancies.**

4.4.3 *Hyperglycosylated hCG*

60 samples from fractions at the peaks of hCG immunoactivity were assayed for H-hCG, 30 from pregnancies which were successful and 30 from pregnancies which failed. In all but 5 of these samples H-hCG was undetectable (<20 ng/mL).

4.5 DISCUSSION

In this study, five major glyco-isoforms of hCG have been identified in early pregnancy on the basis of their differing affinities for WGA. These isoforms are expressed throughout early gestations and display a well-ordered progression through early pregnancy and a marked and rapid shift at around eight weeks of gestation. The concentration of maternal serum hCG is known to vary with gestation and pregnancy viability. We have taken this into account by expressing results for each chromatography run as a percentage within each

fraction of the total hCG recovered from the column. Analysis of our data is not, therefore, affected by variations in initial serum hCG concentration.

hCG is detectable during pregnancy in maternal urine and serum following implantation (Skarulis *et al.,* 1992). It consists of two dissimilar subunits, α and β, which are glycosylated and non-covalently bound (Pierce & Parsons, 1981). Glycosylation is considered to play an important role in the bio-availability and bio-activity of glycoprotein hormones in general (Willey, 1999) and hCG in particular (Channing *et al.,* 1978; Moyle *et al.,* 1975; Merz, 1988; Birken *et al.,* 1996). Post-translational glycosylation comprises the addition of two N-linked oligosaccharides to each subunit; on amino acids 52 and 78 (α subunit) and 13 and 30 (β subunit). In addition to N-linked oligosaccharides, 4 O-linked oligosaccharides are located within the hCG-β–COOH terminus (Bahl, 1969b).

As previously described H-hCG is the predominant glyco-isoform detected in early pregnancy. This early H-hCG form declines rapidly to be replaced by hCG, which then becomes the predominant form (Cole, 2007). Replacement of H-hCG by hCG is a continuous process. Both hCG and H-hCG have potential for multiple structural variations not only between but within their oligosaccharide moieties (Kovalevskaya *et al.,* 1999; Sasaki *et al.,* 2008). By the time of the luteal-placental shift the contribution of H-hCG to total hCG immunoactivity is minimal, so the variable glycosylation of total hCG at this gestation cannot be accounted for on the basis of H-hCG alone.

Glyco-isoforms of hCG have been demonstrated in normal pregnancy, failing pregnancy and trisomy 21 pregnancy by a variety of methods (Skarulis *et al*., 1992; Cole *et al*., 1997; Kovalevskaya *et al*., 2002b; Diaz-Cueto *et al*., 1996; Nemansky *et al*., 1998). The structural basis for this heterogeneity has usually been attributed to variation in glycosylation though the precise basis for these differences has varied according to techniques employed and gestational age. Analysis of serum hCG charge isoforms has indicated changes between 10-11 and 35-38 weeks gestation (Diaz-Cueto *et al*., 1996). Urine and serum hCG, together with the free α-subunit, increase in core fucosylation and branching of complex oligosaccharides as pregnancy progresses from 7-12 weeks through to 28-32 weeks gestation (Skarulis *et al*., 1992; Nemansky *et al*., 1998). These studies report differences in hCG glycoslyation between temporarily distinct

stages of gestation. We have concentrated on short, consecutive stages of early pregnancy in order to investigate how isoforms of hCG develop. Our data show significant changes between early first and early second trimesters with a marked shift at 8 - <12 weeks gestation.

The molecular basis for the resolution of these isoforms is a function of their variable N-acetylneuraminic acid (NeuNAc) and/or GlcNAc content, as inferred from the known oligosaccharide affinities of WGA. Saccharides which are bound specifically by WGA are 2-acetamido-2-deoxy-D-glucose (GlcNAc) and its β-1,4-linked oligomers, and N-acetylneuramic acid (NeuNAc) (Bhavanandan & Katlic, 1979; Monsigny et al., 1980; Ming-Chuan, 1992). With reference to hCG, GlcNAc residues are located within N- and O-linked oligosaccharides (Bahl, 1969b) and there are two NeuNAC residues on the N-linked oligosaccharides of the α- and β-subunits (Kessler et al., 1979).

We have shown an altered spectrum of hCG oligosaccharide structure at around 8 weeks gestation in normal early pregnancy which appears to coincide with the luteal-placental shift in progesterone production. This relatively sudden change in five isoforms, identified by WGA affinity chromatography, contrasts with the gradual change from H-hCG to hCG which is known to occur. This shift to the left in isoform expression is not only observed with advancing gestation, but is also seen when comparing pregnancies that fail with pregnancies that are viable. Failed and failing pregnancies seem to make this shift prematurely. The significance of these findings is yet to be established.

Other studies have demonstrated variable expression of a single glyco-isoform in early successful and failed pregnancies. These studies have used specific antibodies to C5 hCG (monoclonal antibody B152) which is a hyperglycosylated structure related to choriocarcinoma hCG. Relatively high levels of this isoform have been described in the first 5-6 weeks gestation, with a subsequent decline in expression as pregnancy progresses but which persists in failing pregnancy (Kovalevskaya et al., 1999; Kovalevskaya et al., 2002b; Yoshimoto et al., 1979; Dean et al., 1980; Sutton-Riley et al., 2006). Hyperglycosylated hCG differs from hCG in its cell of origin and putative functions (Cole, 2007). How the expression of H-hCG relates, if at all, to changes in the physiological spectrum of hCG isoforms in early, continuing pregnancy or contributes to the aberrant

pattern of hCG which we have described in failed pregnancies is not known. We have shown that the progressive expression of hCG in the first 16 weeks of continuing pregnancies does not represent any significant H-hCG. Changes in H-hCG alone do not account for our findings; differences in relative expression of all 5 isoforms are seen through normal and 3 of 5 failing pregnancies. Whether over expression of H-hCG in failing pregnancy is an isolated phenomenon or is associated with variable expression of other isoforms is not known.

Variable binding of hCG to WGA has also been reported in quantitative rather than qualitative work (Abushoufa et al., 2000). Isoelectric focusing studies have also been reported, but this technique is not specific for glycosylation. Another feature of previous studies is the use of urinary hCG, which is known to have undergone desialation (Birken et al., 1996). Less data are available for serum hCG glycoforms.

Our own data indicate a spectrum of WGA-bound glyco-isoforms each of which undergoes changes between early first and second trimesters. The significance of this altered spectrum of hCG oligosaccharide structure at around 8 weeks gestation in normal early pregnancy is not clear. It appears to coincide with the luteal to placental shift in progesterone production, which occurs at approximately the 7th to the 9th week of gestation (Csapo et al., 1973).

Variation in hCG glycosylation has been shown to affect signal transduction but not hormone-receptor binding (Puett et al., 1996) so, although heterogenous hCG receptors have been described, it would seem that they are not a pre-requisite for differences in hCG biological activity. The variations in hCG glycosylation which we have described may result in different biological effect whilst acting through the same hCG receptors. Changing hCG biological activity in early pregnancy does not appear to correlate with the expression of hyperglycosylated hCG (Cole, 2007). Our findings cannot be explained on the basis of changes in the relative proportions of H-hCG to hCG alone nor on the quantity of hCG applied to, or recovered from, WGA columns, and suggests that the expression of appropriate hCG isoforms is a pre-requisite for a successful outcome not only in very early pregnancy but also throughout the first trimester.

We have described a reproducible and clinically applicable method for identifying hCG glyco-isoforms in maternal serum. The method that we have developed is rapid and requires minimal sample volume. The concentration of maternal serum hCG which we have shown can be resolved into isoforms (54-109,274 U/L) encompasses the full range seen in clinical practice.

4.6 CONCLUSION

Using lectin-affinity chromatography we have demonstrated hCG glyco-isoforms in maternal serum and have identified differences with gestational age and also by pregnancy outcome. We have observed a shift to the left in isoform expression with advancing gestation, which failed and failing pregnancies seem to make prematurely. We have shown that changes in H-hCG alone do not account for these findings.

This work provides us with further insight into the endocrinology of early pregnancy and possible mechanisms of early pregnancy failure and the role of hCG structure. It forms the basis of a means by which we can increase our understanding of the role of hCG isoforms in the physiology of pregnancy.

4.7 SUMMARY OF GLYCOSYLATION OF MATERNAL SERUM HCG IN EARLY PREGNANCY

- Variable glycosylation of hCG can be identified by lectin-affinity chromatography with WGA.
- hCG glyco-isoform expression changes with gestational age.
- hCG glyco-isoform expression differs in viable as compared to non-viable pregnancies at similar gestations.
- The variations in glycosylation identified cannot be accounted for by changes in H-hCG alone.

CHAPTER 5. PREDICTION OF SPONTANEOUS RESOLUTION OF PREGNANCIES OF UNKNOWN LOCATION USING NOVEL BIOCHEMICAL MARKERS

5.1 INTRODUCTION

The introduction of transabdominal ultrasound to gynaecology significantly improved the management of women with suspected ectopic pregnancy. The combination of a positive pregnancy test and the absence of an intra-uterine gestational sac on sonographic examination became generally accepted as an indication for laparoscopy. These observations, however, are not specific to ectopic pregnancies and can also be seen in intrauterine pregnancies (IUPs) that are too early to visualise, and in complete miscarriages (Ankum *et al.,* 1993). With the introduction of high-resolution transvaginal probes the diagnosis of ectopic pregnancy has become more accurate, with up to 90% visualised ultrasonographically (Condous *et al.,* 2005). However, in 8-31% of patients presenting to an EPAU it is still not possible to confirm the pregnancy site by TVS at the first visit (Hahlin *et al.,* 1995; Banerjee *et al.*, 1999; Ankum *et al.,* 1993). The situation, when the location of a pregnancy cannot be confirmed as an IUP or an extrauterine pregnancy on the basis of a transvaginal scan, is currently classified as a 'pregnancy of unknown location' (PUL). This is a descriptive term rather than a pathological diagnosis.

In current practice PULs are managed conservatively (Sagili & Mohamed, 2008). Expectant management is advocated to avoid unnecessary surgical intervention and reduce the number and frequency of follow-up visits to a minimum. An expectant management approach based on serum hCG and progesterone was initially evaluated by Hahlin and co-workers (Hahlin *et al.,* 1995). In their study of 80 women, 45 (56%) pregnancies resolved spontaneously, 16 (20%) were ectopic, 7 (9%) miscarried and the remaining 12 (15%) developed into normal pregnancies. The surgical intervention rate was 29%, one third of these procedures were performed to treat miscarriage. Banerjee *et al.,* (1999), Facey *et al.,* (2006) and Day *et al.,* (2009) have subsequently confirmed that an expectant approach based on hCG and

progesterone levels, is safe and has a high success rate, with intervention rates of 4-9%.

The majority of PULs are failing pregnancies. These include both failing ectopic pregnancies and failing intrauterine pregnancies, which are never visualised on TVS. In a prospective observational study in 2001, Banerjee *et al.* found serum progesterone alone to be as accurate as more complex diagnostic models for the prediction of successful expectant management of PULs, with a positive predictive value of 97% using a cut-off level of 20 nmol/L. For the group with progesterone >20 nmol/L further investigations and follow-up visits are required. Facey *et al.* (2006) in their retrospective evaluation of 103 PULs, identified 7 failed pregnancies in the 28 patients with an initial progesterone level >20 nmol/L. Day *et al.,* (2009) have recently reported a 1.3% intervention rate in women with PULs with progesterone </= 10 nmol/L, but less than 50% of their PULs fell into this category.

