The Effect of Prolongation of Luteal Support with Progesterone following In-vitro Fertilisation Treatments on Pregnancy Outcome.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Medicine

Dr Richard Thomas Russell
MB ChB MRCOG

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Abstract

Over 5 million babies have been born as a result of IVF procedures. Worldwide, over 1 million cycles of IVF are performed annually. The IVF procedure involves ovarian stimulation with the purpose of developing multiple follicles and maximising the potential oocyte yield. As a consequence of high oestradiol levels produced during treatment and the use of GnRH agonists or antagonists, a luteal phase deficiency results. This phenomenon is associated with reduced implantation potential and suboptimal conditions for maintenance of early pregnancy. Luteal support in the form of progesterone or HCG has been demonstrated to improve pregnancy rates after IVF. A number of luteal support protocols have been investigated with progesterone the most commonly used drug. The optimum duration of luteal support has yet to be defined. With no agreement in clinical practice evident, the reported use of progesterone ranges from withdrawing luteal support at confirmation of biochemical pregnancy to continuation beyond 12 weeks gestation. Whilst luteal support is considered a very important aspect of IVF treatment, there is very little evidence to support an optimum duration of use.

The DOLS trial is a prospective randomised double blind placebo controlled trial investigating the effect of additional luteal support beyond confirmation of pregnancy test after assisted conception. Four hundred and sixty seven patients were randomised after confirmation of biochemical pregnancy to receive a further 8 weeks of vaginal progesterone or 8 weeks of placebo. Summary results were to include a primary outcome defined as viable pregnancy at 12 weeks gestation, whilst secondary outcomes were to report on live birth rates, pregnancy associated complications, neonatal outcomes, effect on first trimester serum screening and effect on uterine artery Doppler velocity.

The DOLS trial reported no difference in pregnancy outcome at 12 weeks gestation, with 167/228 (73.3%) women randomised to the extended luteal support treatment arm having a confirmed viable intrauterine pregnancy compared with 167/233 (71.7%) women randomised to the placebo arm of the trial; adjusted risk ratio 0.97 (95%CI 0.87 to 1.09). Similarly live birth rates were not different between the treatment groups; 71.1% versus 70.4% respectively. No effect of extending luteal support beyond positive pregnancy test was observed in reference to complications of pregnancy, neonatal outcome, uterine artery Doppler velocity or antenatal screening outcome.

In conclusion, we have confirmed that continuing luteal support using progesterone beyond confirmation of biochemical pregnancy offers no benefit in terms of pregnancy outcomes. However the extended use of progesterone until 12 weeks gestation does not confer harm. We suggest that all clinics worldwide should consider offering luteal support no further than positive pregnancy test, at which point it can be safely withdrawn without compromising live birth rates and reducing treatment burden.

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The process up to completion of this thesis has been a long and frequently arduous journey; from initial conception of the study, performance of the clinical research, data analysis and thesis writing, my academic abilities and often my patience has been tested to the limit. However it would be wrong of me to suggest that I was the only one who has suffered, or should I suggest suffered at my hands. There is a long list of colleagues and friends who have helped me along the way, and whose assistance I would like to acknowledge.

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Abbreviations

αFP	Alpha feto protein
AMH	Anti mullerian hormone
APS	Anti-phospholipid syndrome
ART	Assisted reproductive technology
βHCG	Beta human chorionic gonadotrophin
cER	Cytosolic estradiol receptor
CES	Cumulative embryo score
CI	Confidence interval
CL	Corpus luteum
cPR	Cytosolic progesterone receptor
CRL	Crown rump length
CRP	C-reactive protein
DMC	Data monitoring committee
EGF	Endothelial growth factor
ELS	Extended luteal support
eNOS	Endothelial nitric oxide synthase
ER	Oestrogen receptor
ESHRE	European society of human reproduction and endocrinology
FMD	Flow mediated dilatation
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
GnRHa	Gonadotrophin releasing hormone agonist

HCG	Human chorionic gonadotrophin
HFEA	Human fertilisation and embryology authority
HPO	Hypothalamic-pituitary-ovarian axis
IL-6	Interleukin 6
IUGR	Intrauterine growth restriction
LBR	Live birth rate
LH	Leutenising hormone
LIF	Leukemia inhibitory factor
LPD	Luteal phase deficiency
ICSI	Intra cytoplasmic sperm injection
ICM	Inner cell mass
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IVF	In vitro fertilisation
MHC	Major histocompatibility complex
MHRA	Medicines and healthcare regulatory agency
MIH	Mullerian inhibitory hormone
MMP	Matrix metallproteinases
MSAFP	Maternal alpha-fetoprotein
Muc-1	Endometrial mucin
NHS	National Health Service
NT	Nuchal translucency
OR	Odds ratio

PAPP-A	Pregnancy associated plasma protein A
PCOS	Polycystic ovarian syndrome
PBMC	Peripheral blood mononuclear cells
PI	Pulsatility index
PIBF	Progesterone induced blocking factor
PIL	Patient information leaflet
PR	Progesterone receptor
RCT	Randomised controlled trial
RI	Resistance index
RR	Risk ratio
SHBG	Sex hormone binding globulin
SOP	Standard operating procedure
TGFβ	Transforming growth factor β
Th	T helper cells
TIMP	Tissue inhibitors of metalloproteinases
TSC	Trial steering committee
TNF	Tumour necrosis factor
TRH	Thyrotrophin releasing hormone
UE3	Unconjugated estriol
UK	United Kingdom
VSD	Ventricular septal defect

Definitions

Biochemical pregnancy

Confirmation of a positive pregnancy test (serum or urine), usually performed 16 days after oocyte retrieval.

Biochemical pregnancy loss

A negative pregnancy test subsequent an initial positive test result (without evidence of clinical pregnancy on ultrasound).

Clinical pregnancy

Evidence of clinical pregnancy demonstrated on ultrasound (intrauterine gestation sac with or without evidence of a fetal pole).

Ongoing pregnancy

Evidence of viable intrauterine pregnancy demonstrated on ultrasound (fetal pole with fetal heart pulsations above 100 beats per minute).

Miscarriage

The loss of a clinical or ongoing pregnancy.

Live birth

Delivery of fetus beyond 23^{+6} weeks gestation; defined by calculated last menstrual period.

Gestation

Gestational age as defined by a calculated last menstrual period. The day of oocyte retrieval and subsequent fertilisation is equivalent to 2 weeks gestation as conventionally described using the last menstrual period. Therefore 5 weeks following oocyte retrieval is equivalent to 7 weeks gestation in conventional terms. Gestational age is used in this thesis unless specifically stated.

Chapter 1: Introduction and Literature Search

1.0 Introduction

The review of the literature was performed using MEDLINE, SCOPUS, EMBASE and the Cochrane Database. Papers unavailable electronically were requested through the British Library.

Infertility is defined as failure to conceive after frequent and unprotected sexual intercourse for one or two years (1). The United Nations defines reproductive health as "a state of complete physical, mental and social well-being in all matters relating to the reproductive system and to its functions and processes" (2). Infertility should, therefore be considered to be a disease process worthy of investigation and treatment (1).

The prevalence of infertility in the United Kingdom (UK) is between 1 in 6 and 1 in 7 couples (3), affecting approximately 3.5 million people (4). The prevalence of infertility is estimated to be 5.5%, 9.4% and 19.7%, at ages 25-29 years, 30-34 years and 35-39 years respectively and can be attributed to a number of causes (figure 1).

The main causes of in infertility in the UK are (approximate prevalence in % terms); (5-7)

- Ovulatory disorders (25%)
- Tubal damage (20%)
- Male factors (30%)
- Uterine or peritoneal disorders, for example endometriosis (10%)
- Unexplained (25%)

In approximately 40% of cases, causes for infertility are found in both the male and female.

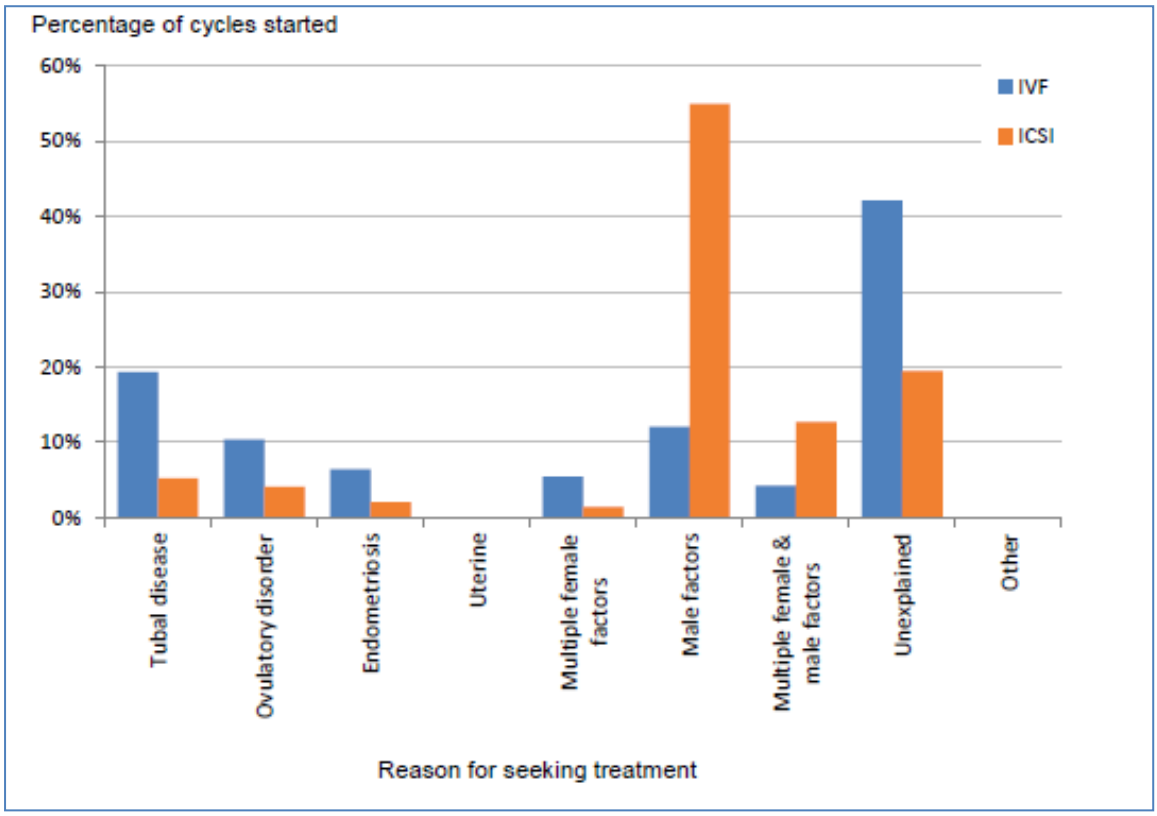

Figure 1. Percentage of couples seeking IVF / ICSI treatment, by reason for seeking treatment in 2011. Fertility Treatment in 2011, Trends and Figures. HFEA. www.hfea.gov.uk (4)

Thorough and appropriate investigation of patients is critical to identify the cause of infertility. Once established, treatment can be classified into three main categories;

1. Medical treatment to restore fertility (for example drugs to induce ovulation)
2. Surgical treatment to restore fertility (for example laparoscopic ablation of endometriosis)
3. Assisted Reproduction Technology (ART) (for example in-vitro fertilisation (IVF) with or without intracytoplasmic sperm injection (ICSI)).

The IVF procedure involves:

- Attainment of sperm from the male, usually obtained through masturbation and occasionally using surgical sperm retrieval techniques.
- Attainment of oocytes (eggs) from the female patient, usually by ultrasound guided transvaginal aspiration of ovarian follicles which have developed as a consequence of ovarian stimulation with exogenous gonadotrophins.
- The male and female gametes are approximated using either straight-forward IVF procedures or ICSI (a process where individual oocytes are injected with individual sperm). If fertilisation occurs, the embryo will undergo a period of developmental observation under strict laboratory conditions.
- The developing embryo is replaced into the woman's uterus using a trans-cervical catheter. Embryo implantation is anticipated shortly after replacement.
- Gestational support for maintenance of pregnancy is then required to prevent early pregnancy loss. Progesterone and human chorionic gonadotrophin are most commonly used.

- Confirmation of viable clinical pregnancy with observation of an appropriately positioned pregnancy and presence of fetal heart pulsations on ultrasound scan.

In 2010 57,957 cycles of IVF or ICSI treatments were performed in addition to 3,911 cycles of donor insemination in the UK. In 2011, 74 UK HFEA licensed clinics treated a total of 48,147 women and performed a total of 61,276 cycles of IVF (47.1%) or ICSI (52.9%) treatments, and 4,091 cycles of donor insemination (4). The increase of 4.3% in number of cycles performed between 2010 and 2011 is indicative of the trend in provision of treatments over the last two decades (figure 2).

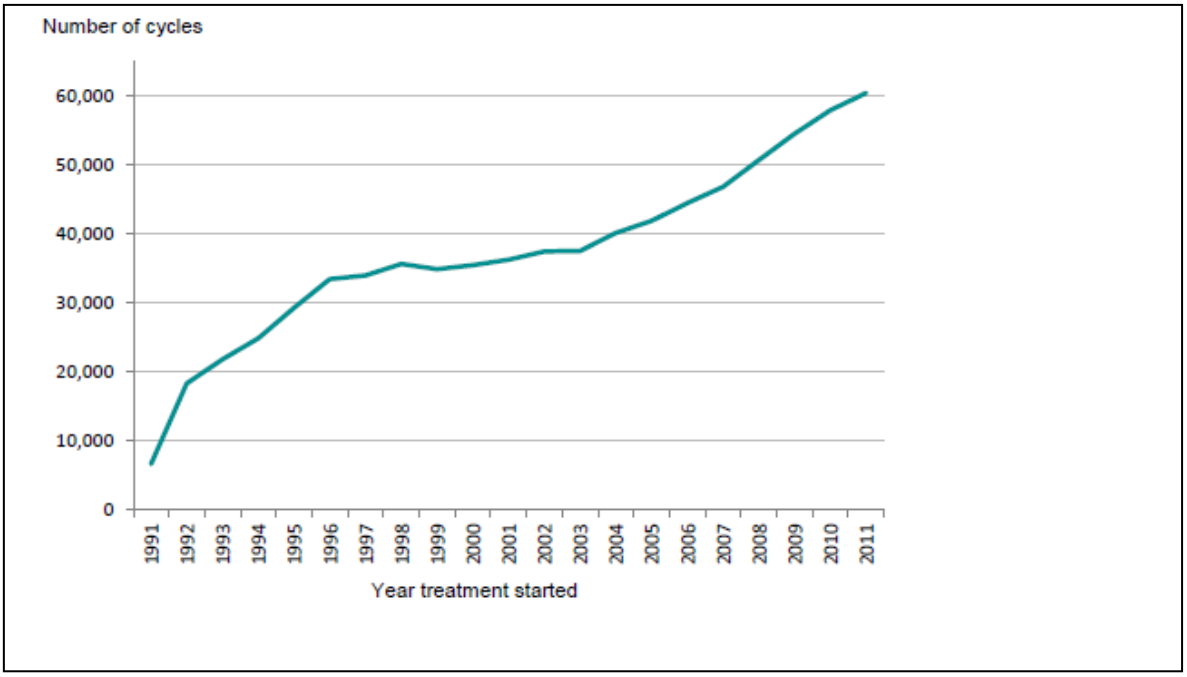

Figure 2. Number of IVF cycles performed each year, 1991 to 2011. Fertility Treatments 2011, Trends and Figures, HFEA 2011. www.hfea.gov.uk (4)

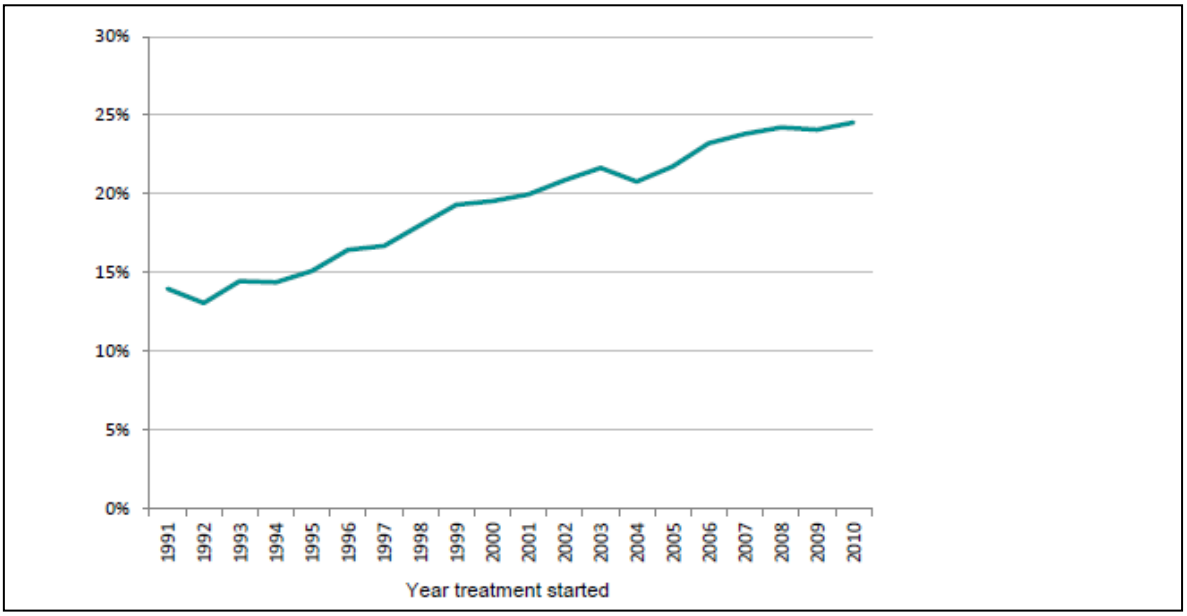

Figure 3. Live birth rate per IVF cycle started; 1991 – 2010. Fertility Treatments 2011, Trends and Figures, HFEA 2011. www.hfea.gov.uk (4)

A similar increase is observed worldwide with the number of children born after ART now exceeding 5 million. The estimated number of children born per year in the latest world report from 2003 ranged between 173,000 and 230,000 (8). The most recent report from the European Society of Human Reproduction and Embryology (ESHRE) reporting births in 2006 attributed to ART ranged from 0.8 to 4.1% of the total national birth cohorts, with more than 3% of all national births resulting from IVF procedures in several countries (9). Multiple deliveries are reported to effect between 5.7 and 38.3% of ART conceived pregnancies and dependent on nationally adopted embryo transfer policies and healthcare provision. Significant reductions in multiple pregnancy rates have been observed in countries with reasonable IVF reimbursement policies (10-12).

Although IVF is a relatively new procedure, technological advances and procedural refinements have resulted in steady increases in successful outcome. Between 1991 and 2010, the live birth rate (LBR) from ART increased from 14.0% to 24.5% per cycle started (4). However the overall success rate remains disappointing (figure 3).

The overall expenditure on IVF is difficult to determine. In the UK there has never been an agreed tariff for treatments provided for by the National Health Service (NHS), with costs approved at a local level. Conversely, over half of all cycles performed are on a self funding basis with significant variation in tariffs between providers. Based on recent health economic models, the average cost of IVF was reported to be £3123 per cycle. In crude terms 61276 cycles performed in the UK in 2011 is equivalent to treatment expenditure in excess of £191 million. This is likely to be a significant underestimation as some clinics report tariffs in excess of £10,000 per cycle. These figures also do not include costs

associated with infertility investigations, intrauterine insemination, ovulation induction and the additional costs associated with the use of donor gametes. Working on a presumption of a similar tariff structure and 1 million cycles performed each year worldwide, expenditure could be in excess of £3 billion. The market value of the IVF industry in the United States alone in 2008 was estimated to be $4.04 billion (13).

1.1 Ovarian Function and Steroidogenesis

The production of oestrogen and progesterone by the ovary corresponds to the cyclical or episodic release of an oocyte. Prior to ovulation, the principle hormone produced is oestrogen (oestrogen dominance / follicular phase) which acts to prepare the female reproductive tract for receiving sperm and fertilization of the oocyte. After ovulation, progesterone production predominates (progesterone dominance / luteal phase) which prepares the endometrium for implantation and support of the embryo should fertilization occur.

The adult ovary consists of interstitial tissue consisting of glandular tissue (interstitial glands), set in stroma. The interstitial tissue surrounds follicles which in turn and through an interconnecting series of events coordinate progress through a menstrual cycle. Gamete production in the female occurs by a process of mitosis, genetic reshuffling, reduction by meiosis and cytodifferentiation during oocyte maturation. The proliferative phase is completed during fetal / neonatal life, when the primordial germ cells or oogonia cease dividing and enter meiosis where they arrest in the first meiotic prophase to become primary oocytes. They do this within the primordial follicle, which consists of flattened mesenchymal cells (granulosa cells) condensed around what is the first oogonium and then the primary oocyte, all enclosed within a basement membrane, the membrane propria. The primordial follicle constitutes the fundamental functional unit of the ovary, and can remain in an arrested state for up to 50 years (14).

During the female reproductive cycle, the primordial follicles are controlled by gonadotrophins; follicle stimulating hormone (FSH) and leutenising hormone (LH) which are

secreted by the anterior pituitary gland under the influence of pulsatile secretions of gonadotrophin releasing hormone (GnRH) produced by the hypothalamus. Fluctuations in the frequency and amplitude of GnRH are important in generating other hormonal changes that are responsible for the cyclical pattern of menstruation. The frequency of pulses is increased by oestrogens and decreased by progesterone and testosterone. The frequency increases during the late follicular phase of the cycle culminating in an LH surge. During the secretory phase, pulsatility frequency decreases secondary to an increase in circulating oestrogen and progesterone. At the time of the mid-cycle surge, the sensitivity of gonadotropes to GnRH is greatly increased because of the exposure to GnRH pulses of the frequency that exist at the time. This self-priming effect of GnRH maximises the LH surge response (14).

There are extensive feedback mechanisms, both negative and positive that exist within the hypothalamic-pituitary-ovarian axis. During the early follicular phase inhibin levels are low whilst FSH begins to increase promoting follicular recruitment and growth. LH secretion is inhibited by the increasing levels of oestrogen produced by maturing follicles. However at 36-48 hours before ovulation, the oestrogen feedback mechanism becomes positive initiating a LH surge. During the luteal phase, the secretion of LH and FSH are inhibited due to high circulating levels of oestrogen, progesterone and inhibin (14).

The primordial follicle once recruited in the menstrual cycle undergoes three stages of development on route to ovulation;

1. Transition to a primary or preantral follicle, followed by

2. Transition to a secondary or antral follicle (also called a vesicular or Graafian follicle), and finally
3. Transition to a preovulatory follicle in the run up to ovulation.

1.1.1 Phase 1: Primordial to Preantral Transition

The earliest preantral phase is characterized by an increase in diameter of the primordial follicle from 20µm to between 200-400µm. A major part of this growth occurs in the primary oocyte which increases in diameter to its final size of 60-120µm *(figure 4a, 4b).* The oocyte growth is not characterized by reactivation of meiosis, rather the chromosomes are actively synthesizing large amounts of ribosomal and mRNA, the latter being used to build organelles and to generate stores of protein, all of which are essential for the later stages of oocyte maturation and for the first few days after fertilization (14).

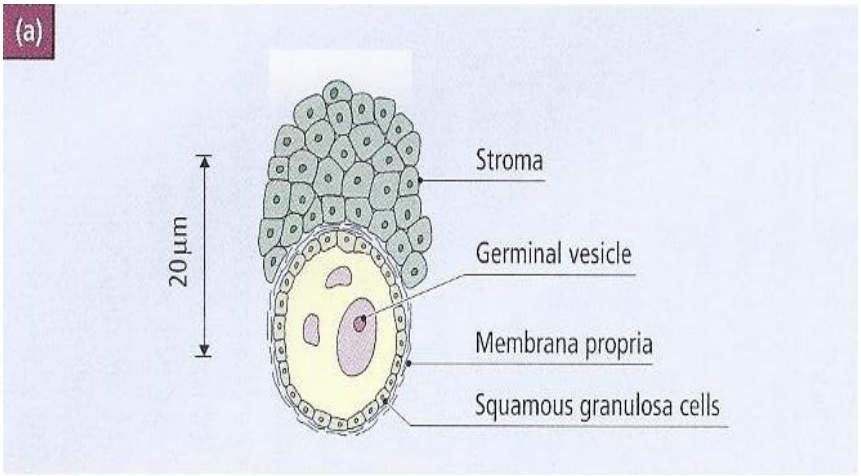

Figure 4a. Schematic representation of primary oocyte. Reproduced from Essential Reproduction, Blackwell Publishing (14).

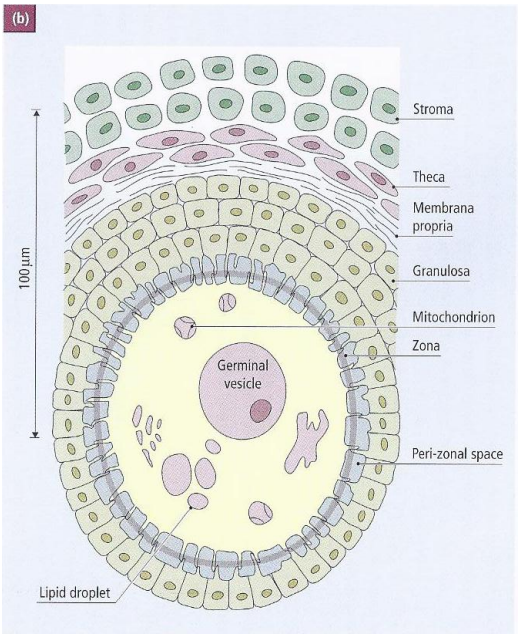

Figure 4b. Schematic representation of the primary oocyte.
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During early growth of the oocyte, glycoprotein is secreted and condenses to form a translucent acellular layer called the zona pellucida. The zona separates the oocyte from the surrounding granulosa cells which divide to become several layers thick (figure 4b). However contact between granulosa cells and the oocyte are maintained via granulosa cytoplasmic processes, which penetrate the zona and form gap junctions at the oocyte surface. Gap junctions also form in increasing numbers between adjacent granulosa cells, thus providing the basis for an extensive network of intracellular communications. Amino acids and nucleotides are transported through this network to the oocyte where they are incorporated into macromolecules. This nutritional network is of vital importance as the granulosa layer is completely avascular. In addition to oocyte growth and granulosa cell proliferation, the preantral follicle increases in size and complexity through the condensation of the ovarian stroma on the outer membrane propria. This loose matrix of spindle shaped cells is called the theca of the follicle *(figure 4b)*. With further proliferation and differentiation the thecal cells can be distinguished into two distinct layers; an inner

glandular, highly vascular theca interna, surrounded by an outer fibrous capsule, the theca externa *(figure 4c) (14).*

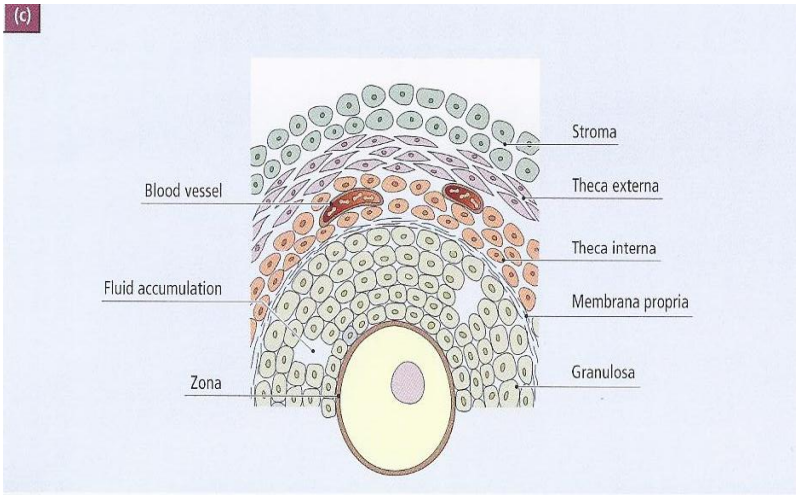


Figure 4c. Pre-antral follicle demonstrating theca interna and theca externa. Reproduced from Essential Reproduction, Blackwell Publishing (14).

1.1.2 Phase 2: Preantral to Antral Transition

In this transition the granulosa cells continue to proliferate resulting in further increases in follicular size. Follicular fluid begins to accumulate between these cells, which is mostly composed of granulosa cell secretions, including mucopolysaccharides and serum transudate. This fluid further coalesces to form a single follicular antrum, marking the beginning of the antral phase of development *(figure 5d).* Fluid continues to accumulate and contributes to an increase in follicular size whilst the granulosa cells continue to proliferate.

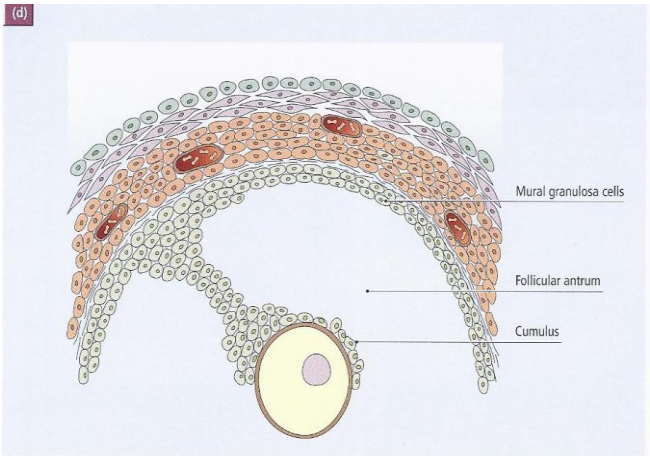


Figure 4d. The antral follicle. *Reproduced from Essential Reproduction, Blackwell Publishing (14).*

The oocyte does not increase in size but continues to synthesize RNA and turnover protein. As the follicular antrum grows the oocyte is suspended in fluid surrounded by a dense mass of granulosa cells called the cumulus oophorus. It is connected to the rim of peripheral or mural granulosa cells only by a thin stalk of cells. It is now ready to enter the preovulatory phase on approach to ovulation (14).

The mechanism by which primordial follicles begin to develop is poorly understood. Preantral follicular development can occur independently of any direct extra-ovarian control, although growth hormone is generally a facilitator for early follicle growth and survival, probably mediated by IGF-1 production from granulosa cells. Intra-ovarian cytokines and stem cell factor initiate oocyte growth in the primordial follicles. However further follicular support requires external support from the pituitary. In a situation whereby this does not occur, usually prior to 2mm in developmental size, apoptosis occurs within the oocyte and the surrounding granulosa cells. This is followed by invasion by macrophages and leukocytes causing scar formation, a process known as atresia. Atresia is

prevented by FSH and LH binding. These hormones bind to FSH and LH receptors respectively that begin to appear in the late preantral and early antral follicles. FSH is sufficient for initial follicular growth, but LH assists further antral expansion. In the antral follicles, only theca interna cells bind LH, and granulosa cells bind FSH with differing binding producing a differing response. The antral follicles, under the influence of gonadotrophins release increasing amounts of steroids; oestradiol 17β and oestrone, androstenedione and testosterone. It is the theca cells which are under the influence of LH synthesize androgens from cholesterol and acetate. In contrast, granulosa cells are unable to form androgens, but can with a supply of androgens produce oestrogens, a process called aromatization. This process is controlled by FSH. Androgens act as a substrate for conversion to oestrogen, and also act with FSH to stimulate proliferation of granulosa cells, follicular growth and aromatase activity. Thus the rising thecal output of androgens fuels a massive increase in oestrogen biosynthesis. This is further controlled by positive feedback process as oestrogens themselves stimulate granulosa cells to proliferate. This positive feedback culminates in a feedback surge towards the end of antral expansion (14).

During the antral phase, several cytokines are stimulated by circulating gonadotrophins and then mediate or modulate the actions of steroids or gonadotrophins. There seems to be a balance between cytokines (IGF's, inhibins) that support follicular progression, acting in cooperation with FSH and androgens, and cytokines that depress follicular development or promote atresia (MIH, TNFα, leptin and IGFBP's). Both inhibin A and B are produced by the granulosa cells; inhibin B under the control of FSH, and inhibin A under the control of FSH and LH. Therefore the ratio of Inhibin A: B rises as the follicle expands to peak at ovulation.

Thus both oestrogen and the Inhibin A:B ratio can be utilized a tools for markers of antral expansion (14).

1.1.3 Ovulation

The expanding antral follicle continues to rely on exogenous hormones, and requires an LH surge to trigger ovulation. The LH surge coincides with the appearance of LH receptors on the outer granulosa cells. If an LH surge occurs when both the granulosa and thecal cells can bind LH, then entry into the preovulatory phase of growth occurs, if it does not the antral follicle dies. The LH surge causes expulsion of the oocyte from the follicle at the time of ovulation and triggers changes within the endocrinology of the follicle, which results in the transition to a functioning corpus luteum.

Within 12 hrs from the beginning of the LH surge, the nuclear membrane surrounding the arrested chromosome breaks down and the arrested meiotic prophase ends, culminating in progress through the first meiotic division and producing the secondary oocyte which contains half the number of chromosomes but retains almost all of the cytoplasm. The unused half of the chromosomes forms the first polar body which subsequently becomes redundant. The chromosomes in the secondary oocyte subsequently undergo a second meiotic division and come to lie on the second metaphase spindle. Meiosis arrests again and the oocyte is ovulated in this arrested metaphase state. Progress through to the second meiotic arrest is accompanied by cytoplasmic maturation. The intimate contact between the oocyte and the granulosa cells of the cumulus is broken down by withdrawal of the cytoplasmic processes. The golgi apparatus synthesize lysosomal like granules, which

migrate towards the surface of the oocyte to assume a sub-cortical position. Protein synthesis continues with new and distinctive proteins synthesized to prepare the oocyte for fertilization. The LH surge initiates meiotic and cytoplasmic maturation (14).

LH acts directly on the follicle causing rapid expansion to 25mm or more in diameter as a consequence of increasing follicular fluid. Accompanying the rapid production of follicular fluid, there is a loosening of the intercellular matrix between the cortical layers and granulosa cells and an increase in total blood flow to the follicle. The pre-ovulatory growth in follicular size is accompanied by changes in steroid secretion. Within 2 hours of the LH surge there is a transient rise in oestrogenic and androgenic output followed by a decline. This rise results from the thecal layer which appears transiently stimulated and hyperaemic. The outer cells of the granulosa layer stop converting androgens to oestrogens and instead synthesize progesterone. LH stimulates progesterone synthesis via newly acquired LH receptors. These cells conversely lose the ability to bind oestrogen and FSH but gain the capacity to bind progestogens. Thus by becoming responsive to LH to produce progesterone and in addition having a positive feedback mechanism, there is an exponential rise in progesterone levels from the follicle just prior to ovulation. The rising progesterone levels have three important consequences; firstly it depresses growth of the less mature follicles, secondly is responsible for ovulation itself, and thirdly promotes the transition to the prostagenic phase of the ovarian cycle (14).

By the end of the preovulatory phase, rapid expansion of the follicular fluid has resulted in a relatively thin rim of mural granulosa cells, basement membrane and thecal cells to which the oocyte is attached by a thinning stalk of granulosa cells. The increasing size of the follicle

and its position in the ovarian cortex causes a bulge at the ovarian surface. At the point of ovulation this bulge becomes avascular. Following a breakdown in connective tissue, the follicle ruptures. The follicular fluid carries the oocyte and its surrounding mass of cumulus cells to the transport system of the reproductive tract; the fallopian tubes, subsequent to possible fertilization. The biochemistry of this ovulatory event involves proteolytic enzymes; matrix metalloproteinases (MMPs), their natural tissue inhibitors (TIMPs) and also the serine proteases; plasmin and plasminogen activator which are under the influence either directly or indirectly of LH, progesterone and prostaglandins (14).

The post ovulatory follicle is composed of a fibrin core, surrounded by several collapsed layers of granulosa cells, enclosed within a fibrous outer thecal capsule; the corpus luteum.

1.1.4 The Corpus Luteum

Within the follicular antrum, the fibrin core undergoes fibrosis over a period of days. The membrane propria between the granulosa and thecal layers break down and blood vessels invade. The granulosa cells hypertrophy to form large lutein cells, which contain mitochondria, smooth endoplasmic reticulum, lipid droplets, Golgi apparatus and a carotenoid pigment called lutein. This transformation is called leutenization and is associated with an increase in progesterone production up to 20 times that seen in the follicular phase. The thecal cells form smaller lutein cells which produce progesterone and androgens and appear richer in LH receptors. Progesterone, 17α hydroxyprogesterone and small amounts of 17β oestradiol are produced by the corpus luteum. Inhibin A is also produced which in turn stimulates progesterone production and oxytocin.

The conversion of a follicle to a corpus luteum is dependent on a high LH surge to provoke ovulation and initiate luteal conversion. LH is subsequently required for maintenance of corpus luteum function. In humans progesterone also has a luteotrophic effect with appropriate receptors detected on granulosa cells from the preovulatory stage (14).

Luteolysis or luteal regression involves collapse of lutein cells, ischaemia and progressive cell death with a consequential fall in serum progesterone. Luteotrophic function continues provided there is adequate luteotrophic support. The support garnered by low levels of LH during the luteal phase is sufficient to sustain a normal length of luteal phase, however to extend this beyond 10-12 days a second exponential rise of LH activity is required. This is discussed in the subsequent text. Without this second surge in LH activity the corpus luteum regresses with a corresponding fall in serum progesterone preceding the onset of menstruation.

Progesterone secretion by the corpus luteum is essential for maintenance and survival of early pregnancy. Surgical excision of the corpus luteum (luteectomy) before 7 weeks gestation uniformly precipitates an abrupt decrease in progesterone resulting in miscarriage (15). When luteectomy is performed more than 27 days after last menstrual period (i.e. 8 weeks or later), there is a slight and transient decrease in progesterone levels, but the pregnancy continues (15). Exogenous progesterone supplementation after early luteectomy (before 7 weeks gestation) prevents otherwise inevitable miscarriage. These early studies suggested that prior to 7 weeks gestation, progesterone is primarily derived from the corpus luteum and almost entirely from the trophoblast after 9 weeks gestation. In between these times and to varying extent, both are responsible for progesterone secretion, this transition

period is described as the luteo-placental shift (16). In a pragmatic example of clinical use, progesterone receptor antagonists induce abortion if given within the first 7 weeks of pregnancy (17).

1.2 Implantation

Implantation is a critical step in the establishment of successful pregnancy, requiring synchronization between the developing embryo (blastocyst) and the endometrium. Bidirectional communication utilising endocrine, paracrine and autocrine signals exist between the embryo and endometrium.

Following fertilisation, the zygote undergoes cell division (figure 5). During each cell division the number of cells (blastomeres) is anticipated to double. After 4 cell divisions 16 cells occupy the zygote surrounded by the zona pellucida which is now referred to as a morula. The blastocyst subsequently develops from the morula, usually five days after ovulation. As cell division continues, blastomeres change their shape and align themselves against each other, forming a fluid filled cavity (blastocoele). The blastocyst comprises an inner cell mass (ICM) which subsequently forms the embryo, and an outer layer of cells called the trophoblast which gives rise to the extra-embryonic structures including the placenta.

Six days after fertilisation, the embryo containing 100-200 cells hatches form the zona pellucida exposing its outer aspect of syncitial trophoblasts to the adjacent luminal epithelium of the endometrium (18).

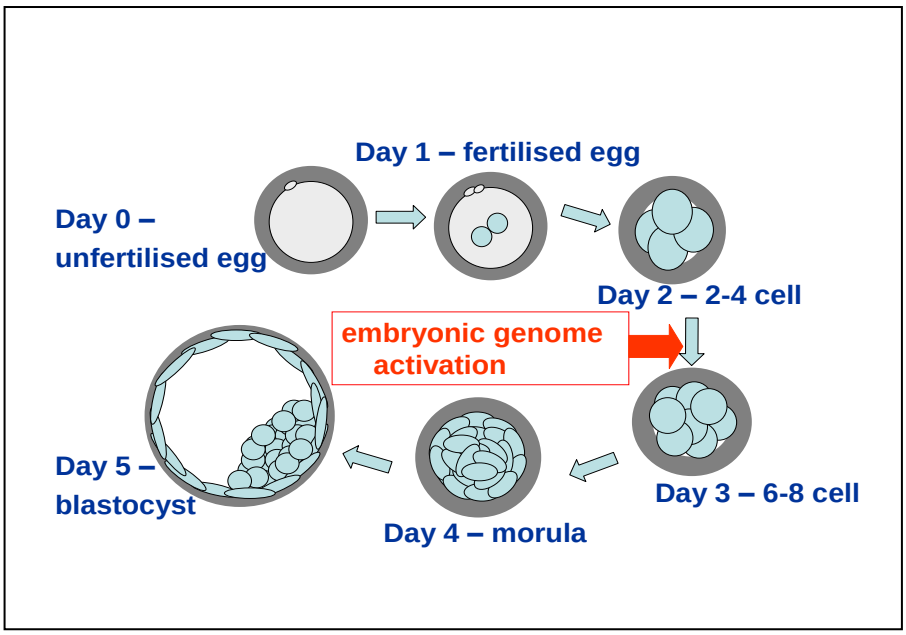


Figure 5. Stages of embryo development. Reproduced from the Textbook of Minimal Stimulation IVF, Milder, Mildest or Back to Nature, Jaypee Brothers Medical Publishing (18).

1.2.1 The Endometrium

The endometrium consists of epithelial, stromal and vascular elements which undergo complex patterns of growth and morphological and functional differentiation in anticipation of pregnancy (19, 20). The endometrium thickens in response to follicular oestrogen, and becomes receptive towards embryo implantation in response to progesterone following ovulation and formation of a corpus luteum (21). In the mid-late secretory phase of the menstrual cycle, differentiation and secretory transformation of the glandular epithelial cells followed by decidualisation of the stromal compartment occurs. The implantation window is a narrow "window of opportunity" when the endometrium is considered receptive to a functional blastocyst. Early studies examining hysterectomy samples from newly pregnant women identified 34 embryos (eight free floating before day 19 of the menstrual cycle, and 26 attached (all after day 21) (22). Studies have suggested that the window of endometrial receptivity extends from post-ovulatory days 6-10 (corresponding to cycle day 20-24). Wilcox et al. confirmed the timing of this window in normal fertile patients and demonstrated a higher risk of miscarriage with delayed implantation (23). If synchrony between the endometrium and the embryo are impaired then a delay in histological maturity of the endometrium could extend the time of non-receptivity leading to failed implantation and pregnancy loss (24). This concept has been termed luteal phase deficiency (LPD) and will be discussed in more detail later in this chapter. In the clinical context, other factors including the presence of endometriosis or hydrosalpinges have been demonstrated to result in aberrant expression of endometrial biomarkers, leading to reduced implantation (25-29).

Implantation in humans involves three stages; (i) apposition of a competent blastocyst and a receptive endometrium, (ii) adhesion of the embryo to the epithelium, and (iii) penetration of the embryo through epithelium and basal lamina with invasion of uterine vasculature (30).

