UNIVERSITY OF SOUTHAMPTON

Thesis of Julian Michael Jenkins

The development and influence of functional ovarian cysts during *in vitro* fertilisation cycles.

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Doctor of Medicine

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

OBSTETRICS AND GYNAECOLOGY

Doctor of Medicine

THE DEVELOPMENT AND INFLUENCE OF FUNCTIONAL OVARIAN CYSTS DURING *IN VITRO* FERTILISATION CYCLES.

by Julian Michael Jenkins

The thesis studied the development of functional ovarian cysts during pituitary downregulation, and the influence of functional ovarian cysts during IVF cycles in relation to steroidogenic activity.

Reviewing 780 IVF cycles with buserelin commenced in the luteal phase and human menopausal gonadotrophin administered from day 4 of the ensuing menses 53 cycles with ovarian cysts were identified. Of the 53 cycles with cysts day 4 serum oestradiol concentrations were significantly ($p<0.05$) higher in cancelled cycles than cycles proceeding to oocyte retrieval, but no significant difference was seen in day 4 serum progesterone and androstenedione concentrations. Twenty three of the latter 53 cycles had a day 4 serum oestradiol >200 pmol/l (95th centile for cycles without apparent cysts). These 23 cycles had a significantly higher cancellation rate ($p<0.05$) and fewer grade 1 embryos per oocyte retrieval ($p<0.05$) than the other 30 cycles with cysts.

Of a further 288 IVF cycles studied, functional ovarian cysts (with day 4 serum oestradiol >200 pmol/l) developed in 16 cycles during pituitary downregulation. Cysts were aspirated in 10 of the latter 16 cycles. Oestradiol, androstenedione and progesterone concentrations were measured in 9 of the latter cyst aspirates. The cyst aspirates had significantly lower progesterone ($p<0.001$), higher androstenedione ($p<0.01$) and similar oestradiol concentrations compared to 10 follicular fluid samples collected at the time of oocyte retrieval. Further support that the cysts were follicular cysts came from a significant ($p<0.01$) rise in serum oestradiol concentrations despite a significant ($p<0.01$) fall in serum progesterone concentrations from the day buserelin had been commenced to day 4 of the IVF cycle. Following cyst aspiration there was a significant ($p<0.05$) fall in serum oestradiol concentrations, and 9 of the 10 patients proceeded to oocyte retrieval resulting in 5 conceptions.

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I am deeply indebted to Dr Fred Anthony for his tireless support and encouragement particularly with regard to experimental design, laboratory procedures and data analysis. I am also grateful to Dr Peter Wood for his invaluable advice and assistance with the androstenedione assays.

This thesis has been a clinical study on patients during in vitro fertilisation cycles. Success requires a team effort and I wish to acknowledge the work and support of my fellow clinician Dr David Davies, two outstanding embryologists Miss Stephanie Gadd and Mr Richard Watson, a dedicated laboratory technician Mrs Irene Pace, and all the other members of the team whose aim is to help infertile couples.

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Publications

Jenkins, J.M., Davies, D.W., Anthony, F.W., Wood, P., Gadd, S.C., Watson, R.H. and Masson, G.M., 1992, The detrimental influence of functional ovarian cysts during in vitro fertilisation cycles. *Human Reproduction*, **7**, 776-780.

Jenkins, J.M., Davies, D.W., Anthony, F.W., Wood, P., and Masson, G.M., 1992, The influence of functional ovarian cysts on IVF cycles related to serum steroid levels. *British Journal of Obstetrics and Gynaecology*, **99**, 526.

Scientific Meeting Presentations

Jenkins, J.M., Davies, D.W., Anthony, F.W., Masson, G.M., Gadd, S.C. and Watson, R.H., 1990, The detrimental effect of functional ovarian cysts on buserelin/human menopausal gonadotrophin IVF cycles. Presented at the First International Scientific Meeting of the Royal College of Obstetricians & Gynaecologists, Singapore (**see footnote**[1]).

Jenkins, J.M., Davies, D.W., Anthony, F.W., Wood, P. and Masson, G.M., 1991, The influence of functional ovarian cysts on IVF cycles related to serum steroid levels. Presented at the Blair Bell Research Society Meeting, Belfast.

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[1] **Above paper was presented with the young MRCOG award by the RCOG.**

Jenkins, J.M., Lee, A., Davies, D.W., Anthony, F.W., Gadd, S.C. and Masson, G.M., 1991, Relationship between follicular activity preceding IVF and the outcome of buserelin/HMG cycles. Presented at the 8th annual conference of ESHRE, Paris, France.

Jenkins J.M. and Anthony F., 1990, The role, if any, of biochemical monitoring in assisted reproduction. Presented at the meeting of the Association of Clinical Biochemists, Southampton.