The main difficulty therefore, with expectant management of pregnancies of unknown location is the lack of robust selection criteria, which reliably predict the likelihood of successful spontaneous resolution of the pregnancy, particularly when the initial progesterone is >20 nmol/L.

hCG and progesterone are the most commonly used biochemical markers of the luteal-trophoblastic axis, hCG is released by the trophoblast within 2 weeks of fertilization and then maintains the progestagenic activity of the corpus lutem. Over the last 10 years several new biochemical markers of the luteal-trophoblastic axis have been described. IGFBP-1 is one of the most important decidual secretory products, with important roles at the embryo-maternal interface in the regulation of placental development (Matsumoto *et al.,* 2008), embryo implantation (Irwin & Giudice, 1998) and fetal growth (Ben Lagha *et al.,* 2006). Inhibin A is produced by the corpus luteum during the luteal phase of the ovarian cycle (Muttukrishna *et al.,* 1994) and is also produced by the syncytiotrophoblast in early pregnancy. The clearance of inhibin A is fast with a short half-life of around 45 minutes (Muttukrishna *et al.,* 1997). Inhibin pro-αC is produced by the corpus luteum (Lockwood *et al.,* 1997) and is thought to play a role as a paracrine and endocrine regulator of placental function. These markers may be useful in monitoring the luteal-trophoblastic axis in pregnancy.

Elson *et al.* (2005a) found that the presence of a raised level of IGFBP-1 and low levels of inhibin A were associated with an increased chance of successful expectant management of miscarriage. Inhibin A has also been used to predict pregnancy loss in women with recurrent miscarriage (Muttakrishna *et al.,* 2002), IVF pregnancies (Hauzman *et al.,* 2004), and threatened miscarriage (Johns *et al.,* 2007). Kirk *et al.* (2009) found that serum inhibin A levels may be useful in predicting failing PULs.

We have hypothesised that by combining clinical information, ultrasound findings and these newer biochemical markers we could predict the spontaneous resolution of pregnancies of unknown location. The aim of this study was to test this hypothesis.

5.2 SUBJECTS & METHODS

This was a prospective observational study of 129 women from Sunderland Royal Hospital, Sunderland, and King's College Hospital, London. Clinically stable women with a positive pregnancy test (Clearview HCG II^{TM}) and no evidence of an intrauterine or extrauterine pregnancy on transvaginal scan were eligible to take part. Patients who chose to take part were provided with an appropriate information leaflet, oral explanation and a consent form by one of the investigative team. A sample of blood was taken, spun and frozen in the laboratory and analysed for hCG, progesterone, inhibin A (Diagnostic Systems Laboratories, USA), inhibin proαC (Oxford Bio-Innovation, Oxford, UK) and IGFBP-1 (Diagnostic Systems Laboratories, USA).

Women were managed in accordance with the relevant unit policy. These are based on the measurement of serum progesterone and β-hCG levels as outlined in Table 10. Follow-up was continued until the pregnancy had spontaneously resolved, viability was confirmed or other treatment was required. Clinical data was collected from patient's medical records and the hospital patient database.

Progesterone (nmol/L)	hCG (IU/L)	Likely diagnosis	Follow-up
<20	>25	Resolving pregnancy	Urine pregnancy test in 7 days
20-60	>25(<1000)	High risk of ectopic	Serum hCG in 2 days
>60	<1000	Normal pregnancy	Repeat scan when hCG > 1000
>20	>1000	Ectopic	Repeat scan ASAP +/- laparoscopy

Table 10. Protocol used for management of women with PUL

5.2.1   Sample size

Eight variables were used in the regression analysis and therefore the sample size calculated allowed for 10 events per variable i.e.: at least 80 pregnancies (Altman, 1999).

5.2.2   Statistical analysis

A database was established to record the women's age, parity, number of previous early pregnancy losses, gestational age from menstrual dates, the presence of pain and bleeding, endometrial thickness as measured on ultrasound, serum biochemical measurements, pregnancy outcomes and time to diagnosis and resolution of the pregnancy. The outcomes were dichotomised into 'spontaneous resolution of PUL' and 'other outcome' categories and statistical analyses were carried out using SPSS version 16.0 (SPSS, Chicago, IL, USA). Normality was tested and comparison of means of continuous variables was performed using Mann-Whitney or Student t-tests depending on data distribution. Proportions were compared using Yates corrected χ^2 test. A value of $P < 0.05$ was considered statistically significant.

A decision tree was developed using the classification and regression trees method. The stopping rules for the iterative process were that the tree should have a maximum of five levels, a minimum of five cases were to be present for a split to be calculated and any given split should not generate a group with less than two cases. These allow sequential analysis of variables to predict whether the PUL would spontaneously resolve or not.

The multivariate logistic regression analysis was performed with spontaneous resolution as the dependent variable. The objective of the model building process was to obtain a "good fit" for the data, with the least number of independent variables.

The regression equation was derived by the forward stepwise selection of variables using the likelihood ratio test for determining which variables to include in the model. The goodness of fit for the model was tested using the Hosmer and Lemeshow test. A non-significant P-value (0.978) suggested a favourable goodness of fit.

Substitution into the regression model with actual values for each case allowed the calculation of the probability of spontaneous resolution for each individual.

 Receiver operating characteristic (ROC) curves were then constructed to describe the relationship between the sensitivity and false-positive rate for different values of these probabilities, and area under the ROC curves (AUC) were computed.

5.3 RESULTS

129 women with a positive pregnancy test and no evidence of an intra-uterine or extra-uterine pregnancy on transvaginal scan consented to take part in the study. Data sets were incomplete in 20 cases and these were excluded from further analysis. Of the 109 cases included in the data analysis, 77 (71%) were recruited from Sunderland Royal Hospital, Sunderland, and 32 (29%) from King's College Hospital, London.

```
                    ┌─────────────────────────────────────┐
                    │ Total number PULs recruited = 129   │
                    └─────────────────────────────────────┘
                                   │
                                   │          ┌─────────────────────────────┐
                                   ├──────────│ Incomplete data sets = 20   │
                                   │          └─────────────────────────────┘
                                   │
                    ┌─────────────────────────────────────┐
                    │     Complete data sets = 109        │
                    └─────────────────────────────────────┘
                                   │
                   ┌───────────────┴───────────────┐
         ┌───────────────────────────┐   ┌───────────────────────────┐
         │ Spontaneously resolving   │   │   Other outcomes = 33     │
         │ PULs = 76                 │   │                           │
         └───────────────────────────┘   └───────────────────────────┘
```
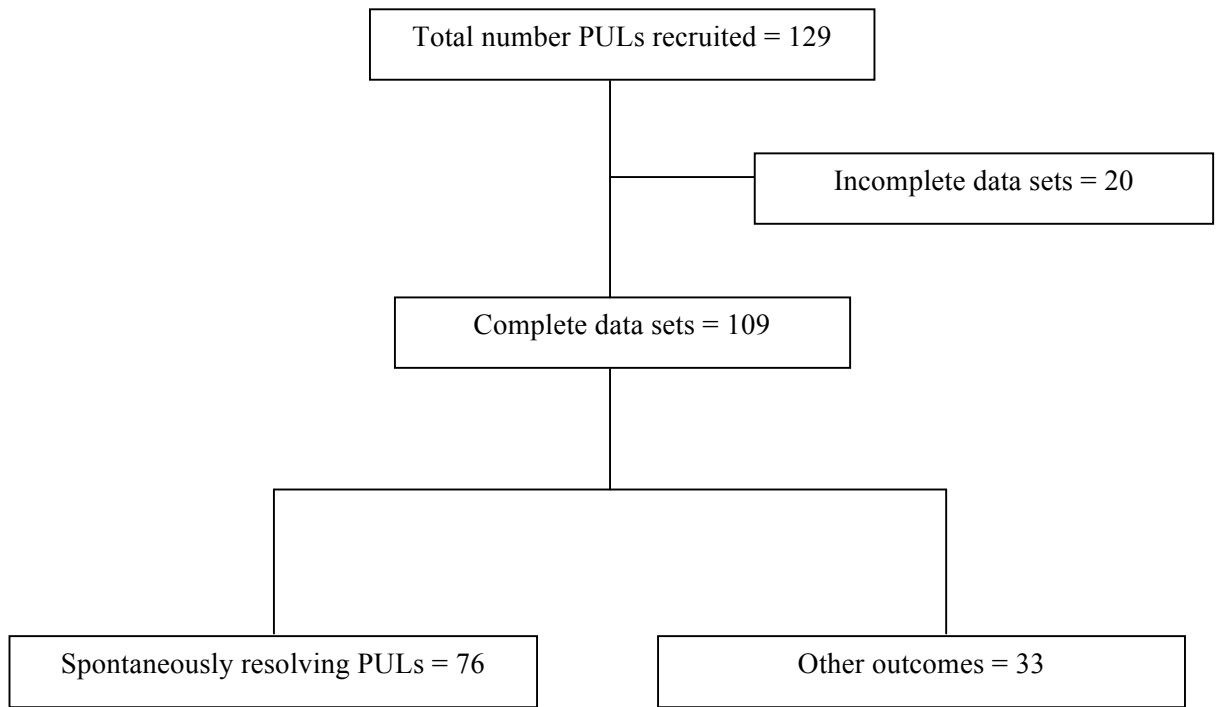
Figure 16. PUL Study recruitment flow chart.

Of the remaining 109 women, 10 (9%) were asymptomatic and 99 (91%) had
abdominal pain (62%) and/or vaginal bleeding (92%). The characteristics of the
study group in terms of their age, parity and number of previous early
pregnancy losses are shown in Table 11.

Characteristic (*n*=109)	
Maternal age (years)*	27.6 (7.2)
Parity#	1 (0-1)
Early pregnancy losses#	0 (0-1)

*Data distributed normally with values given as the mean and standard deviation; #data distributed non-parametrically with values given as the median (25th to the 75th interquartile range).

Table 11. Maternal characteristics

Of the 109 women 91% did not require any further treatment and 9% did require medical (3%) or surgical (6%) intervention. The majority (70%) were retrospectively labelled as spontaneously resolving PULs i.e. the pregnancy was never visualised on TVS and resolved spontaneously. These took 7-18 days to spontaneously resolve (median 7 days) i.e. for the urinary pregnancy test to become negative. The final clinical diagnoses made for all of the cases and the time taken to make the diagnoses are shown in Table 12.

Clinical diagnosis	Number (%)	Time to diagnosis (days)
Resolving PUL	76 (70%)	N/A
Persistent PUL[#]	2 (2%)	15. 5 (13-18)
Miscarriage*	7 (6%)	16. 3 (8. 3)
Ectopic pregnancy[#]	8 (7%)	6 (2. 3-9. 5)
Viable IUP*	16 (15%)	13. 1 (5. 6)

*Data distributed normally with values given as the mean and standard deviation; [#]data distributed non-parametrically with values given as the median (25th to the 75th interquartile range).

Table 12. Clinical diagnoses at completion of follow-up and time to diagnosis

Variable	Outcome		P
	Spontaneous resolution n=76	Other outcomes n=33	
hCG (IU/L)[#]	242 (47.3-87)	573 (276-1478)	0.002
Progesterone (nmol/L)[#]	5 (3.28-7.38)	46 (30.10-81.4)	<0.001
Inhibin A (pmol/L)[#]	7.65 (0-16.207)	16.6 (11.39- 30.47)	<0.001
Inhibin ProαC (pmol/L)[#]	178.7 (77.2-353.0)	200.0 (90-564.8)	0.112
IGFBP1 (μg/L)[#]	13.41 (8.09-23.72)	7.0 (3.91-14.79)	0.02
Maternal age (years)[*]	27.1 (7.79)	28.6 (5.6)	0.27
Gestational age (weeks)[*]	7.46 (2.42)	5.8 (2)	0.01
ET (mm)[#]	7.3 (5.02-10.95)	10.0 (6.8-15.10)	0.04

*Data distributed normally with values given as the mean and standard deviation; [#]data distributed non-parametrically with values given as the median (25th to the 75th interquartile range).