1.2.1.1 Apposition and Adhesion

Apposition is relatively unstable and follows the initial interaction of the embryo and the apical surface of the luminal epithelium of the endometrium (31-33). In much the same way that the embryo must hatch from its zona pellucida to expose surface receptors and adhesion molecules, the surface of the endometrium undergoes changes that render it receptive to embryonic interaction. The glycocalyx is predominantly made of endometrial mucin (Muc-1) (34). Mucins are high molecular glycoproteins that protect the upper reproductive tract from bacterial colonisation. Muc-1 is present throughout the menstrual cycle making its role in implantation unclear, however it is thought to act as scaffolding for cell adhesion proteins that are required for embryo attachment (35). Microvilli on the apical surface of the syncytiotrophoblasts interdigitate with micro-protrusions from the apical surface of the uterine luminal epithelium, known as pinopodes, which are associated with increased endometrial receptivity (36). Pinopodes appear to be a preferred site for embryo attachment (37, 38), and may have a temporal association with the window of implantation (39-41). Stable adhesion is characterised by increased physical interaction between the blastocyst and epithelium. This process may involve the activation of signalling cascades and remodelling of the uterine epithelium. Interactions between the trophoblast and the lateral aspects of the endometrial cells have been described with resulting disruption of the

junctional complexes between the endometrial cells and invasion of the trophectodermal processes which project into these spaces (42, 43).

Recently, research has aimed at identifying biomarkers of endometrial receptivity that are able to identify normal biologic processes, pathogenic processes or pharmacological responses to a therapeutic intervention. However, their discussion is beyond the scope of this thesis.

1.2.1.2 Invasion

At 10 days after the syncytiotrophoblast has penetrated the uterine epithelium, it is completely embedded in the stromal tissue of the uterus, with the uterine epithelium re-growing over the implantation site. The cytotrophoblasts invade the entire endometrium and inner third of the myometrium (interstitial invasion) and penetrate the uterine vasculature (endovascular invasion). This process establishes a primitive utero-placental circulation and marks a shift from which the embryo now obtains metabolic support from the maternal extracellular fluid coming primarily from the endometrial glands. The maternal and fetal circulations lie in close proximity and exchange nutrients and waste. During endovascular invasion the cytotrophoblast cells breach the termini of the spiral arteries driven by oxygen tension (44), with resultant uterine arterioles that are hybrid structures composed of fetal and maternal cells. Cytotrophoblast invasion transforms maternal arterioles from small-bore, high resistance vessels, to large bore and low resistance vessels capable of meeting the fetal demands for maternal blood flow. In healthy pregnancies the extravillous cytotrophoblast cells invade as far as the inner third of the myometrium. The

health of both the fetus and the mother are contingent upon regulating the extent of trophoblast invasion. Early implantation is regulated by the interplay of matrix metalloproteases and tissue inhibitors of these proteases. Deep invasion is regulated by interplay between Th1 cytokines that prevent invasion and Th2 cytokines that enhance implantation.

1.2.2 Factors Influencing Implantation

1.2.2.1 Steroid Hormones

Regulation of early implantation is a complex process mediated by several growth factors, cytokines and adhesion molecules within the uterus and the pre-implantation blastocyst. However, oestrogen and progesterone are thought to play a significant part in this process. Progesterone acts on an oestrogen-primed endometrium in preparation for implantation. The cyclical features of endometrial proliferation and differentiation are the consequence of sequential exposure to oestradiol and progesterone, produced by the developing ovarian follicle and corpus luteum respectively. The preovulatory increase in secretion of 17β-oestradiol promotes proliferation and differentiation of uterine epithelial cells. A subsequent rise in progesterone secretion induces proliferation and differentiation of stromal cells (30). Oestrogen and progesterone act primarily through their nuclear receptors, the oestrogen receptor (ER) and progesterone receptor (PR) respectively which in turn result in activation or repression of downstream target genes (45). The most common and best understood receptors are the oestrogen receptors, ER-α and ER-β and the progesterone receptors PR-A and PR-B (46). Steroid hormones are required to coordinate the receptivity of the endometrium; both oestrogen and progesterone are required for

endometrial receptivity, and progesterone expression is essential for implantation and maintenance of early pregnancy. Observations by Lessey et al. demonstrated an absence of ER and PR receptors from the endometrial glandular epithelium at the mid to late luteal phase during the implantation window, suggesting that these genes are not required for implantation, but rather required to generate the molecular profile that is essential for embryo attachment and implantation (47). ER-α is known to be the primary mediator of oestrogen signalling in the uterus with knockout studies in mice rendering them infertile (48). In the functional layer of the endometrium, ERα expression increases in both glandular and stromal cells in the proliferative phase, whilst declining in the secretory phase owing to suppression by progesterone. In the basal layer, ERα is present in glandular and stromal cells throughout the cycle (49). Similar studies have shown PR-A as the major mediator of progesterone signalling in the female reproductive tract (50). Both ER and PR are up-regulated during the follicular phase by ovarian oestradiol and subsequently down-regulated in the luteal phase by progesterone acting at both the transcriptional and post-transcriptional level (51). Observations have demonstrated a significant decline in PR expression in the glands of the functional layer of the endometrium (the region which is shed at menstruation) with transition from the proliferative to the secretory phase of the cycle. In contrast PR expression persists in the stroma of the functional layer, particularly in areas in close proximity to uterine vasculature. The basal layer is differentially regulated as the glands and stroma of the deeper zones express PR throughout the cycle. These differences between the superficial and basal layers are likely to be functionally important.

ERβ is present in endometrial endothelial cells and indicates that oestrogen may act directly on endometrial blood vessels and may be involved in endometrial angiogenesis and vascular permeability changes during the cycle. Progesterone receptors are absent from the vascular

endothelium of the spiral arteries, which suggests the effects of progesterone withdrawal on these vessels, which play a role in menstrual induction are likely to be mediated by the PR-positive perivascular stromal cells (52, 53).

1.2.2.2 Integrins

Integrins are a large family of cell adhesion molecules which function as receptors for the extracellular matrix components laminin, fibronectin, perlecan, thrombospondin and osteopontin. Multiple heterodimers of integrins are expressed on the embryo and the endometrium and are considered functionally important during apposition, attachment and invasion of the embryo (54). Whilst full discussion of the exact role of integrins are outside the scope of this thesis, it is noted that altered expression of integrins in the human endometrium, i.e. a reduction in the expression of certain integrins in the presence of endometriosis and hydrosalpinges results in reduced implantation potential (26, 28, 55).

Several integrin heterodimers are expressed on the pre-implantation blastocyst, most notably avβ3 and α5β1. α5β1 is expressed on trophoblast cells and transported to their apical surface on blastocyst activation, suggesting a role for initial attachment after hatching (56). Cytotrophoblast invasion is a delicate balance between members of the integrin family; integrins avβ3 and α1β1 promote invasion, whereas α5β1 restrain it (57). It is likely that the cytotrophoblasts modulate their adhesion molecule expression in a stepwise fashion to a profile similar to that of endothelial cells, enabling the cytotrophoblast to assume characteristics similar to those of maternal vascular cells (58).

1.2.2.3 Matrix Metalloproteinases

The invading cytotrophoblast regulates the expression of matrix metallproteinases (MMP's) secreted by the embryo and tissue inhibitors of metalloproteinases (TIMP's) secreted by the endometrium (59, 60). Metalloproteinases regulate cytotrophoblast invasion through up-regulation, whilst tissue inhibitor expression provide a mechanism to restrict cytotrophoblast invasion (61). Complex expression of MMP's and TIMP's in maternal decidual cells adjacent to the invading blastocyst also exist (62).

1.2.2.4 Growth Factors

Many growth factors are expressed in the luminal epithelium during the window of implantation and are often increased at the site of embryo apposition. These include epidermal growth factors, insulin-like growth factors, vascular endothelial growth factors, fibroblast growth factors and platelet derived growth factors (54).

The expression patterns of endothelial growth factor (EGF) ligands and receptors within the endometrium suggest a local paracrine / autocrine signalling pathway necessary for embryo attachment. HB-EGF is expressed in luminal endometrial cells and expression levels peak during the implantation window, whilst ErbB-4 is expressed in the trophectoderm of the peri-implantation blastocyst (63, 64). In mouse models HB-EGF is regulated by oestrogen and progesterone with a specific increase in levels at the site of blastocyst apposition (65).

Insulin-like growth factors regulate cell growth, differentiation, and metabolism in multiple tissues. Ligand availability is modulated by IGF binding proteins (IGFBP's). The expression

pattern of IGFBP-1 and IGF-II suggest an autocrine / paracrine signalling response between the decidua and the trophoblast that regulates trophoblast invasion. IGFBP-1 is expressed in secretory endometrium whilst IGF-1 and IGF-II are expressed in the invading cytotrophoblast (66). IGFBP-1 can alter the invasiveness of cytotrophoblast cells in vivo (67). Vascular endothelial growth factors induce endothelial cell proliferation and increase vascular permeability. VEGF is expressed throughout the menstrual cycle with peak levels in the glandular epithelium during the secretory phase. Oestrogen has been demonstrated to increase VEGF expression (68). In mice models there is an increase in VEGF expression in the luminal epithelium and adjacent stroma at the implantation site.

1.2.2.5 Leukemia-Inhibitory Factor

Leukemia-inhibitory factor (LIF) is a member of the IL-6 cytokine family and known to have an important role in decidualisation and implantation (69). In the proliferative phase of the menstrual cycle, LIF expression is not detected. However LIF mRNA and protein can be detected throughout the secretory phase in the glandular and luminal epithelium. Its levels peak in the late secretory phase between cycle day 19 and 25, corresponding with the ideal implantation window. The responsiveness of LIF-R to LIF is dependent on oestrogen and progesterone activity. Progesterone, HB-EGF and TGF-β may all regulate LIF secretion (70). Targeted disruption in mice of the LIF gene renders implantation impossible due to failure of decidualisation despite ovulation and fertilisation. LIF is also thought to be necessary to mediate the effects of oestrogen on the endometrium during the period of implantation, suggesting that LIF is a target of oestrogen in the endometrium (71). Clinically it has been noted that women with unexplained infertility or recurrent miscarriage have decreased

levels of LIF expression in the endometrium compared with fertile controls (72). Women treated with antiprogestins display decreased LIF expression in the endometrium (73).

1.2.2.6 Hox genes

Hox genes are transcription factors that are thought to play a critical role in implantation in humans. Hoxa-10 and Hoxa-11 are expressed in adult endometrial glands and stroma (74). Their regulation and expression increases in the mid-late secretory phase during the window of implantation and remain elevated thereafter (75, 76). Both Hoxa-10 and Hoxa-11 are regulated by oestrogen and progesterone by direct binding of their receptors to their respective gene promoters (77). Infertile patients associated with endometriosis fail to demonstrate a mid-luteal increase in Hoxa-10 or Hoxa-11, whilst Hoxa-10 levels are decreased in patients with polycystic ovarian syndrome, fibroids and hydrosalpinges (78-81). The data suggests an important role of Hox genes in endometrial receptivity, a requisite step for successful implantation.

1.2.2.7 Prostaglandins

Prostaglandin biosynthesis is required for successful implantation. Arachidonic acid is converted to prostaglandin H_2 by the enzyme cyclooxygense which exists in two isoforms: COX-1 and COX-2. In the endometrium COX-1 production increases in response to progesterone and oestradiol 17β, with levels decreasing in the mid-luteal phase of the menstrual cycle in anticipation of implantation (81). In contrast COX-2 which is not steroid dependent is restricted to the implantation site and depends on the presence of a

competent blastocyst (81, 82). Interleukin-1 in conditioned medium of human embryos induces COX-2 gene expression (83).

1.2.2.8 Immunological Factors

Immune tolerance of the invading trophoblast tissue by the maternal immune system is one of the most perplexing functions of implantation. Trophoblasts are presumed to be essential to this hemi-allograft tolerance because they lie at the maternal-fetal interface where there is direct contact with the maternal immune system. Trophoblastic tissue does not seem to possess classical major histocompatibility complex (MHC) class II molecules, but the cytotrophoblasts upregulate a non-classical MHC class 1b molecule, HLA-G as they invade the uterus (84). The exact mechanism of their function has not been elucidated but may include up-regulation of inhibitory immunoglobulin-like transcript 4, a HLA-G receptor that is expressed on macrophages and a subset of natural killer lymphocytes (85-89). Maternal decidual lymphocytes are abundant in the uterus during pregnancy with the majority being CD56+ natural killer (NK) cells (up to 70%) (90). However unlike peripheral blood lymphocytes they have low cytotoxic activity. These lymphocytes are directly recruited by the invading cytotrophoblast via chemokine secretion (91, 92). Local factors that inhibit cytotoxicity against the invading trophoblastic tissue for this localized immunosuppression include cytotrophoblast-derived interleukin-10 (93). A similar effect is observed with progesterone that regulates the migration and proliferation of immune and inflammatory cell populations in the endometrium (86). There is also growing evidence that progesterone plays a significant role in establishing an adequate endometrial environment for the early stages of pregnancy.

During pregnancy, the maternal immune system is modulated by progesterone via control of cytokine production (94). In normal pregnancies there is a shift in the decidua from cellular immune response (Th1 cytokines) to humoral immunity (Th2 cytokines) which may be driven by the hormonal stimuli associated with pregnancy (95). Decidual T cells do not seem to produce a detrimental response against extravillous trophoblast; however the mechanism for this remains unclear. Furthermore, $CD4^{+}CD25^{+}$ T regulatory cells have been described in first trimester decidua and these may suppress T cell responses to trophoblast antigens (96).

Immunological recognition of pregnancy results in up-regulation of progesterone receptors on activated lymphocytes (97). In the presence of progesterone, lymphocytes of pregnant women synthesize a 34-kDa protein known as progesterone-induced blocking factor (PIBF) (98), which mediates both the immunomodulatory and anti-abortive properties of progesterone (94, 99-101). It has been postulated that a significantly increased Th1 cytokine expression may be responsible for reproductive failure (102). It has also been noted that the activation of peripheral blood mononuclear cells (PBMC) with trophoblast antigens confirmed that women with idiopathic recurrent spontaneous pregnancy loss have a Th1 type cytokine profile, characterised by the production of interleukin (IL)-2, tumour necrosis factor (TNF) and interferon ϒ (IFNϒ) (103).

PIBF expression on maternal lymphocytes increases as a result of pregnancy and that the stimulus for PIBF induction occurs immediately after implantation (104). Szekeres-Bartho et al. demonstrated that a low percentage of PIBF-positive lymphocytes is inversely related to natural killer (NK) cell activity, preterm labour and pregnancy loss (105). This possible

association between Th1 and Th2 dominance and recurrent miscarriage has resulted in attempts to manipulate this balance and suppress the cell mediated immunity. Progesterone has been proposed to act as an immunological suppressant blocking Th1 activity and inducing Th2 cytokines (IL-4 and IL-10). Raghupathy et al. demonstrated this effect with use of orally administered dydrogesterone which has a high affinity for progesterone receptors, and very similar in its physiological effects and molecular structure to endogenously produced progesterone. It was noted that progesterone successfully down-regulated Th1 cytokines and stimulated Th2 cytokines, resulting in a shift towards Th2-type immunity. Dydrogesterone was also noted to induce PIBF production (88). El-Zibdeh et al. transferred this approach to a clinical benefit, and demonstrated a significant improvement in the chances of a successful pregnancy in women with recurrent miscarriage (106).

Kalinka et al. suggested that inducing PIBF production could be the indirect mechanism by which dydrogesterone improves pregnancy outcome (87).

1.3 Luteal Phase Deficiency

1.3.1 Definition

A luteal phase defect is classed as an ovulatory dysfunction. It is defined by a defect in the corpus luteum to produce sufficient quantities of progesterone in the luteal phase of the menstrual cycle, or an inability of the endometrium to respond to circulating levels of progesterone (24, 107, 108). A variety of mechanisms may be involved which result in a luteal phase deficiency (LPD), and consequently inadequate transformation of the endometrium, resulting in defective implantation, placentation, embryonic development and subsequent pregnancy failure (109-111).

1.3.2 Presentation

Luteal phase deficiency has been associated with;

- Infertility (112, 113)
- First trimester pregnancy loss (114)
- Short cycles (115-118)
- Premenstrual spotting (119)
- Anorexia and eating disorders (120)
- Excessive exercise (121)
- Stress (122, 123)
- Polycystic ovarian syndrome (PCOS) (124)
- Endometriosis (125)
- Ageing (126)
- Inadequately treated 21-hydroxylase deficiency (127)

- Thyroid dysfunction and hyperprolactinaemia (128)
- Ovulation stimulation alone (129)
- Ovulation induction with or without GnRH agonists and ART (130)
- Postpartum period with significant weight loss or extreme exercise (131)
- Randomly in normal menstrual cycles (117)
- Renal transplantation (132)
- Increased beta endorphin levels (133)
- Lactation (134)
- IVF treatment

A “short luteal phase” was initially described as an interval of 8 days or fewer from the LH peak to the onset of menstruation (117). This has been associated with low follicular FSH levels, altered follicular FSH/LH ratios or abnormal FSH and LH pulsatility that may result in reduced oestradiol and progesterone levels (117, 118, 135-137). However, short luteal phases can occur in young healthy women with regular cycles, questioning its very relevance.

Abnormalities in GnRH, FSH and LH pulsatility are evident during recovery from amenorrhoea and which may result in diminished luteal oestrogen and progesterone secretion (121, 138-140). Consequently, diminished LH pulsatility resulting in a diminished progesterone secretion may be problematic in ovulation induction cycles in women with hypothalamic amenorrhoea (140, 141). On this assumption, it is reasonable to propose that any disruption of the hypothalamic-pituitary-ovarian (HPO) axis could disrupt the endometrial milieu.

An example of this HPO disruption can be observed in PCOS and its proposed effect on endometrial receptivity and resulting implantation failure (79). Hyperinsulinaemia associated with PCOS induces hyperandrogensism via a direct effect on the ovary and indirect effect on the liver which reduces the production of sex hormone binding globulin (SHBG), with the effect of increasing free circulating testosterone (142). Insulin has been demonstrated to alter the synthesis and pulsatility pattern of LH and FSH secretion, hindering ovulatory functions (142). In PCOS women, GnRH pulsatility is increased, favouring LH secretion over FSH with a constant increase in GnRH pulsatility throughout the menstrual cycle. In women with LPD there is a similar GnRH derangement, but with GnRH pulse frequency normal or increased in the follicular phase and suppressed in the luteal phase (107). Plasma concentrations of insulin and LH have been positively correlated during the luteal phase (143). Increased androgen and insulin levels could also have a direct effect on the endometrium (144-148), and may explain the poorer reproductive outcomes in patients with PCOS (149). However, this finding has been disputed by several authors (150-152). In ovulatory women with PCOS, there is a defect in luteal phase progesterone secretion which may also contribute to reduced fertility (153). The mechanism behind this is thought to arise from a defect in granulosa cell function and progesterone production secondary to high serum insulin levels (154).

In a similar fashion, hyperprolactinaemia is associated with a disruption of GnRH pulsatility by acting on GnRH neuronal prolactin receptors or by increasing hypothalamic dopamine and opioid peptide levels with consequent reduction in steroid production (155-157). Whilst higher levels of prolactinaemia can lead to anovulation, more subtle levels may impact on endometrial receptivity, 16% of women with LPD demonstrate hyperprolactinaemia (158).

Thyroid disorders also affect the HPO axis. Thyrotrophin releasing hormone (TRH) in hypothyroidism may cause hyperprolactinaemia by stimulating prolactin production and secretion.

Obese patients have also been demonstrated to have elevated LH pulsatility and urinary progesterone metabolites compared with control subjects (159). Whether this abnormality contributes to a lower fecundity rates remains to be proven.

Advanced maternal age has been associated with inadequate luteal function and is associated with reduced progesterone and oestradiol metabolites in the luteal phase (160, 161). It is unclear to what extent this contributes to the lower fertility rates that are associated with advancing maternal age.

Antimullerian hormone (AMH) is a member of the transforming growth factor-β (TGF-β) super-family and is produced by the granulosa cells of preantral and early antral follicles. AMH plays a major role in the inter- and intra-follicular coordination. AMH is now principally used as a marker of ovarian reserve during fertility investigation. It is widely recognised that AMH levels are markedly higher in patients with PCOS compared to healthy controls (162-164). Several studies have demonstrated that high AMH levels can negatively impact on the function of granulosa theca lutein cells (31), reduce the expression of LH receptors (165), and affect the activity of aromatase in cultured human granulosa cells (166).

Endometriosis patients and older patients have been shown to demonstrate progesterone resistance, suggesting that a longer duration of luteal support may be required (167).

There is increasing evidence that abnormal folliculogenesis may result in impaired function of the corpus luteum. Biologically it becomes plausible that by optimising folliculogenesis and pre-ovulatory follicular dynamics using ovulation induction, the function of the resulting corpus luteum improves and possibly fertility outcome.

1.3.3 Luteal phase deficiency associated with IVF

Assisted reproduction is a treatment option to overcome the luteal phase defect observed in non-IVF cases, however IVF procedures are also associated with luteal phase deficiency (168).

IVF cycles utilising GnRH agonists have been associated with luteal phase deficiency. GnRH agonists cause LPD by prolonged desensitisation of pituitary LH secretion, for up to 3 weeks after down-regulation is achieved (169-173). As progesterone production from the corpus luteum is dependent on LH stimulation from the pituitary (short loop negative feedback) a luteal phase deficiency results (172, 174). GnRH agonists have also been shown to result in a decrease of LH receptors on granulosa cells and have a direct effect of suppressing granulosa cell production of oestradiol and progesterone (175). Inadequate endometrial development was observed after ovarian stimulation with HMG and GnRH agonist that were not supplemented in the luteal phase (176). The use of GnRH antagonists has also been associated with a LPD and reduction in pregnancy rates (171, 177-179), however in contrast to GnRH agonists the recovery of LH production from the pituitary is more rapid following cessation of medication (180, 181), and milder in its effect (182). This initially led to the belief that subsequent luteal support after a GnRH antagonist cycle would be unwarranted

(183). Further studies identified a significant reduction in luteal length with reduced pregnancy rate (171, 177), concluding that additional luteal support be considered mandatory in these cases too (177, 180, 184, 185). The administration of HCG in patients receiving GnRH antagonists results in normalisation of the luteal phase (186). Friedler et al. demonstrated that the luteal phase characteristics and dynamics of IVF cycles in both agonist and antagonist cycles are similar (187).

During IVF treatments, exogenous stimulation to promote multi-follicular development results in supra-physiological steroid levels which directly inhibits LH release by means of a negative feedback mechanism at the level of the hypothalamic-pituitary axis (188-190). Supraphysiological levels of sex steroids may also cause a profound modification of endometrial receptor dynamics. Advanced histological maturity is more common in IVF cycles compared to controls (191-194). Elevated levels of post ovulation progesterone may also suppress the GnRH pulse generator resulting in diminished LH output and subsequent impaired luteal function (107). Hence without adequate luteal support, a shorter luteal phase occurs with impairment of implantation and promotion of endometrial shedding and menstruation (169). Observations of peri-ovulatory serum oestradiol and luteal phase progesterone levels were significantly higher in HMG stimulated cycles in patients undergoing IVF, but were significantly higher in pregnant compared to non-pregnant patients. A decline in both plasma steroids during the mid-luteal phase was also observed in pregnant patients, suggesting some degree of corpus luteum deficiency (195). Early luteal regression was confirmed by other studies utilising either clomiphene citrate or human menopausal gonadotrophin cycles, suggesting that luteal support may be beneficial (196, 197). However, other studies have failed to confirm that LPD occurs in all cycles and in all

patients that receive human menopausal gonadotrophin alone or in combination with clomiphene citrate (198-203). It has been suggested that a decrease in the endometrial concentration of cytoplasmic progesterone receptors, rather than a decrease in plasma progesterone concentrations may be attributable to a cascade of events leading to implantation failure (191, 204, 205). Forman et al. found a negative correlation between both preovulatory oestradiol and day 16 progesterone and the concentration of cytosolic progesterone receptor (cPR), while advanced endometrial maturity tended to be associated with low concentrations of cPR. In a further study, analysis in natural cycles were characterised by low levels of cytosolic oestradiol receptors (cER) and high cPR, whereas in stimulated cycles the concentration of both receptors were greatly reduced (191).

As part of the IVF procedure and after ovarian stimulation, follicular aspiration of the oocyte also results in the aspiration and depletion of granulosa cell numbers that are responsible for progesterone secretion (176, 206, 207). However a significant detrimental effect on steroid production following follicular aspiration has been refuted by Kerin et al., who demonstrated no impairment of steroid production when a single follicle was aspirated (208). There may also be a hypothetical short loop process involving HCG to suppress LH release.

1.3.4 Incidence & Diagnosis

Diagnostic tests for luteal phase deficiency are based on the following physiological observations (209);

1. The normal luteal phase is relatively fixed at 12-14 days.

2. Progesterone levels peak in non-pregnancy cycles 6-8 days after ovulation.
3. Progesterone is secreted in pulses.
4. The endometrial response is a reflection of follicular phase oestrogen and luteal phase progesterone.
5. Once implantation occurs, progesterone secretion by the corpus luteum is dependent upon rising HCG levels.
6. Failure of HCG levels to increase directly causes corpus luteum failure and a decline in serum progesterone levels (210).

The following have been proposed as a method for diagnosing LPD.

1.3.4.1. Basal Body Temperature

Small increases in basal body temperature (BBT) result from increases in serum progesterone subsequent to ovulation. An observed temperature elevation of less than 11 days has been suggested to identify patients with poor ovulatory function (209). However it is well recognised that some women have monophasic BBT patterns despite confirmed ovulation. The use of BBT is not significantly robust enough to reliably define the time of ovulation and should no longer be advocated (211).

1.3.4.2 LH Surge Detection

Detection of the LH surge at mid cycle aims to define the time from ovulation to onset of menses. An interval of 11-13 days is considered normal whilst an interval of 8 days or fewer is considered evidence of a short luteal phase (117, 212). However short luteal phases may

also occur in healthy fertile women (117). Ovulation kits demonstrate a false positive surge in 7% of cycles in women with regular menstrual cycles (213).

1.3.4.3 Progesterone Levels

Progesterone is secreted in pulses that reflect the generation of LH pulses from the pituitary gland; however progesterone levels have been shown to fluctuate 8-fold within a 90 minute period resulting in great variance in serum concentrations observed in the mid and late luteal phases. Levels as low as 2.3ng/mL and as high as 40.1ng/mL have been observed within relatively short time intervals spanning a single secretory pulse (214). Consequently, single or serial progesterone measurements have limited clinical utility and may not be a true reflection of luteal function. Progesterone levels are expected to peak 6-8 days after ovulation in non-conceptus cycles (210). However as previously discussed, the detection of an LH peak is unreliable (210). Due to the non-linear pattern of progesterone secretion there is no standard characterisation of progesterone secretion in normal fertile women (215), with no minimum progesterone level defining “fertile” luteal function. Random progesterone levels should therefore be considered unreliable as a diagnostic tool. A study by Usadi et al. investigated the theory that low progesterone levels lead to inadequate endometrial development. The results however suggested no impact in lowering progesterone to 3-10ng/ml was apparent from histological analysis (216). In the clinical situation of a failing intra-uterine pregnancy or ectopic pregnancy, low or inadequately rising progesterone levels are often reported. However the progesterone level is likely a reflection of failing HCG levels and consequent corpus luteum stimulation, secondary to an abnormal pregnancy. Low serum progesterone levels should be used as a tool to identify

pregnancy viability and not as an indicator to initiate supplemental exogenous progesterone. Urinary pregnanediol, a progesterone metabolite has been identified as a surrogate marker of corpus luteum function; however its use has yet to be validated in clinical practice.

1.3.3.4. Endometrial Histology

Histological assessment of uterine receptivity was first described by Noyes et al. in 1950 with subsequent revision in 1975 (20, 217). Noyes criteria has become a gold standard for endometrial dating and diagnosis of luteal phase deficiency (217). Luteal phase deficiency is diagnosed by a deviation of endometrial maturity from that expected for the phase of the menstrual cycle by two days (217). A maturation delay of the endometrium is thought to interrupt normal implantation and placentation (141). However, with subjective uncertainties regarding the detection of the preceding LH peak, a potential flaw in the process arises (218). Histological biopsy has been demonstrated to be an imprecise tool for differentiating fertile from non-fertile women, with histological parameters not validated in practice (219). In two randomised controlled trials involving healthy, regularly menstruating and fertile women, endometrial maturation was delayed in 25% of biopsy cycles with high inter-observer and intra-observer variability of histological diagnosis (218, 220). In a subsequent randomised controlled trial involving 847 women with regular menstrual cycles, 49% of mid-luteal and 35% of late luteal biopsies were “out of phase”, with no difference detected between fertile and non-fertile women (221). Similar findings were confirmed by subsequent studies; concluding that histological dating performs poorly as a predictor of endometrial receptivity (218).

The true incidence of LPD remains uncertain. In a study of 1630 women being evaluated for infertility only 4% had documented LPD, whilst no cases were identified in fertile women requesting tubal reanastomoses (222). Similarly Li et al. suggested that LPD was present in 14% of infertility patients compared with 4% in fertile controls. The same study also suggested a significantly higher incidence of LPD in patients with endometriosis and unexplained infertility compared with other infertility causes (223). The reported incidence of LPD in infertile women has a wide detection rate (1.9% to 60%), however much of the observed variability has been attributed to changes dependent on the stage of the menstrual cycle in which the biopsy was obtained, and in combination with the previously described inter-observer variability (224, 225). Davis et al. reported an incidence of LPD of 26.7% of patients using a 2-day lag versus 6.6% if a 3-day lag was used (226). For confirmed diagnosis, 2 consecutive out of phase biopsies are required, however studies of second biopsies found a highly variable rate of abnormality, ranging between 20 and 80% (227). Balasch et al. demonstrated that the rate of out of phase biopsies was no different in infertile women than occur by chance alone (228, 229).

The fundamental principle of LPD is that a delay in endometrial maturity is directly related to a lower level of circulating progesterone, through inadequate corpus luteum production. In a study by Usadi et al., two doses of intramuscular progesterone were given on the background of supplemental oestradiol following suppression of ovarian function with a GnRH agonist. Both “model” cycles were compared to the natural cycle in study participants. There was no observed impact of lowering the progesterone to 3-10ng/ml and histological dating (216). Alternative markers of endometrial function have been proposed including biochemical, morphological and molecular markers (213, 215, 230-234). However

these have yet to be validated in clinical practice and have not yet been able to identify fertile from sub-fertile women. Endometrial protein expression appears to differ in subjects with lowered progesterone replacement, suggesting a potentially more subtle deficiency (216).

The usefulness of histological dating of the endometrium in diagnosing luteal phase deficiency is no longer considered appropriate, and its routine use in clinical practice should not be advocated (1, 209). However the existence of luteal phase deficiency remains a treatment dilemma for clinicians (221).

1.4 Treatment of Luteal Phase Deficiency

The aim of treating luteal phase deficiency is to normalise menstrual irregularities and treat any reversible cause that may be attributable as a causative factor. For example, the normalisation of thyroid function, prolactin level, body mass index and extreme exercise regimes. Otherwise treatment has largely been considered empirical with the aim of promoting endometrial maturation and improving endometrial receptivity. In turn this should result in improved implantation and early pregnancy development. However as luteal insufficiency has been defined by surrogate end points such as serum progesterone levels or out of phase endometrium without absolute correlation to poor fertility outcomes (228, 229, 235, 236), the only practical way to diagnose or define LPD would be to demonstrate an improvement in live birth rates through treatment. In non-IVF cycles, ovulation induction has been considered a treatment for LPD. Guzick et al. used ovulation induction as a treatment in women with a previous out of phase endometrial biopsy. The

luteal phase biopsy was corrected in 8 out of 10 women with more than one pre-ovulatory follicle and 2 out of 8 women with a single follicle. Does ovulation induction improve infertility by inducing multiple ovulation, or does it correct LPD as a direct effect (237)? Ovulation agents like clomiphene citrate are known to have an anti-oestrogenic effect on the endometrium which may reduce the likelihood of implantation. Supraphysiological steroid levels may also negatively affect implantation.

Previously, the quality of the oocyte and the subsequent development of the embryo were considered largely independent of the surrounding follicle. Emerging data suggests that oocyte secreted factors are interdependent with follicular maturation processes (figure 6)(238). Through the multi-factorial regulation of ovulation at a cellular level, the release of inferior quality oocytes may be prevented by the integration of endocrine follicular or oocyte signals indicating suboptimal cycle quality (239). These mechanisms will have an impact on luteal phase quality via embryo developmental competence, with resulting HCG stimuli and the secretory quality of the corpus luteum resulting indirectly from the follicular maturation process.

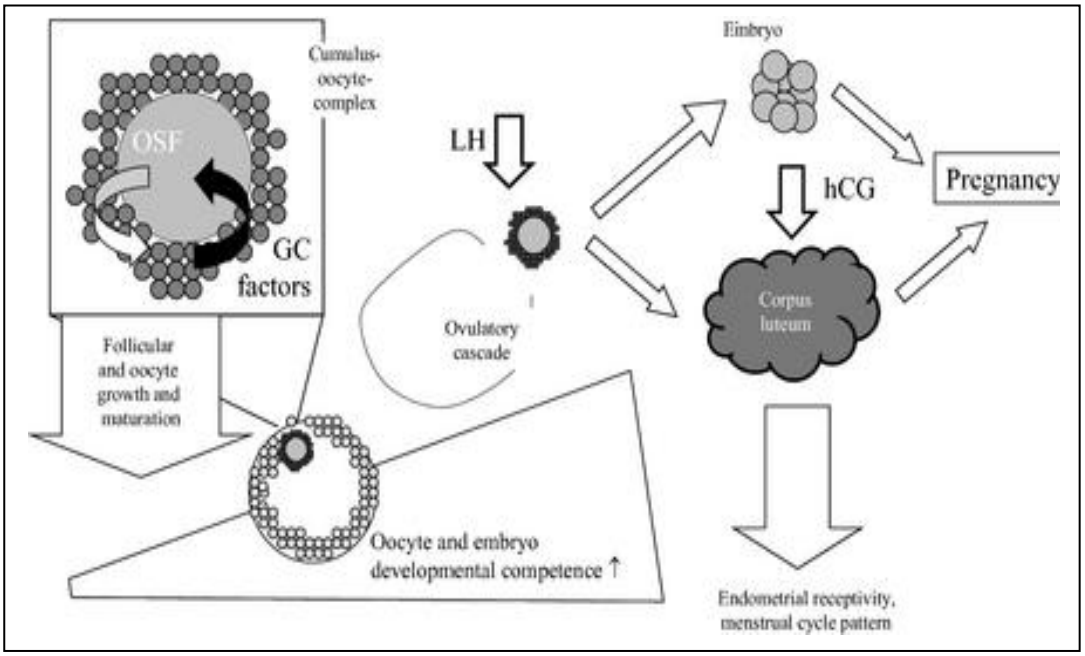


Figure 6. Interdependence of follicular maturation, oocyte and embryo development and function of the corpus luteum (238).

There are four principle pharmacological agents that are used for luteal support after IVF either alone or in combination; 1) HCG, 2) Progesterone, 3) Oestradiol, 4) GnRH agonists. A number of meta-analyses have consistently reported a benefit of luteal support after IVF (169, 170, 185, 240). The more recent Cochrane review by van der Linden et al. includes the most robust methodology and is considered the most influential resource for guiding clinical practice (185).

1.4.1 Human Chorionic Gonadotrophin

The luteal phase begins on the day of LH surge which causes ovulation. During the luteal phase the corpus luteum undergoes a morphological and biochemical change called "leutenization". Under the influence of LH, granulosa cells produce progesterone which transforms the endometrium into a secretory state ready for implantation. After

implantation, trophoblastic tissue from the placenta secretes HCG which maintains and preserves corpus luteum function and production of progesterone and oestradiol (241-244).

1.4.1.1 HCG versus placebo or no treatment

In a recent meta-analysis of 5 studies which included 746 patients, reporting 140 clinical pregnancies; HCG versus placebo or no treatment was not demonstrated to improve clinical pregnancy rate (OR 1.30, 95%CI 0.90 to 1.88) (245-249). However, a significant difference in ongoing pregnancy rate in favour of HCG was observed; OR 1.75 (95%CI 1.09 to 2.81). However only three of the studies included reported ongoing pregnancy, with only 81 ongoing pregnancies and 527 participants (246-248). No difference in miscarriage rates were reported from two studies included; OR 0.67 (95%CI 0.15 to 3.09) (246, 248). The incidence of multiple pregnancy was not reported as a significant effect; OR 1.30 (95% CI 0.90 to 1.88) (245-249). The incidence of ovarian hyperstimulation syndrome showed a significant effect in favour of placebo; OR 0.28 (95%CI 0.14 to 0.54), however this result was only based on a single study (247). Only one study, Beckers et al. reported live birth rates with only 6 events in 38 participants; OR 2.25 (CI 0.37 to 13.80), suggesting no apparent benefit (246).

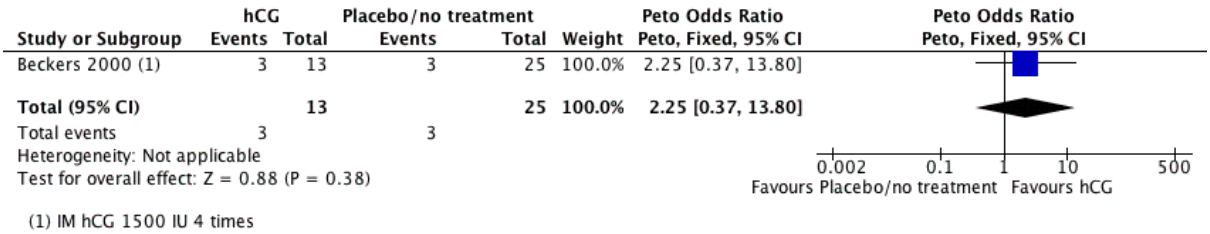


Figure 7. Forest plot comparing: HCG versus placebo or no treatment, outcome: Live birth (185)

1.4.2 Progesterone

Exogenous progesterone supplementation is commonplace, directly replacing progesterone that would normally be produced by the corpus luteum. Progesterone is most commonly administered by intravaginal or intramuscular routes and has previously been used orally. More recent preparations not in common use include vaginal reservoirs, subcutaneous injection and nasal sprays. Clinician and patient preference often dictate use.

1.4.2.1 Progesterone versus placebo or no treatment

In a recent Cochrane review the effect of progesterone compared with placebo or no treatment was analysed following assisted conception cycles (185). A meta-analysis of the seven studies included reported a significant effect on clinical pregnancy in favour of progesterone; OR 1.83 (95%CI 1.29 to 2.61) (245, 248, 250-254). Similarly, a positive effect was observed for five studies included the ongoing pregnancy rate; OR 1.87 (95%CI 1.19 to 2.94) (248, 250, 252, 253, 255). No difference in miscarriage rate was reported based on three included studies; OR 0.84 (95%CI 0.33 to 2.11) (248, 252, 255). No studies reported on rates of OHSS. Only one study reported live birth rate which suggested a significant effect in favour of progesterone; OR 2.95 (95%CI 1.02 to 8.56). However only 17 live births were reported from 156 participants(250).

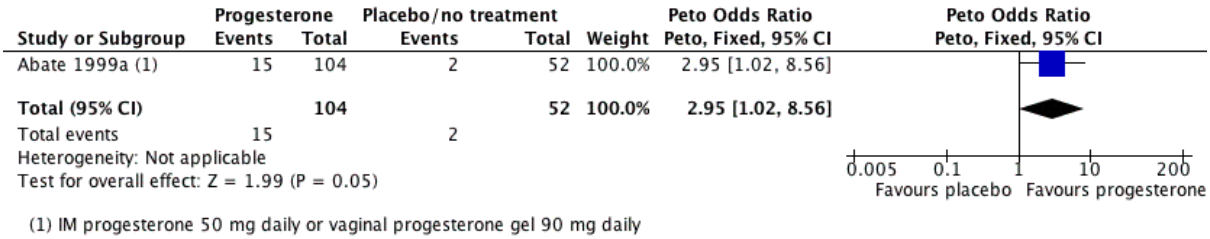


Figure 8. Forest plot of comparing progesterone versus placebo or no treatment, outcome live birth (185)

1.4.2.2 Progesterone versus HCG

Many studies have looked at pregnancy outcome comparing HCG and progesterone as luteal support. In a meta-analysis (185) of ten studies no difference was observed between the treatment groups in terms of clinical pregnancy rate; OR 1.14 (95%CI 0.90 to 1.45) (245, 248, 256-262) or ongoing pregnancy rate; OR 1.09 (95%CI 0.66 to 1.80)(248, 259, 263). No difference in miscarriage rates were reported; OR 10.75 (95% CI 0.39 to 1.44) (248, 257-260). Evidence from 4 studies showed no effect on the incidence of ovarian hyperstimulation syndrome, although the results should be interpreted with caution as there was a disagreement between studies (256, 259-261). The live birth rate was only reported for 2 studies, both of which suggested no difference in outcome between HCG and progesterone exposed groups; OR 2.43 (95%CI 0.84 to 6.97) (257, 259)

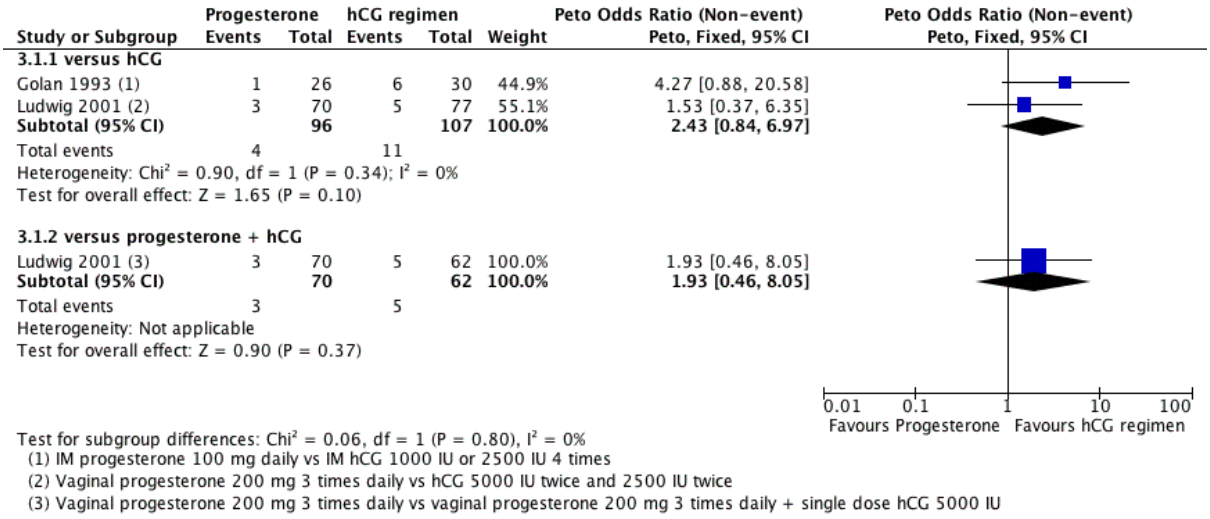


Figure 9. Forest plot comparing progesterone versus HCG regimens outcome for live birth rate (185).

1.4.2.3 Progesterone versus progesterone + HCG

Several authors have postulated a benefit of adding HCG to progesterone. In the Cochrane review, no benefit was observed in terms of clinical pregnancy rate, ongoing pregnancy rate

or live birth rate. In keeping with previous studies there was no difference in OHSS rates, although the authors suggested that this finding should be interpreted with caution (185).