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Jenkins, J.M., Anthony, F.W., Wood, P., Rushden, D., Masson, G.M. and Thomas, E., 1992, The development of functional ovarian cysts during pituitary downregulation. Presented at the joint meeting of the Society for the Study of Fertility and the British Fertility Society, Bristol University.

Abbreviations

ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cm	centimetre
$P450_{scc}$	cholesterol side chain cleavage system
$P450_{c17}$	17-hydroxylase/c17,20 lyase
$P450_{aro}$	aromatase system
FSH	follicle stimulating hormone
HCG	human chorionic gonadotrophin
HMG	human menopausal gonadotrophin
^{125}I	radioactive isotope of iodine
IRMA	immunoradiometric assay
IVF	in vitro fertilisation
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
LHRHa	luteinising hormone releasing hormone agonist
mm	millimetre
MRCOG	Member of the Royal College of Obstetricians and Gynaecologists
NS	not significant
OR	oocyte retrieval
pH	logarithm to the base 10 of the reciprocal of the hydrogen-ion concentration
RCOG	Royal College of Obstetricians and Gynaecologists
RIA	radioimmunoassay
SD	standard deviation
TSH	thyroid-stimulating hormone
UK	United Kingdom

Abbreviations

SI Units	Système International d'Unités
mmol/l	10^{-3} moles per litre
µmol/l	10^{-6} moles per litre
nmol/l	10^{-9} moles per litre
pmol/l	10^{-12} moles per litre

Conversion between SI Units and Imperial Units

Progesterone	nmol/l	=	ng/ml x 3.18
Oestradiol	pmol/l	=	pg/ml x 3.67
Androstenedione	nmol/l	=	ng/ml x 3.5

INTRODUCTION

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This thesis considers the influence of functional ovarian cysts during IVF cycles, and the mechanism by which cysts may develop during the treatment. The term "functional ovarian cyst" is commonly used to refer to an ovarian cyst arising from physiological dysfunction, which may be idiopathic or secondary to pathology or medical treatment. However, the steroidogenic activity of functional ovarian cysts varies considerably. It is this steroidogenic activity of functional ovarian cysts, which this thesis particularly addresses with relation to the development of the cysts and their influence on the outcome of IVF cycles.

In the first successful human in vitro fertilisation cycle an oocyte was collected during a natural cycle (Edwards and Steptoe, 1979). It was later established that pregnancy rates could be increased if there were more embryos available for replacement, and thus controlled ovarian hyperstimulation with gonadotrophins +/- clomiphene prior to oocyte retrieval was introduced (Fischel, 1986). Unfortunately during controlled ovarian hyperstimulation oestradiol feedback from multiple follicles resulted in premature luteinisation and cancellation of between 10-30 % of IVF cycles (Loumaye, 1990). To overcome the problem of high endogenous LH concentrations and premature luteinisation LHRH analogues were introduced to downregulate the pituitary gland prior to ovulation induction with gonadotrophins (Flemming *et al.* 1982) and controlled ovarian hyperstimulation with gonadotrophins preceding IVF (Porter *et al.*, 1984). When LHRH agonists were first used for the latter purpose there was little mention made of the development of functional ovarian cysts during their administration (Flemming *et al.*, 1982, Porter *et al.*, 1984). Subsequently reports emerged to confirm that functional ovarian cysts may develop during LHRH agonist administration (Meldrum *et al.*, 1988, Feldberg *et al.*, 1989). Controversy existed whether

spontaneous functional ovarian cysts influenced IVF cycles (Thatcher *et al.*, 1988, Hornstein *et al.*, 1989), and it seemed that functional cysts developing during LHRHa administration may not have a significant influence on the outcome of IVF cycles (Ron-El *et al.*, 1989, Sampaio *et al.*, 1991). However, this thesis will critically evaluate the literature and present studies to show that functional ovarian cysts may detrimentally influence IVF cycles.

The thesis presents 4 studies. Study 1 considers whether there is a relationship between steroidogenic activity of functional ovarian cysts and an influence on IVF cycles. Study 2 investigates the development of functional ovarian cysts during LHRH agonist administration. Study 3 investigates steroidogenesis within functional ovarian cysts. Study 4 uses the knowledge derived of the development and influence of functional ovarian cysts to establish effective clinical management of patients with functional ovarian cysts during IVF cycles.

The thesis is presented in 4 sections - introduction, methods, results/discussions and a conclusion. References cited in all 4 sections are grouped together at the end of the thesis. At the start of each section there is an outline of its content.

The introduction reviews the literature covering the scope of the thesis from which are derived 3 hypotheses. The objectives of the studies subsequently presented in the thesis are defined.