Table 13. Comparison of measured variables in spontaneous resolution and other outcomes

Table 13 shows the different variables in women when compared according to outcome. There were significant differences in serum hCG, progesterone, inhibin A and IGFBP-1 levels, and in gestational age and endometrial thickness between women who required further treatment and those who did not. This was not affected by adjusting the progesterone levels for the difference in assays. There was no significant difference between the pre and post adjusted progesterone data sets.

None of the women suffered from excessive vaginal bleeding or required blood transfusion. A patient who presented with bleeding at 6 weeks gestation and had an endometrial thickness of 13 mm required a laparotomy and admission to

ITU with a septic miscarriage 23 days later. There was one case of ruptured ectopic pregnancy which was diagnosed 8 days after initial presentation with serum progesterone level of 8 nmol/L and hCG 945 IU/L. This was managed laparoscopically.

Serum progesterone was found to have a statistically significant coefficient and was therefore included in a logistic regression model. Natural logarithm of serum progesterone levels was used to achieve conformity to linear gradient. The probability of successful expectant management was then calculated using the formula:

Probability of spontaneously resolving PUL = $1/(1+e^{-z})$

Where $z = 1.998 \times lnprog - 6.126$

With this model, at a cut-off value of 10% probability of spontaneous resolution, prediction of spontaneous resolution was made with sensitivity of 72% (95% CI 61-82) and specificity of 94% (95% CI 85-102). A comparison of ROC curves (see Table 14 and Figure 17) showed that the serum progesterone alone performs significantly better than the logistic regression model and all other individual parameters.

The novel biochemical markers were not useful in the decision tree analysis for predicting spontaneous resolution of PULs (see Figure 18). The decision tree, which includes serum progesterone and hCG levels, predicts 94% of spontaneous resolution (sensitivity 99%, specificity 73%, positive predictive value 89%, negative predictive value 96%).

Variable	AUC	Standard Error	P
hCG	0.686	0.052	0.002
Progesterone	0.926	0.029	<0.001
Inhibin A [#]	0.714	0.056	<0.001
Inhibin ProαC	0.607	0.061	0.80
IGFBP1 [#]	0.343	0.061	0.011
LR progesterone	0.914	0.031	<0.001

Table 14. Results of AUC analysis for the use of different hormonal variables to predict spontaneous resolution of PUL.

ROC Curve

Source of the Curve
- lrprog
- prog
- hCG
- inhA
- inhproaCRl
- IGFBP1

Diagonal segments are produced by ties.
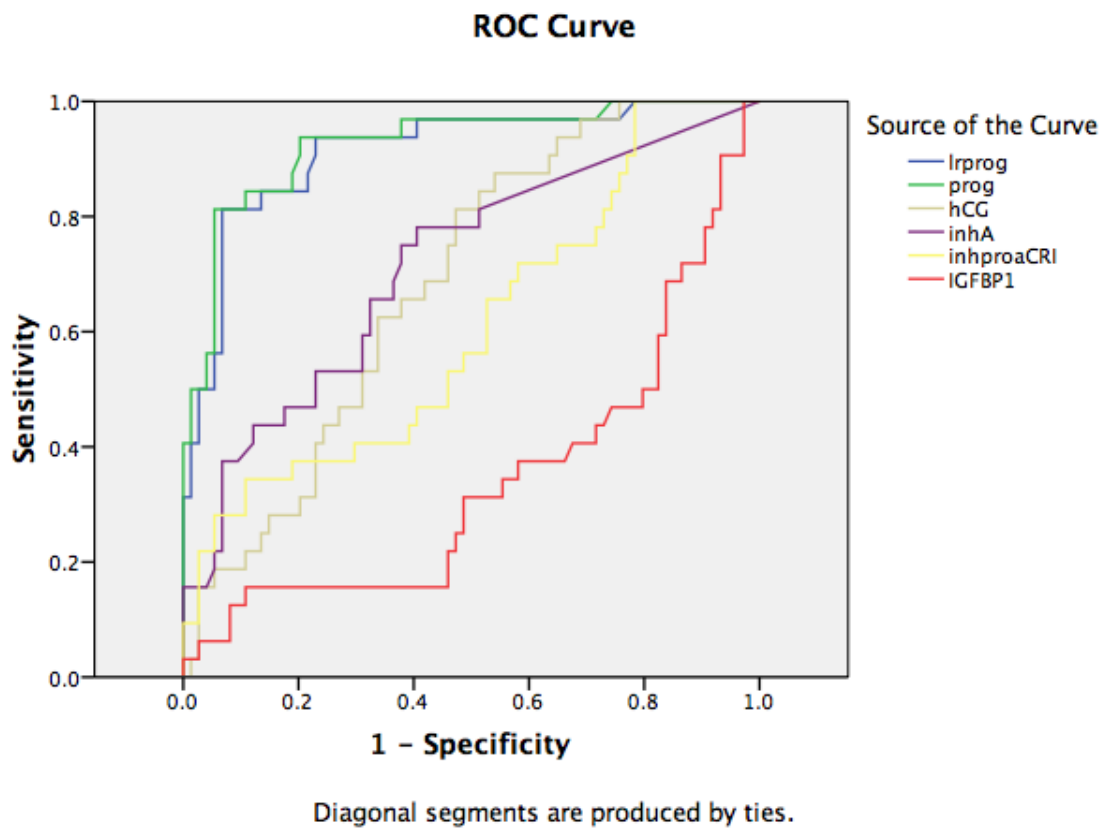
Figure 17. Receiver Operating Characteristics curves demonstrating the performance of the biochemical markers and the logistic regression model in their ability in predicting correctly which pregnancies will resolve without the need for intervention.
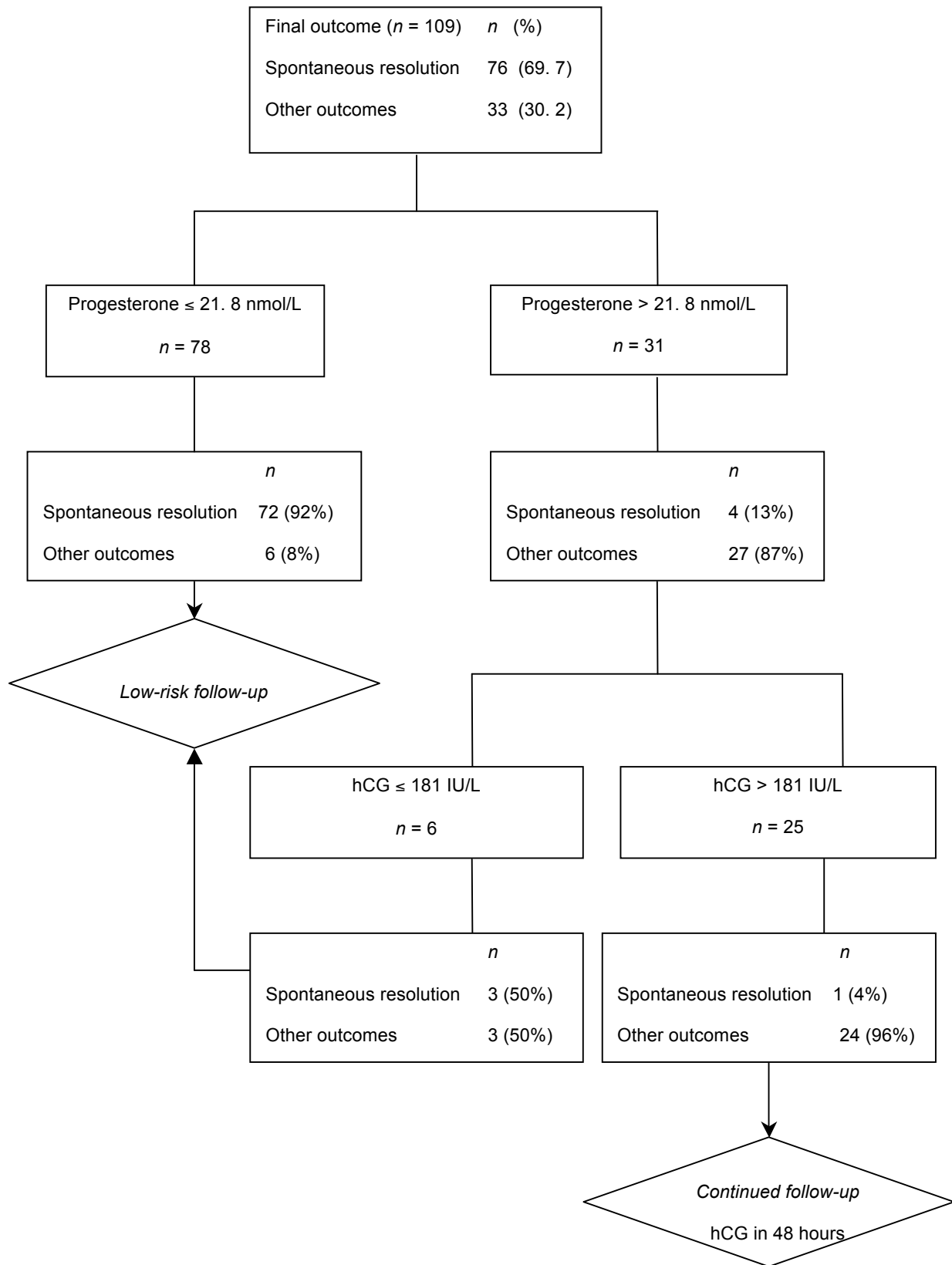
Figure 18. Decision tree analysis for expectant management of PUL

5.4 DISCUSSION

Our study has confirmed that the majority of women with a PUL have a spontaneously resolving pregnancy that is never identifiable on USS and requires no further intervention. The proportion of women with spontaneously resolving PULs was 70%, which is similar to the rates found in previous studies (Banerjee *et al*., 2001; Condous *et al*., 2004). The frequencies of normal intrauterine pregnancies, miscarriages and ectopic pregnancies and our intervention rate were also similar to those in previous reports (Banerjee *et al*., 2001; Condou*s et al*., 2004).

The aim of this study was to investigate the usefulness of novel biochemical markers in the role of predicting spontaneous resolution of PULs. The results presented show that serum progesterone concentrations are still the best single predictor of spontaneous resolution and therefore those pregnancies that require no further intervention. There is however no difference in serum progesterone between the intrauterine and ectopic pregnancy groups, which adds to the large body of evidence that serum progesterone levels are not useful in predicting the location of a pregnancy.

Inhibin A was found to be the next most useful marker of spontaneously resolving PULs after progesterone. All pregnancies with an inhibin A less than or equal to 11 pmol/L were spontaneously resolving PULs, reflecting either reduced amount of trophoblast or non-functioning trophoblast. Inhibin A seems to reflect the trophoblast dynamics more accurately than hCG which may be due to its shorter half-life. These findings are consistent with that of Elson *et al.* (2005a) who found that in the expectant management of miscarriage, unmeasurable inhibin A was strongly associated with successful expectant management.

 In their recent study Kirk *et al.* (2009) also found that inhibin A levels were significantly lower in spontaneously resolving PULs than in intrauterine or ectopic pregnancies. In this study they found inhibin A levels to be a stronger predictor of failing PUL than we have, although this difference is likely to be explained by the smaller proportion of failing PULs in their cohort and their use of missing value imputation in their statistical analysis. In both studies novel biochemical markers were not better at predicting PUL outcome than

progesterone, and like hCG, inhibin A levels were not useful at predicting the location of a pregnancy.