1.4.3 Oestradiol

Progesterone and oestrogen are produced by the corpus luteum although the latter is produced in relatively smaller amounts. Several authors suggested a benefit of combining progesterone with oestrogen. Only one included study reported on live birth. Eleven events were reported in 100 participants with no beneficial effect reported between treatment allocations; OR 1.13 (95%CI 0.43 to 2.94) (264). No evidence of benefit was observed in clinical or ongoing pregnancy rates; OR 1.25 (95%CI 0.99 to 1.59) and OR 1.00 (95%CI 0.77 to 1.31) respectively (264-271). The incidence of miscarriage and OHSS were not significantly different.

1.4.4 Gonadotrophin releasing hormone agonist

The prolonged use of GnRH agonists results in pituitary desensitisation, thus preventing premature LH surge during IVF cycles. Whilst initially resulting in a flare effect on pituitary receptors, the down regulation effect can be observed after more than 14 days. Therefore the use of GnRH agonists in luteal support seems somewhat confusing. It is likely that down-regulation of GnRH receptors may not be totally abated 8 days after cessation of GnRH agonist injections, with further administration causing a flare effect leading to release of pituitary LH, which in turn stimulates progesterone production from the corpus luteum. The use of GnRH antagonists which enable rapid reversal of GnRH receptor status may benefit from luteal support in the form of GnRH agonists.

Three studies included in the Cochrane review suggest a benefit of adding GnRH agonists to progesterone when compared to progesterone alone, reporting higher live birth rates; OR 2.44 (95%CI 1.62 to 3.67) (272-274). Clinical and ongoing pregnancy rates were also higher, although there was considerable heterogeneity between all study groups.

1.5 Progesterone as Luteal Support

1.5.1 Route of administration and pharmacokinetics of progesterone

1.5.1.1 Oral Preparation

Oral progesterone is subject to extensive first pass metabolism of the liver, resulting in the side effects of somnolence and the induction of hypnotic effects similar to the tranquilizing effects seen with benzodiazepines (275). Traditionally the oral bioavailability of progesterone is less than 10% (276); however with the introduction of micronized progesterone preparations, an efficient system of delivery and enhanced bioavailability has been achieved (277, 278). Micronization of progesterone to particle sizes of less than 10µm increases the available surface area of the drug and enhances the aqueous dissolution rate and intestinal absorption. Suspension in oil and packaging in a gelatine capsule has also been shown to further enhance bioavailability (279).

Serum progesterone concentrations following oral administration of progesterone are highly variable; however important trends are apparent. After oral administration of progesterone, the maximum concentration (C_{max}) and area under the curve (AUC) are proportional to the administered dose within the dose range 100-300mg. Peak plasma levels of progesterone however vary among individuals and between studies. The mean plasma level achieved with oral progesterone doses of ≥100mg are at least comparable to those obtained in the luteal phase. Progesterone levels remain elevated for up to 12 hours and do not return to baseline until at least 24 hours after final oral dose (280). When micronized progesterone is administered orally at doses of 100-300mg, the maximum concentration is achieved within 2-3 hours. There appears to be a differential effect of dose and time to peak

concentrations, indicating that the pharmacokinetics (i.e. absorption and emanation) of micronized progesterone are dose-independent. Consistent with pharmacokinetic dose proportionality, the mean (±SD) terminal half-life for 100mg, 200mg and 300mg doses of oral micronized progesterone are similar (18.3 ± 3.5 hours, 16.8 ± 2.3 hours and 16.2 ±2.7 hours respectively (281, 282).

The pharmacokinetic parameters appear to be unchanged after 5 days of dosing, suggesting there is not a significant accumulation of progesterone or alteration of progesterone metabolism with multiple dosing. When micronized progesterone is orally administered in twice daily doses (100mg in the morning, 200mg in the evening) for 5 days, plasma levels of progesterone remain significantly elevated throughout each 24 hour period and for up to 36 hours after the final dose. Progesterone levels do not fall to baseline values until 84 hours after the last dose (283).

Frishman et al. evaluated the effects of oral micronized progesterone on luteal phase defects (200mg three times daily). Seven patients who previously had a luteal phase defect corrected by vaginal progesterone suppositories were included in this study. Treatment was initiated 3 days after documented rise in basal body temperature. Endometrial biopsies revealed that all seven patients had normal in-phase profiles. Serum progesterone levels measured after 4 days of treatment showed that there was a significant level of serum progesterone peak (approximately 50mg/nL) 2 hours after dosing. The primary adverse effect was drowsiness which was experienced by 4 patients. However five patients preferred oral therapy to vaginal preparations (284).

1.5.1.2 Vaginal and Intramuscular Preparations

Levine and Watson compared the pharmacokinetics of an oral micronized progesterone preparation (Progestin) and a vaginal preparation (Crinone 8%) (285). The results demonstrated that the vaginal gel was associated with a higher maximum serum progesterone concentration and 24 hour area under the curve for drug concentration vs. Time (AUC_{0-24}). This signifies a higher total progesterone exposure over a 24 hour period for a single dose of progesterone administered vaginally compared to one dose given orally. It was concluded that vaginal administration of progesterone results in greater bioavailability with less relative variability than oral progesterone.

	Crinone 8%, 90mg (n=6)	**Progesterone Capsules, 100mg (n=6)**
C_{MAX} (ng/mL)	10.51 ± 0.46	2.20 ± 3.06[a]
Dose-normalised C_{MAX} (ng/mL/mg)	0.12 ± 0.005	0.02 ±0.031[a]
$C_{Avg(0-24)}$ (ng/mL)	5.55 ± 0.61	0.14 ± 0.22[a]
T_{MAX} (h)	7.67 ± 3.67	1.00 ± 0.41[a]
AUC_{0-24} (ng.h/mL)	133.26 ± 14.61	3.46 ± 5.15[a]
Dose-normalised (ng.h/mL/mg)	1.48 ± 0.16	0.035 ± 0.052[a]

SD = standard deviation; C_{MAX} = maximum concentration; $C_{AVG(0-24)}$ = Mean drug concentration over 24 hours after administration; T_{MAX} = time to maximum concentration; AUC_{0-24} = area under concentration-time curve to 24 hours after administration. [a] P<0.05, Student's t-test

Table 1. Pharmacokinetic parameters of progesterone after administration of Crinone 8% vaginal gel 90mg, and oral progesterone capsules 100mg (285).

As previously detailed, oral administration of progesterone yields poorly sustained plasma progesterone concentrations (286). Serum progesterone levels are higher after intramuscular administration than after intra-vaginal administration (287). However plasma progesterone levels have often been considered a poor marker of bioavailability compared with endometrial progesterone levels. Endometrial levels of progesterone are significantly higher in the intra-vaginal group compared with the intramuscular group (median 14.1, range 8.5 to 59.4, 95%CI 9.89 to 38.79 and median 1.2, range 0.5 to 13.1; 95%CI -0.48 to 7.39) ($p<0.05$).

The intramuscular administration of progesterone (an oil based solution) requires daily painful injections which has been associated with local inflammation, sterile abscess formation and damage to the sciatic nerve which may result in sensory or motor impairment of the lower extremity (288). Effective luteal support may necessitate several weeks use (289). Rarer side effects also include eosinophilic pneumonitis, which typically manifests 3 weeks following the first injection (290). Intramuscular progesterone is carried in an oil base derived from sesame oil which has been associated with a rare risk of severe allergic reaction. In cases where there is known allergy it is recommended to switch to a peanut oil preparation (291). The preservative used is benzyl alcohol and may be the aetiology for an allergic reaction (290). However, some patients may prefer the convenience of once daily injections.

The effects of differing progesterone formulations and dosages have been compared for effect at the endometrial level. In 43 women with primary ovarian failure who underwent 75 hormone-substituted cycles, vaginal application of 200mg of micronized progesterone

every 8 hours was enough to induce secretory transformation of the endometrium on day 21 (day 8 of progesterone therapy). The use of oral progesterone therapy (100mg every 8 hours) and intramuscular progesterone (50mg twice daily) resulted in an inadequate response and heterogenous endometrium respectively (292).

In a similar study the pharmacokinetics and endometrial levels of progesterone after vaginal and intramuscular administration were compared (293). In the study, 20 agonadal subjects received 200mg of transvaginal micronized progesterone every 6 hours or 50mg of IM progesterone twice daily. Mid-luteal (day 7 of progesterone therapy) endometrial biopsies obtained from women who received vaginal progesterone showed similar histological maturity and oestrogen and progesterone receptor content as those that received intramuscular progesterone. Further, intravaginal progesterone resulted in higher endometrial progesterone concentrations (11.5ng/mg of protein) compared with intramuscular progesterone (1.4ng/mg of protein). Full secretory transformation of the endometrium was achieved with serum progesterone levels of 1-3ng/mL after vaginal administration but not after intramuscular or nasal delivery (294, 295). The progesterone concentration difference in the uterine cavity was probably a consequence of a “uterine first-pass effect” (296). A uterine distribution mechanism could be involved with diffusion from the vaginal / uterine vein to the artery. Progesterone concentration in the uterine artery was found to be higher than in the radial artery after vaginal administration (297). However, Bulletti et al. demonstrated a local direct vagina to uterus transport process with Crinone gel, which results in preferential progesterone uptake of by the uterus (298). In a study by Fanchin et al., Crinone given twice daily in combination with physiological amounts of oestradiol was shown to produce endometrial changes similar to those normally present

during the luteal phase, despite the presence of sub-physiological plasma progesterone levels (296).

Endometrial progesterone concentrations reach a steady state within 5 hours after vaginal administration (298). Vaginal preparations are able to achieve similar histological changes in the endometrium as those after an intramuscular injection. These effects occur despite lower serum levels compared with levels achieved with the intramuscular route (299).

Side effects experienced by patients using intravaginal preparations include; local irritation, discharge and dyspareunia (300). However, a majority of patients are prefer vaginal preparations to intramuscular preparations as they are easier to administer (301).

A large volume of research has looked at the beneficial effects of intramuscular administration over vaginal or rectal administration. However no studies have demonstrated any benefit of either route in terms of pregnancy rates, miscarriage rates, OHSS rates or live birth rates (185, 245, 251, 302-304).

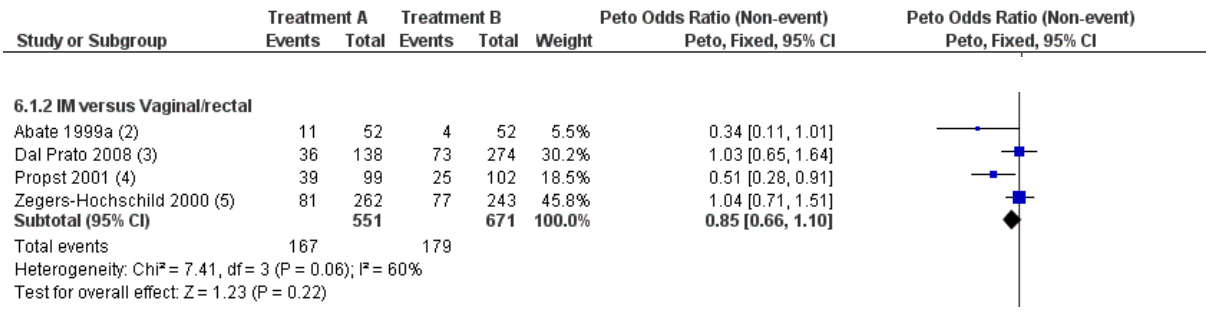


Figure 10. Forrest plot comparing intramuscular progesterone and vaginal/rectal progesterone outcome: live birth rate (185)

No difference in pregnancy rate or live birth rate was observed between vaginal and rectal administration of progesterone. (185, 305, 306).

1.6 Duration of Luteal Support

Luteal support is commonly practiced worldwide in the majority of stimulated IVF cycles. Whilst studies have suggested that progesterone is as effective as HCG in maintaining pregnancy, the latter is associated with a higher incidence of ovarian hyperstimulation syndrome (169). Current preference tends to favour the use of progesterone. Whilst the benefit of luteal support remains unchallenged (169, 170, 185, 190, 240, 307-309), and with no preparation or route of administration proven superior, the optimum duration of luteal support remains to be demonstrated.

Luteal support during IVF can be initiated on the day of HCG administration, day of oocyte retrieval, the day after oocyte retrieval or day of embryo transfer without compromising implantation or pregnancy rates (310, 311). A delay in commencing luteal support beyond 6 days after oocyte retrieval negatively impacts on the pregnancy rate (312). A number of studies have attempted to address the optimal duration of treatment.

In 1992 Prietl et al. randomised a small number of patients at the time of positive pregnancy test to receive further intramuscular oestradiol valerate and 17 alpha hydroxyprogesterone caproate and continued until 12 weeks gestation, or no further treatment. In the treatment arm the ongoing clinical pregnancy rate at seven weeks gestation was 89%, compared with 59% in the control group (313). Although only 120 patients were involved in the study, a

significantly higher proportion of pregnancies were ongoing at 7 weeks gestation after treatment with progesterone and oestradiol ($p<0.01$). However, the authors utilised a very heterogenous study group, utilising clomiphene, clomiphene & HMG, GnRH agonist and HMG alone during ovarian stimulation. Luteal support prior to confirmation of pregnancy used either HCG or progesterone dependent on the risk of developing OHSS. The combination of oestradiol and progesterone as luteal support makes it difficult to attribute effect to outcome. Randomisation procedures were suboptimal and live birth rates were not reported.

In 1998, Stovall et al. performed a retrospective study that suggested luteal support (progesterone) could be safely withdrawn at 6 weeks gestation in a subgroup of patients with high serum progesterone levels above 60ng/ml achieved using intramuscular progesterone. Luteal support remained withdrawn provided the serum progesterone level was maintained above 30ng/ml. Subsequent live birth rates were comparable (314).

In one of the earliest studies, Schmidt et al. in 2001 suggested withdrawal of vaginal progesterone at the time of positive pregnancy test did not affect the miscarriage rate or live birth rate (63% in the progesterone withdrawal group versus 64% in extended luteal support group). Whilst this trial had 200 patients in each treatment arm, it was a retrospective study (315).

In the first prospective randomised study Anderson et al. (2002) demonstrated no significant benefit of extending luteal support beyond biochemical pregnancy. Three hundred and three patients having used luteal support until confirmation of biochemical

pregnancy were randomised to either receive an additional 3 weeks of luteal support (up to 7 weeks gestation) or no further support. Recombinant FSH was used in during ovarian stimulation and patients with heavy bleeding were excluded. In the study group (no additional luteal support) versus the control group (additional luteal support), the miscarriage rate was 4.6% and 3.3%, ongoing pregnancy rate 88.7% and 90.8% and live birth rate 78.7% and 82.4% respectively. No statistical differences in gestational age at birth or birth weight were reported. Episodes of bleeding in the non-progesterone group were lower than in the progesterone treatment group; however this outcome did not reach statistical significance. No effect on live birth rate was apparent (316). This study substantiated the earlier report by Schmidt (317).

In 2008, Aboulghar et al. reported no difference in extending luteal support beyond 7 weeks gestation (318). Patients with a confirmed fetal heart rate at 6-7 weeks, having received LPS up until that point were considered eligible to be included in the study. Trial eligibility criteria were those women <39 years of age having undergone a long GnRH agonist protocol. Patients whose partners were azoospermic or had themselves experienced bleeding prior to ultrasound examination were excluded. Prior to recruitment patients received either vaginal progesterone (11%) or intramuscular progesterone (89%). Luteal support was continued according to initial usage. No significant differences in bleeding episodes were reported (15.9% in patients who continued additional luteal support compared to 20.8% in the early cessation group) (OR 0.72 95%CI 0.38 to 1.36). No difference in the miscarriage rate up to 20 weeks gestation was reported; 4.6% versus 4.8% (OR 0.94; 95%CI 0.3 to 3.01). Live birth rates were not reported.

In 2010, Goudge et al. reported no effect on live birth rate when luteal support was withdrawn at pregnancy test or continued until 7-8 weeks gestation (319). Forty six patients were randomised to receive additional support, and 51 patients were randomised to cease support at positive pregnancy test. This study utilised a combination of highly purified and recombinant gonadotrophins along with a small (<5%) number of cycles utilising GnRH antagonists alongside a majority of GnRH agonist cycles. Intramuscular progesterone was used as treatment intervention. Inclusion criteria included patients in their first cycle of treatment and less than 37 years of age. Patients were only randomised to withdraw luteal support if their serum progesterone was >15ng/mL. If lower, additional support was continued. The pregnancy rate between the treatment and control group was 63% versus 62.7% (p=0.976), ongoing pregnancy rate 58.7% versus 51% (p=0.446) and live birth rate 52.5% versus 49% (p=0.839); therefore no difference in outcome was observed.

In 2011, Kyrou et al. published a similar trial in GnRH antagonist cycles, randomising 200 patients to receive vaginal progesterone (200mg TDS) until pregnancy test or continue to 7 weeks gestation. Eligibility criteria included patients with a serum βHCG greater than 75IU/L 14 days post embryo transfer and a doubling in βHCG levels 48 hours later. Patients with PCOS, endometriosis (American Fertility Score stage 3 or greater) or azoospermia were excluded. No difference in ongoing pregnancy rates were reported (82% versus 73%, P=0.175; difference 9%, 95%CI 2.6 to 20.3). No differences in the incidence of miscarriage or vaginal bleeding were reported. Live birth rates were not reported (320).

In 2012, Kohls et al. reported no difference in pregnancy rates when luteal support was withdrawn at 5 weeks gestation compared to 8 weeks gestation. This randomised

prospective trial included patients aged less than 40 years, but were excluded if any bleeding occurred before the first ultrasound scan. Patients were randomised at first ultrasound to receive an additional 3 weeks of vaginal progesterone or no treatment. GnRH antagonists and recombinant gonadotrophins were utilised during controlled ovarian stimulation. The miscarriage rate in singleton pregnancies did not differ (5/80 versus 6/79; p=0.75) whilst bleeding episodes were more frequent in the early progesterone cessation group (18.0 ± 2.6 versus 7.2 ± 1.3 episodes). Comprehensive live birth rates were not reported (321).

In a recent meta-analysis no differences in miscarriage rate, ongoing pregnancy rate or live birth rate were reported with early luteal support cessation (322).

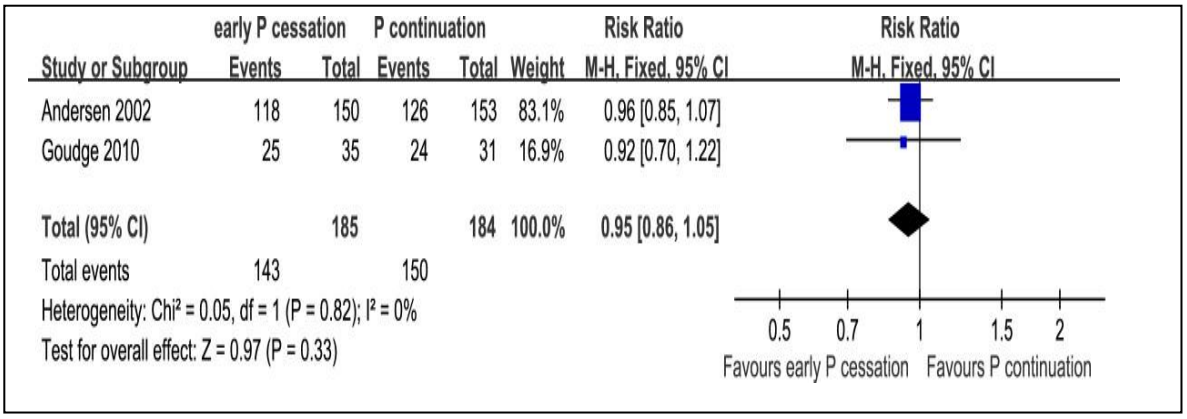


Figure 11. Live birth rate of women who underwent early P cessation versus P continuation after IVF/ICSI (322).

In the most recent Cochrane review, no separate analysis was undertaken looking at optimum duration of support (185). Instead several sub-group analyses were performed. Of the seven studies included in the progesterone versus placebo or no treatment, 3 studies withdrew luteal support at positive pregnancy test, with the chance of clinical pregnancy OR

1.40 (95%CI 0.74 to 2.64) (245, 248, 254). Four studies extending treatment up to 12 weeks reported a benefit with an OR 2.07 (95%CI 1.35 to 3.17) (250-253). In a second sub group analysis, including 6 trials comparing progesterone versus HCG regimes, 5 studies stopped luteal support at pregnancy test and reported no difference in outcome; OR 1.01 (95%CI 0.78 to 1.54) (245, 248, 257, 259, 260). One study extended luteal support up to 12 weeks; OR 1.15 (95%CI 0.47 to 2.82). No recommendation about the optimum duration of luteal support was made by the review authors.

The National Institute of Clinical Excellence (NICE) recently published guidelines that included advice about the optimum duration of luteal support. Three studies were included in their analysis, Goudge 2010, Nyboe Anderson 2002 and Kyrou 2011. These studies were deemed very low, low and very low quality. NICE concluded that luteal support for an extended duration did not appear to result in clinical benefit or cause harm compared with a shorter duration of luteal support. However it was conceded that the evidence in this area was limited. The group conceded that it was biologically plausible that luteal support may be of benefit up to 8 weeks after embryo transfer, after which time the pregnancy should be self-supporting. Women should be informed that there is no evidence to recommend luteal support beyond 8 weeks.

The American Society of Reproductive Medicine also opted for a more conservative approach, recommending luteal support is probably not necessary beyond 8-10 weeks gestation.

Despite emerging evidence that luteal support is unnecessary beyond pregnancy test, the vast majority of clinics worldwide continue to utilise an extended regime, further details of which are explored in chapter 2.

1.7 Uterine Artery Doppler

1.7.1 Background

Prior to the introduction of Doppler ultrasound assessment, uterine artery blood flow could only be measured through invasive techniques or those using radio-dilution (323). However using these methods, the arcuate arteries rather than the uterine arteries, and blood flow rather than impedance was measured. Whilst the term “resistance” is commonly used in describing Doppler, it is in fact “impedance” that is measured, this being the combination of forward and reflected blood flow. The advent of colour Doppler has made vessel location more precise (324).

With the widespread use of ultrasound to assess the uterine artery, abnormal waveforms and high impedance have been consistently associated with poorer pregnancy outcomes including fetal hypoxia and low birth weight (325, 326).

1.7.2 Physiology of Doppler Measurements

Non-invasive testing of placental perfusion can be achieved with the use of Doppler ultrasound to measure uterine artery pulsatility index (327). PI is a measure used to describe the shape of the flow waveform in a blood vessel. It is a measure of the variability of blood velocity in a vessel, equal to the difference between the peak systolic and minimal diastolic velocities divided by the mean velocity during a cardiac cycle (328). Increased PI reflects increased impedance to blood flow in the uterine arteries and is thought to reflect the failure of the trophoblastic invasion of the spiral arteries and their conversion into low-

resistance vessels, with consequent impairment in uteroplacental function (327). Vascular changes in the uterine artery can be detected by uterine artery Doppler as early as 5 weeks gestation (329, 330).

High impedance or the presence of diastolic notches indicates inadequate trophoblastic invasion (330). The conversion of spiral arteries into uteroplacental arteries plays a basic role in the establishment of the physiological placental blood supply. Abnormal and inadequate conversion of the branches of the uterine arteries into low-resistance uteroplacental vessels, due to poor extravillous trophoblastic invasion is associated with intrauterine growth restriction (IUGR) and placentation problems (330, 331). Impaired trophoblastic invasion of maternal spiral arteries is associated with increased impedance to flow as demonstrated by uterine artery Doppler waveform measurement (332, 333).

Similarly, umbilical artery blood velocity reflects the function of the placental tertiary villous tree (334). Anatomically, the capillaries of the gas exchanging mature intermediate and terminal villi have the greatest potential to influence total fetal—placental vascular impedance (335). Reduced villous development correlates with abnormal umbilical artery Doppler velocimetry, being severe in cases of absent or reversed end diastolic flow (336).

1.7.3 Histopathology Associated with Uterine artery Doppler

Normal implantation is characterised by endovascular and perivascular trophoblast infiltration of the maternal uterine arteries, which results in their conversion to large, flaccid conduits capable of high compliance (337). It is the loss of smooth muscle and elastin within

the spiral arteries which enable them to dilate and result in increased capacity for greater volumes of maternal blood, which sustain the increasing requirements of the placenta and fetus (338). Trophoblastic invasion of both the lumen and the adventitia of the spiral arteries with the loss of smooth muscle results in the creation of an intramural trophoblast and fibrinoid layer in its place, which subsequently becomes re-endothelialised. Invasion is believed to occur in two phases, initially involving only the superficial myometrium, but progressing in to the deep myometrium from 4 weeks gestation (339). Brosens et al. demonstrated that in myometrial placental beds associated with preeclampsia, these adaptive changes were not universal and that "normal" spiral arteries without these adaptive changes were present (340). In some pathology specimens, physiological changes were confined to the decidual segments leaving the myometrial segments undisturbed (341).

Whilst an increase in uteroplacental resistance is associated with an increased pulsatility index and early diastolic notching (342), it has been proposed that this early diastolic notch may be the result of an abnormal uterine artery wall, and independent of placental obstructive measurements (343). These resultant effects were accentuated with a reduction in the radius of the uterine artery whilst changes in mean arterial pressure had little effect on waveform appearance. In vivo models have confirmed a linear correlation between an increase in uterine vascular resistance and increase in uterine artery pulsatility index (344).

1.7.4 Clinical Use of Doppler Measurements

Early studies suggested that abnormal uterine waveforms may be a predictor of preeclampsia and adverse pregnancy outcome (345). It was realised that women with proteinuric hypertension and a deviation of the waveform of >2SD (peak systolic velocity and early diastolic notching) on the normogram of a cardiac cycle were associated with fetal hypoxia and low birth weight (326). A failure of adaption of the spiral arteries is a distinctive feature in pregnancies complicated with preeclampsia, however this is also evident in pregnancies complicated by non-proteinuric gestational hypertension (346), chronic hypertension (347) and IUGR with evidence of preeclampsia or hypertension (348). In a study by Medazi et al., all pregnancies which resulted in normal weight infants demonstrated complete trophoblastic invasion of the spiral arteries. In comparison 55.3% of pregnancies complicated by IUGR failed to demonstrate this normal pregnancy adaption (330). Placenta obtained from pregnancies with confirmed IUGR and with abnormal umbilical uterine artery Doppler velocimetries were demonstrated to have a significantly increased number of villous infarcts, cytotrophoblast proliferation and thickening of the villous trophoblastic basal membrane. Abnormal placental bed pathology was significantly associated with abnormal uterine artery velocimetry (OR 33.7, $p<0.001$) (330). Early diastolic notching is likely due to wave reflection, and is indicative of abnormally high placental bed resistance in the third trimester (349).

Doppler assessment alone is a poor predictor of complications associated with impaired placentation (350). However it has a strong negative predictive value. Women with

abnormal Dopplers will have uneventful pregnancies, whilst women with complicated pregnancies are unlikely to have normal uterine Doppler waveforms.

Acute atherosis of maternal vessels, with vascular necrosis, foam cell and perivascular leucocyte infiltration are features demonstrated in abnormal placental beds (351). However these findings do not independently correlate with poor pregnancy outcome, due to a sufficient collateral supply of unaffected vessels allowing sufficient blood flow (331). Villous infarction, villous hypovascularity, terminal villous fibrosis, increased syncytiotrophoblast knotting, cytotrophoblast proliferation and abruption placentae have also been associated with poor placentation (352-354). Placental examination of pregnancies complicated with intra-uterine growth restriction, those exhibiting extensive hypoxic damage or haemorrhagic changes were six times more common in cases where uterine artery Doppler velocimetry was abnormal, whilst not being associated with maternal hypertension or proteinuria (355).

The underlying pathology of preeclampsia has not been fully elucidated, but abnormal placentation and endothelial dysfunction are involved. Failure of remodelling of the placental trophoblasts results in hypoxic insult of the placenta. Inadequate vascular remodelling during placentation reduces blood flow from the uterus to the placenta and increases vascular resistance. Whilst there is some predictive value of Doppler assessment in first and second trimesters (356) (357), single measurements in the first trimester have not proven to be clinically useful (358, 359). However effective screening for preeclampsia can be achieved by measuring the pulsatility index (PI) at 11-13 weeks gestation, in combination with maternal history, blood pressure, serum pregnancy associated plasma

protein-A and placental growth factor (360). Early detection of potential problems and treatment with aspirin and antihypertensive medication may improve pregnancy outcome (361). It is estimated that combined screening could predict 90% of cases of preeclampsia occurring before 34 weeks and 45% of cases of late onset preeclampsia (362, 363).

Placental abruption is believed to result from impaired placentation at an early gestation (331). In the 8th gestational week the transformation of the spiral arteries to low resistance arteries begins (364). Several Doppler studies performed in the first trimester have demonstrated an impedance to flow in the uterine arteries and subsequent development of pre-eclampsia (365-371).

More recent studies have suggested that whilst uterine artery pulsatility indices are independent of age, differences between non-fertile and fertile patients have been demonstrated (324, 372, 373). Blood flow resistance in the uterine arteries has been shown to be predictive of implantation with IVF (374, 375). In women with recurrent pregnancy loss, the non–pregnant pulsatility index (PI) and the impedance in the uterine arteries are increased (376-378). This pre-pregnancy circulatory profile reflects a high resistance to uterine flow. This is a comparable picture seen in pregnancies complicated with pre-eclampsia. In formerly preeclamptic women, the non-pregnant uterine artery PI inversely correlates with venous compliance which in turn correlates with plasma volume (379). Along with venous compliance, plasma volume represents the cardiovascular and venous reserve capacity (380). Venous reserve capacity represents the ability of the venous system to adapt to arterial demands. A mismatch between venous reserve capacity and arterial demands is known to correlate with adverse pregnancy outcome. It has been suggested that a low

venous compliance relates to restricted uterine perfusion that may affect the uterine environment to such an extent that it influences embryonic, placental and uterine spiral artery development and may increase the incidence of pregnancy loss and hypertensive complications of pregnancy (381).

1.7.5 Extra-placental factors that affect Pulsatility Index

It has been proposed that extra-placental factors may influence abnormal uterine artery Doppler impedance, which cannot be solely explained by abnormal uteroplacental vessel histopathology. Abnormal uterine artery PI is more common in women whose placental bed biopsies show absent or incomplete physiological adaption. Complete physiological adaption is also absent in one third of biopsies from women with normal uterine artery Doppler and normal pregnancy outcome. Similarly, one third of women with abnormal uterine artery Doppler and either IUGR or preeclampsia did not have pathological changes on examination of placental bed biopsy (333).

First trimester trophoblast with high uterine impedance already shows less endovascular invasion than in those whom adaption to a low impedance flow occurs (382). Whilst it is generally accepted that placentation occurs in the first and early second trimesters, some of the placentation may continue into the late second trimester.

Flow velocity is influenced by the ejection systolic pulse, arterial wall compliance, blood stream inertia and downstream peripheral resistance (383). Whilst the placenta and placental bed correlate well with downstream resistance, the uterine artery waveform is

also a reflection of maternal vascular tone. This association is demonstrated in women with preeclampsia who have a higher long term risk of cardiovascular disease, and in women with traditional cardiovascular risk factors pre-pregnancy that have a higher risk of preeclampsia (384). Patients with early onset or severe preeclampsia have poorer endothelial function for at least two years postpartum, whilst post partum cardiac function is similarly compromised (385-387). Flow-mediated dilatation (FMD) is considered the gold standard non-invasive assessment of endothelial function. Lower FMD values indicate endothelial dysfunction. Patients exhibiting bilateral uterine artery notches without preeclampsia demonstrate reduced FMD (388). In women with confirmed preeclampsia, those with bilateral notches had a lower FMD than those with normal waveforms, implying endothelial dysfunction. High levels of high sensitivity C-reactive protein (CRP), a marker of systemic inflammation were found to be higher in pre-eclamptic women with abnormal uterine waveforms compared to those with normal waveforms (389). More recent surrogate markers of endothelial health and arterial stiffness include arterial pulse wave reflection and aortic pulse wave velocity, with higher levels associated with increased uterine artery impedance (390).

Maternal heart rate can affect uterine artery pulsatility index, demonstrating a significant negative correlation (391). In clinical practice this variable is not routinely adjusted for, however by doing so, both positive and negative predictive values improve (392).

A variation in impedance of the uterine arteries has been demonstrated to alter according to the menstrual cycle in response to oestradiol and progesterone levels (324, 372, 393-395).

Uterine impedance falls progressively during the luteal phase of the menstrual cycle when serum progesterone levels are highest. A more pronounced effect is evident in the vessel ipsilateral to the corpus luteum. This reduction in impedance observed in the secretory endometrium around the implantation window is strongly correlated with the plasma levels of progesterone rather than oestradiol.

Habara et al. also reported that uterine artery PI was inversely correlated with serum progesterone levels. Administration of dydrogesterone reduced the pulsatility index in the presence of adequate or high oestrogen levels (376, 396). The relaxation of uterine muscle by progesterone may be involved in the reported improvement of uterine perfusion (376).

Studies utilising oestrogen with or without cyclical progestin have shown to inhibit progression of atherosclerosis in animal models. This effect appears to be mediated by the endothelium (397). Oestrogen also appears to upregulate oestrogen receptors in the vessel wall resulting in reduced smooth muscle replication and intima thickening (398). Oestrogen improves vasoreactivity, in part mediated by nitric oxide possibly by reducing circulating levels of inflammatory markers including vascular cell adhesion molecules and intracellular adhesion molecules (398, 399). Oestrogen also increases basal blood flow, decreases resistance and increases vessel diameter in post menopausal women as demonstrated in ultrasound studies assessing flow mediated vasodilatation (400-402). Oestradiol and progesterone have also been postulated to have an effect on systemic arterial compliance, however the results of studies are inconsistent (403-406). In a study of 110 women undergoing controlled ovarian stimulation, oestradiol or progesterone levels were not associated with changes in blood viscosity (407).

Patients with PCOS have been demonstrated to have higher uterine artery impedance compared to women with regular menstrual cycles (408, 409). It is not clear whether chronically raised LH levels or hyperandrogensism are responsible for these observed changes. The direct vasoconstriction and stiffening effects of hyperandrogenaemia on the vessel wall along with hyperinsulinaemia are thought to be potential mechanisms of vascular damage.

Nitric oxide is generated in the endothelium by endothelial nitric oxide synthase (eNOS) and is essential for maintaining endothelial function and vascular tone (410). Decreased concentrations of nitric oxide metabolites are detected in women with preeclampsia compared to normal pregnancies (411, 412), and lower levels of eNOS have been found in women with raised uterine artery impedance (413). However, and contrary to expectation there is raised eNOS expression in the placentas of women with bilateral uterine artery notches (414). These findings are consistent with findings that flow mediated dilatation in response to nitric oxide is impaired in preeclampsia (415), which may manifest itself as raised pulsatility index through increased vascular tone via endothelial dysfunction.

Anti-hypertensives have generally not been shown to effect uterine artery PI (416-422), with the exception of alpha methyldopa which results in a significant decrease in uterine artery impedance (423). Therapeutic doses of nifedipine used in tocolysis have also demonstrated a significant decrease in PI (424).

Interestingly, normal uterine artery Doppler parameters have been recorded in extra-uterine pregnancies (425, 426). This phenomenon, with an absence of placental bed

contributing to downstream resistance confirms factors external to the placenta may be responsible for the uterine artery waveform.

Malaria appears to cause increased resistance in the uteroplacental vasculature, although this remains reversible with clearance of the parasite (427). This may suggest a role of pro-inflammatory cytokines and local vascular damage, unrelated to the adaption of the spiral arteries to pregnancy, resulting in abnormal uterine artery waveforms.

Uterine artery impedance and waveform changes in the postpartum period are complex and often difficult to predict (428-432), however it is clear that the normal non-pregnant uterine waveform can take several weeks to return to normal following removal of the placenta. This may suggest that other factors, possibly hormonal factors may affect the responsiveness of the endothelium, beyond the trophoblastic effect.

1.7.6 Resistance Index (RI)

Measurement of resistance index is another calculation that can be derived from the Doppler waveform. In a similar fashion to pulsatility index it is reflection of abnormal vascular impedance and is associated with disorders of abnormal placentation and poor pregnancy outcome.

Resistance Index is calculated using the peak systole divided by the sum of systole and diastole (RI = systole / (systole + diastole). The resistance index is also known by the name Pourcelot index.

A fall in the uterine artery resistance index (RI) may begin as late as the luteal phase of menstrual cycle and continues to decrease in the first trimester of pregnancy through the second trimester, slowing during the third trimester (433, 434).

Gestational Age (weeks)	**Uterine artery RI**
6	0.83
7	0.80
8	0.82
9	0.79
10	0.78
11	0.74
12	0.70

Table 3. Resistance indices of a normal uteroplacental circulation (329)

Chapter 2: Current Practice of Luteal Support Following IVF Treatment

2.0 Establishing Current UK Practice

Safe clinical practice based on robust evidence is essential for maintaining high standards of care. Although IVF is considered by many to be a relatively new treatment there is a wealth of published data available to coordinate patient care. The evidence for luteal support was presented in chapter 1 of this thesis. However, the optimum duration of luteal support is not clear. There appears to be lack of well conducted studies available to guide clinicians.

In the concept stage of the DOLS trial, personal observations suggested that clinical practice was based more on "professional comfort" than an evidence base. It is understandable that in the absence to the contrary and that the withdrawal of luteal support prematurely may potentially undermine the hard work that resulted in achieving pregnancy, it is unsurprising that clinicians and patients are unwilling to withdraw luteal support without reassurance.

As part of this research project, three questionnaires were planned to establish "luteal support" practice in the UK at three defined end points:

1) A survey at the beginning of the DOLS trial.
2) A second survey performed immediately after the closure of the DOLS trial, but prior to the trial results being analysed.
3) A third survey planned two years after publication of the DOLS trial results.

The questionnaires would serve as a dataset of current practice both at the beginning of the trial and closure of the trial, thus establishing whether there has been a change in practice independent of the DOLS trial. The final questionnaire would establish the impact of the trial results on IVF practice.

The only published data reporting luteal phase support in the UK was published in 2008. Cyclogest was reported as preferred drug preparation (55%), followed by HCG (38%), Gestone (42%) and Crinone (25%). Although the article was not explicit in discussing its results it infers that some respondents were using multiple drugs in their luteal support regimens. Cessation of luteal support ranged from 24.1% at confirmation of biochemical pregnancy test and 48% at 12 weeks gestation (435).

2.1 Luteal support practice in the UK (2008)

2.1.1 Materials and Methods

In December 2008, a written questionnaire (Appendix A) regarding luteal support practice was sent to 73 IVF clinics in the United Kingdom, identified through the HFEA website. Respondents were invited to self identify on the return form, included with a pre-paid addressed envelope.

2.1.2 Results

Seventy three IVF clinics were contacted, of which 50 responded, a response rate of 68.5%. All 50 clinics who responded used some form of progesterone as principle luteal support.

2.1.2.1 Preferred First Line Luteal Support Preparation

The majority of IVF clinics, 49/50 utilised vaginally or rectally administered preparations; 41 clinics used Cyclogest, 3 clinics used Uterogestan and 5 clinics used Crinone. Two clinics that primarily used Cyclogest also offered Crinone as a treatment option. All clinics surveyed were able to offer Cyclogest as either principle choice or a second line alternative.

Principle Luteal Support Agent	**Number of units (%)** **(n=50)**
Cyclogest pessary (PV/PR)	41 (82%)
Crinone 8% gel(PV)	5 (10%)
Gestone / Prontogest (IM)	1 (2%)
Uterogestan (PV)	3 (6%)
HCG	0

Table 1. Reported first choice preparation for luteal support (2008).

One clinic reported using an intramuscular preparation as principle preparation; however 6 units were able to offer an intramuscular preparation as an alternative. The most commonly used intramuscular progesterone dose was 50mg daily, with the exception of one clinic that utilised 100mg daily as a standard. Two of the clinics surveyed reported using a combination of vaginal progesterone and HCG, although it was not clear from the response whether this was used in combination in all patients, or were used separately based on clinical indications.

The most common dosage of Cyclogest was 400mg twice daily, and was utilised by 43 of the 50 clinics that used Cyclogest. The table below reports the differing doses of Cyclogest commonly used.

	Dosage	**Frequency of Administration**	**Number of IVF Units (n=50)***
Cyclogest	200mg	BD	4 (8%)
	400mg	OD	2 (4%)
	400mg	BD	43 (86%)
	400mg	TDS	1 (2%)

Table 2. Preferred Cyclogest dosing regimens (2008).
**All 50 clinics had the potential to prescribe Cyclogest as either first choice or alternative preparation.*

2.1.2.2. Duration of Luteal Support

The greatest variation in practice is observed in the prescribed duration of luteal support. One unit did not include an answer to this question; therefore the responses are reported for 49 clinics.

Gestation luteal support withdrawn	**Number of units (%) (n=49)***
Biochemical pregnancy confirmation	12 (24.5%)
Clinical pregnancy confirmation (approx 6-7 weeks gestation)	3 (6.0%)
8 weeks gestation	2 (4.0%)
9 weeks gestation	1 (2.0%)
10 weeks gestation	5 (10.2%)
12 weeks gestation	**24 (49.0%)**
13 weeks gestation	1 (2.0%)
14 weeks gestation	0
16 weeks gestation	1 (2.0%)
28 weeks gestation	0

Table 3. Utilised duration of luteal support (2008).
**One clinic did not provide an answer to this question.*

The most common duration for luteal support was up to 12 weeks gestation, with 49% of respondents. A little over 24% percent of clinics withdrew luteal support on confirmation of

biochemical pregnancy, and eleven clinics (22%) used luteal support up until 7 to 10 weeks gestation, a time supposed to correspond to the luteo-placental shift.

2.2 Luteal support practice in the UK (2013)

2.2.1 Materials and Methods

A second survey was performed between 17th June and 5th July 2013 (Appendix B). Contact details for all IVF centres in the United Kingdom that held a license to perform IVF/ICSI were identified through the HFEA website. Summary details including number of cycles performed by each clinic for the previously reported year (July 2010 to June 2011) were also recorded. In order to maximise the response rate, a telephone interview was conducted with a member of the medical or senior nursing team, regarding luteal support practice in fresh autologous IVF cycles in each clinic.

2.2.2 Results

Seventy four IVF clinics were contacted, and all agreed to be interviewed. This was equivalent to a 100% response rate. The reported results were based on 58,016 cycles of fresh autologous IVF / ICSI cycles during the period July 2010 to June 2011. For 5 IVF clinics, no information about the annual number of cycles was available. However, all clinics provided luteal support and had an agreed luteal support policy. It was assumed that all Satellite and Transport IVF centres had similar policies to their affiliated licensed IVF centre.

2.2.2.1 Preferred First Line Luteal Support Preparation

Principle Luteal Support Agent	**Number of units (%)** **(n=74)**
Cyclogest pessary (PV/PR)*	63 (85.1%)
Crinone 8% gel(PV)	8 (10.8%)
Gestone / Prontogest (IM)	2 (2.7%)
Uterogestan (PV)	1 (1.4%)
HCG	0

Table 4. Reported first choice preparation for luteal support (2013).

**Two clinics decided on Cyclogest or HCG as principle choice depending on whether the patient had a poor ovarian response to stimulation (preference to HCG) or normal / high response (preference to Cyclogest).*

Of the 8 clinics that offer Crinone as first line luteal support, 1 IVF clinic offered an intramuscular preparation as an alternative.

Two clinics utilised intramuscular preparations in preference to all other preparations, however one clinic also offered a vaginal preparation if requested by the patient.

Of the 63clinics that use Cyclogest as preferred preparations:

- 4 clinics offer Crinone as an alternative
- 9 clinics offer intramuscular progesterone as an alternative
- 5 clinics offer Uterogestan as an alternative
- 2 clinics offer HCG as an alternative dependent on clinical criteria.
- 4 clinics offer either Crinone or an intramuscular preparation as an alternative

Two clinics surveyed considered utilising HCG in preference to Cyclogest in patients with a low risk of ovarian hyperstimulation syndrome, low numbers of retrieved oocytes or previous implantation failure.

The most commonly utilised preparation was found to be Cyclogest with 63 clinics offering it as first choice agent with one additional clinic offering it as a second choice alternative. Overall, 73 of the 74 clinics surveyed had the capacity to offer a vaginal preparation either in preference or on request.

Drug	**Dosage**	**Frequency of Administration**	**Number of IVF Units (n=63)**
Cyclogest	200mg	BD	1 (1.6%)
	400mg	OD	1 (1.6%)
	400mg	**BD**	**58 (92.1%)**
	400mg	TDS	3 (4.8%)

Table 5. Preferred Cyclogest dosing regimens (2013).

92.1% of clinics that utilised Cyclogest as the preferred choice for luteal support prescribed 400mg twice daily.

2.2.2.2 Duration of Luteal Support

In the 2013 survey, thirty of the seventy four clinics withdraw luteal support at 12 weeks gestation, representing 40.5% of clinic responses, 22868 cycles and 39.4% of all IVF cycles performed. Eighteen clinics withdraw luteal support at confirmation of biochemical pregnancy test, representing 24.3% of all clinics and 17442 cycles or 30% of all cycles performed in the UK. Sixteen clinics currently withdraw luteal support around the time of first ultrasound confirmation of ongoing clinical pregnancy (6-8 weeks by calculated LMP), representing 21.6% of all clinics, 10457 cycles and 18% of all cycles performed. Six percent of clinics currently extend luteal support beyond 12 weeks gestation, 4111 cycles in total, with the longest duration reported as 28 weeks gestation.

Gestation luteal support withdrawn	**Number of IVF Clinics (%) (n=74)**
Biochemical pregnancy confirmation	18 (24.3%)
Clinical pregnancy confirmation (approx 6-7 weeks gestation)	9 (12.1%)
8 weeks gestation	7 (9.4%)
9 weeks gestation	2 (2.7%)
10 weeks gestation	3 (4.0%)
12 weeks gestation	**30 (40.5%)**
13 weeks gestation	2 (2.7%)
14 weeks gestation	2 (2.7%)
16 weeks gestation	0
28 weeks gestation	1 (1.3%)

Table 6. Duration of luteal support (2013).

As part of the data collection, the authors looked at whether luteal support provided by National Health Service (NHS) clinics differed from that practiced in privately run clinics. Only two IVF clinics in the UK treated only NHS funded patients (4.00% of all cycles performed), and both extended luteal support until 12 weeks gestation. Of the 15 clinics that treated only private patients (16.7% of all cycles), 3 clinics extended luteal support until biochemical confirmation of pregnancy, one clinic until 8 weeks gestation, and 11 clinics up

to or beyond 12 weeks gestation. The remaining 57 units treated both NHS and privately funded IVF cycles; 15 clinics withdrew luteal support at biochemical pregnancy, 15 clinics extended support until 6-8 weeks gestation, 5 clinics until 9 or 10 weeks gestation and 20 clinics up to or beyond 12 weeks gestation.

2.3 Comparing the 2008 and 2013 surveys of practice

In the 2008 survey, 50 of the 73 clinics contacted responded to the questionnaire, a response rate of 68.5%. In the 2013 survey, seventy four assisted conception units were identified, all of which agreed to be interviewed, a response rate of 100%.

The data collected in the 2013 survey corresponded to a total of 58,016 cycles of fresh autologous IVF/ICSI treatments performed between July 2010 and June 2011.

Cycles per year	**Survey 2008 (responses=50)**	**Survey 2013 (responses=74)**	**p-value**
< 500	16	26	0.847
500–1000	20	28	0.852
1000–1500	4	9	0.559
>1500	10	11	0.474

Table 7. Characteristics of clinics surveyed. (p-value: fisher exact test)

2.3.1 Preferred Luteal Support Preparation

The preferred principle agent for luteal support is summarised in Table 8. In the UK, Cyclogest pessaries appear to be the preferred option, with 82% of clinics in 2008 and 85.1% of clinics in 2013 using this as first choice preparation. Statistically there appears to have been no change in this preference between the two surveys.

A majority of units offer an alternative luteal support agent depending on patient request and preference. The choice of preferred luteal support agent was not significantly different between the two surveys.

Principle Luteal Support Agent	**Survey 2008 Number of units (%)**	**Survey 2013 Number of units (%)**	**p-value**
Cyclogest pessary (PV/PR)	41 (82%)	63 (85.1%)	0.804
Crinone 8% gel(PV)	5 (10%)	8 (10.8%)	1.000
Gestone / Prontogest (IM)	1 (2%)	2 (2.7%)	1.000
Uterogestan (PV)	3 (6%)	1 (1.4%)	0.302
HCG	0	0	na

Table 8. Comparison of first choice preparation for luteal support in 2008 and 2013. (p-value: fisher exact test)

2.3.2 Preferred Dose of Luteal Support

Currently, the preferred dose of Cyclogest used in practice is 400mg PV/PR twice daily. This remained unchanged between the surveys (86% vs. 92.1%). A summary of alternative dosing regimens are summarised in table 9.

Drug	Dosage	Frequency	Survey 2008 Number of IVF Clinics (%)	Survey 2013 Number of IVF Clinics (%)	p-value
Cyclogest	200mg	BD	4 (8%)	1 (1.6%)	0.157
	400mg	OD	2 (4%)	1 (1.6%)	0.565
	400mg	**BD**	**43 (86%)**	**58 (92.1%)**	**0.350**
	400mg	TDS	1 (2%)	3 (4.8%)	0.647

Table 9. Comparison of preferred Cyclogest dosing regimens 2008 and 2013. (p-value: fisher exact test).

2.3.3 Duration of Luteal Support

In 2008, 49% of clinics withdrew support at 12 weeks gestation. In the 2013 survey, thirty of the seventy four clinics withdrew luteal support at 12 weeks gestation, representing 40.5% of clinic responses, 22868 cycles and 39.4% of all IVF cycles performed. No significant change was noted between surveys.

Eighteen clinics withdraw luteal support at confirmation of biochemical pregnancy test, representing the practice of 24.3% of all clinics and 17442 cycles or 30% of all cycles performed in the UK. This practice was again consistent with the 2008 survey. Sixteen clinics currently withdraw luteal support around the time of first ultrasound confirmation of ongoing clinical pregnancy (6-8 weeks by calculated LMP), representing 21.6% of all clinics, 10457 cycles and 18% of all cycles performed. Once again this figure is consistent with findings in the 2008 survey.

Six percent of clinics currently extend luteal support beyond 12 weeks gestation, 4111 cycles in total, with the longest duration reported as 28 weeks gestation.

A comparison of all respondents suggested that there had not been a significant change in duration of luteal support in the last 5 years. However, the authors were able to identify 47 clinics that responded to both surveys, making a direct spatial comparison of their luteal support policy possible. Thirty one (66%) of these clinics reported no change in their practice towards duration of luteal support between the survey times; 11 clinics consistently maintained luteal support until confirmation of biochemical pregnancy, 15 clinics until 12 weeks gestation and 5 clinics between 8 and 10 weeks gestation.

Five clinics (11%) reported an increase in routine duration of luteal support; 2 clinics increased luteal support from biochemical pregnancy until ultrasound confirmation of clinical pregnancy at 6-7 weeks gestation. One clinic extended luteal support from an initial 7 weeks to 9 weeks gestation, and 2 clinics increased their duration of support from 7 or 9 weeks gestation to beyond 12 weeks.

In contrast eleven (23%) IVF clinics reduced their standard duration of luteal phase support. One IVF clinic reduced usage from 16 weeks to 12 weeks gestation. Two IVF clinics reduced usage from 12 weeks gestation to 8 or 9 weeks gestation, and four clinics reduced their routine usage from 12 weeks gestation to confirmation of clinical pregnancy. One IVF clinic reduced support from 8 to 6 weeks gestation. Two clinics reduced their luteal support from 10 to 8 weeks gestation and one clinic reduced use from 10 weeks gestation to confirmation of biochemical pregnancy.

Gestation Luteal Support Withdrawn	**Survey 2008 Number of IVF Centres (%) (n=49)***	**Survey 2013 Number of IVF Centres (%) (n=74)**	**p-value**
Biochemical pregnancy confirmation	12 (24.5%)	18 (24.3%)	1.000
Clinical pregnancy confirmation (approx 6-7 weeks gestation)	3 (12.2%)	9 (12.1%)	0.359
8 weeks	2 (8.2%)	7 (9.4%)	0.315
9 weeks	1 (2.0%)	2 (2.7%)	1.000
10 weeks	5 (10.2%)	3 (4.0%)	0.264
12 weeks	**24 (49.0%)**	**30 (40.5%)**	**0.458**
13 weeks	1 (2.0%)	2 (2.7%)	1.000
14 weeks	0	2 (2.7%)	0.517
16 weeks	1 (2.0%)	0	0.398
28 weeks	0	1 (1.3%)	1.000

Table 10. Comparison of preferred duration of luteal support 2008 and 2013. (p-value: fisher exact test)
**One clinic did not provide an answer to this question.*

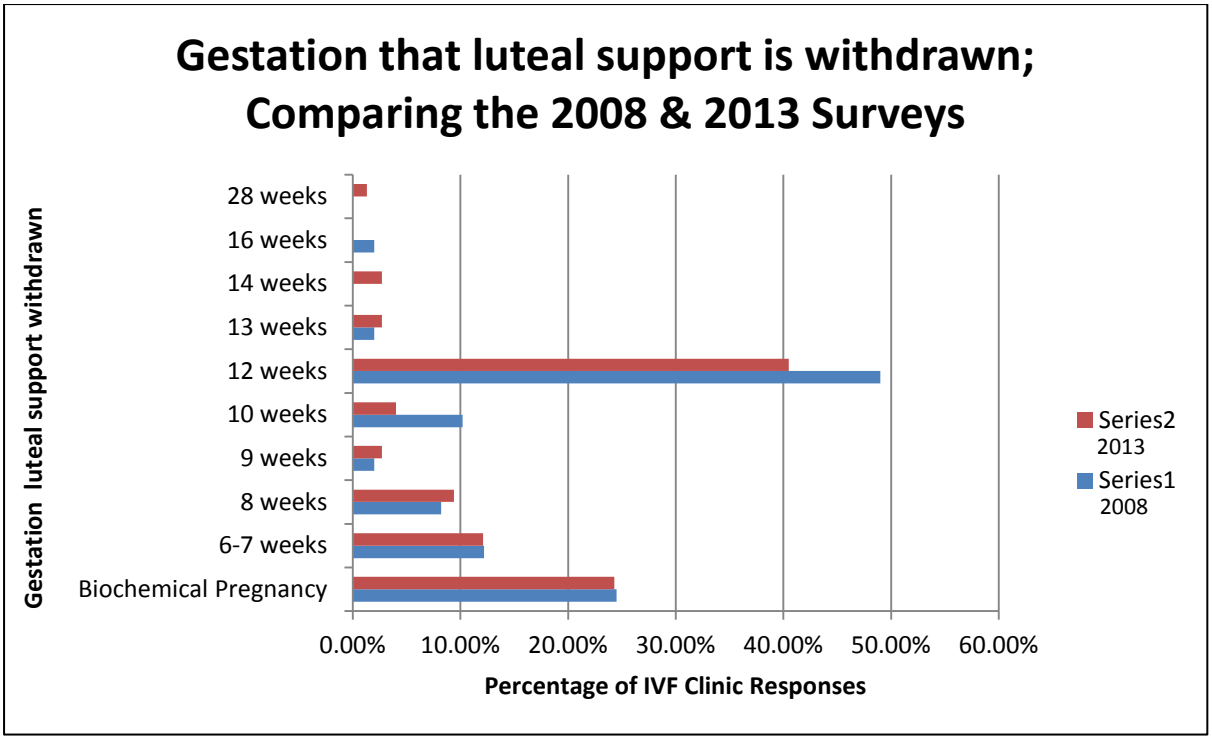


Figure 1. Schematic representation comparing duration of luteal support practice in the 2008 & 2013 surveys.

2.4 Discussion

Our study suggests that the supplementation of the luteal phase following IVF is considered mandatory, with all clinics surveyed using luteal support. The majority of clinicians in the UK appear to use Cyclogest 400mg twice daily administered vaginally or rectally until 12 weeks gestation. This is consistent with evidence that suggests vaginal preparations are better tolerated by patients and avoid the inherent complications associated with the use of intramuscular preparations and HCG (1, 240, 288-290). However, worldwide, usage is likely influenced by a combination of factors; product licensing agreements, drug availability, drug cost and physician preference. In Europe there is a reported preference for vaginally administered products in contrast to the United States where the use of intramuscular progesterone is more common (436).

Our survey however, has clearly highlighted a lack of agreement regarding the optimum duration of luteal support after IVF treatment. Twenty four percent of clinics discontinue progesterone support after confirmation of biochemical pregnancy, whilst over 47% of clinics continue luteal support beyond 12 weeks gestation.

Whilst a direct comparison between identified clinics demonstrated some change in duration of luteal support, these were not unidirectional and could at best be described as modest changes without significantly impacting overall practice. It is unclear why these changes had occurred between surveys. Whilst there has been a growing body of evidence to suggest prolonged progesterone supplementation may be unnecessary (315, 316, 318-320, 437), we suspect that clinicians continue to argue that this evidence still remains to be substantiated in larger clinical trials. Whilst putative attempts to reduce the duration of treatment have been observed, these reductions are minimal. It is more perplexing to understand why several clinics have increased their standard duration of luteal support. With easier dissemination of information over the internet, is this shift in practice a response to pressure from patients, or quite simply clinicians trying to maximise any variable that may improve treatment outcome, whilst absolute evidence to the contrary is absent?

As clinicians, we should adhere to the principle of “primum non nocere”. Whilst exogenous progesterone is generally considered safe, there continues to be concerns that prenatal progesterone exposure may be linked to urogenital malformation in male offspring (438,

439). Ideally, we should utilise the safest drug at the lowest dosage for the shortest period of time possible to achieve the desired effect.

In the five years between surveys there has been little evidence of change in duration of luteal support, despite a number of key studies being published. Our response rate in this current survey could not be more comprehensive, and accurately depicts current UK practice. A potential weakness of our study is a lower than ideal response rate in our 2008 survey, however we believe the quantity of data available is sufficiently robust to make reliable comparisons. Contrary to popular belief there was no apparent influence of practice between NHS and privately resourced clinics.

2.5 Worldwide trends in Luteal Support

In September 2009 IVF-worldwide.com reported the results of a survey of luteal phase support practice. Ninety seven clinics from 35 countries responded to the survey (Europe 17, South America 5, Far East Region 3, Asia 3 and USA including Canada 7). Participating centres reported an average number of treatments below 1000 cycles per year. Nine centres reported performing more than 1000 cycles, 3 centres more than 2000 cycles, and 2 centres more than 3000 cycles annually. The report encompassed 51,155 IVF cycles (436).

A recent update to this survey was reported in June 2012. Four hundred and eight units from 82 countries responded to the survey (USA & Canada 52, Europe 185, Central & South America 46, Asia 89, Australia & New Zealand 14, Africa 22). Participating centres reported an average of 700 cycles per year. Forty two IVF clinics reported between 1000 and 2000 cycles per year and 14 IVF clinics reported between 2001 and 4000 cycles. Eight IVF clinics reported performing more than 4000 cycles per year. The report encompassed 284,600 IVF cycles. The results of the survey are reported below.

2.5.1 Preferred luteal support preparation

A comparison of practice between the two surveys suggests that vaginal progesterone preparations remain the most popular route of luteal support, with over 64% and 77% of clinics responding in this way. Interestingly there appears to be a reduction in the number of clinics using HCG as luteal support (4% and 0%) with a fall in the number of clinics reporting intramuscular progesterone use; 13% in 2009 compared with 4.6% in 2012.

Criteria	**Survey 2009** **51155 cycles**	**Survey 2012** **284600 cycles**
Vaginal progesterone cream / gel	64%	77%
IM progesterone	13%	4.6%
HCG	4%	0%
Oral progesterone	2%	0.5%
Vaginal progesterone in combination with IM progesterone	15%	17.5%
Vaginal progesterone in combination with oral progesterone	1%	
HCG in combination with any type of progesterone	1%	Not reported

Table 11. Worldwide preferred luteal support agent (436).

2.5.2 Preferred vaginal preparation

Preference of vaginal progesterone formulation is presented in the table below.

Vaginal Preparation	**Survey 2012** **284600 cycles**
Vaginal tablets (e.g. Uterogestan)	44.0%
Vaginal gel (e.g. Crinone)	25.0%
Vaginal suppository (e.g. Endometrin or Cyclogest)	15.0%
Combination of the above	14.0%

Table 12. Preferred vaginal progesterone formulation (436).

2.5.3 Duration of Luteal Support

The proportion of clinics withdrawing luteal support at conformation of biochemical pregnancy was similar in both surveys, with 13 and 15% in 2009 and 2012 respectively. In 2009, 22% of clinics withdrew luteal support at confirmation of ongoing pregnancy, compared with 13% of clinics in 2012. The 2009 report omitted a category reporting cessation of luteal support at 8-10 weeks making a comparison difficult beyond this gestation. In the 2009 survey, 66% of clinics reported using luteal support beyond clinical pregnancy and up until 12 weeks gestation. In 2012 the comparable figure was 72%.

Criteria	**Survey 2009** **51155 cycles**	**Survey 2012** **284600 cycles**
Until positive βHCG	13%	15%
Until FHR is recognised	22%	13%
Until 8-10 weeks gestation	Not reported	44%
Until 10-12 weeks gestation	66%	28%

Table 13. Worldwide survey of duration of luteal support in 2009 and 2012.

Comparing the two surveys overall, there has been some change in observed practice. HCG does not appear to be used anymore and vaginal preparations are used in preference to intramuscular or oral preparations. Interpreting whether there has been any significant change in the duration of luteal practice is more difficult to confirm due to the difference in reporting, however it seems that as similar minority of clinics continue support up until biochemical pregnancy confirmation. The majority of clinics withdraw luteal support between 7 and 12 weeks gestation. However, significant geographic variations in practice were noted. In Asia, Europe, North America and Australia, luteal support was withdrawn at 12 weeks gestation in 81.3%, 53.5%, 59.6% and 93.8% of cycles respectively (436).

Aboulghar et al. (2008) reported practice from 21 leading IVF centres from around the world. Luteal phase support was withdrawn on the day of positive pregnancy test in 8 clinics, 2 weeks after pregnancy test in 4 clinics, between 2 and 4 weeks after pregnancy test

in 5 clinics, at 9, 10 and 11 weeks gestation in 3 clinics and in 1 clinic at 12 weeks gestation (318).

In summary, there is a significant variation in practice of luteal support after IVF procedures, both in terms of preferred luteal support agent, dosage and most significantly in its duration of use. This disparity is not only evident from our surveys of UK practice but also appears consistent comparing worldwide practice.

Chapter 3: Rationale for the DOLS trial

The DOLS trial is a pragmatic, prospective double blind placebo controlled trial comparing 2 weeks luteal support with 10 weeks luteal support using progesterone following IVF treatment, and pregnancy outcome, i.e. withdrawing luteal support at 4 weeks gestation or continuing until 12 weeks gestation.

The vast majority of clinicians use luteal support after IVF treatment and consider it an important part of successful treatment. There is considerable evidence that luteal support improves pregnancy rates compared to placebo or no treatment. The administration of human chorionic gonadotrophin, progesterone and recently gonadotrophin releasing hormone agonists have been all been successfully used for luteal support. Clinicians tend to favour progesterone in preference to HCG due to a lower incidence of OHSS, whilst vaginal progesterone preparations appear to be preferred by patients.

In the previous chapter, we demonstrated a significant variation in luteal support practice. Currently in the UK, 24% of clinics withdraw progesterone luteal support after confirmation of biochemical pregnancy, 12% of clinics at confirmation of pregnancy, 17% of clinics at a time which theoretically coincides with the luteoplacental shift and at 12 weeks gestation in 41% of clinics. A similar pattern of practice has also been reported worldwide. It is not immediately obvious why this discrepancy exists. There have been a limited number of studies which have attempted to consolidate a gestation which it is considered safe to withdraw luteal support. However, the majority of studies have been criticised for their suboptimal design and reporting. The national advisory committees; NICE, ASRM and ESHRE have consequently failed to provide definitive advice, allowing a huge discrepancy in practice to exist.

The importance of progesterone during pregnancy is well documented. Early studies demonstrated the importance of the corpus luteum and progesterone production until the luteoplacental shift. Luteal phase deficiency is recognised as a consequence of GnRH agonist and antagonist effect at the pituitary level, whilst it is also appreciated that supraphysiological oestradiol levels have a detrimental effect on the endometrium. Exogenous progesterone supplementation has been shown to surmount these problems and result in improved implantation and pregnancy rates. The luteoplacental shift begins around 8 weeks gestation with placental progesterone production independent by 12 weeks gestation. The benefit of exogenous progesterone during the first trimester has a plausible biological rationale.

The premise of luteal support is to improve the implantation rate and maintenance of ongoing pregnancy. Miscarriage is an inevitable consequence of abnormal pregnancy, but many gestations fail to continue despite being chromosomally normal. By maximising the potential of luteal support, it may be possible to reduce the incidence of miscarriage and obviate the physical, psychological and financial implications of both management of miscarriage and subsequent infertility treatment. It could be argued that a 10% reduction in pregnancy loss is significant and would offset the cost and treatment burden associated with additional progesterone.

Sub-optimal progesterone levels have been associated with pregnancy failure. Whilst it is possible that pregnancy failure may be secondary to falling serum progesterone, it is more likely that falling serum progesterone levels are a consequence of a failing pregnancy. Is it possible that exogenous progesterone administration could overwhelm the innately low

progesterone production associated with a chromosomally abnormal pregnancy? Is it plausible that extended luteal support could then result in a higher proportion of high risk prenatal screening results and a higher incidence of fetal abnormality?

Successful pregnancy begins with successful placentation. A number of biomarkers are used in antenatal screening tests and are also surrogate markers of placentation. PAPP-A and free βHCG are produced by the invading syncytiotrophoblast and associated with the degree of invasion and placental mass. Is it possible the quantification of these analytes and / or nuchal translucency measurement could be affected by duration of progesterone exposure? Do the algorithms used in quantifying a screening risk need to be altered as a consequence? The DOLS trial aims to report on the incidence of chromosomal abnormality and trends in screening, as per progesterone exposure.

The research team considered exploring potential reasons for effect if a longer duration of luteal support conferred benefit. Uterine Doppler velocity indices have long been associated with abnormal placentation and a reflection of abnormal down-stream impedance. A reduction of uterine artery velocity indices is well documented with advancing gestation, whilst a reduction in vascular tone has been reported during the luteal phase of the menstrual cycle when higher progesterone levels prevail. Is it possible that duration of exogenous progesterone has a direct effect on placentation as reported by uterine artery velocity and pregnancy outcome?

Is it possible that prolonged progesterone exposure could impact on pregnancy outcome? Pre-eclampsia is associated with poor placentation which is turn associated with reduced

birth weight and poor neonatal outcome. Falling progesterone levels have been implicated in preterm labour. Prenatal progesterone exposure has been associated with fetal malformations, most commonly hypospadias and clitoromegaly. Is it possible that duration of first trimester progesterone exposure affects the incidence of these pregnancy complications and a consequence of altered placentation?

Chapter 4: Methodology & Materials

4.0 Trial Details

Trial Title

Duration of luteal support with progesterone pessaries to improve the success rates in assisted conception: a randomised controlled trial. The DOLS trial.

Trial Description

A pragmatic, prospective, double blind and placebo controlled single centre randomised controlled trial.

Trial Objective

To investigate whether extending luteal support with progesterone pessaries beyond confirmation of clinical pregnancy following IVF confers any benefit on pregnancy outcome and reduce the incidence of pregnancy failure. All trial participants were recruited following confirmation of biochemical pregnancy after embryo transfer, i.e. at 4 weeks gestation, and having already received two weeks of progesterone as luteal support. Participants were subsequently randomised to receive either 8 additional weeks of progesterone or placebo, i.e. until 12 weeks gestation.

Investigation site

The trial was performed at the Hewitt Fertility Centre, part of the Liverpool Women's NHS Foundation Trust Hospital, one of the largest assisted conception clinics in the UK.

Trial Sponsor

Liverpool Women's NHS Foundation Trust Hospital

Ethical Approval

Ethical approval was obtained from the Mersey Regional Ethics Committee and the Liverpool Women's NHS Foundation Trust R&D Committee prior to the trial start date. The trial was conducted according to GCP guidelines, the NHS Research Governance Framework and the Declaration of Helsinki.

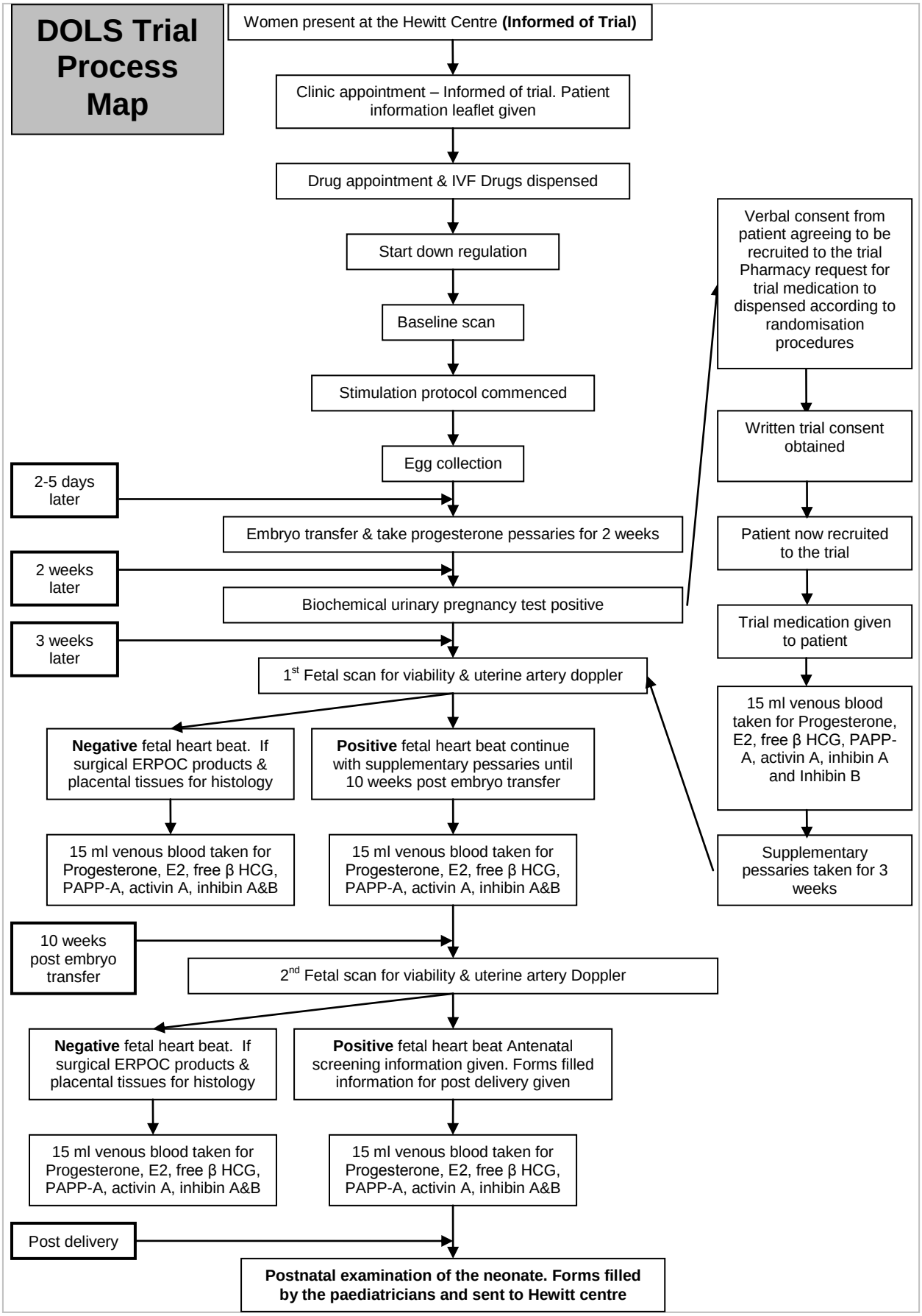
ISRCTN Registration Number: 05696887

Eudract No: **2006-000599-33**

Mersey ethics committee registration number: **06/MRE08/17**

Liverpool Women' Research and Development Trials number: **0646**

The DOLS trial protocol has been published in Trials (440).


DOLS Trial Process Map
Women present at the Hewitt Centre (Informed of Trial)
Clinic appointment – Informed of trial. Patient information leaflet given
Drug appointment & IVF Drugs dispensed
Start down regulation
Baseline scan
Stimulation protocol commenced
Egg collection
2-5 days later
Embryo transfer & take progesterone pessaries for 2 weeks
2 weeks later
Biochemical urinary pregnancy test positive
3 weeks later
1st Fetal scan for viability & uterine artery doppler
Negative fetal heart beat. If surgical ERPOC products & placental tissues for histology
Positive fetal heart beat continue with supplementary pessaries until 10 weeks post embryo transfer
15 ml venous blood taken for Progesterone, E2, free β HCG, PAPP-A, activin A, inhibin A&B
15 ml venous blood taken for Progesterone, E2, free β HCG, PAPP-A, activin A, inhibin A&B
10 weeks post embryo transfer
2nd Fetal scan for viability & uterine artery Doppler
Negative fetal heart beat. If surgical ERPOC products & placental tissues for histology
Positive fetal heart beat Antenatal screening information given. Forms filled information for post delivery given
15 ml venous blood taken for Progesterone, E2, free β HCG, PAPP-A, activin A, inhibin A&B
15 ml venous blood taken for Progesterone, E2, free β HCG, PAPP-A, activin A, inhibin A&B
Post delivery
Postnatal examination of the neonate. Forms filled by the paediatricians and sent to Hewitt centre
Verbal consent from patient agreeing to be recruited to the trial Pharmacy request for trial medication to dispensed according to randomisation procedures
Written trial consent obtained
Patient now recruited to the trial
Trial medication given to patient
15 ml venous blood taken for Progesterone, E2, free β HCG, PAPP-A, activin A, inhibin A and Inhibin B
Supplementary pessaries taken for 3 weeks

4.1 Pre-Trial Patient Management

Prior to trial recruitment all women had a treatment regime based on their age, body mass index, cause of infertility, ovarian reserve testing and previous response to treatment. All treatment and management decisions were made according to the clinics Standard Operating Procedure's (SOP's) and were independent of the DOLS trial.

Pituitary down-regulation was achieved using subcutaneous Suprafact® 0.5mg/day (Buserelin; Sanofi-aventis, UK) a GnRH agonist initiated in the mid-luteal phase of the preceding menstrual cycle and continued until the day before triggering ovulation. Down regulation was considered effective with the endometrial lining measured <5mm in antero-posterior diameter and with no follicular development greater than 6mm in size. A minimum of 14 days treatment was used.

All women were treated with Menopur® (Ferring Pharmaceuticals Ltd, UK), a highly purified human menopausal gonadotrophin (HMG). A starting gonadotrophin dose between 150-600 IU was prescribed according to standard clinic protocols and adjustments considered according to ovarian response. The response to stimulation was monitored using transvaginal ultrasound follicular tracking. When 2 follicles measuring at least 16mm were attained, final maturation was triggered using 5000 IU HCG, Profasi® (Serono, UK) or Gonasi® (Amsa, Italy).

Transvaginal oocyte retrieval was performed 36 hours after HCG administration under ultrasound guidance and conscious sedation. Single or double lumen oocyte retrieval needles were used as indicated by the ovarian response.

Suitable oocytes were fertilised (IVF or ICSI) according to clinical indication. Laboratory procedures, culture medium and embryo surveillance were performed in accordance with standard laboratory operating procedures.

Embryo transfer was performed 2, 3 or 5 days post oocyte retrieval. The timing of embryo transfer was based on the quality of the embryo(s) available for transfer and the patient's age. Embryo transfer was performed without ultrasound guidance.

The day after oocyte retrieval, all patients initiated luteal phase support with vaginal or rectal administration of 400mg progesterone twice daily (Cyclogest®; Actavis UK Ltd, UK). Patients were instructed to continue luteal support until 16 days post oocyte retrieval at which point they were asked to perform a urinary pregnancy test.

4.2 Patient Selection and Recruitment

All patients attending the Hewitt Centre were considered to be potential DOLS trial candidates. In accordance with good clinical practice guidelines, all patients received information about the DOLS trial prior to recruitment. Several opportunities were used to inform patients about the trial;

1) Ahead of the first IVF clinic appointment, all patients were required to attend a patient information evening, an opportunity to familiarize themselves with treatment processes through a series of presentations. The DOLS trial was presented at these meetings.

2) All patients received an information pack that included the appropriate treatment consent forms. A "DOLS Trial Patient Information Leaflet" (PIL) was included.

3) Patient Information Leaflets were circulated throughout the Hewitt Centre reception area, procedure recovery rooms and clinic rooms.

4) DOLS trial poster displays were erected in clinical and reception areas informing patients about current recruitment levels. This information was updated every 3-4 weeks.

5) The television screen in the clinic reception area was programmed to broadcast regularly updated information about the trial.

6) The DOLS Trial was discussed with patients before their embryo transfer with a patient information leaflet given prior to discharge.

4.3 Eligibility Criteria

Specific inclusion criteria and absence of exclusion criteria were conditions of trial participation.

Inclusion Criteria

- Women who had been treated using the long down-regulation protocol as part of their IVF cycle, i.e. with Buserelin®.
- Women who had been treated using Menopur® as part of their IVF stimulation protocol.
- Women who had used Cyclogest pessaries (400mg PR/PV) twice daily for the previous 2 weeks.
- Women who had a positive pregnancy test.
- Women who were willing to enter the trial.

Exclusion Criteria

- Women who had previously been recruited to the DOLS trial. *
- Women who had a transport IVF cycle, i.e. not completed all of their treatments in the Hewitt Centre.
- Women who were undergoing frozen embryo transfer.
- Women who had poorly controlled asthma (>1 hospital admission in the last year.)
- Women who had diabetes and on insulin treatment.
- Women who had epilepsy and on anti-epileptic treatment.
- Women who had hypertension and on anti-hypertensive treatment.
- Women who had renal dysfunction and under the care of a nephrologist.
- Women who had cardiac disease and under the care of a cardiologist.
- Women who had severe liver impairment or jaundice.
- Women who had breast cancer or received treatment for breast cancer within the

past year.

- Women who had a current or previous history of thromboembolism.

*This criteria was added following the first Data Management Committee Meeting when it became apparent that six women had been recruited to the DOLS trial in their previous pregnancy.

4.4 Recruitment of Trial Participants

At the end of each day, the case notes for all patients who had undergone an embryo transfer were cross-referenced to determine the patients potential eligibility for trial recruitment should they become pregnant. A DOLS trial "potential recruitment log" was updated daily. When the patient reported a positive pregnancy test, a subsequent telephone call to the patient by the research team was made to discuss trial processes, recruitment and confirm potential trial eligibility. Three potential outcomes were possible;

1) Trial eligibility criteria not met. A routine pregnancy scan was arranged at 7 weeks gestation and the outcome recorded in the DOLS trial register.
2) Trial eligibility criteria met, but the patient did not wish to join the trial. A routine pregnancy scan was arranged at 7 weeks gestation and the outcome recorded in the DOLS trial register.
3) Trial eligibility criteria met and the patient wished to join the trial.

- Outcome entered into the DOLS Trial Register.
- A "verbal agreement to join the DOLS trial" form was completed and signed by a

member of the trial team during a telephone conversation and placed in the trial participation consent pack. This form acted as a record of the patients' intent to formally complete the written DOLS trial consent form when they attended the IVF unit to collect their trial medication.

- A time was confirmed with the patient to attend the unit to complete the trial consent forms and collect the trial medication. This had to be completed within 24 hours of using their last Cyclogest pessary.
- A DOLS Trial pharmacy prescription was completed with patient identifying details and stratified for age (<37 or >37 years). This was delivered to the Liverpool Women's Hospital Clinical Trials Pharmacy Unit, with instructions to prepare and dispense the trial medication for an agreed time. The trial drug pack was collected by the trial team prior to the patients' appointment.

4.5 Pharmacy Procedures & Randomisation Procedures

In pharmacy, the woman's correct age stratification was confirmed by a trained member of the clinical trials pharmacy unit. A study number was then allocated to the woman according to their age stratification and in a sequential recruitment order, for example the first three women were identified with the study numbers 1001, 1002 and 1003 etc. (see section 4.14.2 for more details). The study number was then referenced with a pre-specified randomisation sequence that indicated treatment allocation. The randomisation sequences and hence treatment allocation had been prepared by the trial statistician before the trial began and held in confidence by the clinical trials pharmacy unit. For further details regarding allocation concealment please refer to section 4.14.3.

The pharmacy subsequently dispensed either active medication or placebo medication depending on treatment allocation. Each patient received a white 16oz cardboard dispensing carton, containing 140 pessaries encased in plain white plastic packaging. Both the active group and placebo group received the equivalent number of medicinal products in the same packaging. A medicinal product information sheet was included with the trial medication.

The study material was stored in the clinical trials room in the pharmacy department and dispensed in accordance with standard dispensing procedures, on an individual patient basis and in line with good clinical practice.

4.6 Trial Medication

The trial medication was manufactured and supplied by Actavis UK Ltd without cost. Additional funding was provided by Actavis UK Ltd (£20,000) towards trial set up costs.

Actavis had no involvement in the design of the study, data collection, data handling, data analysis, study interpretation, drafting of manuscripts or decisions to publish.

The placebo was manufactured to appear identical in appearance to the progesterone pessary. Pessaries were packaged in identical non-identifiable white plastic casings in strips of 5 (figures 1-5).

Figure 1. Appearance of trial drug packaging containing progesterone pessaries.

Figure 2. Appearance of trial drug packaging containing placebo pessaries.

Figure 3. Direct comparison of the appearance of the trial medication. Progesterone on the left and placebo on the right.

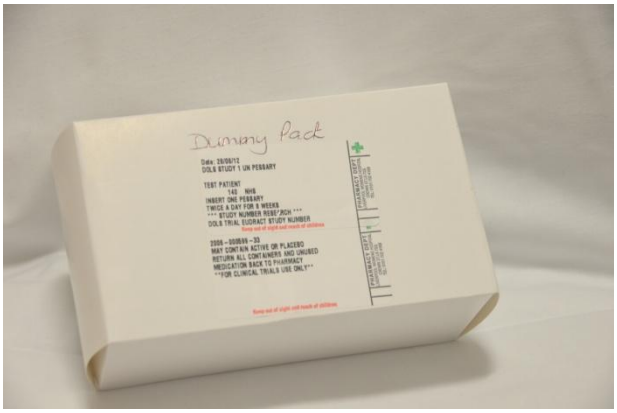


Figure 4. Plain white 16oz pharmacy box that trial medication was packaged in.

Figure 5. Example of DOLS trial pharmacy pack.

4.7 First Trial Visit – Trial recruitment

The first trial visit occurred upon confirmation of biochemical pregnancy, i.e. at 4 weeks gestation. To finalise recruitment to the trial, several processes occurred;

- The woman's identity was confirmed.
- The woman's eligibility for trial participation was confirmed.
- Written consent was obtained from the woman and countersigned by a member of the trial team in triplicate. One copy of the consent form was attached to the completed eligibility criteria form and filed in the DOLS Trial consent form folder, a second copy was filed in the woman's hospital case notes, and a third copy was given to the woman. At this point the patient was considered "recruited" to the trial.
- A declaration sticker indicating that "This patient has been recruited to the DOLS Trial" was placed on the front of the woman's hospital case notes, and chronologically within the notes.
- The Trial Data Collection form was completed using the woman's hospital notes and reproductive unit database, with any missing data completed by direct patient questioning.
- The DOLS study number (pharmacy randomisation number) present on the pharmacy drug pack was entered on all trial documentation along with the woman's identifying details.
- An appointment for a scan to confirm pregnancy viability was arranged at seven weeks gestation.
- A venous blood sample was drawn from the woman's ante-cubital fossa (2 x 7.5mls tubes) and transported to the laboratory in trial specific laboratory forms.

- The trial drug pack was given to the woman with confirmation of instructions for use.
- An opportunity to answer any questions was given.

Completion of Comparative Data Parameters

Data collection sheets were completed using the patients case notes, direct patient questioning and from the IDEAS computer database system.

- Name, date of birth, address
- Pregnancy test type; urine or blood
- Day of pregnancy test; number of days from oocyte retrieval
- Age (age at oocyte retrieval)
- BMI (body mass index) weight (kg) / height (m^2)
- Primary infertility (no previous female pregnancy achieved) or secondary infertility (previous female pregnancy achieved)
- Cause(s) of infertility; male factor, advanced maternal age, PCOS, endometriosis, tubal factor, unexplained, fibroids, same sex couple
- Number of IVF/ICSI cycles; i.e. number of oocyte retrievals including current cycle
- Previous ART pregnancy; all pregnancies achieved by patient through IVF/ICSI; biochemical pregnancy, miscarriage, ectopic pregnancy, termination of pregnancy, live birth
- Previous non-ART pregnancy, all pregnancies achieved by patient by natural conception; biochemical pregnancy, miscarriage, ectopic pregnancy, termination of pregnancy, live birth
- Duration of infertility; in months
- FSH, LH, Prolactin, AMH; most recent blood results within 12 months of the current

treatment cycle.

- Present treatment cycle; IVF or ICSI including reason for requiring ICSI
- Total gonadotrophin dose used during this cycle.
- Total number of days of gonadotrophin stimulation
- Number of oocyte(s) retrieved
- Number of oocyte(s) fertilised
- Number of embryos frozen; surplus embryos cryopreserved from this cycle of treatment
- Day of embryo transferred; day 2, day 3 or day 5
- Date of embryo transfer; number of days from oocyte retrieval
- Number of embryos transferred
- Grade of embryo(s) transferred; see later grading and scoring
- Clinician performing embryo transfer
- Catheter used; manufacturer and length of catheter
- Embryo transfer procedure; straightforward, outer sheath, stylet, volsellum
- Bleeding between embryo transfer and study recruitment with quantification of red or brown loss.

4.8 Second Trial Visit - First Pregnancy Scan

The second trial visit was arranged to confirm pregnancy viability at 7 weeks gestation.

Ultrasound Confirmation of Pregnancy Viability

A transvaginal ultrasound scan was performed in all patients (Toshiba Nemio SSA-550A Diagnostic Ultrasound System (Toshiba Medical Systems Corporation, Japan) utilising 2-D technology and 6 MHz probe.