To our knowledge this is the first time that IGFBP-1 and inhibin proαC levels have been investigated in the management of PUL. Two theories exist for the role of IGFBP-1 in early placentation. One is that higher levels of IGFBP-1 inhibit binding of the trophoblast to the decidua, the other that there is overproduction of IGFBP-1 by the decidua in response to defective implantation. In miscarriage high levels are associated with an increased chance of successful expectant management (Elson *et al.,* 2005a). It is thought that the high levels of IGFBP-1 reflect a defect in the attachment of the trophoblast to the decidua and thus an increased chance of spontaneous expulsion of retained products of conception. In our study IGFBP-1 tended to be higher in the spontaneously resolving PULs than in other outcome groups, although not significantly so. IGFBP-1 was less than or equal to 16 ng/mL in every PUL which was subsequently found to be a viable intrauterine pregnancy. This again suggests that high levels of IGFBP-1 reflect defective implantation.

We have not found inhibin proαC levels to be helpful in differentiating spontaneously resolving PULs from those that need further investigation. Inhibin proαC is known to be a produced by the corpus luteum (Fowler *et al*., 1998) and levels fall after administration of mifepristone in termination of pregnancy (Lahiri *et al*., 2003). It has been suggested that inhibin proαC may be responsible for maintaining luteal production of progesterone. We would therefore expect lower levels to be associated with spontaneously resolving PULs but our findings have not confirmed this.

Persisting PULs are PULs where the serum hCG levels fail to decline, where there is no evidence of trophoblast disease, and the location of the pregnancy cannot be identified by ultrasound or laparoscopy. In general the serum hCG levels are low (<500 IU/L) and have reached a plateau. Such cases have been successfully treated with methotrexate (Condous *et al*., 2004). Biochemistry (hCG and progesterone) has been shown not to be reliable for predicting persisting PUL (Condous *et al*., 2002). We had only two cases of persistent PUL in our series. We were therefore unable to make any conclusions regarding the usefulness of the novel biochemical markers in this group. As

persisting PULs are relatively uncommon, a large multicentre study would be required to investigate this further.

In this study novel markers of the luteal-trophoblastic axis have not been more useful than traditional markers in the identification of spontaneously resolving PULs. Unlike miscarriages, PULs are a heterogenous group including both viable and failing intrauterine and extrauterine pregnancies. A high progesterone level reflects a normally functioning corpus luteum and placenta of a viable pregnancy (Banerjee *et al.,* 1999) which can occur in both intrauterine and ectopic pregnancies. We have confirmed that in PULs a low progesterone level indicates a failing corpus luteum and therefore a spontaneously resolving pregnancy. As the newer markers may more specifically reflect the functioning of the trophoblast, differences between the various specific types of pregnancy may have been lost in the analysis as 'spontaneously resolving PULs' and 'other outcomes'. Numbers of cases within the outcomes grouped together as 'other' were too small to allow individual analysis. In the expectant management of PUL however, the clinically important division is between pregnancies that are spontaneously resolving and those that need further investigation.

Endometrial thickness was significantly lower and gestational age significantly higher in the spontaneously resolving PULs compared to the other outcome groups. It has previously been shown that chorionic villi may be present in 69-100% of miscarrying women with an endometrial thickness of less than 5 mm (Rulin *et al.,* 1993, Kurtz *et al.*, 1991). It would appear that in PULs, as in miscarriages, the smaller amount of trophoblast present is associated with spontaneous resolution of the pregnancy.

PULs which are early viable intrauterine pregnancies are not visible on scan because they are too small to visualise as a result of their early gestational age. If a pregnancy is a PUL at an increased gestational age then it is more likely to be an abnormal pregnancy. This explains the increased gestational age in the spontaneous resolution group.

Day *et al*. (2009) have recently proposed a modified clinical protocol for the management of PUL. They have shown that clinically stable patients with a serum progesterone level of less than 10 nmol/L can be safely discharged from

active follow-up after their initial visit. In our study we had 63 such cases. Although 59 (94%) of these did resolve spontaneously, three cases of ectopic pregnancy and one miscarriage were subsequently diagnosed, all of which required further intervention. We would therefore recommend that follow-up pregnancy tests continue to be performed to allow identification of these cases.

Decision tree analysis is a useful clinical decision making tool and forces decision-makers to make the bases for their decisions explicit (Thornton, 1990). The decision tree analysis as described in this study provides information that can be used both for patient selection and counselling. Using this approach every clinically stable women with a PUL would have their follow-up strategy decided based on initial hCG and progesterone, minimizing the need for repeat blood-sampling at 48 hours, as in the two-stop hCG ratio model favoured by Condous' group. This would reduce visits, delay, and patient anxiety whilst the second hCG result is awaited. It may also be more cost effective, with no reduction in sensitivity.

5.5 CONCLUSION

In this study we have shown that inhibin A, IGFBP-1 and inhibin proαC, novel biochemical markers of the luteal-trophoblastic axis, are not clinically useful in the prediction of spontaneous resolution of PULs. Our data confirm that serum progesterone is the best single marker, and that a model using a combination of serum hCG and progesterone is the most effective approach to predict spontaneous resolution of PULs in a single visit. Further investigation is needed into the use of novel biochemical markers in the management of more specific pregnancy outcome groups such as ectopic pregnancies.

5.6 SUMMARY OF PREDICTION OF SPONTANEOUS RESOLUTION OF PUL USING NOVEL BIOCHEMICAL MARKERS

- The novel biochemical markers inhibin A, IGFBP-1 and inhibin proαC are not clinically useful in the prediction of spontaneous resolving PULs.

- Serum progesterone remains the best single marker for predicting spontaneously resolving PULs.

- A model using a combination of serum hCG and progesterone is the most effective approach to predict spontaneous resolution of PULs in a single visit.

CHAPTER 6. PREDICTION OF SUCCESSFUL EXPECTANT MANAGEMENT OF MISCARRIAGE AND ECTOPIC PREGNANCY USING NOVEL BIOCHEMICAL MARKERS

6.1 INTRODUCTION

Early pregnancy complications include miscarriage, ectopic pregnancy and pregnancy of unknown location (PUL). In recent years the management of these patients has changed with a shift in focus from a surgical approach to one based on an expectant or 'watch and wait' policy (RCOG, 2006). PUL will not be discussed further in this chapter as it is dealt with in full in Chapter 5.

6.1.1 Miscarriage

Miscarriage is a common problem with approximately one in seven confirmed pregnancies ending in miscarriage in the first trimester (Regan *et al.,* 1989) and accounts for approximately 50 000 inpatient admissions in the United Kingdom annually (DoH stats, 2005) (Sagili & Divers, 2007). Miscarriage and its treatment can have both medical and psychological consequences. While maternal death is rare after miscarriage, particularly in the first trimester, it has featured in previous Confidential Enquiries into Maternal Deaths in the UK, particularly after surgical procedures in association with sepsis.

Surgical evacuation of retained products of conception has been the standard procedure for patients with incomplete or missed miscarriage for more than 60 years. Although still the method of choice when bleeding is excessive, vital signs are unstable or infected tissue is present in the uterine cavity, the surgical and anaesthetic risks, the psychological distress (Lok & Neugebauer, 2007) and the increased cost (Petrou *et al.,* 2006), make it less advantageous in other cases. In the last decade medical management with antiprogesterone and/or prostaglandins has become popular with success rates quoted from 40-95% (Sagili & Divers*,* 2007) varying with the regimen used and the type of miscarriage. The commonly used drug misoprostol is not licensed for use in the management of miscarriage and side effects include diarrhoea, fatigue and vaginal bleeding.

Expectant management of miscarriage has been shown to be safe and effective. A review by Butler (2005) showed that expectant management is successful within 2-6 weeks without increasing the complications in 80-90% of women with incomplete spontaneous miscarriage and 65-75% of women with missed miscarriage. The MIST trial did not show any increase in infection rates with expectant management as compared to medical or surgical management, rates were low (2-3%) regardless of treatment modality although expectant and medical management were associated with a higher number of unplanned admissions and unplanned surgical evacuation procedures (Trinder *et al.*, 2006).

6.1.2 Ectopic Pregnancy

Ectopic pregnancy is still a major problem in the United Kingdom, with around 30,000 cases diagnosed annually. The overall rate of ectopic pregnancy (11/1000 pregnancies) and the mortality rate (0.3/1000 ectopic pregnancies) had been relatively static in the UK from 1991 to 2005 but had fallen to 0.17/1000 ectopic pregnancies in the recent maternal mortality report (CMACE, 2011). It is hoped that this trend will continue.

Many ectopic pregnancies detected in modern practice would not have been diagnosed in the past and are likely to have resolved without any treatment. Historically ectopic pregnancies were diagnosed at the time of surgery, often in women who were not aware that they were pregnant. Now that home pregnancy tests can detect hCG levels at less than 25 IU/L, a pregnancy can be diagnosed before a menstrual period has been missed and as a consequence women present at earlier gestations. The widespread use of dedicated early pregnancy assessment units with high resolution transvaginal ultrasonography, allow early diagnosis of pregnancy location and are likely to have contributed to this increase in the diagnosis of relatively clinically insignificant ectopic pregnancies. Despite this increase in the sensitivity of non-invasive tests for ectopic pregnancy and the detection of very mild forms of the condition, patients and clinicians generally still consider ectopic pregnancy to be a life-threatening condition. As a result some sort of therapeutic intervention is usually used

whenever the diagnosis of an ectopic pregnancy is made, regardless of the clinical presentation.

The management of tubal ectopic pregnancy in the presence of haemodynamic instability should clearly be by the most expedient method, surgery (RCOG, 2006). The options for the treatment of haemodynamically stable women, however, also include medical management and expectant management.

Single dose intramuscular methotrexate is the most commonly used medical treatment for ectopic pregnancies in the UK (RCOG, 2004). Methotrexate is a cytotoxic drug that binds to the enzyme dihydrofolate reductase and therefore interferes with DNA synthesis and disrupts cell multiplication. Success rates range from 65-95% (Kirk *et al.,* 2006) with the studies varying widely in their inclusion criteria. The reproductive outcomes after methotrexate treatment have been studied and one study has shown that overall subsequent intrauterine pregnancy rates were higher and ectopic pregnancy rates lower after methotrexate than salpingostomy (Fernandez *et al.,* 1998). Economic comparisons have also shown benefits of methotrexate over the surgical management of ectopic pregnancies (Mol *et al.,* 1999; Sowter *et al.,* 2001).

Expectant management of tubal ectopic pregnancies was first evaluated by Lund in 1955. In his series of 119 patients only 68 (57%) were successfully treated expectantly and 51 patients required surgery for persistent symptoms or large intraperitoneal haemorrhage (Lund, 1955). This was before laparoscopy and ultrasound were available.

Since then a number of studies, using more stringent inclusion criteria, have reported much more successful expectant management of ectopic pregnancies (65-95%). Low hCG levels, a decreasing trend in hCG levels, the absence of an ectopic gestational sac and a longer time from the last menstrual period are associated with more successful expectant management (Trio *et al.,* 1995; Atri *et al.,* 2001). In one study an hCG level of <1000 IU/L was chosen as the optimal cut-off identifying 88% of women whose ectopic pregnancies spontaneously resolved (Trio *et al.,* 1995). In 2004 Elson *et al.* described decision-tree analysis to predict spontaneous resolution of tubal ectopic pregnancies and therefore aid selection of appropriate management (Elson *et al.,* 2004). Initial serum βhCG level was again the best individual predictor of

outcome (96% success with βhCG </= 175 IU/L) but progesterone level, gestational age and average diameter of the ectopic were used to predict spontaneous resolution where initial βhCG >175 IU/L.