The following ultrasound data were collected

- Number of intra-uterine gestation sacs
- Presence of a yolk sac in each gestation sac
- Presence of a fetal pole in each gestation sac
- Measurement of crown rump length (CRL) of each fetal pole. The CRL was defined as the length of the fetus from the top of its head to bottom of the torso. It is measured as the largest dimension of the embryo, excluding the yolk sac and its extremities.
- Estimated gestational age calculated using the CRL (onboard software calculation)
- Confirmation of presence of fetal cardiac pulsations (absent, < 100bpm, >100bpm)
- Presence of pelvic free fluid which may suggest a diagnosis of ovarian hyperstimulation syndrome or ruptured ectopic pregnancy
- Presence of andexal masses which may suggest a diagnosis of ruptured ectopic pregnancy or heterotopic pregnancy.
- Calculation of uterine artery pulsatility index and resistance index.

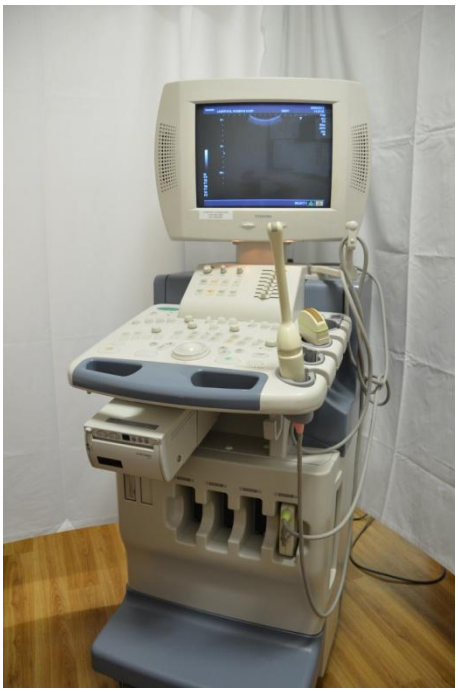


Figure 6. Toshiba Nemio SSA-550A Diagnostic Ultrasound System

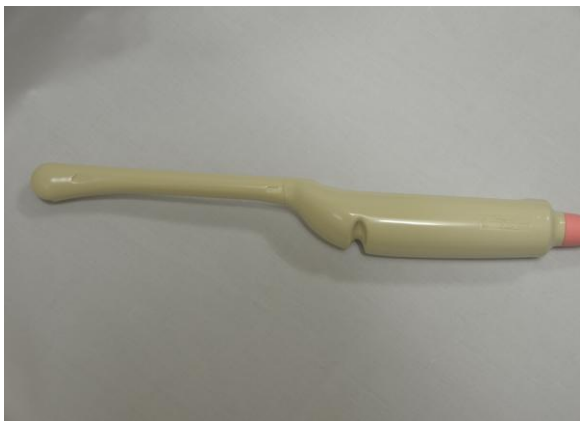


Figure 7. Toshiba 6 Mhz transvaginal ultrasound probe.

Additional Data

- Continued compliance with trial medication was determined by direct patient questioning. If non-compliance was reported, the date of last pessary was recorded.
- The route of pessary administration of the trial medication was recorded; vaginal, rectal or both.
- The woman's intended hospital trust for antenatal care was recorded.
- A subjective visual score that determined the severity of side effects was completed for individual symptoms of nausea, bloating, vaginal discharge and vaginal irritation. The visual score ranged from a minimum score of 0 (no symptoms) to a maximum score of 10 (worst possible severity of symptoms).

Additional Processes

- If an ectopic pregnancy was suspected or confirmed, or miscarriage diagnosed, treatment proceeded in accordance with hospital guidelines.
- 15mls of venous blood was obtained from the woman and transported to the laboratory with trial specific laboratory forms, as previously described.
- A further ultrasound scan was arranged at 12 weeks gestation.
- The woman was given instruction about the processes of antenatal referral and information about the availability of first and second trimester prenatal screening tests.
- A HFEA early pregnancy outcome form was completed electronically and submitted to the HFEA.

4.9 Third Trial Visit - Second Pregnancy Scan

The third trial visit was arranged to confirm ongoing pregnancy viability at 12 weeks gestation.

Ultrasound Confirmation of Ongoing Pregnancy

A transvaginal ultrasound was performed in all patients (Toshiba Nemio SSA-550A Diagnostic Ultrasound System (Toshiba Medical Systems Corporation, Japan) utilising 2-D technology and 6 MHz probe as previously described.

The following ultrasound data were collected

- Number of intra-uterine gestation sacs
- Presence of a yolk sac in each gestation sac
- Presence of a fetal pole in each gestation sac
- Measurement of crown rump length (CRL) of each fetal pole. The CRL is the length of the fetus from the top of its head to bottom of the torso. It is measured as the largest dimension of the embryo, excluding the yolk sac and its extremities.
- Estimated gestational age, as calculated by crown rump length.
- Confirmation of fetal cardiac pulsation(s) (absent, < 100bpm, >100bpm)
- Presence of pelvic free fluid that may suggest a diagnosis of ovarian hyperstimulation syndrome or ruptured ectopic pregnancy
- Presence of andexal masses that may suggest a diagnosis of ectopic pregnancy or heterotopic pregnancy.
- Calculation of uterine artery pulsatility index and resistance index.

Additional Data

- Continued compliance with trial medication was determined by direct patient questioning. If non-compliance was reported, the date of last pessary was recorded.
- The route of pessary administration of the trial medication was recorded; vaginal, rectal or both.
- A subjective visual score that determined the severity of side effects was completed for individual symptoms of nausea, bloating, vaginal discharge and vaginal irritation. The visual score ranged from a minimum score of 0 (no symptoms) to a maximum score of 10 (worst possible severity of symptoms).
- The woman's intention towards first or second trimester serum screening in this pregnancy.
- Confirmation of the hospital where antenatal care had been requested.

Additional Processes

- If a miscarriage was diagnosed, treatment proceeded in accordance with hospital guidelines.
- 15mls of venous blood was obtained from the woman and transported to the laboratory with the trial specific laboratory forms, as previously described.
- A DOLS Trial Birth Details and Examination of the Newborn proforma complete with patient and trial identification details was given to the patient with a prepaid addressed envelope. Instruction was given that the birth details could be completed by the patient or their midwife, but the examination of the newborn details should be completed by their midwife, attending physician or paediatrician.
- The woman was subsequently discharged from active trial participation and asked to

discontinue trial medication. Surplus trial medications were returned to the clinical trials pharmacy unit.

4.10 Third Trimester Phone Call

All patients were contacted by the research team at 34 weeks gestation. Patients were asked to confirm their planned hospital for birth and whether any prenatal screening tests had been done. Any complications of pregnancy were also recorded.

4.11 Delivery and Birth Details

All patients were asked to return the DOLS Trial Birth Details and Examination of the Newborn data sheets that they had been given. Missing or unclear data was checked by a retrospective review of the patients' hospital records.

After the birth of the final trial participant, hospital notes were requested from all participating hospitals and comprehensively reviewed. All birth details were rechecked and complications of pregnancy recorded. Second trimester screening results, ultrasound abnormalities and maternal and neonatal discharge details were also recorded.

4.12 Outcome Measures

4.12.1 Primary Outcome

The primary outcome measure was defined as the proportion of all randomised women that continue successfully to a viable pregnancy (defined as at least one fetus with FHR >100 beats minute) on transabdominal / transvaginal ultrasound at 12 weeks gestation (i.e. at 10 weeks post embryo transfer and at the end of 8 weeks supplementary trial treatment).

4.12.2 Secondary Outcomes

1. Viable pregnancy rate (defined as at least one fetus with FHR>100 beats per minute) on transvaginal ultrasound at 7 weeks gestation (i.e. at 5 weeks post embryo transfer and at the end of 3 weeks supplementary trial treatment).

2. Levels of serum markers (Oestradiol, progesterone, Inhibin A, Inhibin B, Activin A, free βHCG and PAPP-A) taken at confirmation of biochemical pregnancy, 7 weeks gestation and 12 weeks gestation.

3. Uterine artery Doppler velocity waveform at 7 and 12 weeks gestation.

4. A comparison of side effect severity between treatment groups using a visual analogue score completed at 7 and 12 weeks gestation. Symptoms include; nausea, bloating, vaginal discharge and vaginal irritation.

5. Antenatal Down's syndrome screening outcomes. The results of all antenatal Down's syndrome screening tests were recorded, including individual serum quantification for the double test, triple test, quadruple test, nuchal translucency, and combined screening.

6. Neonatal outcomes. All babies undergo a routine neonatal examination following birth. Neonatal abnormalities were reported using the birth details trial form. Excessive androgenisation would be an important adverse outcome of supplementary progesterone which would be detected by routine neonatal examination.

Long term follow-up of children may be conducted as a separate study. As part of the written consent process, trial participants agreed to be contacted as required.

Pregnancy, birth and neonatal details were sometimes captured in hospitals other than our own. Local clinical and R&D contacts were identified to help facilitate case note retrieval and secure the necessary approvals.

7. Continued compliance with trial medication was determined by direct patient questioning. If non-compliance was reported, the date of last pessary was recorded. The effect of compliance on the primary outcome may be required during analysis of the trial results.

4.13 Preparation and Analysis of Serum hormones

4.13.1 Sample Preparation

After drawing, the blood samples were transported to the laboratory for processing. Each sample was assigned a trial bar code number which was matched with the woman's identification details, trial randomisation number and date of blood procurement. The samples were spun at 3000 rpm for 10 minutes (Beckman J-6B High Capacity Centrifuge; Beckman Coulter) to separate the serum. Five serum aliquots of 1ml volume were then stored at -20°C.

4.13.2 Serum Analyses

Oestradiol was measured in plasma using an electrochemiluminescence immunoassay (ECLIA) on a Roche Elecsys 2010 Analyser (Lewes UK). The assay has a sensitivity of 18 pmol/L established from precision profiles (22% coefficient of variation of duplicates) a measuring range of 18 - 15,780 and a CV of <10% across the range 80 - 10,000 pmol/L.

Progesterone was measured in plasma using an electrochemiluminescence immunoassay (ECLIA) on a Roche Elecsys 2010 (Lewes UK). The assay has a sensitivity of 0.095 ng/mL established from precision profiles (22% coefficient of variation of duplicates), a measuring range of 0.095 - 60.0 and a CV of <4% across the range 8.0 - 60.0 ng/mL.

Free βHCG was measured in plasma using a solid phase two-site fluoroimmunoassay (FIA) on an Auto Delfia® immunoassay system supplied by Perkin Elemer (Bucks, UK). The assay

has a sensitivity of 1.5 ng/mL established from precision profiles (22% coefficient of variation of duplicates), a measuring range of 1.5 - 200 ng/mL and a CV of <5% across the range 11.0 - 100.0 ng/mL.

PAPP A was measured in plasma using a solid phase two-site fluoroimmunoassay (FIA) on an Auto Delfia® immunoassay system supplied by Perkin Elmer (Bucks, UK). The assay has a sensitivity of 5.0 mU/L established from precision profiles (22% coefficient of variation of duplicates), a measuring range of 5.0 - 10,000 mU/L and a CV of <3% across the range 100 – 5,000 mU/L.

Inhibin A was measured in plasma using a 2 step enzyme linked immunosorbent assay (ELISA) supplied by Beckman Coulter ® (California, USA). The assay has a sensitivity of 5.0 pg/mL established from replicates of the zero standard (20 repeated measurements within one assay), a measuring range of 5 - 1000pg/mL and CVs of 5.4% at 89.3pg/mL and 6.1% at 375.4pg/mL.

Inhibin B was measured in plasma using a Gen II 3 step enzyme linked immunosorbent assay (ELISA) supplied by Beckman Coulter® (California, USA). The assay has a sensitivity of 2.0 pg/mL established from replicates of the zero standard (20 repeated measurements within one assay), a measuring range of 2.6 - 1000 pg/mL and CVs of 6.4% at 12.5 pg/mL, 6.2% at 86.0 pg/mL and 4.4% at 368.4 pg/mL.

Activin A was measured in plasma using a Quantikine 3 step Enzyme Immunoassay (EIA) supplied by R&D Systems Europe (Abingdon, UK). The assay has a sensitivity of 0.75 pg/mL established from replicates of the zero standard (20 repeated measurements within one assay), a measuring range of 4 - 1000pg/mL and CVs of 7.2% at 99.7 pg/mL, 7.4% at 288.3 pg/mL and 6.5% at 504.5 pg/mL.

4.14 Statistical Analyses

A statistician was contracted to advise the trial team about the appropriate power calculations and sample size required.

4.14.1 Power calculation

Internal data obtained from IVF treatments initiated at the Hewitt Centre at the time of trial conception, reported a biochemical pregnancy rate of 32.5%. Approximately 80% of patients with a biochemical pregnancy will progress to a viable clinical pregnancy at 7 weeks gestation and confirmed on ultrasound scan; a 25.8% clinical pregnancy rate. These results were based on then current clinical practice that allowed withdrawal of luteal support at confirmation of biochemical pregnancy. We suggested that a 10% absolute increase in pregnancies reaching viable clinical pregnancy at 12 weeks gestation would be clinically important and therefore justify use of an additional 8 weeks of luteal support with progesterone, i.e. a 10% absolute reduction in the pregnancy loss rate from 20% to 10% following confirmation of biochemical pregnancy.

A two group chi-squared test with a 5% two-sided significance level will have 80% power to detect the difference between a control group proportion 80% and a treatment group proportion 90% (odds ratio of 2.151) when the sample size in each group is 210.

Given that the method of analysis is logistic regression with age as a factor, patients were stratified into two groups; those less than 37 years old and those older than or equal to 37 years old. It is recognised that age is an important variable when discussing success rates in assisted conception. To account for this, stratification was planned to optimise the distribution of patients of all ages. A suitable sample size would be 230 per group. It was anticipated that drop-outs and withdrawals would be minimal due to the highly motivated nature of our patients.

We aimed to randomise 460 women in total with complete follow up, but anticipated recruiting 480 women to achieve this.

The DOLS trial was a pragmatic trial and therefore data was analysed on an “intention to treat” basis; i.e. women were to be analysed according to the treatment group to which they were randomised whether or not they actually comply with treatment. Stopping treatment did not constitute withdrawal from the study.

4.14.2 Randomisation Sequences

The randomisation sequences were prepared for the trial by Anna Hart (trial statistician) using random sized blocks (2, 4 and 6) using the statistical package available at www.randomization.com. Two separate randomisation sequences were prepared; for women less than 37 years of age and women older than 37 years. The first list for women less than 37 years old were given trial numbers beginning with the prefix "1" (total 360, 180 per group) and the second list for women older than 37 years old who were allocated a trial number beginning with the prefix "2" (total 180, 90 per group).

4.14.3 Allocation Concealment

The randomisation sequences were kept in the Pharmacy Trials Unit. Only pharmacy personnel had access to them and hence treatment allocation. The trial investigators were to be kept blind to all randomisation sequences and treatment allocations. During database entry, the randomisation lists were made available. Patients were allocated to either Group A or Group B for the purpose of statistical analysis. The drug allocation for either group was unknown until after the trial results had been reported. It was therefore possible for the statisticians to remain blinded to treatment allocation whilst analysing the results.

4.14.4 Statistical Analysis

Anna Hart was contracted as the trial statistician during the design and development of this project. However, due to a conflict in contractual obligations she was unable to commit to performing the statistical analysis. It was therefore necessary to arrange alternative

statistical support. After consultation, Pollyanna Hardy and John Townend representing the NPEU Clinical Trials Unit at the University of Oxford agreed to assist development of the statistical analysis plan and perform the analysis. However it was possible for both Anna and Pollyanna to liaise directly during a handover period, which resulted in a small number of amendments that were initially published in the trial protocol. The statistical analysis plan is included as Appendix 3.

Demographics and other clinical variables

Other variables e.g. describing the groups at baseline or the clinical progress of the women were described using summary statistics only.

Primary Outcome

The primary outcome (proportion of viable pregnancies at 12 weeks gestation) was summarised with a relative risk and 95% confidence interval. The primary analysis was performed using logistic regression and taking into account the age of the woman (i.e. the stratification variable).

If other important prognostic factors were imbalanced between the 2 groups the robustness of the findings were to be investigated by including other covariates in the logistic regression.

Although it is anticipated that withdrawal rates will be low, there may be missing values. A range of clinically plausible scenarios were to be used to impute missing values in order to test the robustness of the findings. Details of these processes were to be approved by the

DMEC once the magnitude of the problem was known. The statistician may have then needed to be un-blinded, in order to carry out realistic conservative analyses.

The effect of compliance (as a suitably-coded factor) was to be investigated by testing the interaction between treatment group and compliance.

Secondary outcomes

These were to be exploratory analyses producing 99% confidence intervals. They were to be unadjusted for multiple analyses. The interpretation was to take multiple testing and the consequent likelihood of type I errors, into account qualitatively.

All results were to be reported descriptively (i.e. number and percentage or mean and standard deviation/median and interquartile ranges). All measures of effect were to be adjusted for the stratification factor of age of the woman (less than 37 years old and older than or equal to 37 years old at randomisation). Binary outcomes were to be analysed using log binomial regression models, with corresponding results presented as adjusted risk ratios with associated confidence intervals. If the model did not converge, log Poisson regression models with robust variance estimation were to be used. Continuous outcomes were to be analysed using linear regression models, also adjusting for the stratification factor of age of the woman. Results were to be presented as adjusted differences in means with associated confidence intervals.

For outcomes relevant to the woman, data was to be analysed using the number of eligible women correctly randomised as the denominator. For outcomes relevant to foetuses and

the infant, the denominator was the number of foetuses and live births (respectively) of eligible women correctly randomised. Adjustment for birth outcomes, Doppler indices and serum analyses are to be adjusted for the clustering effect of multiple pregnancies. At 7 and 12 weeks gestation, adjustment for the number of viable fetuses present at each gestation, and for birth details adjustment for the number of babies born beyond 24 weeks gestation.

For serum hormone measurements below the detection limit at any point, the values were set to half of the detection limit for the purposes of calculating means or differences between means. These were; free βHCG <2.14 replaced with 1.07, PAPP-A <48.0 replaced with 24.0 and Inhibin B <2.6 replaced with 1.3.

Continuous outcomes were analysed by analysis of covariance and dichotomous variables by logistic regression. In a similar way to the analysis of the primary outcome these analyses were to stratify for age, and the robustness of findings were to be examined by including other potentially important prognostic covariates. A range of plausible values were to be imputed for missing values. Where a large proportion of values are missing (typically >25%) only descriptive statistics will be provided.

If any variables are highly skewed, leading to doubts about the validity of the parametric analyses, non-parametric analyses were to be carried out or bootstrapping used.

Outcome assessment was carried out with the trial team and statisticians remaining blinded to the treatment allocation. Treatment groups were analysed according to either Group A or Group B only. Only after final analysis was treatment allocation revealed.

4.15 Data management

A Data Monitoring Committee (DMC) for the trial was established, independent of trial personnel. The inaugural meeting was to determine the stopping rules for the trial and define the terms of reference. Regular meetings were established for the duration of the trial with the option of open and closed sessions so the DMC members could perform an interim data analysis if required. The DMC was responsible for providing information to the Trial Steering Committee (TSC).

Un-blinding of the trial remained the responsibility of the Chief Investigator. Should any Suspected Unexpected Serious Adverse Reaction (SUSAR) have occurred, it was possible to un-blind using a 24 hour telephone line within the pharmacy department. The on-call pharmacist had access to un-blind the trial.

Members of the Data Monitoring Committee:

Professor Siladitya Bhattacharya MD MBBS MRCOG *(chair)*

(Sub-specialist in reproductive medicine)

University of Aberdeen

Dr Steve Roberts *(statistician)*

Senior Lecturer in Medical Statistics

University of Manchester

Mr Murray Luckas MD, MRCOG

Consultant Obstetrician and Gynaecologist

Leighton Hospital

Members of the Trial Steering Committee

Mr Luciano Nardo (voting member)

Independent Chair

Consultant Gynaecologist and Subspecialist in Reproductive Medicine

St Mary's Hospital, Manchester

Mr Simon Wood (voting member)

Consultant Gynaecologist and Subspecialist in Reproductive Medicine

Countess of Chester Hospital, Chester

Mr Rafet Gazvani (voting member)

Chief Investigator

Consultant Gynaecologist and Subspecialist in Reproductive Medicine

Liverpool Women's NHS Foundation Trust Hospital, Liverpool

Mrs Yasmin Sajjad

Principle Investigator

Consultant Gynaecologist and Subspecialist in Reproductive Medicine

Liverpool Women's NHS Foundation Trust Hospital, Liverpool

Dr Richard Russell (voting member)

Co-Investigator / Trial Manager

Liverpool Women's NHS Foundation Trust Hospital, Liverpool

Professor Zarko Alfirevic

Professor of Fetal and Maternal Medicine

University of Liverpool, Liverpool

Dr Anna Hart

Trial Statistician

Lancashire School of Health and Postgraduate Medicine (LSHPM)

University of Central Lancashire

Mrs Emma Neill (voting member)

Lay person

The trial statistician was to be made aware of patient assignment to treatment codes (e.g. Group A or Group B), but was to remain blinded to which treatment code is the active ingredient or placebo. The trial statistician was to be involved in the preparation of reports for DMEC. Un-blinding may have become necessary when invoking sensitivity analyses for missing values.

It was necessary during the trial to replace the trial statistician due to the expiration of contracts with the R&D department. Pollyanna Hardy was seconded to oversee the outline statistical analysis plan and analysis of the results.

The trial data was input into a database using the SPSS statistics package (IBM, United States). Single data entry with two comprehensive checks was performed by me. The trial database was locked prior to statistical analysis. Data was transferred to Stata Data Analysis and Statistical Software (StatCorp LP; Texas, USA) using StatTransfer (Circle Systems; Seattle, USA).

Direct access to source data / documents was permitted for trial related monitoring, audits, and IRB regulatory inspections.

Trial monitoring was the responsibility of the research and development manager at Liverpool Women's NHS Foundation Trust Hospital.

4.16 Funding

Drug packs (progesterone / placebo) were supplied by Actavis UK Ltd. Funding was provided by Actavis UK Ltd (£20,000) and The Moulton Foundation (£76,103).

Neither funding body had involvement in the design of the study, data collection, data handling, data analysis, study interpretation, drafting of manuscripts or decisions to publish.

4.17 Embryo Assessment

4.17.1 Embryo Grading

Identifying embryos of good quality and hence implantation potential is crucial for optimizing treatment outcome. With the quantification of embryo quality it becomes possible to identify the embryo(s) with the greatest potential. In December 2007 the Human Fertilisation and Embryology Authority (HFEA) announced their intention to incorporate an outcome based policy into the Code of Practice to reduce the number of multiple births in the United Kingdom (UK) to no more than 10% by 2013. It was recognised that to achieve this, the number of embryos replaced needed to be reduced. Identifying those embryos with greatest implantation and pregnancy potential was imperative. Morphological assessment of cleavage stage embryos and blastocysts is the most commonly utilised method of embryo grading. All embryos at both the cleavage and blastocyst stages of development were graded according to the joint ACE /BFS guidelines (441).

4.17.2 Embryo Scoring

The embryo grading system is a subjective measurement that allows for quantification of appearance of an embryo. However comparison of embryos using these criteria is difficult. Subjectively there are too many permutations that do not lend to comparative analysis. We have therefore used a cumulative substitute system for scoring an embryo, at both the cleavage stage and blastocyst stage that allows for better comparison (442).

4.17.2.1 Cleavage Stage Embryo Grading and Scoring

Each parameter as assessed by the grading system has a corresponding embryo grading score. The cumulative embryo score is the summation of each parameter's individual score.

Blastomere Number	Score	Blastomere Size	Score (+)	Fragmentation	Score (+)
1	1	1	1	1	1
2	2	2	2	2	2
3	3	3	3	3	3
4	4	4	4	4	4
5	5				
6	6				
7	7				
8	8				
9	9				
10	10				
11	11				
12	12				

For example: A cleavage stage embryo graded to be 8(2/3), i.e. 8 (blastomeres), 2 for blastomere size, and 3 for fragmentation = 8+2+3 = cumulative embryo score of 13.

4.17.2.2 Blastocyst Stage Embryo Grading and Scoring

Similarly the cumulative embryo score is the summation of each individual parameter of blastocysts assessment.

Expansion Status	**Expansion Status substitution score**	**ICM Grading**	**ICM Grading substitution score (+)**	**Trophoectoderm Grading**	**Trophoectoderm Grading substitution score (+)**
1	1	A	4	**a**	3
2	2	B	3	**b**	2
3	3	C	2	**c**	1
4	4	D	1		
5	5	E	0		
6	6				

For example: A blastocyst graded 6(A/b), i.e. Expansion status 6 (score 6), ICM grading A (score 4), trophoectoderm b (score 2) = cumulative embryo score 6+4+2 = 12.

A high scoring embryo was defined as an embryo transferred on day 2 with a cumulative embryo score (CES) ≥9 and ≤ 12; on day 3, a CES ≥ 14; and on day 5 (blastocyst) a CES ≥ 9 and ≤ 12

A morula or compacting morula is usually the lowest quality blastocyst possible and not part of the embryo grading or scoring process. In this methodology both a morula and a compacting morula are assigned a cumulative embryo score of 1, based on the assumption that the poorest blastocyst can have a CES of 2.

4.18 Uterine Artery Doppler Assessment

Uterine artery measurements were obtained from all trial patients at 7 and 12 weeks gestation, in accordance with the scheduled visits described in the DOLS protocol.

4.18.1 Method for Measuring Uterine Artery Doppler

Uterine artery Doppler measurements were obtained using a Toshiba Nemio ultrasound machine, with a 6 mHz transvaginal ultrasound probe. A mid-sagital section of the uterus and cervical canal was obtained and the internal cervical os identified. The transducer was tilted to one side towards each paracervical region in turn. Using colour flow mapping the uterine arteries were identified running alongside the uterus and cervix. Pulsed wave Doppler was then applied to obtain flow velocity waveforms from the ascending branch of the uterine artery at the point closest to the internal os. When applying colour Doppler, dimensions of the colour box and velocity scale were optimised. Application of the pulsed wave Doppler was applied with the sampling gate adjusted to 2mm and an appropriate angle of intonation. When three consecutive waveforms were obtained, inbuilt computer software calculated the relative Pulsatility Index (PI) and Resistance Index (RI) of each uterine artery from the waveform tracing.

All Doppler assessments were performed by a single operator to minimise inter-operator variability.

Chapter 5: Main Results

The DOLS trial recruited patients between 28th November 2008 until 31st March 2010, and between 4th March 2011 and 23rd May 2012. The break in recruitment was attributable to the availability of trial medication. It became apparent that the "use by date" for the placebo medication expired near the end of the first round of recruitment. Although additional medication was requested from Actavis UK Ltd, it was not manufactured and delivered until March 2011.

A total of 467 patients were randomised and completed the trial. Six patients were excluded from the primary analysis because they were randomised twice, in two consecutive pregnancies. Only the first randomisation was included in the statistical analysis, therefore 461 patients were included in the primary analysis. No patients were lost to follow up.

The Consort diagram is presented in figure 1.

The statistical analysis of the results was performed with patients allocated to either trial medication allocation "Group A" or allocation "Group B". Analysis was performed with both the trial team and statisticians blind to the treatment drug allocated to each group. The results of the statistical analysis were reviewed and considered by a team of senior reproductive clinicians and embryologists prior to the blinding being revealed. Once a consensus agreement was reached regarding the clinical significance of the trial outcome, the drug allocation was revealed.

5.1 Consort Data

Between 28/11/2008 to 31/03/2010 and 04/03/2011 to 23/05/2012, 5446 women were assessed for eligibility and considered for the trial; a total recruitment time of 31 months.

Four thousand five hundred and eighty two women were excluded who did not meet the eligibility criteria.

Eight hundred and sixty four women met the eligibility criteria. Four hundred and sixty nine patients were randomised. Two patients were randomised in error and did not receive trial medication. Therefore 467 patients were successfully randomised and recruited to the trial.

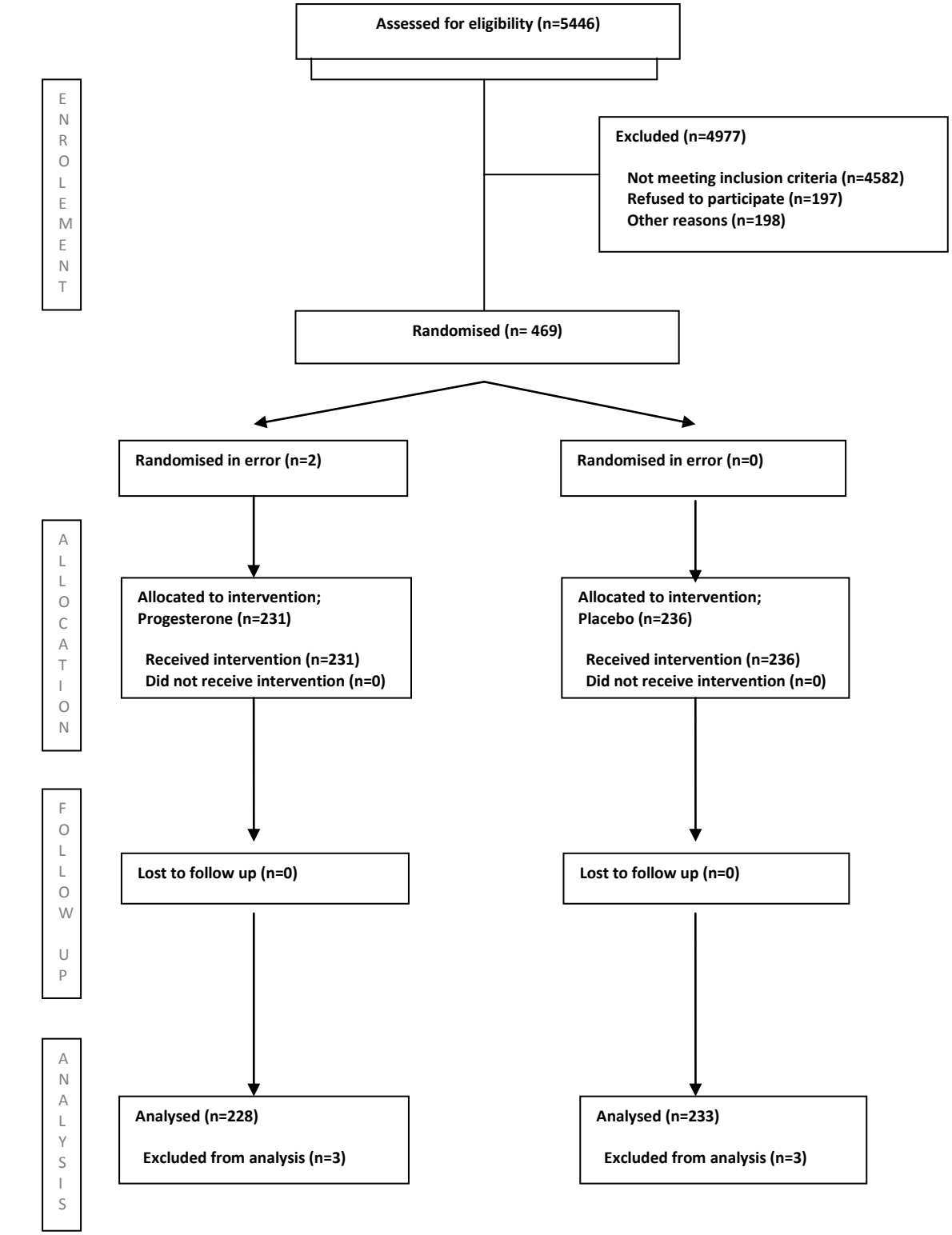
Three hundred and ninety seven women who met all eligibility criteria were not randomised;

- 197 patients refused to participate;
    - Distance from study site too far – 49 patients
    - Did not want to use additional pessaries – 7 patients
    - No reason given – 141 patients
- 198 women were not randomised for other reasons;
    - Unable to give adequate consent due to lack of translation – 8 patients
    - Unable to contact to discuss trial further, or unable to attend within the time frame required (24 hours since last pessary) – 23 patients
    - Failure to inform trial team of positive pregnancy test – 69 patients
    - Reasons not documented – 98 patients

- Two patients were randomised in error and subsequently not recruited to the trial

No patients were lost to follow up.

Consort Diagram – The DOLS Trial



5.2 Demographic Characteristics

The demographic characteristics of the trial participants at randomisation are summarised in Table 1.

Background Information

Two hundred and twenty eight patients were randomised to the extended progesterone arm of the trial, whilst 233 patients were randomised to the placebo arm of the trial.

The mean age at randomisation was 32.7 ± 4.3 years in the extended luteal support (ELS) group compared with 32.7 ± 4.3 years in the placebo group. Allocation according to age stratification was also similar. In the ELS group 176 patients (77.2%) of patients were under 37 years of age at randomisation compared with 183 patients (78.5%) in the placebo group. Fifty two patients (22.8%) patients were over 37 years of age in the ELS group compared with 50 patients (21.5%) in the placebo group.

The mean body mass index (BMI) was similar in both groups; 24.5 ± 3.4 in the ELS group and 24.2 ± 3.4 in the placebo group.

In the ELS group 146 (64.0%) patients were defined as having primary infertility, compared with 147 (63.1%) of patients in the progesterone group. Consequently the number of patients with secondary infertility was also similar; 36.0% versus 36.9%.

In the ELS group, 25.9% and 10.1% had one and more than previous pregnancy, compared with 26.2% and 10.7% respectively in the placebo group. Of the patients who had one or more previous pregnancies, the number of previous pregnancies conceived resulting from previous IVF was similar comparing the groups. In the ELS group 59 (72%) patients had no previous IVF pregnancies, whilst 21 (25.6%) patients and 2 (2.4%) patients had one or two previous IVF pregnancies respectively. This compared with 65 (75.6%), 17 (19.8%) and 4 (4.7%) patients in the placebo group.

A patient was considered parous if they had a previous delivery beyond 24 weeks gestation or a delivery that was registered as a live birth. The majority of patients in both treatment groups had not previously had a delivery; 208 (91.2%) patients in the ELS group compared with 208 (89.3%) patients in the placebo group. Eighteen (7.9%) patients and 2 patients (0.9%) the ELS group had one or more deliveries previously. This compared to 24 (10.3%) and 1 (0.4%) patient in the placebo group, respectively.

The number of IVF cycles between the two groups was compared. In the ELS group, 158 patients (69.3%) had received only one cycle of assisted conception treatment including the current treatment, compared to 142 patients (60.9%) in the placebo group. Seventy patients (30.7%) and 91 patients (39.1%) had received previous IVF cycles in the ELS and placebo groups respectively.

The duration of infertility in the ELS group and the placebo group was 36 months (IQR 30-54) and 42 months (IQR 30-60) respectively.

The reported causes of sub-fertility were relatively similar in both trial arms. In the ELS arm and placebo arm respectively the main causes were; 93 (40.8%) and 117 (50.2%) patients reporting a male factor component; 51 (22.4%) and 47 (20.2%) patients with tubal factor infertility; 20 (8.8%) and 29 (12.5%) patients with endometriosis, 39 (17.1%) and 27 (15.9%) patients with polycystic ovarian syndrome; 55 (24.1%) and 46 (19.7%) patients with unexplained infertility. Table 1 includes the less frequently reported causes of subfertility.

IVF Treatment Details

In the ELS group 137 patients (60.1%) had IVF performed and 91 patients (39.9%) had ICSI performed. This compared to 126 (54.1%) patients who had IVF and 107 (45.9%) patients who had ICSI performed in the placebo group.

The total dose of gonadotrophins used during ovarian stimulation was similar between the treatment groups. The mean dose of stimulation was 2974 IU ± 1218 in the ELS group and 2992 IU ± 1312 in the placebo group. The total duration of stimulation was also similar. In the ELS group the median duration of stimulation was 10.5 days (IQR 10-12) compared with 11 days (IQR 10-12) in the placebo group.

The median number of oocytes retrieved was similar in the two groups; in the ELS group, 10 oocytes (IQR 7-14) were retrieved compared to 12 oocytes (IQR 7-15) retrieved in the placebo group. The median number of oocytes that fertilised was also comparable. In the ELS group an average of 6 oocytes fertilised (IQR 4-9), similar to the 6 fertilised oocytes (IQR 3-9) fertilised in the placebo group.

The timing of embryo transfer occurred on days 2, 3 or 5 in 25.9%, 43.4% and 30.7% of treatment cycles in the ELS group, and 26.1%, 42.9% and 30.9% respectively in the placebo group.

The mean number of embryos transferred was 1.58 ± 0.49 in the ELS group and 1.61 ± 0.49 in the placebo group. Ninety five (41.7%) patients had a single embryo transfer in the ELS group compared with 92 (39.5%) patients in the placebo group. A similar proportion of patients had a dual embryo transfer; 133 (58.3%) patients in the ELS group compared with 141 (60.5%) patients in the placebo group. No patients had more than two embryos replaced.

A similar number of high scoring embryos were transferred between the two trial arms, in the ELS group 0.96 ± 0.78 high scoring embryos were transferred per cycle compared with 0.85 ± 0.80 high scoring embryos in the placebo group. The proportion of transfers involving no high scoring embryos was 71 (32.3%) in the ELS group and 91 (40.1%) in the placebo group. Eighty seven cycles (39.6%) in the ELS group involved the transfer of one high quality embryo, whilst 78 (34.4%) cycles in the placebo group had one high scoring embryo replaced. Sixty two patients (28.2%) had two high scoring embryos replaced in the ELS group compared with 58 (25.6%) patients had two high scoring embryos replaced in the placebo group.

In comparing the mean number of supernumerary embryos cryopreserved after embryo transfer; 0.82 ± 1.47 embryos were cryopreserved in the ELS group and 0.81 ± 1.63 embryos in the placebo group.

Baseline pituitary hormone profiles and AMH levels were comparable between the two treatment arms. Mean follicle stimulating hormone (FSH) levels were 6.92 ± 2.00 IU/L in the ELS treatment arm and 6.91 ± 2.13 IU/L in the placebo treatment arm. Mean leutenising hormone (LH) was reported as 6.51 ± 5.19 IU/L in the ELS arm and 6.32 ± 3.10 IU/L in the placebo arm. Mean serum prolactin levels were 298 ± 112 IU/L in the ELS arm and 304 ± 115 IU/L in the placebo arm.

Antimullerian hormone (AMH) levels were 11.0 ± 8.42 pmol/L in the ELS group compared to 12.1 ± 12.0 pmol/L in the placebo group. Only 53 patients had AMH estimation.

At randomisation, the median serum oestradiol level was reported as 2280 pmol/L in the ELS trial arm, and 2180 pmol/L in the placebo arm. Median serum progesterone levels were comparable between the two treatment arms; 35.8 ng/mL in the ELS group, and 38.1 ng/mL in the placebo group. Median inhibin A levels were 116 pg/ml in the ELS group, compared with 96.8 pg/mL in the placebo group. Median inhibin B levels were 8.38 pg/mL in the ELS arm and 9.06 pg/mL in the placebo arm. Median activin A levels were 352 pg/mL in the ELS group and 324 pg/mL in the placebo trial arm.

Serum free βHCG was present in similar quantities in both groups, with a median and interquartile range of <2.14 (<2.14 and <2.14) in the ELS arm and <2.14 (<2.14 and <2.14) in the placebo group. Median and interquartile ranges of serum PAPP-A was reported as <48 (<48 and <48) in the ELS arm and <48 (<48 and <48) in the placebo group.

	Progesterone n=228	**Placebo n=233**
Age at randomisation (years) mean ± sd	32.7 ± 4.26	32.7 ± 4.28
< 37 years	176 (77.2%)	183 (78.5%)
≥ 37 years	52 (22.8%)	50 (21.5%)
BMI (kg/m^2) – mean ± sd	24.5 ± 3.4	24.2 ± 3.4
Type of infertility		
Primary	146 (64.0%)	147 (63.1%)
Secondary	82 (36.0%)	86 (36.9%)
Any previous pregnancy		
None	146 (64.0%)	147 (63.1%)
1	59 (25.9%)	61 (26.2%)
>1	23 (10.1%)	25 (10.7%)
If one or more previous pregnancies, no of previous ART pregnancies		
0	59 (72.0%)	65 (75.6%)
1	21 (25.6%)	17 (19.8%)
2	2 (2.4%)	4 (4.7%)
Parity[a]		
None	208 (91.2%)	208 (89.3%)
1	18 (7.9%)	24 (10.3%)
>1	2 (0.9%)	1 (0.4%)
Number of IVF/ICSI attempts (including current cycle)		
1	158 (69.3%)	142 (60.9%)
>1	70 (30.7%)	91 (39.1%)
Duration of infertility - median (IQR)	36 (30 to 54)	42 (30 to 60)
Cause of infertility *(more than one can be completed per patient)*		
Male factor	93 (40.8%)	117 (50.2%)
Tubal factor	51 (22.4%)	47 (20.2%)
Endometriosis	20 (8.8%)	29 (12.5%)
PCOS	39 (17.1%)	37 (15.9%)
Anovulation	3 (1.3%)	3 (1.3%)
Advanced maternal age	7 (3.1%)	4 (1.7%)
Unexplained	55 (24.1%)	46 (19.7%)
Same sex couple	1 (0.4%)	2 (0.9%)
Single female	0	1 (0.4%)

Others	0	3 (1.3%)
Current treatment details		
IVF	137 (60.1%)	126 (54.1%)
ICSI	91 (39.9%)	107 (45.9%)
Total dose gonadotrophin (IU) – mean ± sd	2974 ± 1218	2992 ± 1312
Total duration gonadotrophin (days) - median (IQR)	10.5 (10 to 12)	11 (10 to 12)
Number oocytes retrieved - median (IQR)	10 (7 to 14)	12 (7 to 15)
Number oocytes fertilised - median (IQR)	6 (4 to 9)	6 (3 to 9)
Day of transfer		
Day 2	59 (25.9%)	61 (26.2%)
Day 3	99 (43.4%)	100 (42.9%)
Day 5 (blastocyst)	70 (30.7%)	72 (30.9%)
Number embryos transferred – mean ± sd	1.58 ± 0.49	1.61 ± 0.49
1	95 (41.7%)	92 (39.5%)
2	133 (58.3%)	141 (60.5%)
Number high scoring embryos[b] transferred – mean ± sd	0.96 ± 0.78	0.85 ± 0.80
0	71 (32.3%)	91 (40.1%)
1	87 (39.6%)	78 (34.4%)
2	62 (28.2%)	58 (25.6%)
Number of supernumerary embryos cryopreserved – mean ± sd	0.82 ± 1.47	0.81 ± 1.63
Baseline hormone levels within last 12 months– mean ± sd		
FSH (IU/L)	6.92 ± 2.00	6.91 ± 2.13
LH (IU/L)	6.51 ± 5.19	6.32 ± 3.10
AMH (pmol/L)	11.0 ± 8.42	12.1 ± 12.0
Prolactin (IU/L)	298 ± 112	304 ± 115
– median (IQR)		
FSH (IU/L)	6.7 (5.7 to 8.1)	6.7 (5.6 to 7.9)
LH (IU/L)	5.45 (4.2 to 7.3)	5.8 (4.5 to 7.4)
AMH (pmol/L)	10.6 (3.7 to 14.6)	8.43 (5.7 to 13.8)
Prolactin (IU/L)	277 (219 to 372)	282 (222 to 368)

Baseline serum levels at randomisation		
median (IQR)		
Oestradiol (pmol/L)	2280 (732 - 4630)	2180 (743 - 4410)
Progesterone (ng/mL)	35.8 (15.5 - 79.5)	38.1 (18.5 - 60.4)
Free BHCG (ng/mL)	<2.14 (<2.14 - <2.14)	<2.14 (<2.14 - <2.14)
PAPP-A (mU/L)	<48 (<48 - <48)	<48 (<48 - <48)
Inhibin A (pg/mL)	116 (38.9 - 223)	96.8 (41.9 - 229)
Inhibin B (pg/mL)	8.38 (4.5 - 14.3)	9.06 (4.98 - 14.4)
Activin A (pg/mL)	352 (276 - 448)	324 (256 - 414)

[a] Number of previous pregnancies registered as live birth, >24 weeks, or registered as a live birth.

[b] High scoring embryo is defined as an embryo transferred on Day2 with a cumulative embryo score (CES) >=9 and <=12; on day 3, a CES >=14; and on Day5 (blastocyst) a CES >=9 and <=12

[c] AMH was carried out in place of FSH and LH during the later stages of the study. N= in a and b

Table 1. Demographic characteristics - counts and percentages unless otherwise stated

5.3 Primary Outcome

The primary outcome of the trial was defined as a pregnancy with at least one fetus with a fetal heart rate >100 bpm confirmed on transvaginal or trans-abdominal ultrasound scan at 12 weeks gestation, i.e. at the end of 8 weeks supplemental trial medication. The viable pregnancy rate at 12 weeks gestation was reported to be 167 / 228 (73.3%) in the ELS group and 167/233 (71.7%) in the placebo group. No statistical significance was observed as demonstrated by an adjusted risk ratio of 0.97 (95% CI 0.87 to 1.09). The result is summarised in Table 2.

	Progesterone n= 228	**Placebo n=233**	**Adjusted[b] RR (95% CI)**
Viable pregnancy[a] at 12 weeks gestation	**167 (73.3%)**	**167 (71.7%)**	**0.97 (0.87 to 1.09)**

[a] At least one fetus with FHR >100 beats minute on transvaginal ultrasound at 10 weeks post embryo transfer / 12 weeks gestation (i.e. at the end of 8 weeks supplementary trial treatment).
[b] Adjusting for age of woman at randomisation

Table 2: Primary outcome – viable pregnancy at 12 weeks gestation.

5.4 Secondary Outcomes

5.4.1 Viable pregnancy outcome at 7 weeks gestation

One of the secondary outcomes of the trial is the proportion of viable pregnancies, defined as at least one fetus with a fetal heart rate >100 bpm confirmed on transvaginal or trans-abdominal ultrasound scan at 7 weeks gestation, i.e. after 3 weeks supplemental trial medication. The viable pregnancy rate at 7 weeks was reported in 175/228 (76.8%) women in the ELS group and 174/233 (74.7%) women in the placebo group. No statistical significance was observed; adjusted risk ratio 0.97 (99% CI 0.84 to 1.11). The result is summarised in Table 3 below.

	Progesterone n=228	**Placebo n=233**	**Adjusted[b] RR (99% CI)**
Viable pregnancy[a] at 7 weeks gestation	**175 (76.8%)**	**174 (74.7%)**	***0.97 (0.84 to 1.11)***

[a] At least one fetus with FHR >100 beats minute on transvaginal ultrasound at 5 weeks post embryo transfer / 7 weeks gestation (i.e. at the end of 3 weeks supplementary trial treatment).
[b] Adjusting for age of woman at randomisation

Table 3: Secondary outcome – viable pregnancy at 7 weeks gestation.

5.4.2 Pregnancy Outcomes

All patients randomised to the trial had a confirmed biochemical pregnancy. A biochemical pregnancy loss was defined as a subsequent negative pregnancy test (serum or urine) after commencement of trial medication without ultrasound evidence of clinical pregnancy. Biochemical pregnancy loss occurred in 33/228 (14.5%) patients in the ELS arm of the trial and 46/233 (19.8%) patients in the placebo arm of the trial; adjusted risk ratio (1.37 99%CI 0.80 to 2.34).

Miscarriage before 12 weeks gestation was defined by ultrasound evidence of an intrauterine pregnancy which did not progress beyond 12 weeks gestation. In the ELS group 25/228 (11%) patients reported miscarriage prior to 12 weeks gestation. In comparison 17/233 (7.3%) miscarriages occurred in the placebo group; adjusted risk ratio of 0.69 (99%CI 0.32 to 1.48).

A similar number of ectopic pregnancies occurred in each group, 3/228 (1.3%) in the ELS arm, and 3/233 (1.3%) in the placebo arm of the trial; adjusted risk ratio 0.97 (99%CI 0.12 to 7.86).

On-going birth multiples were analysed at 12 weeks gestation in each trial arm. Singleton, twin and triplet gestations were evident in 57.5%, 15.4% and <0.5% of patients in the ELS group, respectively (131/228, 35/228 and 1/228). In the placebo arm the corresponding birth multiples were singletons 143/233 (61.4%) and twins 24/233 (10.3%). No triplets were

reported in the placebo arm of the trial. The adjusted risk ratio and confidence intervals are included in Table 4. No significant difference was reported between treatment groups.

Mid-trimester loss (defined as a pregnancy loss between 12 and 23^{+6} weeks gestation) was similar in both groups; 3/228 (1.3%) mid-trimester pregnancy losses occurred in the ELS group compared with 3/233 (1.3%) episodes reported in the placebo group; adjusted risk ratio 0.97 (99%CI 0.12 to 7.86). Two episodes of mid-trimester pregnancy loss reported in the placebo group involved the loss of twin gestations.

One termination of pregnancy was performed for a woman randomised to the ELS trial arm who had a viable dichorionic diamniotic twin pregnancy confirmed at 12 weeks gestation. A routine anomaly scan performed at 20 weeks gestation, confirmed the intrauterine death of one twin with the remaining viable fetus demonstrating hydrocephalus and periventricular cystic changes. Termination of pregnancy was recommended following which a diagnosis of alloimmune thrombocytopaenia was confirmed.

Three stillbirths occurred in the trial cohort, 2 in the ELS arm of the trial and 1 in the placebo arm. One stillbirth was diagnosed prior to the onset of labour at term, with no identifiable cause at post mortem. Another pregnancy resulted in the stillbirth of a first twin at 32 weeks gestation resulting from cord prolapse; the second twin was born alive. A third stillbirth occurred at 26^{+5} weeks gestation after prolonged rupture of membranes which resulted in a spontaneous breech delivery with head entrapment and subsequent fetal demise.

One neonatal death occurred in a pregnancy to a woman allocated to the ELS arm of the trial. A precipitate vaginal delivery of DC / DA twins at 26^{+1} weeks gestation resulted in the live birth of both babies but neonatal death of the first twin during the early neonatal period.

A total of 379 babies were born to women who participated in the trial, 194 in the ELS group and 185 in the placebo group. In the ELS arm of the trial, 130 (57%) singleton live births and 32 (14%) sets of twins were reported. In the placebo arm of the trial 143 (61.4%) singletons were live born alongside 21 (9%) sets of twins. The risk ratio and 99% confidence interval were not significant for each pregnancy multiple; 1.08 (99%CI 0.88 to 1.31) and 0.63 (99%CI 0.32 to 1.25) for singleton and twin live births respectively. No triplet live births were reported in either treatment group.

The live birth rate defined as any woman with one or more live births per treatment allocation was reported as 162 cases in the extended progesterone support group and 164 cases in the placebo group; RR 0.98 (99%CI 0.84 to 1.15).

The live birth rate per embryo transferred is 0.57 ± 0.43 and 0.54 ± 0.42 in the ELS and placebo allocations respectively. The adjusted mean difference suggested no significant difference in outcome. Table 4 summarises the pregnancy outcomes.

	Progesterone n = 228	**Placebo n = 233**	**Adjusted[a] RR (99% CI)**
Biochemical loss	33 (14.5%)	46 (19.7%)	**1.37 (0.80 to 2.34)**
Miscarriage (<12 weeks)	25 (11.0%)	17 (7.3%)	**0.69 (0.32 to 1.48)**
Ectopic pregnancy	3 (1.3%)	3 (1.3%)	**0.97 (0.12 to 7.86)**
Birth multiples at 12 weeks[b]			
Singleton	131 (57.5%)	143 (61.4%)	**1.07 (0.87 to 1.30)**
Twins	35 (15.4%)	24 (10.3%)	**0.67 (0.35 to 1.27)**
Triplets	1 (0.44%)	0	**n/a**
Mid-trimester loss (12 – 23^{+6} weeks)	3 (1.3%)	3 (1.3%)	**0.98 (0.12 to 7.97)**
Termination of pregnancy	1 (0.4%)	0	**n/a**
Stillbirth	2 (0.9%)	1 (0.4%)	**0.48 (0.02 to 11.1)**
Neonatal Death	1 (0.4%)	0	**n/a**
Total live births	194	185	
Singleton	130 (57.0%)	143 (61.4%)	**1.08 (0.88 to 1.31)**
Twins	32 (14.0%)	21 (9.0%)	**0.63 (0.32 to 1.25)**
Triplets	0	0	**n/a**
Live birth per embryo transferred	0.57 ± 0.43	0.54 ± 0.42	**-0.03 (-0.13 to 0.73)[c]**

[a] Adjusting for age of woman at randomisation

[b] Number of foetuses at 12 weeks gestation

[c] Adjusted mean difference

Table 4. Summary of pregnancy outcomes.

5.4.3 Labour and Delivery

The mean gestational age of delivery was 271 ± 19.1 days in the ELS group, a similar result reported in the placebo group; 271 ± 16.8 days; adjusted mean difference -2.05 (99%CI -6.87 to 3.20).

The proportion of patients delivering preterm, very preterm and extremely preterm are summarised in Table 5. No significant difference was observed between the groups.

Spontaneous labour occurred in 88 (53%) pregnancies in the ELS group, and 85 (50.9%) pregnancies in the placebo group; adjusted risk ratio 0.91 (99%CI 0.70 to 1.18). Labour was induced in 53 (31.9%) confinements in the ELS group and in 50 (29.9%) confinements in the placebo group; adjusted risk ratio 0.93 (99%CI 0.61 to 1.43). Scheduled elective caesarean section was performed in 18 (10.8%) pregnancies in the ELS group and in 22 (13.2%) pregnancies in the placebo group; adjusted risk ratio 1.27 (99%CI 0.60 to 2.69). Planned caesarean section indicated for placenta praevia was reported in 5 (3.1%) pregnancies in the ELS group compared with 6 (3.7%) pregnancies in the placebo group; adjusted risk ratio 1.14 (99%CI 0.25 to 5.31).

Vaginal delivery was achieved in 101 (61.6%) births in each treatment group; adjusted risk ratio 0.97 (99%CI 0.79 to 1.19). Cesarean section was required in 63 (38.4%) pregnancies in each treatment arm; adjusted risk ratio 1.03 (99%CI 0.75 to 1.43).

A summary of labour and delivery details are summarised in Table 5.

	Progesterone n = 167	Placebo n = 167	**Adjusted[ab] RR (99% CI)**
Gestational age at delivery:			
mean ± sd	271 ± 19.1	271 ± 16.8	-2.05 (-6.87 to 3.20)
37^{+0} - 43 weeks	131 (79.9%)	136 (82.9%)	
34^{+0} – 36^{+6} weeks	27 (16.5%)	18 (11.0%)	
28^{+0} – 33^{+3} weeks	3 (1.8%)	9 (5.5%)	
24^{+0} – 27^{+6} weeks	3 (1.8%)	1 (0.6%)	
Onset of labour			
Spontaneous	88 (53.0%)	85 (50.9%)	0.91 (0.70 to 1.18)
Induced	53 (31.9%)	50 (29.9%)	0.93 (0.61 to 1.43)
Elective cesarean	18 (10.8%)	22 (13.2%)	1.27 (0.60 to 2.69)
Mode of delivery			
Vaginal delivery	101 (61.6%)	101 (61.6%)	0.97 (0.79 to 1.19)
Cesarean section	63 (38.4%)	63 (38.4%)	1.03 (0.75 to 1.43)
Cesarean section for placenta praevia	5 (3.1%)	6 (3.7%)	1.14 (0.25 to 5.31)

[a] Adjusting for age of woman at randomisation
[b] Adjusted for the effect of multiple births

Table 5. Summary of labour and delivery details.

5.4.4 Antenatal Pregnancy Complications

Any complication of pregnancy was considered an important outcome. Table 6 summarises the results of 334 women who had a pregnancy progress beyond the first trimester.

Women were assigned a diagnosis of threatened preterm labour if they received steroids and tocolysis. The incidence between the treatment allocations was not significantly different. Three events (1.8%) occurred in the ELS group and 2 events (1.2%) in the placebo group; adjusted risk ratio 0.68 (99%CI 0.67 to 7.01).

Cholestasis of pregnancy, pregnancy induced hypertension, thrombosis and gestational diabetes occurred in 25 patients (15%) in the ELS arm of the trial and 25 patients (15%) in the placebo arm of the trial; adjusted risk ratio 1.28 (99%CI 0.68 to 2.40).

Cholestasis of pregnancy, defined by the presence of raised bile acids and abnormal liver function tests and requiring pharmacological intervention or delivery was similar between the treatment allocations; 3 patients (1.8%) in the ELS group and 4 patients (2.4%) in the placebo group.

Pregnancy induced hypertension or preeclampsia, defined as proteinuric hypertension requiring antihypertensive treatment or intervention to deliver was reported in 16 patients in each arm of the trial (9.6% versus 9.6%).

Venous thromboembolism (VTE) was reported in 3 pregnancies that progressed beyond the first trimester. One woman in the ELS group was diagnosed with a deep vein thrombosis in the third trimester. One woman randomised to placebo was diagnosed with a DVT in the second trimester and a cerebro-vascular accident (CVA) in the post natal period. The patient was subsequently diagnosed with anti-phospholipid syndrome (APS) and Protein C deficiency. A third woman, randomised to placebo was investigated for a presumed CVA, but diagnostic tests were inconclusive.

A fourth case involved a patient who was randomised to the progesterone arm of the trial, diagnosed with a superficial vein thrombosis demonstrating forward propagation and requiring anticoagulation at 11 weeks gestation. This woman was also diagnosed with a first trimester miscarriage and was subsequently diagnosed with APS and tested positive for anti-cardiolipin antibodies.

The incidence of gestational diabetes was similarly distributed according to treatment arm; 6 patients (3.6%) in the ELS group and 7 patients (4.2%) in the placebo group. There was no observed difference in the distribution of patients controlled with diet, oral hypoglycaemic agents or insulin comparing study interventions.

Pregnancy admissions were analysed for all randomised women. Admissions for bleeding appeared higher in the placebo group compared with the ELS group, 16 (9.6%) versus 8 (4.8%), however this was not statistically significant. A closer analysis of these admissions revealed that only 3 admissions occurred in the first trimester, and 2 admissions occurred in the 2nd trimester. The remaining admissions occurred in the third trimester.

Admission for abdominal pain (not associated with bleeding) was reported in 7 (4.2%) and 5 (3.0%) patients respectively in the ELS and placebo groups respectively.

Admissions for treatment of pregnancy induced hypertension were similar to the findings reported previously. Fifteen admissions (9.0%) were reported in the ELS group and 13 (7.8%) in the placebo group.

Ovarian hyperstimulation syndrome requiring admission was reported in 2 (1.2%) patients in the ELS group, with no reported incidence in the placebo arm of the trial.

Vomiting requiring admission was reported in 3 (1.8%) and 2 (1.2%) cases in the ELS and placebo groups respectively.

In summary, the occurrence of unplanned admissions demonstrated no statistically significant difference between the study interventions; adjusted risk ratio of 1.14 (99%CI 0.64 to 2.03).

Twenty five patients (15.2%) and 32 patients (19.4%) received at least one steroid injection in the ELS and placebo groups respectively; adjusted risk ratio 1.28 (99%CI 0.68 to 2.40), suggesting no significant difference between the two groups.

	Progesterone	**Placebo**	**Adjusted[ad] RR (99% CI)**
	n = 167	*n = 167*	
Threatened preterm labour[b]	3 (1.8%)	2 (1.2%)	0.68 (0.67 to 7.01)
At least one steroid administration	25(15.2%)	32(19.4%)	1.28 (0.68 to 2.40)
APH	3(1.8%)	7(4.2%)	
Planned caesarean	7(4.2%)	7(4.2%)	
PIH	2(1.2%)	5(3.0%)	
Threatened PTL	4(2.4%)	3(1.8%)	
PLROM	6(3.6%)	7(4.2%)	
Other	3(1.8%)	3(1.8%)	
Antenatal complications	25(15.0%)	25(15.1%)	1.00 (0.51 to 1.96)
Cholestasis	3(1.8%)	4(2.4%)	
PIH / Preeclampsia	16(9.6%)	16(9.6%)	
Thrombotic event[c]	1(0.6%)[c]	2(1.2%)	
Gestational diabetes	6(3.6%)	7(4.2%)	
	n = 228	*n = 233*	
Pregnancy admissions	30(18.0%)	34 (20.5%)	1.14 (0.64 to 2.03)
Bleeding	8(4.8%)	16(9.6%)	
Pain	7(4.2%)	5(3.0%)	
PIH	15(9.0%)	13(7.8%)	
OHSS	2(1.2%)	0	
Vomiting	3(1.8%)	2(1.2%)	

[a] Adjusting for age of woman at randomisation

[b] As quantified by having had tocolysis and steroids

[c] DVT diagnosed in first trimester preceding miscarriage

[d] Adjusted for the effect of multiple births

Table 6: Summary of antenatal complications.

5.4.5 Neonatal Outcomes

Male births accounted for 43.8% of all births (85/194) in the ELS arm of the trial, compared with 51.9% (96/185) in the placebo arm of the trial. No significant difference was observed between the two groups; adjusted risk ratio 1.18 (99%CI 0.89 to 1.55).

Birth weight was not statistically significant between the groups; 3020 ± 701 grams in the ELS group compared with 3060 ± 698 grams in the placebo group; adjusted mean difference 37.1 (99%CI -175 to 249).

The proportion of babies delivered at the extremes of birth weight for gestation was also compared, using customised growth charts. Babies with a birth weight <10th centile for gestation were reported in 33 (17.1%) births in the ELS group and 37 (20.0%) births in the placebo group; adjusted risk ratio 1.18 (99%CI 0.67 to 2.07). Babies born weighing >90th centile for gestation were reported in 10 (5.2%) cases in the ELS group and 17 (9.2%) cases in the placebo group; adjusted risk ratio 1.82 (99%CI 0.68 to 4.90)

The number of babies reported to have an APGAR score <7 at delivery was not different between treatment allocations. Six babies (16.5%) were reported in the ELS group and 3 (1.7%) babies in the placebo group; adjusted risk ratio 0.52 (99%CI 0.94 to 2.82). Neonatal unit admissions were also compared. In the ELS group, 32 (16.5%) newborns were admitted compared with 29 newborns (15.7%) in the placebo group; adjusted risk ratio 0.94 (99%CI 0.46 to 1.92).

Examination of the newborn reported one baby with hypospadias (Prader 4). This patient had been randomised to the progesterone arm of the trial. Another patient who had a second randomisation excluded from the primary analysis also gave birth to a baby with a hypospadias (Prader 4). This patient had been randomised to receive placebo.

Table 7 summarises the neonatal delivery details.

	Progesterone n = 194	**Placebo n = 185**	**Adjusted[a] RR (99% CI)**
Sex			
Male	85 (43.8%)	96 (51.9%)	1.18 (0.89 to 1.55)
Female	109 (56.2%)	89 (48.1%)	0.86 (0.66 to 1.11)
Birth weight (grams) - mean ± sd	3020 ± 701	3060 ± 698	37.1 (-175 to 249)[bc]
Birth centile			
< 10%	33 (17.1%)	37 (20.0%)	1.18 (0.67 to 2.07)[c]
>90%	10 (5.2%)	17 (9.2%)	1.82 (0.68 to 4.90)[c]
External Genitalia Assessment			
Prader 5	84 (43.3%)	96 (51.9%)	n/a
Prader 4	1 (0.5%)	0	n/a
Prader 3	0	0	n/a
Prader 2	0	0	n/a
Prader 1	0	0	n/a
Prader 0	109 (56.2%)	89 (48.1%)	n/a
APGAR <7	6 (3.2%)	3 (1.7%)	0.52 (0.94 to 2.82)[c]
NNU admission	32 (16.5%)	29 (15.7%)	0.94 (0.46 to 1.92)[c]

[a] Adjusting for age of woman at randomisation

[b] Adjusted mean difference

[c] Adjusted for the clustering effect of multiple pregnancy

Table 7. Neonatal Outcomes (denominator is number of live births)

The incidence of fetal abnormalities diagnosed in the antenatal period is summarised in Table 8. Four cases in total were identified, and are reported with trial medication allocation. A ventricular septal defect (VSD) was diagnosed in a pregnancy known to be affected with Down's syndrome (patient randomised to the progesterone group). Three patients were identified with renal abnormalities including; duplex kidney, hypoplastic kidney and multicystic dysplastic kidney disease. The latter case resulted in an intrauterine death of one twin at 32 weeks gestation. Only the pregnancy diagnosed with a duplex kidney belonged to the ELS group. No obvious safety concerns were evident when comparing the study intervention.

Patient	Trial Medication	Pregnancy	Fetal Anomaly
Case 1	Progesterone	Twin 1 (DC/DA)	Duplex kidney
Case 2	Placebo	Singleton	Hypoplastic right kidney
Case 3	Placebo	Twin 1 (DC/DA)	Multicystic dysplastic kidney disease (IUD at 32 weeks gestation)
Case 4	Progesterone	Singleton	Ventricular septal defect, TAMS, (trisomy 21)

Table 8. Fetal problems diagnosed antenatally

Neonatal pathology at the time of hospital discharge is presented in Table 9. No association with treatment allocation was evident. However, morbidity appeared strongly correlated with gestation at delivery.

Patient	Trial Medication	Pregnancy	Gestation at Delivery (weeks $+^{days}$)	Pathology at time of discharge
Case 1*	Progesterone	Singleton	37^{+6}	Ventricular septal defect, TAMS (known trisomy 21)
Case 2*	Progesterone	Twin 1 *(DC/DA)*	37^{+1}	Duplex kidney
Case 3	Placebo	Singleton	38^{+6}	Congenital dislocation of hips
Case 4	Placebo	Twin 1 *(DC/DA)*	35^{+0}	Patent ductus arteriosus, patent foramen ovale
Case 5	Progesterone	Singleton	40^{+4}	Cerebral infarct
Case 6	Placebo	Singleton	39^{+2}	Congenital dislocation of hips
Case 7	Progesterone	Twin 2 *(DC/DA)*	36^{+3}	Dilated bowel (inter-hospital transfer)
Case 8	Progesterone	Singleton	40^{+0}	Hypospadias (prader 4)
Case 9	Progesterone	Twin 2 *(DC/DA)*	26^{+3}	Bronchopulmonary dysplasia, patent ductus arteriosus, metabolic bone disease, bilateral undescended testes. (Twin 1 resulted in neonatal death)
Case 11	Placebo	Twin 1 *(DC/DA)*	26^{+2}	Bronchopulmonary dysplasia, post haemorrhagic hydrocephalus (requiring Omaya reservoir and ventriculo-peritoneal shunt), cystic periventricular leukomalacia, hypoadrenalism, patent ductus arteriosus, neonatal epilepsy, necrotising enterocolitis
	Placebo	Twin 2 *(DC/DA)*	26^{+2}	Bronchopulmonary dysplasia, patent ductus arteriosus, patent foramen ovale, necrotising enterocolitis, inguinal hernia, grade 3 retinopathy.

**Patient included in Table 8*

Table 9. Neonatal pathology at time of discharge

5.4.6 Compliance and Adherence

One woman did not take the trial medication provided. The woman, who had been randomised to the progesterone arm of the trial, subsequently visited her general practitioner securing a prescription for progesterone which she used instead.

Eleven percent of patients in the ELS group utilised the trial medication vaginally compared to 9% of patients in the placebo group. Fifty percent of patients in the ELS group used the pessaries rectally compared to 55.4% of patients in the placebo group. Some patients utilised a combination of rectal or vaginal application; 24.6% of patients in the ELS arm compared to 16.8% of patients in the placebo group. In cases where pregnancy loss occurred prior to the first ultrasound appointment the patients preferred route of pessary use is unknown (14.5% in the ELS arm compared with 18.9% in the placebo arm). Details are summarised in Table 10.

	Progesterone n=228	**Placebo n=233**
None	1 (0.4%)	0 (0.0%)
Vaginal	25 (11.0%)	21 (9.0%)
Rectal	114 (49.6%)	129 (55.4%)
Both vaginal and rectal[a]	56 (24.6%)	39 (16.7%)
Unknown	33 (14.5%)	44 (18.9%)

[a] Any woman who admits to rotating route of use, or who has changed documented route of administration between visits

Table 10. Reported route of pessary usage during trial.

Adherence to the trial medication was recorded at scheduled ultrasound visits (Table 11). At 7 weeks gestation, adherence to trial medication was reported to be 96.4% in the ELS group

and 99.4% in the placebo group. At 12 weeks gestation adherence was reported to be 92.2% and 98.2% respectively (Table 11).

	Progesterone n=167	**Placebo n=167**
Adherence at 7 weeks gestation	161 (96.4%)	166 (99.4%)
Adherence at 12 weeks gestation	154 (92.2%)	164 (98.2%)

Table 11. Adherence to trial medication at 7 and 12 weeks.

5.4.7 Side Effects Profile

Table 12 summarises the severity of side effects reported for each treatment allocation at both 7 and 12 weeks. No significant difference was noted according to treatment allocation.

	Progesterone	**Placebo**	**Median Difference (99% CI)**
At 7 weeks gestation	*(n=196)*	*(n=189)*	
median (IQR)			
Nausea / Vomiting	5 (2 to 6)	4 (2 to 5)	0 (-1 to 0)
Bloated feeling	5 (2 to 7)	5 (2 to 7)	0 (-1 to 1)
Vaginal discharge	2 (0 to 4)	2 (0 to 4)	0 (0 to 1)
Vaginal irritation	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)
At 12 weeks gestation	*(n=167)*	*(n=170)*	
median (IQR)			
Nausea / Vomiting	4 (1 to 6)	4 (1 to 7)	0 (-1 to 1)
Bloated feeling	4 (2 to 7)	4 (2 to 6)	0 (-1 to 1)
Vaginal discharge	2 (0 to 5)	2 (0 to 5)	0 (0 to 1)
Vaginal irritation	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)

Table 12: Side effects reported per treatment allocation. Side effects reported using a visual analogue scale from 0 to 10 (a score of 0 = no symptom, a score of 10 = maximum severity of symptom)

5.4.8 Serum hormones

The comparisons of serum hormone levels were not significantly different between the treatment allocations, with the exception of serum progesterone at 7 weeks gestation. In the progesterone allocation, the serum progesterone levels were higher 60.2 ± 55.2 versus 51.6 ± 53.8 ng/mL; adjusted risk ratio -11.1 (-21.3 to -1.01). Serum oestradiol levels however were similar between treatment allocations.

A detailed comparison of other serum analyses is discussed later in this thesis.

Serum Hormones	**Progesterone**	**Placebo**	**Adjusted[ab] mean difference (99% CI)**
At 7 weeks (mean ± sd)	*(n=196)*	*(n=189)*	
Serum Progesterone (ng/mL)	60.2 ± 55.2	51.6 ± 53.8	-12.3 (-23.9 to -2.58)
Serum Oestradiol (pmol/L)	6520 ± 5961	6160 ± 4342	-578 (-1820 to 397)
Serum free β-HCG (ng/mL)	56.3 ± 48.2	48.7 ± 31.6	-5.24 (-14.7 to 3.03)
Serum Inhibin A (pg/mL)	312 ± 217	288 ± 161	-17.9 (-56.8 to 22.1)
Serum Inhibin B (pg/mL)	11.6 ± 12.5	12.9 ± 16.0	1.11 (-0.99 to 3.58)
Serum Activin A (pg/mL)	605 ± 283	557 ± 250	-31.1 (-92.5 to 30.5)
PAPP-A (mU/L)	90.2 ± 288	72.5 ± 139	-18.2 (-104 to 25.2)
At 12 weeks (mean ± sd)	*(n=167)*	*(n=170)*	
Serum Progesterone (ng/mL)	57.2 ± 46.8	58.6 ± 55.9	2.41 (-14.7 to 16.5)
Serum Oestradiol (pmol/L)	9970 ± 6510	9650 ± 5810	-137 (-1490 to 1550)
Serum free β-HCG (ng/mL)	52.6 ± 42.4	52.5 ± 37.0	5.13 (-5.49 to 14.7)
Serum Inhibin A (pg/mL)	426 ± 273	396 ± 210	-3.14 (-68.4 to 55.0)
Serum Inhibin B (pg/mL)	14.7 ± 15.3	15.1 ± 11.2	-0.52 (-2.85 to 3.05)
Serum Activin A (pg/mL)	1350 ± 694	1260 ± 520	-39.6 (-202 to 119)
PAPP-A (mU/L)	3410 ± 3220	3110 ± 2750	-101 (-623 to 785)

[a] Adjusted for baseline serum hormone values and age of woman at randomisation

[b] Adjusted for number of viable fetuses at gestation sampled

Table 13. - Serum hormone levels at 7 and 12 weeks gestation.

5.5 Excluded Patients

A total of 467 patients were recruited and randomised to the trial. However 6 patients had been randomised on two occasions. Only the outcome of the first trial participation was included in the primary analysis; a modified intention to treat basis.

For completeness, all trial participations were included in a subsequent analysis comparing live birth rate, i.e. all 467 trial episodes. The live birth rate remained unaffected by these inclusions with 167/230 (73.5% in the progesterone group and 171/237 (72.2%) in the placebo group.

The pregnancy outcomes of patients randomised on more than one occasion are presented in Table 14 along with their treatment allocations.

	Participation number	**Treatment**	**Pregnancy outcome**
Patient 1	1st participation	Placebo	Biochemical pregnancy
	2nd participation	Placebo	Live birth
Patient 2	1st participation	Placebo	Biochemical pregnancy
	2nd participation	Placebo	Live birth
Patient 3	1st participation	Placebo	Biochemical pregnancy
	2nd participation	**Progesterone**	Mid-trimester loss
Patient 4	1st participation	Placebo	Live birth
	2nd participation	**Progesterone**	Live birth
Patient 5	1st participation	**Progesterone**	Biochemical pregnancy
	2nd participation	Placebo	Live birth
Patient 6	1st participation	**Progesterone**	Miscarriage
	2nd participation	Placebo	Live birth

Table 14. – Trial outcomes of patients excluded from primary analysis.

Chapter 6: Doppler Velocity Studies

6.1 Hypothesis

Extended luteal support using progesterone may improve pregnancy outcome which may be mediated through an effect on the uterine artery, or be an effect mediated through improved implantation.

6.2 Rationale

Doppler assessment of the uterine arteries is a non-invasive method of measuring placental perfusion utilising the pulsatility index (PI) of the uterine artery and less commonly the resistance index (RI). Increased PI reflects increased impedance to blood flow in the uterine arteries and is thought to reflect the failure of the trophoblastic invasion of the spiral arteries and their conversion into low-resistance vessels, with consequent impairment in uteroplacental function (327). High impedance or the presence of diastolic notches indicates inadequate trophoblastic invasion and is used to evaluate placentation problems (330). High impedance has been associated with conditions arising from sub-optimal placentation such as pre-eclampsia and intrauterine growth restriction (331). Vascular changes in the uterine artery can be detected by Doppler as early as 5 weeks gestation (329). Several studies have also demonstrated a higher pulsatility index reflecting impedance in the non–pregnant uterine arteries of patients with a history of recurrent pregnancy loss (376-378), whilst blood flow resistance has also been reported as a good predictor of implantation success (Steer 1992, Sterzik 1999). Several studies have observed differences in Doppler flow changes during the menstrual cycle with the pulsatility index progressively falling during the luteal phase when serum progesterone levels are highest (324, 372, 393). Pulsatility indices

have been inversely correlated with serum progesterone levels, whilst no effect has been observed with oestradiol levels (376).

The application of progesterone in the luteal phase has been shown to increase uterine blood flow in the presence of adequate oestrogen levels, which may in part be achieved by uterine relaxation (376, 396). Vessel resistance and aortic stiffness have also been associated with endothelial dysfunction and pro-inflammatory conditions (389, 395). Progesterone has significant anti-inflammatory properties that may counter these effects (443).

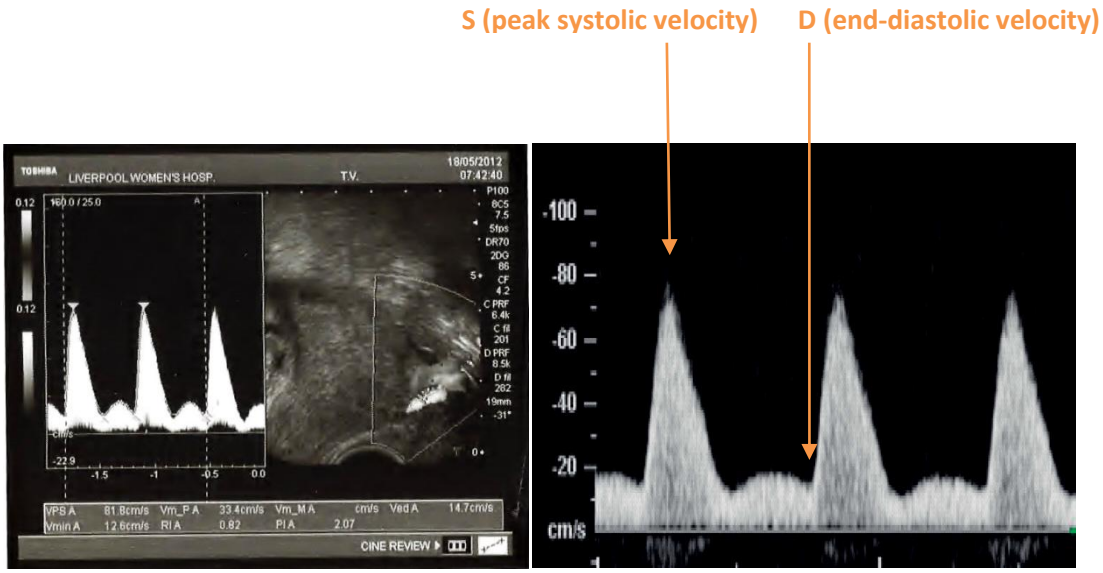
If extended luteal support improves the pregnancy outcome after assisted conception, could one of the above mechanisms be responsible for this effect?

6.3 Method for Measuring Uterine Artery Doppler

Uterine artery Doppler measurements were obtained using a Toshiba Nemio ultrasound machine, with a 6 mHz transvaginal probe. A mid-sagital section of the uterus and cervical canal was obtained and the internal cervical os identified. The transducer was tilted to the side towards each paracervical region in turn. Using colour flow mapping the uterine arteries were identified running alongside the uterus and cervix. Pulsed wave Doppler was then applied to obtain flow velocity waveforms from the ascending branch of the uterine artery at the point closest to the internal os. When applying colour Doppler, the dimensions of the colour box and velocity scale were optimised. Application of the pulsed wave Doppler with the sampling gate adjusted to 2mm and an appropriate angle of intonation was

performed. When three consecutive waveforms were obtained, the inbuilt computer software calculated the relative Pulsatility Index (PI) and Resistance Index (RI) from the waveform tracing for each uterine artery in turn.

The image below is representative of the optimal image obtained of the ascending branch of the uterine artery identified using colour flow mapping. The second image is typical of the captured waveform image from which the PI and RI are calculated.



The PI is calculated as the difference between the peak systolic velocity (S) and the end-diastolic velocity (D), divided by the mean velocity (Vm): PI = (S−D)/Vm.

Resistance Index is calculated using the peak systole divided by the sum of systole and diastole (RI = systole / (systole + diastole).

6.4 Statistical Analysis

Blood velocity parameters were examined for both left and right uterine arteries at each visit. Mean values for PI and RI were calculated from the data obtained from each uterine artery. The mean and standard deviations were calculated and compared using adjusted mean difference with 99% confidence intervals.

6.5 Results

There was no significant difference in the resistance indices and pulsatility indices between the treatment groups at 7 and 12 weeks gestation. Absolute figures and adjusted mean differences are summarised in the table below.

Blood velocity measure	**Progesterone**	**Placebo**	**Adjusted[ad] mean difference (99% CI)**
<u>At 7 weeks gestation</u>	***n = 196***	***n = 189***	
RI[b] – mean ± sd	0.80 ± 0.64	0.80 ± 0.54	-0.01 (-0.02 to 0.01)
PI[c] – mean ± sd	2.18 ± 0.61	2.09 ± 0.42	-0.10 (-0.24 to 0.04)
<u>At 12 weeks gestation</u>	***n= 167***	***n= 170***	
RI[b] – mean ± sd	0.70 ± 0.07	0.70 ± 0.07	0.00 (-0.02 to 0.02)
PI[c] – mean ± sd	1.53 ± 0.39	1.51 ± 0.34	-0.03 (-0.14 to 0.07)

[a] Adjusting for age of woman at randomisation
[b] Resistance index
[c] Pulsatility index
[d] Adjusted for the clustering effect of multiple viable fetuses in the same woman

Table 1. Mean uterine arterial blood velocity at 7 and 12 weeks gestation.

6.6 Discussion

Our study has proved no benefit in supporting the luteal phase with the addition of progesterone beyond pregnancy confirmation. Analysis of pulsatility and resistance indices between the treatment arms has shown no statistical difference, whilst maintaining the expected fall in RI and PI that is associated with progress through the first trimester of pregnancy. Whilst this trial is not sufficiently powered to detect small differences in

complications during pregnancy, the results obtained suggest no protective effect of additional progesterone.

Additional progesterone does not appear to affect the degree of placentation as reflected by Doppler velocity; nor does it appear progesterone has a direct effect on the uterine artery as measured using these Doppler indices.

Further details are discussed in Chapter 9 of this thesis.

Chapter 7: Prenatal Screening

7.1 Hypothesis

Could extending luteal support with progesterone override the biological mechanisms that would otherwise result in the pregnancy loss of a chromosomally abnormal fetus? Is this reflected in the number of pregnancies diagnosed with chromosomal abnormality or those that report high risk screening results? Could there be an effect of treatment on the biological or biochemical parameters that are integral to screening tests involved in the detection of Down's syndrome?

7.2 Background & Rationale

In 1984, Merkatz et al. discovered a relationship between low serum alpha-fetoprotein (α-FP) and Down's Syndrome (444). Progressive innovation has resulted in the development of several prenatal screening tests with improved capability in identifying pregnancies considered at high risk and suitable for consideration of invasive diagnostic testing. Several screening tests have been developed;

1) Double test, performed in second trimester
2) Triple test, utilising maternal age, maternal serum alpha-fetoprotein (MSAFP), unconjugated estriol (uE3) and human chorionic gonadotrophin (HCG), and performed in the second trimester.
3) Quadruple test, utilising maternal age, maternal serum alpha-fetoprotein (MSAFP), unconjugated estriol (uE3), human chorionic gonadotrophin (HCG) and Inhibin A. The quadruple test is performed in the second trimester.
4) Nuchal translucency is performed in the first trimester (11-13^{+6} weeks).

5) Combined test; nuchal translucency, free βHCG and PAPP-A in the first trimester (between 11 and 13^{+6} weeks).

First trimester screening offers earlier detection of potential abnormalities and higher accuracy compared with previous tests. The quadruple test performed between 15 and 20 weeks gestation has a sensitivity of 81% and false positive rate of 7%. First trimester screening has a sensitivity of 85% and false positive rate of 5% (445, 446). If an abnormal pregnancy is identified, earlier intervention is possible. Combined first trimester screening is increasingly available and becoming integrated into clinical practice. In the last few years, the ability to isolate and analyse fetal DNA from the maternal circulation has resulted in the development of commercially available assays such as the Harmony Test™. However this test is not yet part of routine antenatal care.

It is well recognised that for pregnancies conceived through assisted conception, an adjustment for risk is necessary. Free βHCG levels are generally higher, whilst PAPP-A levels are generally lower in ICSI cycles (447). A similar phenomenon was observed in previous screening tests. Unconjugated estriol and AFP levels are usually lower, and Inhibin A, βHCG and free βHCG are usually higher in assisted conception cycles (447), leading to a 2-3 fold increase in false positive rates (448-452).

7.2.1 Nuchal Translucency

It is now generally accepted that measurements of nuchal translucency do not need modification based on the method of conception (447-464).

7.2.2 PAPP-A

PAPP-A is synthesized by the syncytiotrophoblasts and acts by increasing the bioavailability of insulin-like growth factor (IGF). PAPP-A is a protease for IGF binding protein-4, therefore low level of PAPP-A is associated with low levels of free IGF. IGF controls the uptake and transport of glucose and amino acids in trophoblasts and plays a role in invasion of trophoblast into the decidua (465). Low levels of PAPP-A have been associated with adverse fetal outcomes, especially those linked to abnormal trophoblastic invasion such as gestational hypertension, IUGR, fetal demise and preterm delivery (465). Reduced PAPP-A levels are evident with ICSI as opposed to IVF cycles, although the mechanism remains unclear (447, 466).

7.2.3 Free βHCG

HCG is a glycoprotein composed of 244 amino acids and produced by the syncytiotrophoblasts. It maintains pregnancy by stimulating progesterone synthesis from the corpus luteum. A maximum level of 100,000 iu/L is reached at 8-10 weeks gestation and decreases as placental steroid production commences (210). Proteolytic cleavage by trophoblastic macrophages destabilises the βHCG molecule producing free βHCG that is secreted in to the maternal circulation (467). In the first trimester, low levels of free βHCG

(<0.5 MoM) have been associated increased risk of miscarriage and low birth weight. Elevations of free βHCG have not been associated with adverse obstetric outcome. Elevated βHCG levels have been associated with adverse perinatal outcome attributed to hypoxia induced cytotrophoblastic proliferation (465, 468). Decreased free βHCG levels have been associated with spontaneous miscarriage < 24 weeks, fetal demise > 24 weeks and preterm delivery.

The effect of ART on first trimester free βHCG levels have been extensively studied indicating increased, decreased or unaltered free βHCG levels (469). A recent study has observed an effect of four different IVF culture media on serum HCG concentrations on day 15 after embryo transfer in singleton viable pregnancies. These higher free HCG levels are consistent with an increase in the mass of syncitial trophoblast tissue resulting in higher free βHCG levels (470). It has been proposed that the culture media may modulate the epigenetic regulation of the placenta, however this has yet to be confirmed by larger studies (471).

Free βHCG and PAPP-A are synthesized by the invading syncytiotrophoblasts. Placental invasion is in part mediated by serum progesterone levels. Could free βHCG levels and PAPP-A be influenced by increasing serum progesterone levels through exogenous application during the luteal support process? Although it is accepted that NT measurements do not need adjustment for assisted conception cycles, duration of luteal support was not adjusted for in any of the studies. Considering there is a great variation in practice in duration of luteal support, an association remains possible. There is no published evidence suggesting a relationship between progesterone exposure and nuchal thickness.

However, there is also no data confirming that progesterone does not affect NT measurements.

7.3 Materials and Methods

Patients randomised to the DOLS trial were informed of the antenatal screening options available. Comprehensive counselling was undertaken including the possibility of invasive diagnostic procedures. As geographical variation in the provision of antenatal screening existed, patients were asked to discuss the issue further with their antenatal care physician or midwife.

An intention to proceed with prenatal screening was noted after completion of the 12 week ultrasound scan and confirmation of ongoing pregnancy. Confirmation of prenatal testing was secured with each patient during the third trimester.