Expectant management has important advantages over medical treatment as it follows the natural history of disease and is free from the serious side effects associated with methotrexate and the surgical risks of surgery. The original purpose of expectant management of ectopic pregnancy was the preservation of fertility although there is yet no randomised study comparing fertility outcome after expectant management with conservative surgical and medical management observational studies suggest that rates are comparable (Kirk *et al.,* 2006). RCOG guidance now suggests that expectant management is a useful form of treatment for ectopic pregnancy in selected cases (RCOG, 2004). This option is therefore limited to a very small proportion of ectopic pregnancies and success rates vary widely between centres (Cohen & Sauer, 1999).

One of the difficulties with expectant management of miscarriage and ectopic pregnancy is the lack of selection criteria which reliably predict the likelihood of successful spontaneous resolution of pregnancy and therefore successful expectant management. In 2005 Elson *et al.,* found that biochemical markers (including serum hCG, progesterone, inhibin A, IGFBP-1 and inhibin proαC) show significant differences in miscarriages and failed pregnancies (including miscarriages and ectopic pregnancies) that resolve spontaneously, as compared with those that do not (Elson, 2005b). We have hypothesised that the decision-trees developed by Elson (2005b) will predict spontaneous resolution of miscarriages and failed pregnancies in our population. Our aim was to test this hypothesis.

6.2 STUDY DESIGN

This was a prospective observational study over 2 years of women with a diagnosis of miscarriage or ectopic pregnancy identified on ultrasound scan. Women were referred to the dedicated Early Pregnancy Assessment Unit (EPAU) at Sunderland Royal Hospital or King's College Hospital by their general practitioners or hospital consultants because of suspected early

pregnancy complications, or self-referred because of previous early pregnancy losses. All women had a positive urine pregnancy test (Clearview HCG II[TM], Unipath, Bedford, UK). A full history was documented by the EPAU nurse and an ultrasound scan was then carried out by the dedicated EPAU sonographer using a high-frequency transvaginal probe.

A diagnosis of a missed miscarriage was made based on one of the following criteria:

(i) the gestational sac was greater than 20 mm in diameter with no intrauterine contents;

(ii) the fetal crown rump length was greater than 10 mm with no fetal heart rate detected;

(iii) the gestational sac had failed to develop from a previous scan more than 10 days ago.

An incomplete miscarriage was diagnosed in women with a history of bleeding in whom an intrauterine gestational sac had been previously seen on scan or where there was absence of an intact gestational sac and the presence of any amount of visible trophoblast in the uterine cavity. The sonographic features of visible trophoblast are irregular echoes in the midline of the uterine cavity.

A diagnosis of tubal ectopic pregnancy was made when a mass with ultrasound appearance of an ectopic pregnancy was seen in either adnexa, separate from the ovary and corpus luteum.

Clinically stable women with a miscarriage or ectopic pregnancy who met departmental eligibility criteria for expectant management were eligible to take part (see Table 15 and Figure 19). Patients who chose to take part were provided with an appropriate information leaflet, oral explanation and a consent form by one of the investigative team. Routine and study blood samples were taken together. hCG and progesterone were measured immediately, and another sample was spun and frozen in the laboratory and later analysed for 17α-hydroxyprogesterone (Diagnostic Systems Laboratory, USA), inhibin A (Diagnostic Systems Laboratory, USA), inhibin proαC (Oxford Bio-Innovation, Oxford, UK) and IGFBP-1 (Diagnostic Systems Laboratory, USA).

Expectant management of miscarriage

- USS evidence of an intrauterine incomplete or missed miscarriage
- <12 weeks gestation
- No evidence of cardiovascular compromise, active bleeding or pyrexia
- No complicating medical disorders (Hb <10g/dL, immunosuppression, coagulation disorders, concurrent disease likely to result in infection)
- Informed consent with expressed preference for expectant management

Expectant management of ectopic pregnancy

- <3 cm tubal mass on USS with
  - No FH
  - No yolk sac
  - No intraperitoneal blood
- βhCG < 1500 IU
- Patient haemodynamically stable
- No moderate/ severe pain
- Informed consent with expressed preference for expectant management

Table 15. Eligibility criteria for expectant management of miscarriage
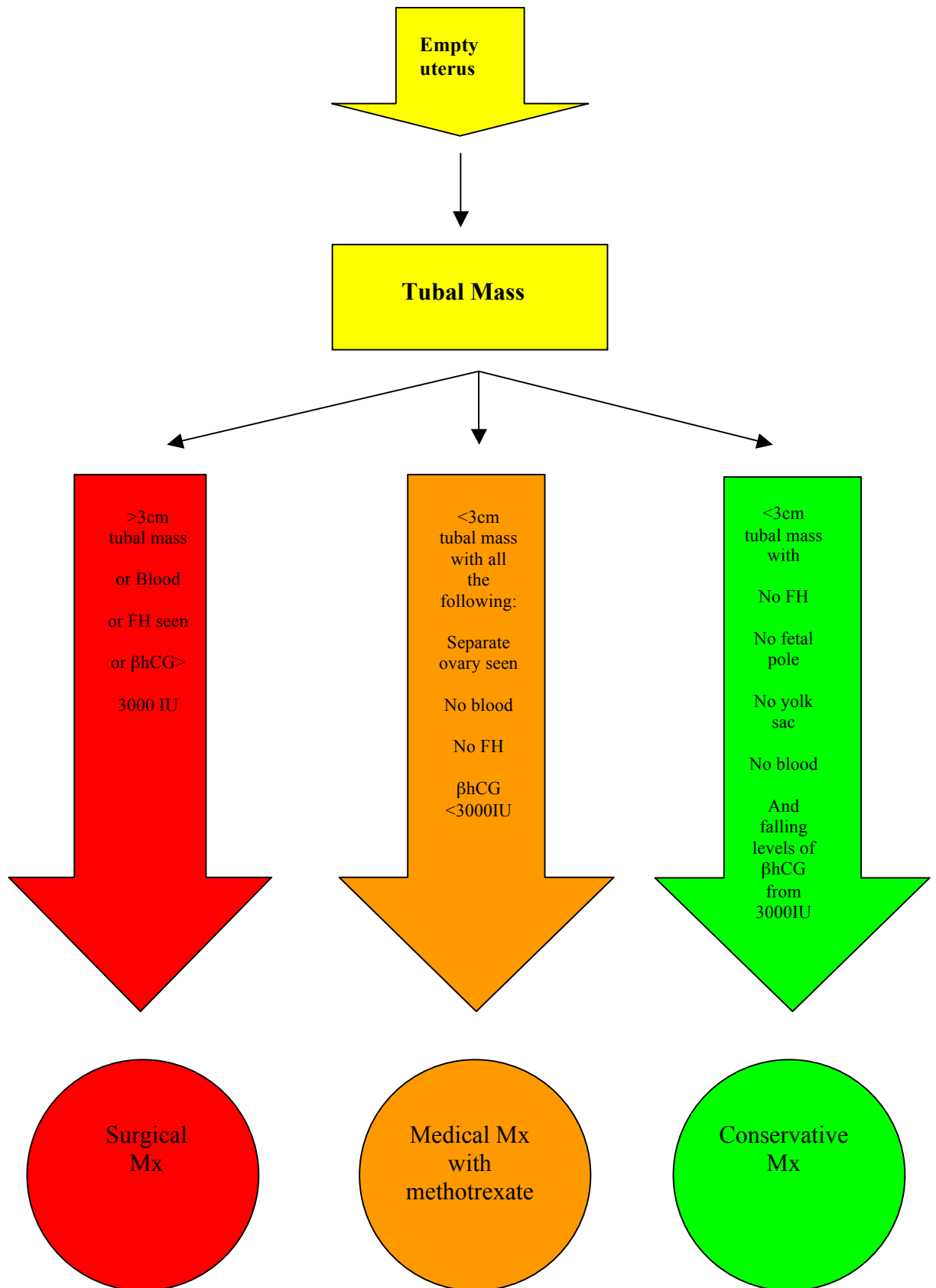
Figure 19. Flow-chart illustrating Trust Protocol "Management of Tubal mass"

Women were managed in accordance with the appropriate unit policy. Follow-up was continued until the pregnancy had spontaneously resolved or other treatment was required.

In the miscarriage arm women were reviewed at 3 weeks and if the gestational sac was still present on USS they were given the option of continuing with expectant management or proceeding with medical or surgical evacuation. If continuing with expectant management they were reviewed again a further 3 weeks later and if the gestational sac was still present on USS they were advised to proceed with medical or surgical evacuation. In the ectopic pregnancy arm of the study, follow-up was continued until the serum hCG was less than 20 IU/L, samples taken at 48 hours, twice weekly for two weeks and then weekly. Expectant management was discontinued if a woman had severe pain or serum hCG was not declining. All women were given open access to the gynaecological ward and the telephone number. Expectant management was discontinued if the women complained of increasing abdominal pain or hCG did not decline on follow-up measurements.

Clinical data was collected from patient's medical records and the hospital patient database and recorded in a Microsoft Excel spreadsheet.

6.2.1 Statistical analysis

Databases was established to record the women's age, parity, number of previous early pregnancy losses, gestational age from menstrual dates, the presence of pain and bleeding, ultrasound findings, serum biochemical measurements, expectant management outcomes, time to resolution of the pregnancy and complications. The outcomes were dichotomised into 'successful expectant management' and 'failed expectant management' categories and statistical analyses were carried out using SPSS version 16.0 (SPSS, Chicago, IL, USA). Comparison of means of continuous variables was performed using Mann-Whitney or Student's–t tests depending on data distribution. Proportions were compared using the Yates corrected χ^2 test. A value of $P < 0.05$ was considered statistically significant. Results were entered

into Elson *et al'*s original decision-trees, and the sensitivities and specificities calculated.

6.3 RESULTS

Study recruitment is summarised in Figure 20. 42 women with a diagnosis of miscarriage were identified on ultrasound scan, opted for expectant management and consented to take part in the study. These were all patients at Sunderland Royal Hospital. In seven women data was incomplete and they were therefore excluded from further analysis. Of the remaining 35 women, 29 (82%) had successful expectant management, three (9%) went on to have medical treatment, and three (9%) had a surgical evacuation. Two women had emergency admissions to the gynaecology ward: one patient woman was admitted with a collapse and had an emergency surgical evacuation; another was admitted with pain and collapse but did not require any further treatment.