Collation of test type and results were collated from the hospital case notes after delivery as described in the trial protocol. If the results were not available, contact was made with the regional laboratories that held the service contracts for screening and the results recorded.

7.4 Results

Not all patients opted for prenatal screening tests, 334 patients had a viable pregnancy recorded at 12 weeks gestation of which 213 patients underwent antenatal screening, representing 63.8% of eligible trial participants.

One hundred and seven patients in the progesterone and 106 patients in the placebo arm of the trial underwent antenatal screening.

A total of 11 patients had a triple test performed (2 in progesterone group, 9 in the placebo group) whilst 34 patients underwent quadruple test (16 in progesterone group and 18 in placebo group). Nuchal translucency only was performed in 64 pregnancies (41 in progesterone group, and 23 in placebo group). Combined screening was reported in 104 pregnancies (48 in progesterone group and 56 in placebo group).

7.4.1 High Risk Pregnancy

The numbers of pregnancies with a screening result greater than 1 in 150 were analysed. Four out of 106 pregnancies (3.8%) in the progesterone group reported a high screening result, compared with 6/106 (5.7%) in the placebo group. An adjusted risk ratio adjusted for the age of participants at randomisation was 1.19 (99%CI 0.22 to 6.49), a non-significant difference.

Similarly the number of patients with a nuchal thickness >3.5mm, and deemed at high risk based on NT measurement alone was equally distributed between the treatment arms, with one patient in each treatment allocation; adjusted risk ratio 0.88 (99%CI 0.03 to 29.6).

Patients considered at high risk by any method of screening were offered invasive prenatal diagnostic testing. In the progesterone arm, 5 patients underwent testing with one confirmed case of trisomy 21. Two patients in the placebo group underwent amniocentesis,

from which no karyotype abnormalities were reported. The results are summarised in the Table 1.

	Progesterone n=107	**Placebo n=106**	**Adjusted[a] RR (99% CI)**
Type of prenatal screening test			
Triple test	2 (1.9%)	9 (8.5%)	n/a
Quadruple test	16 (15%)	18 (17%)	n/a
Nuchal translucency	41 (38.3%)	23 (21.7%)	n/a
Combined screening	48 (44.9%)	56 (52.8%)	n/a
	n=106	**n=106**	
High risk screening result[bc] (<1 in 150)	4 (3.8%)	6 (5.7%)	1.19 (0.25 to 5.58)
Nuchal thickness >3.5mm[c]	1 (1.1%)	1 (1.3%)	0.88 (0.02 to 30.8)
Prenatal Diagnostic Tests			
Chorionic villus sampling	1	0	n/a
Amniocentesis	4	2	n/a

[a] Adjusting for age of woman at randomisation

[b] Baby's were assessed using any of the following: triple test, quadruple test, nuchal translucency only, combined test

[c] Adjusted for the clustering effect of multiple pregnancy

Table 1. Prenatal screening and diagnostic test outcomes.

7.4.2 Nuchal Measurements

The mean nuchal thickness of all fetuses measured was not significant between the treatment arms of the trial. In the progesterone exposed group the mean nuchal thickness was 1.63 ± 0.85 mm, compared with 1.52 ± 0.45 mm, with an adjusted mean difference of -0.13 (99%CI -0.48 to 0.13), suggesting no effect of progesterone exposure. However, overall participant numbers are too small to be absolutely conclusive.

	Progesterone n=106	**Placebo n=106**	**Adjusted[ab] mean difference (99% CI)**
Nuchal thickness (mm) (mean ± sd)	1.63 ± 0.85	1.52 ± 0.45	-0.13 (-0.46 to 0.12)

[a] Adjusted for age of woman at randomisation

[b] Adjusted for the clustering effect of multiple pregnancy

Table 2. Comparison of nuchal translucency measurement.

7.4.3 Serum Free βHCG

Serum free βHCG levels at recruitment to the trial did not differ according to treatment allocation. Free βHCG is produced by enzymatic cleavage of the HCG molecule by the trophoblast. In subsequent assays at 7 and 12 weeks gestation the serum levels of free βHCG are increased compared with baseline levels, probably reflecting the maturation of placental function and increasing enzymatic activity. No significant differences between the extended progesterone and placebo allocation were observed, suggesting no direct effect of exogenous progesterone exposure (Table 3). This is despite serum progesterone levels at 7 weeks gestation being higher in the progesterone group compared to the placebo group.

	Gestation	Progesterone	Placebo	Adjusted[ab] mean difference (99% CI)
Serum free βHCG (ng/mL) Mean (sd)	4 weeks (recruitment)	2.92 (20.9) (n=228)	1.17 (0.56) (n=223)	n/a
Serum free βHCG (ng/mL) mean (sd)	7 weeks	**56.3 (48.2)** (n=196)	**48.7 (31.6)** (n=189)	**-5.24 (-14.7 to 3.03)**
Serum free βHCG (ng/mL) mean (sd)	12 weeks	**52.6 (42.4)** (n=167)	**52.5 (37.0)** (n=170)	**5.13 (-5.49 to 14.7)**

[a]Adjusted for baseline serum hormone values and age of woman at randomisation

[b] Adjusted for the number of viable fetuses at the time of sampling

Table 3. Gestational comparison of free βHCG according to treatment allocation

7.4.4 Serum PAPP-A

Serum PAPP-A levels at trial recruitment did not differ according to treatment allocation. PAPP- A is synthesized by syncytiotrophoblasts. In subsequent assays at 7 and 12 weeks gestation the serum levels of free PAPP-A increase, reflecting placental maturation. No significant difference between the extended progesterone and placebo groups was observed, suggesting no direct effect of exogenous progesterone exposure on PAPP-A levels (Table 4).

	Gestation	Progesterone	Placebo	Adjusted[ab] mean difference (99% CI)
PAPP –A (mU/L) mean (sd)	4 weeks (recruitment)	82.5 (78.7) (n=228)	24 (0) (n=223)	n/a
PAPP –A (mU/L) mean (sd)	7 weeks	**90.2 (288)**	**72.5 (139)**	**-18.2 (-104 to 25.2)**
PAPP –A (mU/L) mean (sd)	12 weeks	**3410 (3220)**	**3110 (2750)**	**101 (-623 to 785)**

[a] Adjusted for baseline serum hormone values and age of woman at randomisation

[b] Adjusted for the number of viable fetuses at the time of sampling

Table 4. Gestational comparison of free PAPP-A according to treatment allocation.

7.5 Discussion

There appears to be no effect of extending progesterone supplementation beyond pregnancy test and up to 12 weeks gestation on a number of parameters that may either individually or in combination affect the outcome of prenatal screening results. Therefore it does not appear that the screening variables including nuchal translucency, serum free βHCG and PAPP-A are affected by extending exogenous progesterone exposure after IVF, and that adjustment based on length of exposure is not warranted. Whilst conceding that the absolute number of subjects included in this analysis is relatively small, there appears to be no suggestion that additional luteal support confers a reduction in pregnancy loss, increase in high risk screening results or higher incidence of chromosomal abnormality.

Further discussion is included in Chapter 9.

Chapter 8: Serum analyses

8.1 Hypothesis

Levels of inhibin A, inhibin B and activin A levels are influenced by the duration of exogenous progesterone exposure observed with differing luteal support regimes.

8.2 Background

Inhibin and activin are polypeptide hormones belonging to the transforming growth factor-β (TGF-β) superfamily.

Inhibin's are heterodimers consisting of a common α-subunit linked to either a β_A subunit (inhibin A) or β_B subunit (inhibin B), linked by disulphide bridges. In women inhibins are secreted by granulosa cells in the ovary and appear to regulate gametogenesis via a negative feedback mechanism on the production of FSH at the level of the pituitary. A local paracrine function has also been noted.

Inhibin A rises in the late follicular phase to reach a maximum in the late luteal phase and peaking at ovulation. Inhibin is predominantly produced by the dominant follicle or corpus luteum (472).

Inhibin B levels rise to a maximum in the mid-follicular phase with a secondary peak at ovulation before falling to basal levels in the luteal phase. Inhibin B is predominantly produced by smaller developing follicles.

Activin's are homo- or heterodimers of the β_A or β_B subunits, giving rise to activin A, B or AB. Activin A is synthesised by placental cytotrophoblast and syncytiotrophoblast (473). There is some evidence that activin A promotes invasion of extra-villous trophoblast (474). It has been suggested that activin A is important for embryonic development (475, 476). During pregnancy, the level of inhibin A is maintained by its secretion from the fetoplacental unit, peaking at 8-10 weeks gestation and subsequently declining until about 20 weeks when it then gradually rises again until term (473, 477).

Inhibins and activins have been shown to regulate GnRH, HCG and progesterone secretion from human placental cells in vitro (478). Inhibin A and activin A concentrations are raised at 15-18 weeks gestation in patients who develop preeclampsia (479). Conversely inhibin A levels are lower in missed miscarriage rates compared with ongoing pregnancy, suggesting that these proteins play a part in placentation (472, 480). These findings were supported by a study confirming that inhibin A, progesterone, oestradiol and HCG levels are lower in failed pregnancies compared with ongoing pregnancy controls, although activin A levels were not significantly different and Inhibin B levels were undetectable in both groups (481). Inhibin A levels are significantly correlated with HCG levels in controls. In sporadic miscarriage, inhibin A was positively correlated with progesterone. In women with a live birth, plasma inhibin A showed a positive correlation with activin A, oestradiol and HCG. In women with subsequent miscarriage, plasma inhibin A was positively correlated with activin A, progesterone, oestradiol and HCG. Serum activin A was also positively correlated with progesterone, oestradiol and HCG. Serum progesterone and HCG were correlated with inhibin A in miscarriage suggesting a similar mechanism and source for these hormones. Activin A has also been associated with preterm labour.

Inhibin A levels are lower in miscarriage patients, whilst activin A is not. This suggests that inhibin A may be a more specific marker of placental function although it is produced by several sources which possibly complicates any analysis (481). Inhibin A levels clear from the circulation within hours compared with HCG which has a longer half life (477), again suggesting that it is a more sensitive marker of placental dysfunction.

Placental dysfunction or failure result in altered levels of inhibin A, B and activin levels. Can these be linked to placental dysfunction and failure of appropriate trophoblast invasion? Is it possible that these levels of hormones which reflect successful placentation are influenced by exogenous progesterone? Conditions of poor placentation have successfully been associated with poor pregnancy outcome. Miscarriage may be the result of a more severe case of failure of placental implantation and placentation (482). Inhibin A is produced by the syncytiotrophoblast, decidua and fetal membranes, and may have a role in regulating embryogenesis and adrenal gland steroidogenesis (210). Elevated inhibin A in the second trimester (>2.0 MOM) has been associated with gestational hypertension, preeclampsia, fetal growth restriction and fetal loss.

Monitoring of serum HCG may not be as reliable in ART pregnancies as it may be raised by exogenous HCG administration used to trigger ovulation. Inhibin A is produced by the feto-placental unit and does not appear to be affected by IVF medication. It also appears to reflect the numbers of implanted embryos, as levels are further elevated in multiple pregnancy. Measurements of Inhibin A and HCG at 6 weeks may be predictive of recurrent miscarriage, with lower levels observed compared with controls.

8.3 Materials and Methods

All patients recruited to the DOLS trial had blood tests drawn at recruitment, 7 weeks and 12 weeks gestation as described in the trial protocol. Samples were processed in accordance with the procedures described in chapter 4.

8.4 Results

8.4.1 Inhibin A

Inhibin A levels at trial recruitment were similar between the interventions, mean 155 ± 148 pg/mL versus 153 ± 159 pg/mL in the ELS and placebo groups respectively. Serum levels at both 7 and 12 week gestations were not significantly different between the treatment allocations. However there was an observed increase associated with advancing gestation in all patients, reflecting increasing placental function (Table 1).

	Gestation	**Progesterone**	**Placebo**	**Adjusted[ab] mean difference (99% CI)**
Serum Inhibin A (pg/mL) mean (sd)	4 weeks (recruitment)	155 (148) (n=228)	153 (159) (n=223)	n/a
Serum Inhibin A (pg/mL) mean (sd)	7 weeks	312 (217) (n=196)	288 (161) (n=189)	-17.9 (-56.8 to 22.1)
Serum Inhibin A (pg/mL) mean (sd)	12 weeks	426 (273) (n=167)	396 (210) (n=170)	-3.14 (-68.4 to 55.0)

[a] Adjusted for baseline serum hormone values and age of woman at randomisation

[b] Adjusted for the number of viable fetuses at the time of sampling

Table 1. Inhibin A levels reported between treatment allocation and gestation

8.4.2 Inhibin B

Inhibin B levels at recruitment were similar between the interventions, mean 13.0 ± 17.2 pg/mL versus 11.6 ± 13.6 pg/mL in the extended luteal support and placebo group respectively. Serum levels at both 7 and 12 week gestations were not significantly different comparing treatment allocation. There was not a significant increase in levels associated with advancing gestation (Table 2).

	Gestation	**Progesterone**	**Placebo**	**Adjusted[ab] mean difference (99% CI)**
Serum Inhibin B (pg/mL) mean (sd)	4 weeks (recruitment)	13.0 (17.2) (n=228)	11.6 (13.6) (n=223)	n/a
Serum Inhibin B (pg/mL) mean (sd)	7 weeks	11.6 (12.5) (n=196)	12.9 (16.0) (n=189)	1.11 (-0.99 to 3.58)
Serum Inhibin B (pg/mL) mean (sd)	12 weeks	14.7 (15.3) (n=167)	15.1 (11.2) (n=170)	0.52 (-2.85 to 3.05)

[a] Adjusted for baseline serum hormone values and age of woman at randomisation
[b] Adjusted for the number of viable fetuses at the time of sampling

Table 2. Inhibin B levels reported between treatment allocation and gestation.

8.4.3 Activin A

Activin A levels at recruitment were similar between the interventions; 378 ± 182 pg/mL versus 342 ± 162 pg/mL in the extended progesterone and placebo group respectively. Serum levels at 7 and 12 weeks gestation were not significant comparing treatment

allocations. There was an observed increase in serum levels with advancing gestation, reflecting increasing placental function (Table 3).

	Gestation	**Progesterone**	**Placebo**	**Adjusted[ab] mean difference (99% CI)**
Serum Activin A (pg/mL) mean (sd)	4 weeks (recruitment)	378 (182) (n=228)	342 (162) (n=223)	n/a
Serum Activin A (pg/mL) mean (sd)	7 weeks	605 (283) (n=196)	557 (250) (n=189)	-31.1 (-92.5 to 30.5)
Serum Activin A (pg/mL) mean (sd)	12 weeks	1350 (694) (n=167)	1260 (520) (n=170)	-39.6 (-202 to 119)

[a] Adjusted for baseline serum hormone values and age of woman at randomisation

[b] Adjusted for the number of viable fetuses at the time of sampling

Table 3. Activin A levels reported between treatment allocation and gestation.

8.4.4 Oestradiol

Oestradiol levels at recruitment were similar between the interventions; 3180 ± 3130 pmol/L versus 3610 ± 5340 pmol/L in the extended progesterone and placebo group respectively. Serum levels at 7 and 12 weeks gestation were not significant comparing treatment allocations. There was an observed increase in serum levels with advancing gestation, reflecting increasing placental function (Table 4).

	Gestation	**Progesterone**	**Placebo**	**Adjusted[ab] mean difference (99% CI)**
Serum Oestradiol (pmol/L) mean (sd)	4 weeks (recruitment)	3180 (3130) (n=228)	3610 (5340) (n=223)	n/a
Serum Oestradiol (pmol/L) mean (sd)	7 weeks	6520 (5961) (n=196)	6160 (4342) (n=189)	-578 (-1820 to 397)
Serum Oestradiol (pmol/L) mean (sd)	12 weeks	9970 (6510) (n=167)	9650 (5810) (n=170)	137 (-1490 to 1550)

[a] Adjusted for baseline serum hormone values and age of woman at randomisation

[b] Adjusted for the number of viable fetuses at the time of sampling

Table 4. Oestradiol levels reported between treatment allocation and gestation.

8.4.5 Progesterone

The serum progesterone levels at randomisation were similar in both treatment allocations; 60.0 ± 68.2 ng/mL versus 63.1 ± 84.6 ng/mL (Table 5).

	Gestation	**Progesterone**	**Placebo**	**Adjusted[ab] mean difference (99% CI)**
Serum Progesterone (ng/mL) mean (sd)	4 weeks (recruitment)	60.0 (68.2) (n=228)	63.1 (84.6) (n=223)	n/a
Serum Progesterone (ng/mL) mean (sd)	7 weeks	60.2 (55.2) (n=196)	51.6 (53.8) (n=189)	-12.3 (-23.9 to -2.58)
Serum Progesterone (ng/mL) mean (sd)	12 weeks	57.2 (46.8) (n=167)	58.6 (55.9) (n=170)	2.41 (-14.7 to 16.5)

[a] Adjusted for baseline serum hormone values and age of woman at randomisation
[b] Adjusted for the number of viable fetuses at the time of sampling

Table 5. Progesterone levels reported between treatment allocation and gestation.

The serum progesterone level at 7 weeks gestation was the only statistically significant difference reported between treatment allocations; 60.2 ± 55.2 ng/mL in the progesterone allocation compared with 51.6 ± 53.8 ng/mL in the placebo allocation; adjusted mean difference -12.3 (99%CI -23.9 to -2.58). Why, when a similar number of ongoing pregnancies were identified in each treatment allocation, were progesterone levels different? Analysing the pattern of progesterone in each treatment group may help understand the cause. In the progesterone allocation group, serum progesterone levels at recruitment, seven weeks and 12 weeks gestation were 60.0 ± 68.2, 60.2 ± 55.2 and 57.2 ± 46.8 ng/mL respectively. In the

placebo allocation group, progesterone levels were 63.1 ± 84.6, 51.6 ± 53.8 and 58.6 ± 55.9 ng/mL. Serum progesterone levels were similar at trial recruitment which is consistent with all patients having received luteal support with progesterone up to the point of trial recruitment. This is not unexpected (Table 5).

At 12 weeks gestation, similar levels of progesterone are observed between treatment allocations, suggesting that the endogenous production of progesterone is now predominant. However, as there is no apparent clinical effect in terms of pregnancy outcome, the analysis of progesterone serum levels becomes a surrogate outcome measure without clinical consequence.

However, is it possible that serum progesterone levels at 7 weeks gestation are reflective of the viability and or number of fetuses at the time of sampling?

The reported serum progesterone levels are adjusted for pregnancy viability; In a subgroup analysis comparing progesterone levels at 7 weeks gestation in non-viable pregnancies only, no statistical significant difference between the progesterone and placebo allocations was apparent; 24.9 ± 19.2 ng/mL and 17.6 ± 18.5 ng/mL in the placebo group; adjusted mean difference -10.97 (99%CI -22.2 to 1.82). This would suggest that despite a non-ongoing pregnancy, serum progesterone levels whilst using exogenous progesterone is no different to the level achieved with placebo usage. However, serum progesterone levels reported in the extended luteal support group was in fact on average 41% higher comparing with the placebo group. This analysis only included 36 patients so it is possible that the comparison is under powered to detect a true statistical significance.

In the second part of the subgroup analysis, the effect of one viable fetus on serum progesterone levels was compared. A total of 274 patients were evenly distributed between treatment allocations. A mean progesterone level of 66.2 ng/mL ± 58.2 was reported in the progesterone group compared with 52 ng/mL ± 49.6 in the placebo group; adjusted mean difference -13.2 (99%CI -25.5 to -0.87), a statistically significant result and similar to the overall serum levels reported at 7 weeks unadjusted for viability. These levels were significantly higher compared with the non–viable pregnancy subgroup analysis. It would however support the concept that exogenous progesterone increases serum levels beyond what is achieved with endogenous production.

In the third part of the sub-group analysis we looked at the comparative effect of more than one viable fetus at viability scan. Seventy patients were included in this analysis (43 in the progesterone group and 27 in the placebo group. Serum progesterone levels were reported as 59.2 ± 51.6 ng/mL in the progesterone group compared with 68.4 ± 76.8 ng/mL in the placebo group; adjusted mean difference -8.66 (99%CI -35.6 to 23.0). Absolute serum progesterone levels were not significant between the allocated treatments and broadly very similar to levels observed in single viable pregnancies. The presence of a multiple pregnancy implies a greater trophoblastic mass which should result in higher levels of HCG secretion. However serum progesterone levels do not appear to be increased, suggesting maximal endogenous progesterone production.

8.5 Discussion

Inhibin A and Activin A are both produced by trophoblast tissue. As the mass of trophoblast increases and the placental matures, serum levels increase. However, no difference and hence no association with duration of exogenous progesterone support was reported. Serum progesterone levels are significantly higher in participants allocated to the progesterone arm, suggesting no direct association between serum progesterone with Inhibin A and Activin A.

Inhibin B levels were consistently low and similar in both treatment arms without significant change with advancing gestation evident. Inhibin B is not produced by the fetoplacental unit and therefore a rise would not be expected. Inhibin B predominantly exerts a negative feedback on FSH production at the level of the pituitary. It is also noted to fall to baseline levels in the luteal phase of the menstrual cycle which corresponds to the time of highest progesterone exposure.

Oestradiol levels appear unaffected by progesterone exposure but increased levels are observed with advancing gestation. Serum progesterone levels are reportedly higher in patients exposed to ongoing exogenous progesterone with levels increased in viable pregnancies.

The results are discussed further in Chapter 9.

Chapter 9: Discussion

9.1 Overall results

The DOLS trial is the world's first randomised placebo controlled trial directly addressing the impact of duration of luteal support after assisted conception.

The results of the trial support the conclusion that extending luteal support beyond confirmation of biochemical pregnancy does not confer benefit. The primary outcome defined as at least one viable pregnancy at 12 weeks gestation, reported 167 ongoing pregnancies (73.3%) in the group allocated to receive extended luteal support and 167 pregnancies (71.7%) in the group allocated to receive placebo; adjusted risk ratio of 0.97 (95%CI 0.87 to 1.09). A similar clinical pregnancy rate at 7 weeks gestation was reported; 175/228 patients (76.8% clinical pregnancy rate) allocated to receive extended luteal support and 174/233 patients (74.7% clinical pregnancy rate) allocated to receive placebo; adjusted risk ratio 0.97 (99%CI 0.84 to 1.11).

The total number of live births reported was not statistically different. One hundred and sixty two deliveries occurred in the progesterone group compared with 164 deliveries in the placebo group; RR 0.98 (99%CI 0.84 to 1.15).

The live birth rate per embryo transferred per treatment allocation was 0.57 ± 0.43 in the extended luteal support group and 0.54 ± 0.42 in the placebo group; adjusted risk ratio -0.03 (99%CI -0.13 to 0.73). There was no reported difference in the incidence of early pregnancy complications observed before 12 weeks gestation. The incidence of unscheduled hospital

admissions, antenatal complications and neonatal outcomes were comparable between treatment allocations.

There was no observed difference in uterine artery Doppler waveform analyses comparing treatment allocations. No effect of duration of treatment was reported in prenatal screening parameters and outcomes, serum markers of placentation, pregnancy complications and neonatal outcome when treatment allocations were compared.

9.2 Strengths of the study

During trial development, there was limited published data available to advocate an optimum duration of luteal support. In the Cochrane review by Daya & Gunby and its subsequent revision by van der Linden et al., similar concerns regarding weaknesses in the included trials were raised (170, 185). Issues concerning appropriate randomisation processes, adequate blinding and concealment of treatment allocation, comprehensive outcome reporting and appropriate sample sizes were all identified. We believe that we have learned from the mistakes of others, and managed to avoid many of the contentious issues observed in previous studies. We are confident our results that can be integrated into clinical practice with certainty.

In the first instance, the research team looked at the validity of the study question. During trial development, only one prospective randomised trial had been published (316). The trial had sufficient participant numbers very similar to our own study but was not placebo controlled. Despite the trial reporting that luteal support beyond pregnancy test may be

unnecessary, no appreciable change in practice was observed. We presented overwhelming evidence in chapter 2 that there is no consistency in practice with regard to the optimum duration of luteal support. Worldwide over 75% of clinics routinely use luteal support with progesterone beyond biochemical pregnancy and up to 12 weeks gestation. Since the first IVF birth 35 years ago, almost 5 million babies have been born as a result of assisted reproductive technologies. With over 1 million cycles performed annually worldwide, the importance of the trial hypothesis and its validity is evident (8).

The DOLS trial received approvals from the institutional review board, regional ethics committee, and the medicines and healthcare products regulatory agency (MHRA). Interim inspections and data monitoring committee meetings were met with approval. The DOLS protocol was published in a peer reviewed journal and the trial was registered in the ISRCTN database (**ISRCTN Registration Number: 05696887)** (440).

To ensure the results of the trial are clinically relevant, trial participation was limited to patients treated with Buserelin as part of a “long cycle” down-regulation, the commonest type of IVF cycle performed. Only patients using Menopur only for ovarian stimulation for considered for the trial, therefore we avoided criticism that would have arisen due to the use of a heterogenous ovarian stimulation regime. Unlike many of the previous studies that included very narrow trial eligibility criteria, we opted for criteria that were representative of the patient population that presents for IVF treatment.

As the trial was a single centred study, all patients were treated according to the same clinical and laboratory standard operating procedures, thus minimising heterogeneity and potential selection bias.

The DOLS trial recruited the largest number of trial participants (461 patients) of almost any trial involving luteal support included in the Cochrane review.

Randomisation and treatment allocation lists were only available to personnel in the clinical pharmacy trials unit. The randomisation lists allocated treatment to either Group A or Group B, therefore maintaining treatment allocation concealment throughout data input and analysis. Allocation concealment was not revealed until after the trial results had been finalised and agreed upon.

The use of a placebo was extremely important. Both the progesterone and placebo pessaries were manufactured by the same supplier to appear identical in appearance and dispensed in non-descript packaging. Therefore the trial was a truly double blind trial which reduced the potential for allocation bias.

The DOLS trial is the first placebo controlled trial looking at the optimal duration of luteal support using a vaginally administered placebo. Two trials comparing progesterone and placebo and pregnancy outcome have been reported previously. The first study compared intramuscular progesterone with placebo whilst the second study compared oral progesterone and placebo (250, 252). Neither study reported results that were statistically significant; however both were included in the Cochrane reviews that concluded a beneficial

effect of progesterone on pregnancy outcome (185). Only 10 of the 69 studies in the Cochrane review reported blinding.

By using a randomisation sequences generated by a third party, allocation concealment and placebo, potential selection and performance bias was minimised. Consequently all patients received similar attention and care during trial participation.

The trial outcomes were clearly defined in the trial protocol. The primary outcome was defined as a viable intrauterine pregnancy with at least one fetal heart rate > 100 bpm on transvaginal or abdominal ultrasound scan at 12 weeks gestation. Secondary outcomes were similarly clearly defined. A pertinent criticism of previous studies was the widespread use of "implantation rate" opposed to "ongoing pregnancy rate" or "live birth rate". Whilst studies in assisted conception have traditionally reported on implantation as outcome, the expectations of both clinicians and patients are now understandably higher. Live birth rate is now considered the gold standard outcome. Only 16 of the 69 studies included in the latest Cochrane review reported live birth rate (185). If one considers evidence for use of luteal support based only on trials that reported live birth, the number of study participants and events becomes very limited. Of the studies comparing luteal support with placebo or no treatment, only two studies reported live birth rate. In the study by Beckers et al. comparing HCG and placebo, 6 live births were reported from a total of 38 participants (177). In the second study comparing progesterone and placebo, 17 live births were reported from a total of 156 trial participants (251). Whilst the former study reported an OR of 2.25, the results included a wide confidence interval (95%CI 0.37 to 13.80). The latter trial reported an OR of 2.95 (95%CI 1.02 to 8.56) in favour of progesterone.

The research team successfully maintained blinding throughout the trial analysis. The results were analysed and presented to the research team analysed by allocation group; “Group A” or “Group B”. Only after an agreement had been reached about the implications and statistical significance of the results was un-blinding performed, minimising potential reporting bias. The trial database was “locked” prior to statistical analysis, with no further changes authorized. All results of all aspects of the trial have been comprehensively reported.

The trial was designed to be analysed on an “Intention to treat” basis, i.e. all patients randomised to the trial would be analysed. In essence our primary analysis was a modified intention to treat analysis, having excluded patients that did not actually fulfil the eligibility criteria. The recruitment of 6 patients on more than one occasion was discussed during the first data committee meeting. An amendment to the eligibility criteria was made clarifying that only first randomisations would be included in the analysis. The pregnancy outcomes of these 6 excluded patients were reported separately and as part of a secondary analysis, an unmodified intention to treat analysis. The inclusion of these 6 patients did not impact on the ongoing pregnancy rate or live birth rate.

If a patient stopped the trial medication after recruitment, it was not considered to be a trial withdrawal. All patients were reviewed at 7 and 12 weeks unless they had an interceding pregnancy loss. There were no withdrawals from the trial, and hence no possibility of attrition bias.

Pregnancy outcome and live birth rate is known for all patients recruited to the trial. Following trial closure, the case notes for all patients were analysed, collating details about pregnancy complications, prenatal screening, birth outcome and neonatal complications. This was performed across all hospital sites that patients received care. This data was principally collected to ensure complete adverse event reporting, but was subsequently used to inform pregnancy outcome reporting. In 3 cases it was not possible to comprehensively analyse all hospital case notes. Three patients ended their confinement outside of the UK. Interviews were conducted with 2 of these patients, whilst the third patient was not contactable. Only a very small number of missing values were apparent after data collection.

The trial team was very keen to report all aspects pregnancy outcome, in particular any possible negative aspects that may be attributable to the treatment intervention, for example the incidence of OHSS, multiple pregnancy, antepartum haemorrhage and preeclampsia etc.

One of the greatest strengths of the trial was that all data collection was performed by one operator; including all counselling, recruitment, completion of trial documentation, ultrasound examinations, telephone interviews and case note review and adverse event reporting. This process reduced the possibility of any inter-observer variation in reporting outcomes.

The SPSS trial database was developed by and all data entered by one operator with single data entry and two comprehensive data checks.

All blood samples were processed, stored and analysed at the end of the trial, in order to minimise batch variation.

9.3 Weaknesses of the trial

The principle weakness of the DOLS trial was the decision to report viable pregnancy at 12 weeks gestation as the primary outcome instead of live birth. During trial development, the standard practice of the majority of IVF trials reported implantation rate or ongoing pregnancy rate as primary outcomes, and was considered standard practice. During trial recruitment there was a shift in research practice for reporting trial outcomes, favouring live birth. Due to the power calculation based on ongoing pregnancy rate, ethics approval, agreed statistical analysis plan and publication of the trial protocol it was not feasible or realistic to alter the primary outcome at this stage. In a retrospective power calculation, based on the proportion of women having at least one live birth, 0.70 in the placebo group, a sample size of 230 women per group would have been sufficient to detect an effect if the proportion was 0.81 or greater (≥11% increase) in the other group with 80% power and an alpha level of 0.05. This suggests that the trial was adequately powered if the initial primary outcome had been defined as live birth.

When performing any type of research and despite optimal planning, potential sources of weakness become apparent. The research team considers the generation of randomisation sequences by a third party, allocation concealment and comprehensive blinding throughout the trial minimised critical steps in the risk of introducing bias. However it is possible that the use of placebo could itself be a potential source of bias, however unlikely. During the trial it was evident that patients had attempted to guess which treatment they had been

allocated. The majority of patients believed they were using the placebo and although the assumptions were without foundation, could this have been a potential source of intervention detection. This is probably unlikely considering the side effects profiles of both treatment allocations were very similar. Would it have been sensible to have asked patients at the end of the trial to guess which treatment they had received? A significantly higher proportion of correct guesses (above 50%) may have suggested that blinding had been inadequate. Patients are more likely to attribute a good outcome or marked side effects to an active treatment, whilst poorer outcomes are likely to be attributed to placebo. It is interesting that the majority of patients who offered an opinion thought they had been given the placebo.

Although the trial was designed to be analysed on an intention to treat basis, we monitored compliance with the trial medication as a secondary outcome. Compliance data was volunteered by patients at surrogate end points. Whilst there was no reason to suspect dishonesty in their responses, it could be argued that a remaining pessary count for each patient would have provided more substantiated compliance information, and should have been more rigorously enforced.

A potential weakness was addressed in the discussion about the trial strengths. An amendment to the trial eligibility criteria was made part way through trial recruitment to prevent patients being recruited on more than one occasion. Only the first randomisation and trial participation was used in the primary analysis. However, all randomisations were subsequently reported in a secondary analysis. In an ideal situation this eligibility criteria should have been made clear from the beginning.

One of the frustrations of the trial was the time needed to complete recruitment. Fifteen months into the study, the trial team were made aware that the expiry date of the placebo medication had been reached and could no longer be dispensed. Consequently, there was a 12 month delay in securing additional medicinal products from the licensee before recruitment could recommence.

A criticism of the trial could arise from the collation of study data and data entry by a single operator. Is it possible that an element of reporting bias could have been introduced? Whilst conceding that dual data entry would be preferential to single data entry with two comprehensive checks, this option was not financially feasible. As data was entered with the operator blind to treatment allocation the possibility of reporting bias appears unfounded. Similarly, it could be argued that using a single person to perform all study procedures was not representative of standard clinical practice which is then not transferable to clinical practice in general. Alternatively, it could be argued that because all procedures were performed by a single operator with significant expertise in their respective field, inter-observer variation and hence reporting bias were minimised, resulting in improved data quality and trial reliability.

Whilst developing the trial protocols, we benefited from the expertise of Dr Anna Hart, a statistician contracted through the hospitals R&D department who provided statistical advice and support. Due to the longevity of trial recruitment, there was an interceding change in contractual commitments between the Trust and Dr Hart's employer. It became apparent that it would not be feasible for Dr Hart to assist with the development of the primary statistical analysis plan as she would be unable to commit to the analysis of results.

A statistics team from the NPEU Clinical Trials Unit in Oxford was approached and subsequently agreed to help with the statistical analysis plan and data analysis. Whilst there was no significant change in the approach to analysis, we acknowledge that a change in statistical team would have been best avoided if possible.

In retrospect I would have liked to have included a serum βHCG assessment at randomisation. Identifying those patients with inherently low serum βHCG levels at recruitment may have provided useful data for a sub-group analysis. Was there a minimal βHCG level that suggested a successful pregnancy outcome was possible? However, the eligibility criteria and processes described in the trial protocol are representative of current practice when reporting a biochemical pregnancy result.

In response to a funding request, a suggestion was made to consider chromosomal analysis of products of conception obtained after failed pregnancy. All material was analysed histologically, but not for karyotype. It would have been interesting to observe any difference in pregnancy loss patterns associated with karyotype abnormalities and treatment intervention. Unfortunately we were unable to secure adequate funding for this.

Actavis UK Limited provided the medicinal products for the trial and an initial £20,000 towards initial start up costs. Additional funds were granted by the Moulton Charitable Foundation (£76,103). Both funding providers remained independent of all trial procedures, analysis of the results, manuscript preparation and decisions to publish.

9.4 Context

The DOLS Trial is the world's first prospective randomised, double blind placebo controlled trial designed to identify the optimal duration of luteal support after assisted conception.

9.4.1 Trial Demographics

The distributions of baseline demographics were described as summary statistics only. Any reported difference in characteristics would have arisen by chance. The distribution of variables were similar in terms of age distribution, body mass index, infertility cause, gonadotrophin use, response to treatment and baseline pituitary blood results. However there are a small number of variables that appear to be unevenly distributed.

The number of previous IVF / ICSI cycles suggested a small discrepancy between the treatment allocations; first cycle treatment accounted for 69.3% of patients in the progesterone group compared with 60.9% of patients in the placebo group. Conversely, a lower proportion of patients had more than one treatment; 30.7% in the progesterone arm compared with 39.1% in the placebo group. This may be consistent with a slightly longer median duration of infertility reported in the placebo group (42 months versus 36 months). Male factor infertility was reported in 40.8% of couples in the progesterone group compared with 50.2% of couples in the placebo group. Instinctively, it would be expected that overall embryo quality would be higher in the placebo group due to the higher incidence of male factor fertility; however this was inconsistent with the higher number of high scoring embryos transferred in the progesterone group.

Of particular importance is the similarity of serum analyses at recruitment, particularly levels of oestradiol and progesterone. All patients received luteal support up until the point of trial recruitment and it would be expected that these parameters be equally distributed, reflecting exogenous administration of progesterone and likely augmented with endogenous progesterone and oestradiol produced by the corpus luteum.

9.4.2 Pregnancy Outcomes

The primary outcome defined as on-going clinical pregnancy at 12 weeks gestation reported no difference in the number of viable pregnancies, one hundred and sixty seven viable pregnancies were confirmed in each treatment arm; adjusted risk ratio 0.97 (95%CI 0.87 to 1.09). A similar secondary outcome result was reported in analysing viable on-going pregnancy at 7 weeks gestation, 175/228 (76.8%) in the extended progesterone group and 174/233 (74.7%) in the placebo group. These results are statistically reliable in concluding that supplemental progesterone does not improve the on-going pregnancy rate at either 7 or 12 weeks gestation.

All trial participants were pregnant at randomisation; a 100% biochemical pregnancy rate. A proportion of patients were diagnosed with a biochemical pregnancy loss. In the progesterone group 33 patients (14.5%) were diagnosed with a biochemical pregnancy loss compared with 46 patients (19.7%) in the placebo group. Although this was not a significant finding (adjusted risk ratio 1.37 (99%CI 0.80 to 2.34) the results suggested an associated higher incidence of biochemical loss in the placebo group.

Confirmed clinical miscarriage was in contrast higher in the progesterone group compared to the placebo group; 25 patients (11%) in the progesterone group compared with 17 patients (7.3%) in the placebo group. This difference did not reach statistical significance either, but suggested a tendency for a higher rate of first trimester clinical pregnancy loss in the progesterone group. Combining biochemical pregnancy loss and clinical miscarriage before 12 weeks gestation there was no difference in the distribution of first trimester pregnancy loss; 58 patients (25.5%) had reported pregnancy loss in patients allocated to the progesterone group compared with 63 patients (27%) in the placebo group. Is it biologically plausible that early luteal supplementation could delay pregnancy loss rather than prevent it, or is this just a random fluctuation of data?

Ectopic pregnancy rates were similar between the treatment arms. Whilst it is appreciated that ectopic pregnancy occurs more frequently after IVF treatments compared to natural conceptions there appeared to be no effect of duration of progesterone exposure. However with only 6 ectopic pregnancies reported in the trial, no absolute conclusion can be made.

Live birth should be considered the ultimate outcome when assessing any outcome of pregnancy. The number of live births, i.e. the number of births resulting in the delivery of at least one baby was similar between the treatment allocations. One hundred and sixty two births occurred in the progesterone group and 164 births in the placebo group. Singleton birth rates were similar between the progesterone and placebo groups, 130 patients (57%) versus 143 (61.4%); adjusted risk ratio 1.08 (99%CI 0.88 to 1.31). The distribution of twin pregnancies was not statistically significant, but overall numbers appeared to suggest a higher incidence of multiple pregnancy in the progesterone group; 32 patients (14%) versus

21 (9.0%); adjusted risk ratio 0.63 (99%CI 0.32 to 1.25). There is no reasonable biological plausibility to suggest why additional progesterone beyond pregnancy test, i.e. two weeks after implantation occurred could influence the twinning rate. Similarly, there was no evidence of an effect of luteal support and multiple pregnancy reported in any of the trials included in the Cochrane review. No triplets delivered. One triplet pregnancy at 12 weeks resulted in a twin live birth, after the demise of one fetus at 15 weeks gestation.

The live birth rate per embryo transfer was similar, 0.57 ± 0.43 versus 0.54 ± 0.42 in the progesterone and placebo groups respectively. The trial was analysed on a modified intention to treat analysis of 461 patients. Six patients had a second randomisation excluded. A secondary analysis using all 467 patients randomised made no statistical difference to the primary outcome or live birth rate.

9.4.3 Serum Progesterone

Serum progesterone levels at 7 weeks gestation was the only statistically significant difference reported between treatment allocations; 60.2 ± 55.2 ng/mL in the progesterone allocation compared with 51.6 ± 53.8 ng/mL in the placebo allocation; adjusted mean difference -11.1 (99%CI -21.3 to -1.01). Why, when a similar number of ongoing pregnancies were identified in each treatment allocation, were progesterone levels different? Analysing the pattern of progesterone in each treatment group may help understand the cause. In the progesterone allocation group, serum progesterone levels at recruitment, seven weeks and 12 weeks gestation were 60.0 ± 68.2, 60.2 ± 55.2 and 57.2 ± 46.8 ng/mL respectively. In the placebo allocation group, progesterone levels were 63.1 ± 84.6, 51.6 ± 53.8 and 58.6 ± 55.9 ng/mL. Serum progesterone levels were similar at trial recruitment which is consistent with

all patients having received luteal support with progesterone up to the point of trial recruitment. This is not unexpected.

At 7 weeks gestation, higher serum progesterone levels are observed in the progesterone allocation group, suggesting that exogenous luteal support results in higher serum levels than that produced endogenously and may be reassuring when looking at compliance. It is more than reasonable to conclude that exogenous progesterone support only results in marginal increases in serum progesterone above that produced endogenously by a combination of corpora lutea and placental production. The luteo-placental shift is known to begin at this gestation. What cannot be appreciated is at what stage of pregnancy or what gestation is this discrepancy more apparent and at which point does it become clinically significant. It is also difficult to ascertain what proportion of serum progesterone is derived from the placenta and what proportion from the corpora lutea.

At 12 weeks gestation, similar levels of progesterone are observed between treatment allocations, suggesting that the endogenous production of progesterone is now predominant. However, as there is no apparent clinical effect in terms of pregnancy outcome, the analysis of progesterone serum levels becomes a surrogate outcome measure without clinical consequence.

However, is it possible that serum progesterone levels at 7 weeks gestation are reflective of the viability and or number of fetuses at the time of sampling?

The reported serum progesterone levels are unadjusted for pregnancy viability; however we know that an equal number of viable pregnancies are distributed between the allocation groups. In a subgroup analysis comparing progesterone levels at 7 weeks gestation in non-viable pregnancies only, no statistical significant difference between the progesterone and placebo allocations was apparent; 24.9 ± 19.2 ng/mL and 17.6 ± 18.5 ng/mL in the placebo group; adjusted mean difference -10.97 (99%CI -22.2 to 1.82). This would suggest that despite a non-ongoing pregnancy, serum progesterone levels using progesterone is no different to that achieved with placebo. However, serum progesterone levels reported in the extended luteal support group was in fact on average 41% higher comparing with the placebo group. This analysis only included 36 patients so it is possible that the comparison is under powered to detect a true statistical significance. It is likely that a failing pregnancy does not produce sufficient HCG stimulation to promote effective steroidogenesis and progesterone production from the corpora lutea. Exogenous progesterone whilst appearing to increase serum progesterone levels above that seen in the placebo group, did not reach the level reported with ongoing pregnancy.

In the second part of the subgroup analysis, the effect of one viable fetus on serum progesterone levels was compared. A total of 274 patients were evenly distributed between treatment allocations. A mean progesterone level of 66.2 ± 58.2 ng/mL was reported in the progesterone group compared with 52 ± 49.6 ng/mL in the placebo group; adjusted mean difference -13.2 (99%CI -25.5 to -0.87), a statistically significant result and similar to the overall serum levels reported at 7 weeks unadjusted for viability. These levels were significantly higher compared with the non–viable pregnancy subgroup analysis. It would

however support the concept that exogenous progesterone increases serum levels beyond what is achieved with endogenous production.

In the third part of the sub-group analysis we looked at the comparative effect of more than one viable fetus at viability scan. Seventy patients were included in this analysis (43 in the progesterone group and 27 in the placebo group. Serum progesterone levels were reported as 59.2 ± 51.6 ng/mL in the progesterone group compared with 68.4 ± 76.8 ng/mL in the placebo group; adjusted mean difference -8.66 (99%CI -35.6 to 23.0). Absolute serum progesterone levels were not significant between the allocated treatments and broadly very similar to levels observed in single viable pregnancies. The presence of a multiple pregnancy implies a greater trophoblastic mass which should result in higher levels of HCG secretion. However serum progesterone levels do not appear to be increased, suggesting maximal endogenous progesterone production.

Implantation leads to an increase in trophoblastic production and secretion of HCG which promotes steroidogenesis from the corpus luteum. Adequate HCG secretion is determined by the health of the developing pregnancy. A suboptimal rise in HCG levels is observed in failing intra-uterine and ectopic pregnancies.

Progesterone dynamics in early pregnancy suggest that progesterone maintains the developing trophoblast during the first 7 to 8 weeks gestation (483, 484). A number of studies have suggested that serum progesterone can be used as a method of predicting abnormal pregnancy in natural conceptions (485-488). Discriminatory progesterone levels ranged from 21.4 to 82.5 nmol/L for predicting normal gestations (488-492). Progesterone

levels <40 nmol/L are likely to be abnormal in contrast to levels >66 nmol/L which are more likely to suggest viability (230). These findings cannot necessarily be extrapolated to assisted conception cycles. Consideration has to be given to the effect of GnRH agonists and antagonists on the ability of the corpus luteum to produce progesterone. Does the presence of multiple corpora lutea as a result of multiple follicular maturation lead to compensatory progesterone levels despite a non-ongoing pregnancy? Finally, what role does exogenous progesterone have in being able to predict viable pregnancy utilising serum progesterone levels?

Whilst a number of studies have reported inconsistent results, Trew et al. reported a large series of patients with a relatively homogenous stimulation protocol with serum progesterone assay at 14 days post oocyte retrieval utilising progesterone as luteal support. Women with viable intrauterine pregnancies had significantly higher serum progesterone (median 430nmol/L) and βHCG (median 150 IU/L) levels compared with those with an abnormal pregnancy (serum progesterone 72 nmol/L and βHCG 41 IU/L; $p<0.001$) or patients who failed to conceive (progesterone 33 nmol/L; $p<0.001$). Women who failed to conceive had consistently low serum levels of progesterone despite exogenous supplementation (493). The results are in agreement with the evidence available for spontaneous conception, suggesting that exogenous progesterone does not interfere with the predictive ability of the progesterone assay (488). No differences between agonist or antagonist cycles were observed and no correlation between the number of retrieved oocytes and serum progesterone levels were found, irrespective of pregnancy outcome.

Correlation between βHCG and progesterone levels was only evident in pregnancies that were abnormal ($r=0.52$; $p<0.001$), but not in ongoing viable pregnancies ($r=0.02$; $p=0.8$),

suggesting suboptimal βHCG levels are produced by abnormal pregnancies leading to lower levels of serum progesterone. Sensitivity and specificity analyses suggested that a level of 103 nmol/L gives a probability of detecting a viable intra-uterine pregnancy of 88.2% with a sensitivity and specificity of 84%. However, considerable overlap in serum progesterone levels was evident between ongoing and non-viable pregnancies.

Vaginal application of progesterone results in sustained plasma levels of progesterone. Vaginal epithelium readily absorbs protein and lipids and it has been suggested that the vagina might have a reservoir effect and that vaginal mucosa might function as a rate-limiting membrane allowing only a finite amount of progesterone to be absorbed (494). Plasma progesterone levels reach a maximum concentration 3-8 hours after vaginal administration, depending on formulation and tend to gradually fall over the next 8 hours (141). Better steady state serum progesterone concentrations are achieved with vaginal preparations suggesting higher local progesterone bioavailability (245). Higher doses of vaginal progesterone are required to achieve similar levels to those observed with intramuscular administration; 100 mg versus 25 mg. Bourgain et al. reported no difference in histological maturity or serum progesterone levels in patients administered either 300 or 600 mg vaginal progesterone (292). Serum progesterone levels are not indicative of local bioavailability (293). In agonadal women undergoing oocyte donation, serum progesterone levels were almost seven times lower as a result of vaginal application (800 mg/day) than as a result of the intramuscular route (100 mg/day), whereas endometrial concentrations were almost 10 times higher after vaginal progesterone than intramuscular progesterone. Peak plasma progesterone concentrations, 8 hours after vaginal administration of 200 mg

micronized progesterone ranged from 4.4 to 181.1 ng/mL, suggesting a person specific variation.

9.4.4 Congenital Abnormalities

Since the birth of the first IVF baby in 1978 concerns about the safety of ART procedures have remained. Higher neonatal morbidity associated with IVF can be classified into two categories; congenital abnormalities and perinatal complications.

Children born as a result of assisted reproductive technologies have reported an excess of congenital abnormalities compared with spontaneous conceptions. A pooled estimate of birth defects in children born from ART is reported as an OR 1.32 (95%CI 1.24 to 1.42) indicating almost a 30% increased incidence compared with spontaneously conceived gestations (495). A recent study by Davies et al. suggested that congenital abnormalities are probably more likely to be related to the couples need for fertility treatment rather than the treatment itself. Children born as a result of IVF did not have an increased risk of congenital abnormality, compared with those conceived as a result of ICSI (496). In comparing ART conceived twins with non–ART twins and with adjustment for zygosity, distribution between the two groups reported the pooled risk of birth defects for ART twins of 1.26 (95%CI 0.99 to 1.60) (495). However it was difficult to separate minor and major congenital abnormalities.

We reported a number of congenital malformations. These were broadly classified as cardiovascular or genitourinary malformations.

One case of ventricular septal defect (VSD) requiring surgical correction was identified antenatally in a patient with confirmed Down's syndrome, a frequently associated finding (497). This patient had been randomised to the progesterone arm of the trial. A VSD accounts for approximately 30-60% of all newborns with a congenital cardiac defect and an incidence of 2-6 per 1000 births.

Three twin pregnancies were reported to have a cardiac defect. In a first twin born at 35 weeks (randomised to the placebo arm) a patent ductus arteriosus and patent foramen ovale was diagnosed which remained evident at the time of discharge. A second twin pregnancy that delivered spontaneously at 26^{+2} weeks (randomised to the placebo treatment arm) reported cardiac defects in both twins; the first twin was diagnosed with a patent ductus arteriosus and the second twin was diagnosed with a patent ductus arteriosus and patent foramen ovale. A third case involving a second twin born at 26^{+3} weeks was also diagnosed with a patent ductus arteriosus. A patent ductus ateriosus may be idiopathic but is commonly associated with preterm delivery.

Major cardiovascular abnormalities are seen in 4.8% of assisted conception pregnancies compared with 3% in naturally conceived babies (p-value <0.001). After adjustment for maternal and fetal factors the increased odds of cardiac malformations in ART offspring is 1.41 (95%CI 1.22 to1.64) (496). Infants born as multiples were are at increased risk of cardiac defects (OR 1.56, 95%CI 1.31 to 1.85). Our study reported an incidence of 5 cardiac abnormalities in a total of 380 live births, approximately 1.3% with no difference observed between the treatment allocations.

Five cases of genitourinary abnormality were reported. In the patients allocated to receive additional progesterone, one case of hypospadias was reported and one case of duplex kidney. In contrast, patients allocated to receive placebo, one case of hypospadias was reported, one case of hypoplastic kidney and one patient diagnosed with multicystic dysplastic kidney disease that was incompatible with life. Genitourinary abnormalities are reported more frequently in babies conceived using assisted conception (OR 1.40 95%CI 1.09 to 1.82), with singletons reporting a higher incidence of genitourinary abnormalities compared with twins (OR 1.57, 95%CI 1.06 to 2.31). The reported incidence of genitourinary abnormalities is no different from that observed in the general population and no difference was observed between treatment allocations.

9.4.5 Neonatal Outcomes

A higher proportion of females were born in the progesterone arm compared with the placebo arm (56.2% versus 48.1%), however it does not seem reasonable to suggest a causative effect of the trial intervention.

Similar numbers of babies required neonatal unit admission between treatment allocations, whilst birth weight and babies born below the 10% centile for gestation were also evenly distributed.

Higher neonatal morbidity rates are associated with antenatal events such as preterm delivery, preeclampsia, low birth rate and antepartum haemorrhage. These complications are higher in multiple pregnancies, which are more common following assisted

reproduction. However, with the implementation of a single embryo transfer policy it is becoming evident that the risk of adverse perinatal outcome is higher in a IVF conceived pregnancies compared to spontaneously conceived gestations, independent of birth multiple (498-500).

9.4.6 Antenatal Complications

A limited number of antenatal complications were reported in our trial. There was no significant difference in the incidence of preeclampsia, gestational diabetes, cholestasis, cesarean section or placenta praevia. However there appeared to be non-significant increased trend of bleeding incidence requiring hospital admission in the placebo group (9.6% versus 4.8%). A number of trials have reported a reduced incidence of first trimester bleeding in patients allocated to receive progesterone compared with placebo allocation (317, 321). However the incidence of bleeding requiring admission in our study was mostly confined to the third trimester, with only 3 admissions in the first trimester.

9.4.7 Uterine Artery Doppler

The analysis of the effect of progesterone supplementation on uterine artery Doppler velocity may have suggested a mechanism of effect if additional progesterone had improved pregnancy outcome. Whilst this study suggested no apparent improvement in outcome, it also reported no measured effect on uterine artery Doppler velocity at either 7 or 12 weeks gestation. A more marked effect of placentation is more likely evident at 7 weeks gestation than 12 weeks. Serum progesterone levels were reportedly higher in the treatment arm compared with the placebo group at 7 weeks gestation, whilst Doppler velocity

measurements remained comparable, suggesting no effect of serum progesterone elevation.

9.4.8 Serum Screening

A number of serum analytes associated with placentation and first trimester serum screening were compared. No statistically significant difference was observed between treatment allocations, suggesting no direct effect of extending exogenous progesterone supplementation. Free βHCG, PAPP-A and activin A which are predominantly produced by the invading trophoblast with serum levels associated with increasing placental mass and progressive implantation. Exogenous progesterone supplementation does not appear to be an influential factor in placentation as measured by these surrogate end points.

It is well recognised that serum screening algorithms have to be modified in IVF pregnancies compared with natural conceptions. Nuchal translucency measurement which is a principle component of combined antenatal screening appears not to be influenced by extending exogenous progesterone supplementation. It is also reassuring that there was no apparent increase in the number of births affected with chromosomal abnormality. The possibility of exogenous progesterone overriding the body's innate ability to reject an abnormal pregnancy seems unfounded. However, this study is not significantly powered to draw definitive conclusions in this regard.

9.4.9 Side Effects

No significant differences between trial participants were reported between treatment allocations with regard to nausea & vomiting, bloating, vaginal irritation and vaginal discharge. This may be consistent with similar serum progesterone levels reported between the treatment groups. Vaginal discharge and irritation, whilst similar between treatment allocations may be due to the matrix composition of the pessary rather than progesterone component. Consequently, compliance between the treatment groups was similar at 7 an 12 weeks (96.4% and 99.4% at 7 weeks and 92.2% and 98.2% at 12 weeks) in the progesterone and placebo groups respectively. The results however suggest a non-significant trend towards better compliance with placebo.

9.5 Interpretation in light of other evidence

Several meta-analyses have confirmed the benefit of luteal support following IVF (169, 170, 185, 240). The use of either HCG or progesterone can be used with similar effect. There appears to be preference for using progesterone rather than HCG which has a lower associated risk of ovarian hyperstimulation syndrome (185, 436).

The DOLS trial reported no difference in clinical pregnancy, ongoing pregnancy or live birth when 2 weeks of luteal support versus 10 weeks luteal support were compared. In the last 20 years a number of studies with similar intention have attempted to address the optimal duration of luteal support. In 1992, Prietl et al. reported the first trial extending intramuscular luteal support with oestradiol and progesterone compared with placebo until 12 weeks gestation. A significantly higher proportion of ongoing pregnancies were reported

in the treatment arm. The trial however utilised a very heterogenous group of patients having used clomiphene, clomiphene and HMG, GnRH agonist and HMG alone during stimulation. Similarly, patients had used either HCG or progesterone prior to trial recruitment. Randomisation procedures were not sufficient and live birth rates were not reported. The combination of oestradiol and progesterone makes it difficult to attribute an effect of outcome to a particular intervention variable (313). This was the first and largest reported cohort of patients suggesting a benefit of extending luteal support and is probably responsible for influencing current clinical practice.

In 1998, Stovall et al. reported the results of a retrospective study that suggested luteal support with progesterone could safely be withdrawn at 6 weeks gestation without detriment to the ongoing live birth rates which were comparable. However, luteal support was only withdrawn if serum progesterone levels remained above 60 ng/ml (314).

In 2001, Schmidt et al. conducted a very similar large retrospective study reported outcomes of 400 patients, comparing withdrawing luteal support with vaginal progesterone at confirmation of pregnancy test or extending luteal support. No difference in miscarriage rate or live birth rate was observed (63% versus 64%) (315). This study was the first to suggest that extending luteal support did not confer benefit, however the study was criticised for its retrospective nature.

Subsequent to the trial by Schmidt et al., a Danish group that included Schmidt conducted the first prospective randomised trial comparing cessation of luteal support at pregnancy test and continuation until first ultrasound at 7 weeks gestation. No differences in

miscarriage rate, ongoing pregnancy rate or live birth rate were observed (78.7% versus 82.4%) (316). The trial excluded patients with bleeding prior to trial recruitment. This could be argued that patients with bleeding prior to pregnancy test are more likely to miscarry, whilst this may be considered to represent an introduction of selection bias, it was consistently implemented. The trial was also not placebo controlled. This was the first prospective trial suggesting no benefit of extending luteal support beyond biochemical confirmation, substantiating the earlier findings by Schmidt et al. (315). This study is currently the most cited article regarding the duration of luteal support and was included in the recent NICE guideline. It was not included in the previous or current Cochrane reviews. Following discussion with the Cochrane review author it appears that omission of this trial was an oversight and it would be included in future updates.

In keeping with the above trial, Aboulghar et al. reported no benefit of extending luteal support beyond 7 weeks gestation if a viable pregnancy was identified (318). Luteal support with progesterone was used, with 89% of patients using intramuscular progesterone opposed to vaginal preparations. It is worth noting that in the worldwide IVF surveys the prevalence of intramuscular preparations fell from 13% in 2009 to 4.6% in 2012, with the latter figure reflecting a response of 408 units from 82 countries. This suggests that whilst the trial results agree that luteal support beyond 7 weeks in not beneficial, the luteal preparation reported is not reflective of “routine” clinical practice.

Three authors have attempted to clarify the optimum duration of luteal support using GnRH antagonist cycles. Goudge et al. in 2010 reported the results of a small trial of 97 patients comparing withdrawal of luteal support at pregnancy test or continuing until 7-8 weeks

gestation. Live birth rates were similar (52.5% versus 49%). Luteal support utilised intramuscular progesterone and a heterogenous down-regulation protocol, with luteal support only being withdrawn if progesterone levels were maintained above 15 ng/ml (319). In 2012, Kohls et al. reported the results of a trial using only GnRH antagonists comparing vaginal progesterone withdrawal at 5 weeks gestation and 8 weeks gestation. The miscarriage rate did not differ between the treatment allocations; however live birth rates were not reported. Patients were also excluded if they had experienced bleeding before pregnancy ultrasound (321). Kyrou et al. randomised 200 patients using vaginal progesterone 200mg TDS to continue luteal support until pregnancy test or continue to 7 weeks gestation. No difference in ongoing pregnancy rate beyond 12 weeks was observed; 82% versus 73%, p=0.175 (95%CI -2.6 to 20.3). Live birth rates were also not reported in this trial (320).

The results of the DOLS trial complement the results reported by Andersen, Schmidt and Goudge, two of which were trials that reported over a decade ago (315, 317, 319). Interestingly, both Kohls and Aboulghar confirmed no benefit in extending luteal support beyond clinical pregnancy. Our trial replaces this threshold and limits the beneficial use of luteal support up until confirmation of biochemical pregnancy (318, 321).

The trial results are consistent with the majority of studies discussed previously, however they are at odds to consensus practice amongst clinicians both in the UK and beyond. The biological plausibility of luteal support at differing gestations has often been rationalised to anecdotal evidence or observed physiological phenomena with incorrect extrapolation. Whilst evidence for accurate diagnosis of luteal phase deficiency is elusive, its existence is

not contested. Controlled ovarian stimulation probably obviates a natural luteal phase deficiency by producing multiple corpora lutea. However, the supraphysiological levels of follicular oestrogen results in negative feedback at the pituitary preventing LH release, whilst promoting advanced endometrial maturity and causing down-regulation of endometrial progesterone receptors. Follicular aspiration during oocyte retrieval was thought to reduce the capacity of the remaining granulosa cells to produce sufficient progesterone, however this has since been disputed (208).

Progesterone is the hormone of pregnancy, and may a have a number of effects at the physiological and immunological level that promote implantation and placentation. Once implantation has occurred βHCG is produced by invading trophoblastic tissue. HCG perpetuates progesterone production by a direct stimulatory effect on the corpus luteum as a consequence of its structural similarity with LH. HCG is used to trigger ovulation, but is known to have a significantly longer duration of effect compared to LH (72 hours versus 20 minutes). It is likely that the interim time between exogenous HCG used to trigger ovulation and endogenous HCG production by the invading trophoblast requires biological support due to the effect of GnRH agonists causing pituitary desensitisation. Once endogenous HCG levels are high enough to drive progesterone production from multiple corporea lutea, further exogenous support appears superfluous. The luteo-placental shift occurs at approximately 7-8 weeks gestation, a point beyond which progesterone is primarily derived from the placenta and becomes independent of progesterone produced by the corpus luteum. A benefit of luteal support beyond this point seems implausible.

9.6 Implications for practice

As clinicians we strive to improve the chance of successful outcome for our patients. Above all else the authors would have liked to have reported a result that transpires to an improvement in live birth rate. Extending luteal support beyond positive pregnancy test does not improve outcome, but neither is it harmful. Our results are consistent with a small number of less conclusive studies that have previously been reported. We believe that the result of this trial has been obtained using the highest quality methodology and adds considerable weight to the argument for early cessation of luteal support.

The DOLS trial was designed to identify the optimum duration of luteal support after IVF. The decision to utilise Cyclogest was fundamental to our trial and a reflection of current clinical practice. The use of progesterone is more common than HCG both within and beyond the UK, most likely due to a lower associated incidence of OHSS. The use of vaginally administered progesterone is used in approximately 77% of IVF cycles and is better tolerated by patients and more convenient. Intramuscular progesterone is used in approximately 4% of all IVF cycles and is associated with discomfort and the potential complications of administration as previously described. All forms of progesterone used as luteal support are associated with side effects. Whilst our trial looked at what we considered to be the four most common side effects, no significant differences between the treatment allocations were reported. Overall, the cessation of progesterone at confirmation of biochemical pregnancy reduces the treatment burden for patients. As clinicians, we should adhere to the principle of “primum non nocere”. Furthermore, whilst exogenous progesterone is generally considered safe, there continues to be concerns that prenatal

progesterone exposure is linked to urogenital malformation in male offspring (438, 439). Ideally we should be utilising the safest drug at the lowest dosage for the shortest period of time possible to achieve a desired effect.

The cost of treatment should also be considered relevant, both at the level of the health service and as an individual. In the UK, the British National Formulary listed cost for Cyclogest 400mg (15 tabs) is £10.18, approximately 67.9 pence per pessary. Using Cyclogest up until pregnancy test (400mg twice daily) requires 32 pessaries, costing £21.72 per cycle. Extending luteal support until 12 weeks gestation is an equivalent cost of £97.78 per cycle. This suggests a potential saving of £76.06 per patient per cycle on the assumption that the cost of medication is provided at cost. Using HFEA figures 61,276 cycles of IVF were performed in 2011. Assuming 75.7% of clinics currently use luteal support beyond pregnancy test and up to 12 weeks gestation, a cost saving involving 46,386 treatment cycles and equivalent of £3.53 million is possible. It has recently been reported that over a million cycles of IVF are performed annually, possibly suggesting a saving in excess of £76 million worldwide. The figures we have presented here are very crude, but make a convincing argument. The financial implication becomes significantly more complicated when different health sectors are considered. The United States is reported to have the most expensive health care provision in the world with drug costs exceeding those of any other country. Cyclogest is not used in the USA, but a comparison using alternative drugs used for luteal support can be used to demonstrate effect. Crinone 90mg PV OD is used in both the UK and USA. In the UK it costs £2.05 per pessary resulting in a cost of treatment up until pregnancy test of £32.80 and until 12 weeks gestation is £147.60. A potential cost saving of £114.80 per patient per cycle. The equivalent cost in America based on an internet

search for the lowest cost of providing Crinone was $19.85 per pessary. A two week supply until pregnancy test would cost $317.60 and extending luteal support until 12 weeks gestation a cost of $1429.20; potentially a saving of $1,112 (approximately £694.75) per patient per cycle. Intramuscular progesterone is used more often in the United States with a cost per vial of $49.00 compared with £4.50 in the UK. Prescription practice varies, so assuming once daily administration, use up until pregnancy test and until 12 weeks pregnancy would cost $784 and $3528 respectively, a potential saving of $2,744 (approximately £1715) per patient per cycle.

Whilst the complexities of these calculations do not take into consideration the vast ranges in observed treatment duration, dosages, drug formulations and pharmaceutical costs, the potential for reducing cost liability of unnecessary treatment becomes apparent.

In summary, we believe that the evidence is now sufficient to rewrite management protocols worldwide and stop luteal support using progesterone beyond biochemical confirmation of pregnancy.

9.7 Implications for research

We have demonstrated no benefit of continuing luteal support with progesterone beyond confirmation of biochemical pregnancy; 16 days after oocyte retrieval. Assuming that luteal support is necessary, could we reduce the dose or duration of luteal support further. We have recently received an approach to be included in a phase III trial to obtain licensing for Cylogest to be used for luteal support after IVF treatment up until the beginning of the

second trimester. Preliminary trial results suggested adequate secretory endometrial transformation using Cyclogest 200mg twice daily (personal communication). Is it possible that this reduced dose would not be detrimental to pregnancy success after IVF? Alternatively exogenous luteal support is required until endogenous production of progesterone is adequately determined by the trophoblastic production of βHCG. At what level of βHCG does the process become self-sustaining? The current trend for blastocyst transfer, 5 days after oocyte retrieval essentially translates to 12 days of luteal support between embryo transfer and pregnancy test. Could reducing the duration further maintain similar pregnancy rates? If substantiated this could indicate a further reduction in treatment costs and burden, although admittedly these would be of moderate impact and significance.

What if the need for luteal support could be obviated completely? As previously discussed, several meta-analyses have concluded that luteal support is beneficial to treatment after IVF, but how strong is that evidence? The Cochrane reviews have significant impact on clinical practice due to the comprehensive methodology utilised. Comparing the included trials in more detail may suggest the evidence for luteal support is not as convincing as first appears. Comparing the effect on live birth rate (the most important outcome of any intervention in assisted reproduction) between HCG and placebo or no treatment, only 1 trial reported live birth rates which included only 38 participants and only 6 live births (246). Comparing progesterone and placebo or no treatment, only one trial reported live birth rates which included only 156 participants and only 17 live births, although considerably weighted towards favouring progesterone; OR 2.95 with a 95% confidence interval approaching borderline non-significance (95%CI 1.02 to 8.56) (251).

Only when including ongoing pregnancy as an outcome does the argument in favour of luteal support gather momentum. Comparing HCG versus placebo or no treatment, 3 studies reported 81 events (49 in HCG group and 32 in placebo group) in 527 participants, suggesting a significant effect in favour of HCG; OR 1.75 (95%CI 1.09 to 2.81) (185). Including trials comparing progesterone versus placebo or no treatment, 91 ongoing pregnancies were reported in 642 participants (61 in progesterone exposed groups and 30 in placebo exposed groups); OR 1.87 (95%CI 1.19 to 2.94) (185).

The evidence for luteal support use is based on a small number of outcomes from a handful of trials with only two smaller trials reporting live birth as an outcome. Is it time that we re-visit the issue of luteal support completely? Whilst advocates would suggest that not using luteal support is unethical (185) others remain open to the possibility that the issue needs further consideration once sufficient evidence from well conducted studies confirming no impact of early cessation of luteal support become available (501). We believe that we have presented sufficient evidence that adds weight to at least opening a debate, whilst it is appreciated that obtaining ethical consent for such a trial and recruiting sufficient numbers of patients may be difficult.

There is currently renewed interest in minimal stimulation IVF treatments with interval replacement of cryopreserved embryos in subsequent unstimulated cycles. Vitrification techniques have now sufficiently improved to consider this process a viable option. Several authors have also noted improved perinatal outcomes using frozen embryos compared with fresh embryo transfer (502). Could the need for luteal support be rendered obsolete if all embryos are replaced in a natural unstimulated cycle?

Progesterone has recently undergone a renaissance of interest in clinical practice. The DOLS trial is not the only study assessing potential benefit on pregnancy outcome. Two large multicentre trials have not been reported. The PROMISE study (ISRCTN92644181) is comparing the effect of progesterone supplementation in early pregnancy in patients with unexplained recurrent miscarriage, and is due to close recruitment in October 2013. The OPPTIMUM trial is observing the effect of prophylactic progesterone treatment in the late 2^{nd} and 3^{rd} trimester to prevent pre-term labour. The results are awaited with interest.

9.8 Conclusion

The DOLS trial is the world's first and largest prospective double blind placebo controlled trial comparing duration of luteal support with vaginal progesterone. We compared luteal support with vaginal progesterone until pregnancy test with use until 12 weeks gestation. We reported no statistically significant benefit in extending luteal support beyond confirmation of biochemical pregnancy with regard to ongoing pregnancy beyond 12 weeks gestation or live birth rate. We also reported no effect on pregnancy outcome, incidence of antenatal complications, neonatal complications, uterine artery Doppler velocity changes or prenatal screening assessment between the treatment allocations. In light of this evidence we strongly advocate the cessation of luteal support at confirmation of biochemical pregnancy. Worldwide the use of progesterone beyond pregnancy test is considered standard practice. Implementing the recommendations resulting from this trial and a number of previously reported studies we are confident that a significant reduction in treatment burden and cost of treatments can be safely achieved without compromising pregnancy outcome.

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Appendix A: Luteal practice questionnaire 2008

Survey of luteal support practice

What do you use for luteal support?

What dose do you use?

When do you withdraw luteal support?

Appendix B: Luteal practice questionnaire 2013

Telephone survey of luteal support practice

IVF Unit		
Contact number		
Number patients per year (HFEA database)		
NHS or private	NHS	Private

Preferred luteal support .....
(Preparation name, dose and route)

Second choice luteal support .....
(Preparation name, dose and route)

Third choice luteal support .....
(Preparation name, dose and route)

Gestation luteal support withdrawn .....

Comments .....

.....

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Appendix C: DOLS Trial Contributors

Mr Rafet Gazvani
Chief Investigator
Development of DOLS Trial Protocol Version 1.0 and 2.0
Secured trial medication and placebo
Approved final draft of trial protocol

Dr Yasmin Sajjad
Principle Investigator
Secured initial ethics approval

Professor Zarko Alfirevic
Development of trial protocol Version 3.0 and subsequent revisions (see below)
Development of statistical analysis plan

Dr Mark Turner
Development of trial protocol Version 3.0 and subsequent revisions (see below)

Dr Anna Hart
Trial Statistician
Power calculation, statistical analysis plan and preparation of randomisation lists

Dr Richard Russell
Co-Investigator
Trial Manager
Development of trial protocol, version 3.0 to version 5.55 (9 protocol version revisions)

- Redesign of trial protocol, adding uterine artery Doppler studies, prenatal screening tests, serum analyses, pregnancy outcomes and neonatal outcomes.
- Redesign of inclusion and exclusion criteria
- Redesign of randomisation procedures, allocation procedures and dispensing procedures.
- Redesign of statistical plan

Collaboration with statistical teams
R&D liaison
Successful application of ethics re-approval(s)
Successful attainment of MHRA approval
Development of Trial Standard Operating Procedures
Development of DMC charter
Development of TSC charter
Development of Primary statistical analysis plan

Negotiating costs and contracts with national laboratories for performing serum analyses
Negotiating contracts with NPEU

Research funding applications; Wellbeing of Women, HTA, Moulton Charitable Foundation.
Securing of research funding from the Moulton Charitable Foundation

All trial procedures for all patients performed by Dr Russell

- Patient recruitment
- Patient consent

- Blood drawing
- Ultrasound examinations, including Doppler measurements
- Counselling regarding prenatal screening availability
- Phone calls to patients in third trimester
- Application for R&D approval for case note review in all hospitals
- Case note review of all notes
- Completion of all trial forms and associated documentation

Reporting of adverse and serious adverse events
Preparations of reports for the DMEC
Preparation for institutional interim R&D inspections
Involved in preparing for an MHRA inspection

Database creation
All data input (single entry with two comprehensive checks)
Preliminary data analysis

Closure of the trial
Preparation of paper for publication
Correspondence to all trial participants once the trial results have been published

Appendix D: Primary statistical analysis plan

The DOLS Trial

Duration Of Luteal Support With Progesterone Pessaries To Improve The Success Rates In Assisted Conception:

A randomised controlled trial

Statistical Analysis Plan for primary paper

EudraCT 2006-000599-33

Ethics ref: 06/MRE08/17

Date: 1st February 2013

Version: iii

Prepared by: Pollyanna Hardy, Senior Statistician, National Perinatal and Epidemiology Unit

Approvals

Trial Statistician			
Print Name:	Pollyanna Hardy	**Signature:**	
Job Title:	Senior Medical Statistician, NPEU Clinical Trials Unit, University of Oxford	**Date:**	
Reviewer			
Print Name:	Dr Richard Russell	**Signature:**	
Job Title:	Trial Manager, Hewitt Centre for Reproductive Medicine, Liverpool Women's NHS Foundation Trust	**Date:**	
Chief Investigator			
Print Name:	Mr. Rafet Gazvani	**Signature:**	
Job Title:	Consultant Gynaecologist, Hewitt Centre for Reproductive Medicine, Liverpool Women's NHS Foundation Trust	**Date:**	

Contents

Introduction

This document outlines the plan for the final data analysis of the DOLS trial for inclusion in the primary peer reviewed publication. Detailed information about the study can be found in the protocol (version 5.5). In brief, this is a pragmatic, double blind, single centre randomised controlled trial to determine if increased duration of luteal support improves the success rates in assisted conception. Following 2 weeks of standard treatment and a positive biochemical pregnancy test, women will be randomly allocated to a supplementary 8 weeks of treatment of vaginal progesterone or 8 weeks of placebo (stratified by age of the woman at randomisation:<37 years old,≥37 years old). The study flow chart is in Appendix A.

Primary outcome

The primary outcome measure is viable pregnancy (at least one fetus with FHR >100 beats minute) on transabdominal / transvaginal ultrasound at 10 weeks post embryo transfer / 12 weeks gestation (i.e. at the end of 8 weeks supplementary trial treatment).

Secondary outcomes

1. Viable pregnancy (at least one fetus with FHR>100 beats per minute) on transvaginal ultrasound at 5 weeks post embryo transfer / 7 weeks gestation (i.e. at the end of 3 weeks supplementary trial treatment).

2. Serum markers: progesterone, Estradiol, , free B-HCG, Inhibin A, Inhibin B, Activin A and PAPP-A at 7 and 12 weeks gestation.

3. Blood velocity in the uterine artery (measured using pelvic Doppler) at 7 and 12 weeks gestation: left and right pulsatility index and resistance index.

4. Side effects: nausea, bloating, vaginal discharge and vaginal irritation (measured using a visual analogue score) at 7 and 12 weeks gestation.

5. For each fetus, Antenatal Down's Screening Outcomes: Serum hormone levels and Nuchal Translucency during treatment measured at 12 weeks of gestation. Antenatal Down's screening results for all patients will be monitored, including individual serum quantification for double, triple, quadruple screening and nuchal

translucency. A high risk fetus will be defined as a fetus with more than 1 in 150 chance of having Down's Syndrome.

6. Neonatal outcomes collected at delivery

Adherence

Adherence with using pessaries at each visit (7 and 12 weeks gestation).

Statistical analysis

The analysis of this trial will be by 'intention to treat', therefore women will be analysed by the groups into which they were randomised regardless of what intervention they received.

All results will be reported descriptively (i.e. number and percentage or mean and standard deviation/median and interquartile range). All measures of effect will be adjusted for the stratification factor of age of the woman (less than 37 years old and older than or equal to 37 years old at randomisation) (Kahan and Morris, 2012). Binary outcomes will be analysed using log binomial regression models, with corresponding results presented as adjusted risk ratios with associated confidence intervals (CI). If the model does not converge, log Poisson regression models with robust variance estimation will be used (Zou, 2004). Continuous outcomes will be analysed using linear regression models, also adjusting for the stratification factor of age of the woman.

If any variables are highly skewed, leading to doubts about the validity of the parametric analyses, non-parametric analyses will be carried out or bootstrapping will be used. Results will be presented as adjusted differences in means with associated CIs.

Analysis populations

For outcomes relevant to the woman, data will be analysed using the number of eligible women correctly randomised as the denominator. For outcomes relevant to foetuses and the infant, the denominator will be the number of foetuses and live births (respectively) of eligible women correctly randomised.

Demographics and clinical variables

Demographic data at baseline and data describing the clinical progress of the women will be described by trial arm using summary statistics only.

Primary outcome

The primary outcome (proportion of viable pregnancies) will be summarised with an adjusted relative risk and 95% confidence interval.

Secondary outcomes

Binary outcomes will be summarised with an adjusted relative risk and continuous outcomes with adjusted mean differences. Corresponding 99% confidence intervals will be presented for all secondary outcomes to reduce the risk of detecting a false positive result due to the large number of outcomes being assessed. Serum markers at 7 and 12 weeks gestation will be analysed adjusting for baseline serum marker values in addition to the stratification factor.

Sensitivity analyses

If important prognostic factors are imbalanced between the two randomised groups the robustness of the findings will be investigated by including these as covariates in the model for the primary outcome and all secondary outcomes.

Results to be included in the Final Analysis Report

The following items will be included in the report:

- CONSORT participant flow chart
- Table of baseline demographic and clinical characteristics by treatment group
- Table of primary and secondary endpoints detailed above by treatment group
- Table of adverse and serious adverse events by treatment group
- Details of serious adverse events

Dummy tables

Table 1: Demographic characteristics - counts and percentages unless otherwise stated

	Progesterone n=	**Placebo n=**
Age at randomisation (years) mean ± sd		
< 37 years		
≥ 37 years		
BMI (kg/m^2) – mean ± sd		
Type of infertility		
Primary		
Secondary		
Any previous pregnancy		
None		
1		
>1		
If one or more previous pregnancies, no of previous ART pregnancies		
0		
1		
2		
Parity[a]		
None		
1		
>1		
Number of IVF/ICSI attempts (including current cycle)		
1		
>1		
Duration of infertility - median (IQR)		
Cause of infertility *(more than one can be completed per patient)*		
Male factor		
Tubal factor		
Endometriosis		
PCOS		
Anovulation		
Advanced maternal age		
Unexplained		
Same sex couple		

Single female Others Current treatment details IVF ICSI Total dose gonadotrophin (IU) – mean ± sd Total duration gonadotrophin (days) - median (IQR) Number oocytes retrieved - median (IQR) Number oocytes fertilised - median (IQR) Day of transfer Day 2 Day 3 Day 5 (blastocyst) Number embryos transferred – mean ± sd 1 2 Number high scoring embryos[b] transferred – mean ± sd 0 1 2 Number of supernumerary embryos cryopreserved – mean ± sd Baseline hormone levels within last 12 months – mean ± sd FSH (IU/L) LH (IU/L) AMH (pmol/L) Prolactin (IU/L) – median (IQR) FSH (IU/L) LH (IU/L) AMH (pmol/L) Prolactin (IU/L)		

Baseline serum levels at randomisation – mean ± sd Oestradiol (pmol/L) Progesterone (ng/mL) Free BHCG (ng/mL) PAPP-A (mU/L) Inhibin A (pg/mL) Inhibin B median (pg/mL) Activin A median (pg/mL) median (IQR) Oestradiol (pmol/L) Progesterone (ng/mL) Free BHCG (ng/mL) PAPP-A (mU/L) Inhibin A (pg/mL) Inhibin B median (pg/mL) Activin A median (pg/mL) Route of pessary administration during trial None Vaginal Rectal Both rectally and vaginally[d] Unknown		

[a] Number of previous pregnancies registered as live birth, >24 weeks, or registered as a live birth.

[b] High scoring embryo is defined as an embryo transferred on Day2 with a cumulative embryo score (CES) >=9 and <=12; on day 3, a CES >=14; and on Day5 (blastocyst) a CES >=9 and <=12

[c] AMH was carried out in place of FSH and LH during the later stages of the study. N= in a and b

[d] Any woman who admits to rotating route of use, or who has changed documented route of administration between visits

Table 2: Primary outcome – viable pregnancy - counts and percentages

	Progesterone n=	**Placebo n=**	**Adjusted[b] RR (95% CI)**
Viable pregnancy[a] at 12 weeks gestation			

[a] At least one fetus with FHR >100 beats minute on transvaginal ultrasound at 10 weeks post embryo transfer / 12 weeks gestation (i.e. at the end of 8 weeks supplementary trial treatment).

[b] Adjusting for age of woman at randomisation

Table 3: Secondary outcome – viable pregnancy - counts and percentages

	Progesterone n=	**Placebo n=**	**Adjusted[b] RR (99% CI)**
Viable pregnancy[a] at 7 weeks gestation			

[a] At least one fetus with FHR >100 beats minute on transvaginal ultrasound at 5 weeks post embryo transfer / 7 weeks gestation (i.e. at the end of 3 weeks supplementary trial treatment).

[b] Adjusting for age of woman at randomisation

Table 4: Secondary outcomes - serum hormone levels at 7 and 12 weeks gestation embryo transfer - mean and sd

Serum Hormones	**Progesterone n=**	**Placebo n=**	**Adjusted[ab] mean difference (99% CI)**
At 7 weeks mean ± sd Serum Progesterone (ng/mL) Serum Oestradiol (pmol/L) Serum free B-HCG (ng/mL) Serum Inhibin A (pg/mL) Serum Inhibin B (pg/mL) Serum Activin A (pg/mL) PAPP-A (mu/L) At 12 weeks mean ± sd Serum progesterone (ng/mL). Serum Oestradiol (pmol/L) Serum free B-HCG (ng/mL) Serum Inhibin A (pg/mL) Serum Inhibin B (pg/mL) Serum Activin A (pg/mL) PAPP-A (mU/L)			

[a] Adjusted for baseline serum hormone values and age of woman at randomisation

[b] Adjusted for number of viable fetuses at time of sampling

Table 5: Secondary outcomes - Blood velocity in the uterine artery at 7 and 12 weeks gestation

Blood velocity measure	Progesterone n=	Placebo n=	Adjusted[ad] mean difference (99% CI)
<u>At 7 weeks gestation</u> RI[b] – mean ± sd PI[c] – mean ± sd <u>At 12 weeks gestation</u> RI[b] – mean ± sd PI[c] – mean ± sd			

[a] Adjusting for age of woman at randomisation

[b] Resistance index

[c] Pulsatility index

[d] Adjusted for the clustering effect of the number of viable fetuses in the same woman

Table 6: Secondary outcomes - Antenatal Down's screening outcomes measured in second trimester (denominator is number of fetuses) - counts and percentages unless otherwise stated

	Progesterone n=	Placebo n=	Adjusted[a] RR (99% CI)
Type of test All tests Triple test Quadruple test Nuchal Translucency only Combined Nuchal Translucency High risk screening result[bd] (>1 in 150) Nuchal Thickness (mm) – mean ± sd[d] >3.5 mm[d] **<u>Number of Pre-natal diagnostic tests</u>** Chorionic Villus Sampling Amniocentesis Trisomy 21 Confirmed			

[a] Adjusting for age of woman at randomisation

[b] Baby's were assessed using any of the following: triple test, quadruple test, nuchal translucency only, combined nuchal translucency

[c] Adjusted mean difference

[d] Adjusted for the clustering effect of viable multiple fetuses in the same woman

Table 7: Pregnancy outcomes

	Progesterone n=	**Placebo n=**	**Adjusted[a] RR (99% CI)**
<u>During pregnancy</u>			
Threatened preterm labour[bf]			
At least one steroid administration[f] APH Planned caesarean section Preeclampsia Threatened preterm labour PLROM Other			
Antenatal Problems[ef] Cholestasis PIH or preeclampsia Thrombotic event Gestational Diabetes			
Reason for pregnancy admissions[ef] Bleeding Pain PIH OHSS Vomiting			
<u>Labour & Delivery</u>			
Gestational age of pregnancy delivery[f]. mean ± sd 37+0 to 43 weeks 34+0 to 36+6 weeks 28+0 to 33+3 weeks 24+0 to 27+6 weeks			

Onset of labour[f] Spontaneous Induced Planned Cesarean Section Mode of delivery[f] Vaginal delivery Cesarean section Cesarean section for placenta praevia Birth Multiples at 12 weeks[c] Singleton Twins Triplets Biochemical pregnancy only Miscarriage (<12 weeks) Ectopic pregnancy Mid-trimester pregnancy loss (12-24 weeks) Termination of pregnancy Stillbirth Neonatal death Total live births Singleton Twins Triplets Live birth per embryo transferred mean ± sd			

[a] Adjusting for age of woman at randomisation

[b] As quantified by having had tocolysis and steroids

[c] Number of foetuses at 12 weeks gestation

[d] Adjusted mean difference

[e] One or more reasons from the list that follows

[f] Adjusted for the effect of multiple births

Table 8: Neonatal Outcomes (denominator is number of live births) - counts and percentages unless otherwise stated

	Progesterone (n=194)	Placebo (n=185)	Adjusted[a] RR (99% CI)
Sex Male Female Birth weight (grams) – mean ± sd [bc] Birth centile[c] <10% >90% External Genitalia Assessment: Prader 5 Prader 4 Prader 3 Prader 2 Prader 1 Prader 0 Apgar score <7[c] SCBU admission[c]			

[a] Adjusting for age of woman at randomisation, and allowing for clustering of multiple births

[b] adjusted mean difference

[c] adjusted for the clustering effect of multiple births

Table 9: Neonatal Problems Diagnosed Antenatally

Patient	Trial Medication	Pregnancy	Fetal Anomaly

Table 10: Neonatal Pathology at time of Discharge

Patient	Trial Medication	Pregnancy	Gestation at Delivery (days)	Pathology at time of discharge

Table 11: Side effects - - median (IQR)

	Progesterone n=	Placebo n=	Median Difference (99% CI)
At 7 weeks gestation Nausea / Vomiting Bloated feeling Vaginal discharge Vaginal irritation At 12 weeks gestation Nausea / Vomiting Bloated feeling Vaginal discharge Vaginal irritation			

Table 12: Adherence to trial medication at 7 and 12 weeks -- counts and percentages

	Progesterone n=	**Placebo n=**
Adherence at 7 weeks gestation Adherence at 12 weeks gestation		