Miscarriages   **Ectopic Pregnancies**

```
┌──────────────────────────┐              ┌──────────────────────────┐
│ Total number miscarriages│              │  Total number ectopics   │
│      recruited = 42       │              │      recruited = 11      │
└──────────────────────────┘              └──────────────────────────┘
           │                                          │
           │   ┌──────────────────────────┐           │   ┌──────────────────────────┐
           ├───│ Incomplete data sets = 7 │           ├───│ Incomplete data sets = 2 │
           │   └──────────────────────────┘           │   └──────────────────────────┘
           │                                          │
   ┌──────────────────────────┐              ┌──────────────────────────┐
   │  Complete data sets = 35 │              │  Complete data sets = 9  │
   └──────────────────────────┘              └──────────────────────────┘
           │                                          │
   ┌───────┴────────┐                        ┌────────┴─────────┐
┌──────────────┐ ┌──────────────┐      ┌──────────────┐ ┌──────────────┐
│  Successful  │ │ Unsuccessful │      │  Successful  │ │ Unsuccessful │
│   expectant  │ │   expectant  │      │   expectant  │ │   expectant  │
│ management=29│ │ management=6 │      │ management=4 │ │ management=5 │
└──────────────┘ └──────────────┘      └──────────────┘ └──────────────┘
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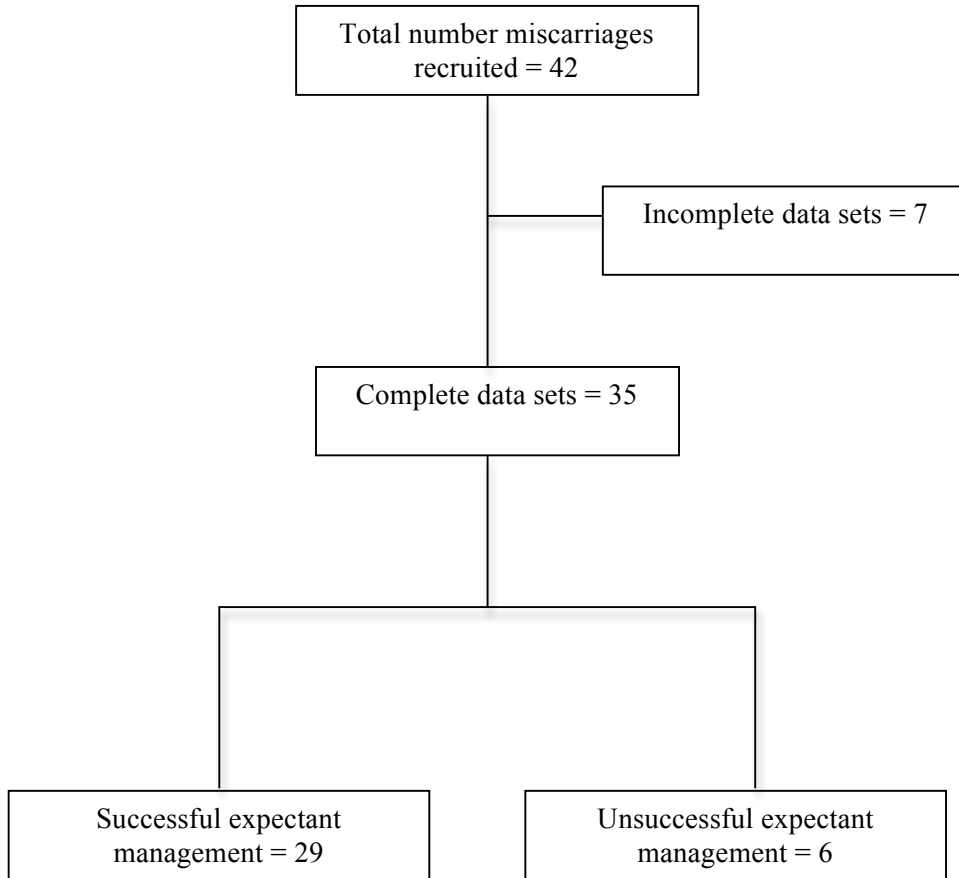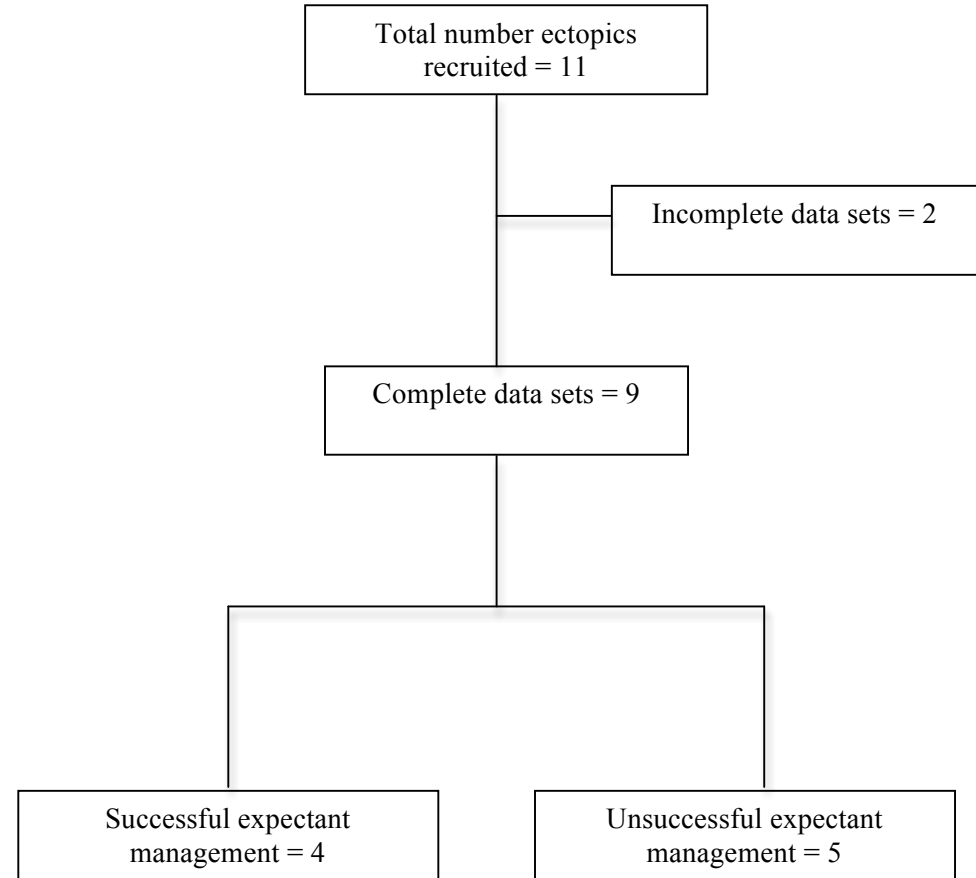
Figure 20. Miscarriage and ectopic pregnancy study recruitment flow chart.

An initial diagnosis of a missed miscarriage was made in 27 (77%) and of incomplete miscarriage in 8 (23%). The median time to pregnancy resolution was 21 days (25th-75th interquartile range 20-24).

Only eleven women with a diagnosis of ectopic pregnancy were identified on ultrasound scan, were suitable for and opted for expectant management, and consented to take part in the study. In two of these women data was incomplete and so they were excluded from further analysis. Of the remaining nine women five (56%) were recruited from King's College Hospital, and four (44%) from Sunderland Royal Hospital. Expectant management was successful in 4 cases (44%), and further treatment was required in five cases (56%). Three of these had surgery (all laparoscopically) and two had medical management with methotrexate. No serious complications occurred. The median mean mass diameter was 18 mm (25th-75th interquartile range 10-20). The mean time to resolution of the pregnancy was 13.1 days (SD 7.3). Progesterone levels were adjusted for the difference between the assays and Mann-Whitney U tests confirmed no statistically significant difference.

Table 16 and Table 17 show the measured variables in the miscarriage and ectopic pregnancy sets when compared by expectant management outcome. The only statistically significant difference between the successful and failed expectant management groups was serum hCG levels in the ectopic pregnancy set.

Variable	Outcome		P
	Successful expectant management n=29	Failed expectant management n=6	
Maternal age (years)[#]	33 (25-38.5)	30.5 (21.8-35.5)	NS
Parity[#]	1 (0-2)	1 (0-3)	NS
Gestational age (weeks)[*]	10 (1.6)	9 (2.3)	NS
Previous early pregnancy losses	0 (0-1)	0 (0-1)	NS
Incomplete miscarriages (%)[**]	17	32	NS
Vaginal bleeding (%)[**]	83	97	NS
Mean diameter POC (mm)[#]	16.3 (9-23)	20.6 (16.6-26.4)	NS
hCG (IU/L)[#]	3270 (606-7830)	8256 (2483-30944)	NS
Progesterone (nmol/L)[#]	1.7 (5.3-2.0)	22.5 (15.0-559.5)	NS
Inhibin A (pmol/L)[#]	24.7 (2.0-44.7)	86.6 (24.0-118.2)	NS
Inhibin ProαC (pmol/L)[#]	190.3 (152.3-260.4)	176.7 (123.9-256.0)	NS
IGFBP1 (μg/L)[#]	27.6 (16.1-36.7)	28.4 (9.6-52.2)	NS
17-OHP (ng/mL)[#]	0.43 (0.12-0.77)	0.41 (0.15-1.1)	NS

*Data distributed normally with values given as the mean and standard deviation; [#]data distributed non-parametrically with values given as the median (25th to the 75th interquartile range). [**]discrete data given as percentage of feature for each final outcome.

Table 16. Comparison of measured variables in miscarriages with successful and failed expectant management

Variable	Outcome		P
	Successful expectant management n=4	Failed expectant management n=5	
Maternal age (years) [*]	28.3 (7.2)	34.4 (6.5)	NS
Gestational age (weeks) [*]	10 (1.6)	9 (2.3)	NS
Parity[#]	0 (0-1)	1 (0-2)	NS
Previous early pregnancy losses	0 (0-2)	0 (0-2)	NS
Vaginal bleeding (%) [**]	75	0	NS
Pain (%) [**]	25	60	NS
Mean diameter mass (mm) [#]	15. 5 (8. 5-21)	18 (10-20)	NS
hCG (IU/L)[#]	56 (42-258)	1497 (661-2063)	0.014
Progesterone (nmol/L)[#]	6. 9 (5. 2-22. 3)	27 (19. 9-45.6)	NS
Inhibin A (pmol/L)[#]	0 (0-0)	0 (0-7)	NS
Inhibin ProαC (pmol/L)[#]	67 (15-421)	253 (54-691)	NS
IGFBP1 (μg/L)[#]	6.5 (2.8-24.4)	6.9 (5.5-44.4)	NS

[*]Data distributed normally with values given as the mean and standard deviation; [#]data distributed non-parametrically with values given as the median (25[th] to the 75[th] interquartile range). [**]discrete data given as percentage of feature for each final outcome.

Table 17. Comparison of measured variables in ectopic pregnancies with successful and failed expectant management

There was no significant difference in time to resolution by expectant management outcome in either the miscarriage or ectopic pregnancy sets. In expectant management of miscarriage the median time to spontaneous resolution was 21 days (25[th]-75[th] interquartile range 20.5-21.5) and 31 days (25[th]-75[th] interquartile range 12.5-43.5) in the failed expectant management group. In the expectant management of ectopic pregnancies median time to pregnancy resolution was 11 days (25[th]-75[th] interquartile range 7.25-14) in the successful group and 16.5 days (25[th]-75[th] interquartile range 5.75-24.25) in the group which required further treatment.

6.3.1 Miscarriages

When our data are put into Elson *et al's* miscarriage decision tree (Elson, 2005a; 2005b) (see Figure 21), successful expectant management is predicted with a sensitivity of 86% (95% CI 74-99) and a specificity of 29% (95% CI 5-62) with a PPV of 83% (95% CI 3-64) and NPV 33% (95% CI 4-71). 91% of cases with an unmeasurable inhibin A level were successfully managed expectantly, compared with 95% in the original study. With higher levels of inhibin A 76% had successful expectant management, compared with 51.5% in the original study.

6.3.2 Failed pregnancies

When our miscarriage and ectopic pregnancy data are used together in Elson's failed pregnancy decision tree (Elson, 2005b) (see Figure 22), successful expectant management is predicted with a sensitivity of 88% (95% CI 76-99) and a specificity of 17% (95% CI 4-38) with a PPV of 74% (95% CI 3-64) and NPV 33% (95% CI 4-71). If hCG < 1800 IU/L then the chance of successful expectant management is 88%, compared with 89% in Elson's original study. When the hCG > 1800 IU/L the chance of successful expectant management is 69%, compared with 44% in the original study.

Figure 21. Expectant management of miscarriage decision tree

Successful expectant management   29 (37)

Failed expectant management   6 (17)

Inhibin A

<=3.9

<>3.9

Successful   8 (20)

Failed   0 (1)

For expectant management

Watch for occasional failure

Successful   21 (17)

Failed   6 (16)

IGFBP-1

<=15

>15

Successful   4 (3)

Failed   2 (9)

For surgery

Successful   17 (14)

Failed   4 (7)

Inhibin pro αC

<=400

>400

Successful   17 (10)

Failed   4 (2)

For expectant management

Watch for occasional failure

Successful   0 (4)

Failed   0 (5)

For expectant management

High chance of failure

Decision tree developed by Elson (2005b) with original numbers shown in brackets

Figure 22. Expectant management of failed pregnancies decision tree

Successful expectant management 33 (55)

Failed expectant management 11 (24)

Decision tree developed by Elson (2005b) with original numbers shown in brackets

hCG

<=180

>1800

Successful 13 (40)
Failed 3 (5)

Successful 20 (15)
Failed 8 (19)

Average lesion diameter

IGFBP-1

<=25

>25

<=15

>15

Successful 13 (34)
Failed 3 (1)

Successful 0 (4)
Failed 0 (6)

Successful 5(3)
Failed 4 (14)

Successful 15 (12)
Failed 4 (5)

For expectant management

Watch for occasional failure

For expectant management

High chance of failure

Gestational age

Inhibin proαC

<=42

>42

<=400

>400

Successful 0 (2)
Failed 0 (1)

Successful 4 (1)
Failed 4 (13)

Successful 15 (9)
Failed 4 (1)

Successful 0(3)
Failed 0 (4)

For expectant management

Watch for occasional failure

For surgery

For expectant management

Watch for occasional failure

For expectant management

High chance of failure

6.4 DISCUSSION

6.4.1 Miscarriages

In this study 83% of expectant management of miscarriage was successful. Previous studies have found success rates ranging from 25% to 91% (Jurkovic *et al.,* 1998; Luise *et al.,* 2002) although the type of miscarriage has been found to be significant, with pregnancies with an intact gestation sac (i.e. missed miscarriages) being less likely to resolve spontaneously. In Elson's original cohort of 54 cases of expectant management of miscarriage (2005a; 2005b), the success rate was 69% with 41% having an initial diagnosis of a missed miscarriage. Our success rate of 83%, with 77% having an initial diagnosis of a missed miscarriage, is therefore better than expected. This may be a result of increasing experience and confidence amongst clinicians with the expectant management of miscarriage, or changing expectations amongst women with miscarriages.

We did not find that type of miscarriage, or any other factor was significantly different between the outcome groups. Inhibin A and IGFBP-1, used in Elson's original decision tree, were again useful for predicting successful expectant management of miscarriages. This again suggests that low levels of inhibin A indicate a small amount of functioning trophoblast (Birdsall *et al.,* 1997; Lockwood *et al.,* 1997) and that inhibin A reflects trophoblastic activity more accurately than hCG (Hauzman *et al.,* 2004), presumably due to its shorter half-life. Elson *et al.,* (2005a) were the first to describe raised levels of IGFBP-1 in association with miscarriage and suggested that that high levels of IGFBP-1 reflect a defect in attachment of the trophoblast to the decidua, thus the products of conception are more easily expelled. This agrees with placental work which has found higher levels of IGFBP-1 mRNA in placentas from pregnancies complicated by pre-eclampsia, suggesting that IGFBP-1 might be associated with impaired trophoblastic invasion (Shin *et al*., 2003). Our findings do not confirm or disprove this theory.

Inhibin proαC was not a useful part of the decision tree in our cohort, as in the cases with a high inhibin A and IGFBP-1, none had an inhibin proαC RI level of more than 400 pmol/L. Previously Illingworth *et al.,* (1996) found lower levels of inhibin proαC-RI to reflect non-viability, and Elson *et al.,* (2005a) found lower

levels of inhibin proαC-RI in successful expectant management of miscarriage. We therefore expected to see lower levels in the successful expectant management group, but this was not demonstrated in our cohort.

6.4.2 Ectopic pregnancies

Our findings confirm Elson's original finding that in ectopic pregnancy, serum hCG is the only significant variable between successful and failed expectant management groups (Elson, 2005b). In our cohort of ectopic pregnancies, all cases with hCG <400 IU/L resolved spontaneously. Lui *et al.,* (1997), Atri *et al.,* (2001), Elson *et al.,* (2005a), Trio *et al.,* (1995) and Korhonen *et al.,* (1994) also found lower initial levels of hCG to be associated with increased success of expectant management. Trio *et al.,* (1995) used receiver operating-characteristics curve analysis to chose hCG < 1000 IU/L as the optimal cut-off, and found it to detect 88% of ectopic pregnancies that resolve spontaneously. In our cohort the success rate of expectant management when serum hCG >1000 IU/L was 0% and when serum hCG < 1000 IU/L was 67%. Expectant management failed however, in two cases with hCG < 1000 IU/L.

When looking at symptoms we noted that all of our ectopic pregnancies with vaginal bleeding were successfully managed expectantly, although this difference did not reach statistical significance. The presence of vaginal bleeding has not previously been described as a marker of successful expectant management and our findings differ greatly from Elson's original study (2005b) where 100% of the ectopic pregnancies requiring surgical intervention had vaginal bleeding. Our observation may be spurious and related to our small numbers, although the presence of bleeding may indicate a tubal abortion, and therefore spontaneous resolution. We also noticed that a higher proportion of our failed expectant management group complained of pain, although again this was not statistically significant. It is likely that the presence of pain contributed to the decision to proceed with active management of the ectopic pregnancy. This therefore may lead to therapeutic bias in interpretation of the results although is reflective of regular clinical practice.

Overall our rate of successful expectant management of ectopic pregnancy was 44%. Other studies have reported success rates of between 48% and 100% (Elson *et al.,* 2004; Ylostalo *et al.,* 1992; Korhonen *et al.,* 1994; Cacciatore *et al.,* 1995; Trio *et al.,* 1995; Lui *et al.,* 1997), however, considering our inclusion criteria, our success rate was lower than expected. This again may be biased by our small numbers, or may be the result of a lack of experience in expectant management of ectopic pregnancy amongst clinicians.

6.4.3 Failed pregnancies

In Elson *et al's* original studies hCG was the best discriminator for successful expectant management of a failed pregnancy. The pregnancies with low hCG levels and a small amount of pregnancy tissue on ultrasound scan were the most likely to resolve spontaneously, both of these measures reflecting the amount of functioning trophoblast. IGFBP-1 was the next best discriminator with high levels reflecting defective attachment to the decidua and an increased chance of successful expectant management. These findings are all confirmed in our population. The rest of the failed pregnancy decision tree is less useful in our cohort, however, as the cut-offs of lesions >25 mm, gestational age <42 days, and inhibin proαC >400 pmol/L were not discriminatory.

6.4.4 Models in the management of early pregnancy failure

Decision-making regarding the treatment of early pregnancy failure is complex. Decision tree analysis produces an easy to follow-pathway which resembles the clinical decision making process. The decision trees can be used for patient selection and counselling, thus enabling women to make informed decisions.

Using the decision tree analysis for the management of miscarriages alone successful expectant management could be predicted with sensitivity of 86% and specificity of 29%. This means that it is excellent at predicting those women who will have a successful management but not very accurate at predicting those whose expectant management will fail. This decision-tree performs nearly as well in our cohort as it did in the cohort from which it was

constructed. It also compares favourably to the logistic regression model proposed by Nielsen *et al.,* (1996) based on the traditional markers hCG and progesterone, and to the use of colour Doppler as advocated by Schwarzler *et al.,* (1999).

The decision tree for failed pregnancies works less well with our data, with 88% sensitivity and only 17% specificity. This again means that the decision tree is good at predicting those women that will have a successful expectant management but is poor at predicting those in which expectant management will fail. In the group for which the decision-tree suggests surgery is indicated, 50% had successful expectant management.

6.5 CONCLUSION

We have shown that many of the discriminatory steps in Elson's decision trees to predict successful expectant management of failed pregnancies and miscarriages are useful in our cohort, despite lack of significant differences between the successful and failed expectant management groups. We have seen that the novel biochemical markers inhibin A, inhibin proαC and IGFBP-1 may be useful in the decision trees, although these are not yet available in routine clinical practice.

6.6 SUMMARY OF PREDICTION OF SPONTANEOUS RESOLUTION OF MISCARRIAGES AND ECTOPIC PREGNANCIES USING NOVEL BIOCHEMICAL MARKERS

- The decision trees using novel biochemical markers inhibin A, IGFBP-1 and inhibin proαC are sensitive but not specific in their prediction of spontaneous resolution of miscarriages and failed pregnancies.

- Serum hCG level remains the best single marker to predict successful expectant management of ectopic pregnancy.

CHAPTER 7. DISCUSSION

The aim of this thesis was to investigate the use of novel biochemical markers in the diagnosis and management of early pregnancy complications and models for their use in clinical practice have been developed and tested. Although there remains a lack of definitive evidence that novel biochemical markers are clinically useful for the diagnosis and management of early pregnancy problems in current practice, our data suggests that further research is necessary.

7.1 METHODOLOGICAL CHALLENGES

There are many methodological challenges when designing and conducting research in patients with early pregnancy problems. The diagnosis of a miscarriage, ectopic pregnancy or a PUL is a distressing and potentially traumatic time for a woman. At the time of diagnosis there is a large amount of clinical information to take in and decisions to make, many women feel unable or unwilling to consider taking part in research for these reasons. The symptoms of bleeding and pain in early pregnancy are very common and can be very worrying for women, as a result many attend emergency services and so not all are seen initially at Early Pregnancy Assessment Units, this can lead to suitable cases being missed. The large number of patients assessed in Early Pregnancy Assessment Units makes it challenging for a small research team to identify all suitable patients, particularly during weekends and public holidays. Excellent working relationships and communication with clinical colleagues are essential.

Expectant management is a fairly novel concept in the management of miscarriage and ectopic pregnancy and patients and clinicians have more experience with medical and surgical management options. As a result, due to counselling techniques and expectations, even when expectant management inclusion criteria are fulfilled, patients often choose to pursue a more active course of management. This has had implications on recruitment for these studies. It already appears however, that this 'culture' is gradually changing, as patients and clinicians build up more experience in expectant management options.

In response to difficulties with recruitment for the expectant management of PUL and ectopic pregnancy studies, we extended our recruitment to King's College Hospital, London. This improved our numbers but has introduced other issues, which we have done our best to address. The study populations covered by the two recruitment centres differ in a number of ways. Although both are inner-city urban areas with high levels of socioeconomic deprivation, the population in Sunderland is fairly static, with a small proportion of ethnic minorities whereas King's College Hospital covers an area with a highly racially mixed and mobile population. Our findings are therefore based on a diverse cohort but we cannot infer that they are applicable for general use.

When recruiting patients from multiple sites it is a concern that the clinical management of the patients could differ between them and therefore that site of recruitment affects the outcome of treatment. Although we cannot exclude this as a possibility, we think this is unlikely to be a significant problem in our studies. The Early Pregnancy Assessment Units at Sunderland Royal Hospital and King's College Hospital have similar approaches to the management of early pregnancy problems and have similar guidelines. There will of course be variation in the application of the guidelines by individual clinicians, but this is reflective of clinical practice, and there was no difference in expectant management outcome between the two recruitment centres.

In the PUL and ectopic pregnancy cohorts the hCG and progesterone analysis was split between the two recruitment centres, and therefore analysed by different methods. Comparisons of the methods by NEQAS and ourselves confirmed that the hCG immunoassays were comparable, and there is a 2 nmol/L difference between the progesterone assays which did not confer any significant difference.

7.2 MODELS IN THE DIAGNOSIS AND MANAGEMENT OF EARLY PREGNANCY PROBLEMS

In this thesis I have developed a decision tree for predicting spontaneously resolving pregnancies of unknown location and have tested models for predicting successful expectant management of miscarriage and failed pregnancy. When formulating a diagnosis or management plan in clinical

practice, a range of factors are combined rather than considered in isolation. This process is mirrored in decision-tree analysis, although the decision-making steps are explicit and evidence-based.

For women found to have a 'pregnancy of unknown location' it is important to balance the importance of reaching a diagnosis with the inconvenience, cost, and potential risks associated with follow-up visits and surgical intervention. Currently used models for the management of PUL use serum hCG ratios at 0 and 48 hours, or serum hCG and progesterone levels, to guide further care. The use of the decision-tree, as described in Chapter 5, can aid with patient selection and counselling. The flow-chart is easy to follow and guides the clinician to the appropriate follow-up strategy based on initial hCG and progesterone levels. Patients can be shown the decision-tree so that they can understand the basis for the strategy of their care and make informed decisions. Using this approach only 23% require 'high-risk' follow-up i.e. a repeat blood sample in 48 hours, and the remaining 77% have an 89% probability of spontaneous resolution. The need for repeat blood sampling at 48 hours is therefore minimized without a reduction in sensitivity.

Expectant management is a useful alternative to medical or surgical intervention in the treatment of miscarriages and ectopic pregnancies. It is an attractive option for women who desire a natural solution and who prefer to avoid hospitals, or fear operations. Many advantages of expectant management have been demonstrated, including improved mental health scores in women undergoing expectant management of miscarriage (Wieringa-de Waard *et al*., 2002), and economic advantages of expectant management of miscarriage over traditional surgical management. The main difficulty with expectant management of both miscarriages and ectopic pregnancies, however, is the lack of criteria which reliably predict the likelihood of spontaneous resolution of the pregnancy, without requiring medical or surgical intervention. The decision-tree models developed by Elson (2005b) for the expectant management of miscarriage and failed pregnancies use serum levels of traditional and novel biochemical markers. A woman can be given a probability of success based on these levels and can therefore make a fully informed decision. We have tested these models and have found them to be useful for predicting successful

expectant management in our cohort, despite differences in baseline factors between this current study and the original findings.

The next step in the development of these models is to test them prospectively in a range of centres, with implementation carefully audited to allow for the differences between ultrasound operators and biochemistry laboratories. This would enable necessary adjustments to be made in order to define the optimal cut-offs for each individual unit and the most appropriate model for general use.

7.3 GLYCOSYLATION OF HCG

Measurements of serum hCG levels are the most commonly used biochemical measurement in the management of early pregnancy problems. We have identified variable glycosylation of hCG by lectin-affinity chromatography, with changes in expression with gestational age and by pregnancy outcome. This suggests that looking only at levels of hCG is too simplistic, and that by analysing the structure of the hCG molecule, in particular the pattern of glycosylation, much more useful information could be obtained. H-hCG, a particular isoform of hCG predominant in very early pregnancy, has already been found to be useful in the prediction of early pregnancy outcome (Sutton-Riley *et al*., 2006). Using lectin-affinity chromatography to look at the overall pattern of glyco-isoform expression has the potential to be a more reliable test, and have wider applicability, it also provides a means by which we can increase our understanding of the role of hCG isoforms in the physiology of pregnancy.

CHAPTER 8. CONCLUSIONS AND FUTURE RESEARCH GOALS

This thesis describes three studies exploring the use of novel biochemical markers in the diagnosis and management of early pregnancy problems:

Lectin-affinity chromatography reveals five major glyco-isoforms of hCG in early pregnancy, the expression of which changes with gestational age and by pregnancy outcome. Further work is required to explore the physiological basis of these findings and the applicability of lectin-affinity chromatography as a clinical test.

The novel markers of the luteo-trophoblastic axis inhibin A, IGFBP-1 and inhibin proαC are found not to be clinically useful in the prediction of spontaneously resolving PULs. A decision tree model using initial serum hCG and progesterone levels is proposed to guide follow-up arrangements. A larger prospective evaluation of this model is required before implementation into clinical practice could be recommended. A large multicentre study could also allow the use of inhibin A, IGFBP-1 and inhibin proαC in predicting specific outcomes of PULs, to be investigated.

The decision trees developed by Elson (2005b), using inhibin A, IGFBP-1 and inhibin proαC, are found to be useful for predicting spontaneous resolution of miscarriages and failed pregnancies. A large prospective multi-centre study is now required to validate these for general implementation. This could also incorporate the multi-centre investigation, which would be needed to recruit sufficient numbers, for an observational study into the use of these novel biochemical markers in the prediction of spontaneous resolution of ectopic pregnancies.

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APPENDICES

APPENDIX A. EXAMPLE PATIENT INFORMATION LEAFLET, CONSENT FORM, GP LETTER AND DATA COLLECTION SHEET

PATIENT INFORMATION LEAFLET

Study of the value of novel biochemical markers in the prediction of successful expectant management of pregnancy of unknown location

We are asking for your help with research that is being conducted in our hospital. Before you decide it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the problem?

You have had an ultrasound scan to check that your pregnancy is developing normally today. Unfortunately we are unable to see your pregnancy on scan. This is a pregnancy of unknown location. There are three possible reasons for this:

1. **A very small normal pregnancy.** The pregnancy may be normal, but too small to be seen on the scan. This usually happens in women who are unsure of their dates or who have long menstrual cycles.

2. **A miscarriage**. The pregnancy may already have been lost with bleeding and cannot be seen on scan. In these cases the pregnancy test may be positive for many weeks after the miscarriage.

3. **An ectopic pregnancy.** The pregnancy may be growing outside the womb (uterus). These pregnancies are usually growing in the Fallopian tubes and may be difficult to see on scan. Sometimes an ectopic pregnancy can be dangerous and this is why we need to monitor you very carefully.

What is the purpose of the study?

In all women with a pregnancy of unknown location we take blood tests to measure pregnancy hormones (βhCG and progesterone) in the blood. This helps us to determine which of the above possibilities is most likely. We call this expectant management as it allows us to avoid unnecessary invasive tests and treatments in many cases. We think that this may be more accurate if we measure some new biochemical markers. These markers have been used in the diagnosis and treatment of other early pregnancy problems but not in pregnancy of unknown location before. If they are more accurate markers we would be able to counsel and treat women with pregnancy of unknown location more effectively.

Why have I been chosen?

Every woman who is found to have a pregnancy of unknown location will be approached to take part in this study.

Do I have to take part?

Participation is voluntary and you may decide not to take part or withdraw at any time without giving a reason. This will not affect your medical care in anyway.

What will happen to me if I take part?

If you choose to take part in our study a blood sample will be taken for the new biochemical markers at the same time as the routine blood tests are taken. No 'extra' blood tests are required. We will then just observe your progress through until a diagnosis is made, and any treatment required is complete. Some of your details (including length of pregnancy, ultrasound scan findings, blood test results, diagnosis, and any treatments required) will be extracted from your medical records and recorded in a research database. Your care will not be any different from normal management, apart from the blood sample mentioned above.

Will taking part in the study affect my care?

As the value of these biochemical markers in investigating pregnancy of unknown location has not yet been investigated, these tests will not affect your care in any way.

What are the possible benefits?

We hope that this study will prove that measuring these biochemical markers can effectively diagnose pregnancy of unknown location. The information we get from this study may help us to improve our care of future patients in your situation. This however cannot be guaranteed.

Will my taking part in the study be kept confidential?

All information the collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

Will I find out the results of the research project?

Once the study is complete we will contact all participants to let you know what the results were.

What should I do if I get pain or bleeding?

If you develop severe pain or bleeding you should contact us immediately. The ward number is **0191 569 9747.** Until the final diagnosis is made we advise you not to travel outside of the local area and to avoid strenuous exercise.

What do I do next?

If you agree to take part in this research project we will ask you to sign a consent form. We will also give you a letter for your doctor. You will be given a copy of this information leaflet, with the contact details, and a signed consent form to keep.

If you have any further questions before or during the study you may contact Dr Maya Chetty on 0191 565 6256, bleep 57519

Thank you for your help.

Patient Identification Number for this study:

CONSENT FORM

Title of Project: Prediction of successful expectant management of pregnancy of unknown location

Name of Researcher: Dr Janine Elson, Dr Maya Chetty

Please initial box

1. I confirm that I have read and understand the information sheet dated .... (version ....) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the research team and that certain details will be recorded in the research database. I give permission for these individuals to have access to my records.

4. I understand that the research notes and database may be looked at by regulatory authorities. I give permission for these individuals to have access to these records.

5. I agree to take part in the above study.

_____   _____
Name of Patient   Signature
Date

_____   _____
Name of Person taking consent   Signature
Date
(if different from researcher)

_____   _____
Researcher   Signature
Date

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Early Pregnancy Assessment Unit

Department of Gynaecology

Sunderland Royal Hospital

Tel: 0191 565 6256 ext 49779

Dear Doctor,

Your patient has consented to take part in our study **"the value of novel biochemical markers in the prediction of successful expectant management of pregnancies of unknown location"**. One blood sample has been taken after the initial ultrasound scan for the analysis of novel biochemical markers.

The study does not involve any additional procedures or hospital visits.

As the value of these markers has not yet been fully evaluated, the care of your patient will not be affected. However if they are found to be of value, they may benefit women in the future.

If you have any questions regarding the study, please contact Dr Maya Chetty on 0191 565 6256, bleep 57519.

Yours sincerely,

Dr Maya Chetty   Miss Janine Elson

Research Fellow   Consultant Obstetrician and Gynaecologist

Ethical approval no: 05/Q0902/63

Date approved: 03/02/06

Study number:

Hospital number:

Date recruited: Fertility pt: Y   /   N

Maternal age: Para   +

LMP: Gestational age:

Bleeding   0   1
Pain   0   1

Endometrial thickness:

Progesterone	
HCG	
17-OHP	
Inhibin A	
Inhibin pro αC	
IGFBP-1	

Outcome: failed PUL   miscarriage   ectopic   viable IUP
Time to diagnosis:
Time to pregnancy resolution:
Further treatment required   0   1
                                   Medical /Surgical

Complications: 0   1
                                 Details:

APPENDIX B. PUBLICATIONS AND PRESENTATIONS

The use of novel biochemical markers in predicting spontaneously resolving 'pregnancies of unknown location'.

Chetty M, Sawyer, E, Dew T, Chapman J, Elson J.

Human Reproduction, 2011; 26(6): 1318-1323.

Development of maternal serum hCG glycosylation in early pregnancy

Chetty M, Johnson AL, Nayar, R, Elson J, Butler SA, Chapman AJ

Manuscript in preparation

Novel biochemical markers to predict spontaneous resolution of pregnancies of unknown location.

Chetty M, Sawyer, E, Dew T, Chapman J, Elson J.

Ultrasound in Obstetrics and Gynaecology, 2009; 34 (suppl. 1): 16 (Abstract)

Platform presentation at 19[th] World Congress on Ultrasound in Obstetrics and Gynaecology, Hamburg (September 2009).

Biochemistry in the diagnosis and management of abnormal early pregnancy

Chetty M, Elson J.

First trimester pregnancy complications symposium. *Clinical Obstetrics and Gynaecology*, 2007; 50(1): 55-66 (Review).

Glycosylation of maternal serum hCG in early pregnancy.

Chetty M, Johnson AL, Elson J, Chapman AJ

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