The methods chapter 2 describes the protocols followed on the Southampton IVF programme and describes the clinical management of the patients during the 4 studies. The methods chapter 3 covers the assays used during the 4 studies.

The results/discussion section consists of 4 chapters with a chapter devoted to the results and discussion of each of the 4 studies in turn.

The concluding chapter draws together all the findings concerning the development and influence of functional ovarian cysts during IVF cycles and discusses future implications of this thesis.

Chapter 1

Literature Review

This chapter reviews the literature relevant to the development and influence of functional ovarian cysts during IVF cycles. The definition of functional ovarian cysts is presented in histological and clinical terms. The current understanding of the normal development of ovarian follicles and the development of the various types of functional ovarian cysts is reviewed. Particular attention is paid to the development of functional ovarian cysts during pituitary downregulation with LHRH agonists, which are now used increasingly during IVF cycles. Ovarian steroidogenesis is reviewed firstly in ovarian follicles then in functional ovarian cysts considering the influence this may have on folliculogenesis. The literature regarding functional ovarian cysts during IVF cycles is assessed with regard to any influence the cysts may have on the IVF cycles and the possible mechanisms of action. Three hypotheses are formulated from the literature and the objectives of the subsequent studies are described.

1.1 Definitions of functional ovarian cysts

Non-neoplastic ovarian cysts can be defined by histological examination into hormonally functional and non-functional types (Robboy *et al.*, 1988). However, in clinical practice histological diagnosis is often not possible as functional ovarian cysts may resolve without surgical intervention. The histological classification of non-neoplastic ovarian cysts will thus first be described followed by consideration of the definition of functional ovarian cysts used in routine clinical practice.

1.1.1 Histological classification of non-neoplastic ovarian cysts (Robboy *et al.*, 1988)

Non-functional ovarian cysts

Serous inclusion cysts form from an invagination of the surface epithelium of the ovary, which becomes separated from the overlying epithelium. The cysts are lined by cuboidal, endometrial, or serous epithelium. These cysts are common and may be single or multiple, but they are usually less than 1 cm in diameter.

Functional ovarian cysts

Follicular cysts arise from graffian follicles undergoing atresia thus they lack an ovum. Although usually small and multiple they may occasionally reach over 5 cms diameter. They have a smooth wall of one or more layers of granulosa cells, with an external layer of thecal cells, although sometimes the epithelial lining is lost. They are usually unilocular containing clear, watery fluid and sometimes fresh or altered blood.

Corpora Lutea cysts are formed by delayed resolution of the cavity of corpora lutea. The wall of the cyst is fibrous or hyaline without a distinct epithelial lining, and is surrounded by a scalloped shell of luteinised granulosa cells.

Theca-Lutein cysts are associated with high levels of circulating gonadotrophins and are due to excessive stimulation of the theca interna. The high levels of gonadotrophins may be due to exogenous gonadotrophin therapy to induce ovulation, pregnancy, hydatidiform mole or choriocarcinoma.

Polycystic ovary syndrome is classically associated with a particular histological appearance. The ovary is enlarged with a thick smooth capsule containing numerous small (around 5 mm diameter) cystic subcapsular follicles separated by hyperplastic stroma. The follicles are lined by thick zones of theca interna, and both the thecal cells and stroma may be luteinised in parts.

1.1.2 Definition of Functional Ovarian Cysts in Clinical Practice

Clinical definition of functional ovarian cysts does not have the advantage of histological examination, and instead usually relies on the patient's history, clinical examination and ultrasound. Ultrasound provides a useful non-invasive method of examining a pelvic mass to determine whether it is likely to be a functional ovarian cyst. By ultrasound a functional ovarian cyst is identified as an intraovarian sonolucent structure (see figure 1.1), but it is necessary to distinguish the latter from a neoplastic cyst, an endometrioma, a non-ovarian structure and a normal follicle.

Ultrasound has been criticised as failing to diagnosis neoplasia in 4% (Herman *et al.*, 1987) to 9% (Meire *et al.*, 1978) of patients with ovarian cysts and incorrectly diagnosing neoplasia in 4% (Meire *et al.*, 1978) to 27% (Herman *et al.*, 1987) of cases. Transabdominal 3.5-MHz to 5-MHz ultrasound probes have a relatively low resolution, high attenuation of the transmitted signal and an inadequate focal length (especially in the obese patient) to correctly identify pelvic structures with a similar acoustic impedance (Thaler, 1988). The latter limitations can to some extent be overcome by using a high-frequency (5 MHz to 7 MHz) transvaginal ultrasound probe (Timor-Tritsch *et al.*, 1988). The features on ultrasound suggestive of neoplasia include solid elements within the cyst, septation of the cyst, papillary projections extending from the inner wall of the cyst and a diameter of greater than 10 cm (Rottem *et al.*, 1990).

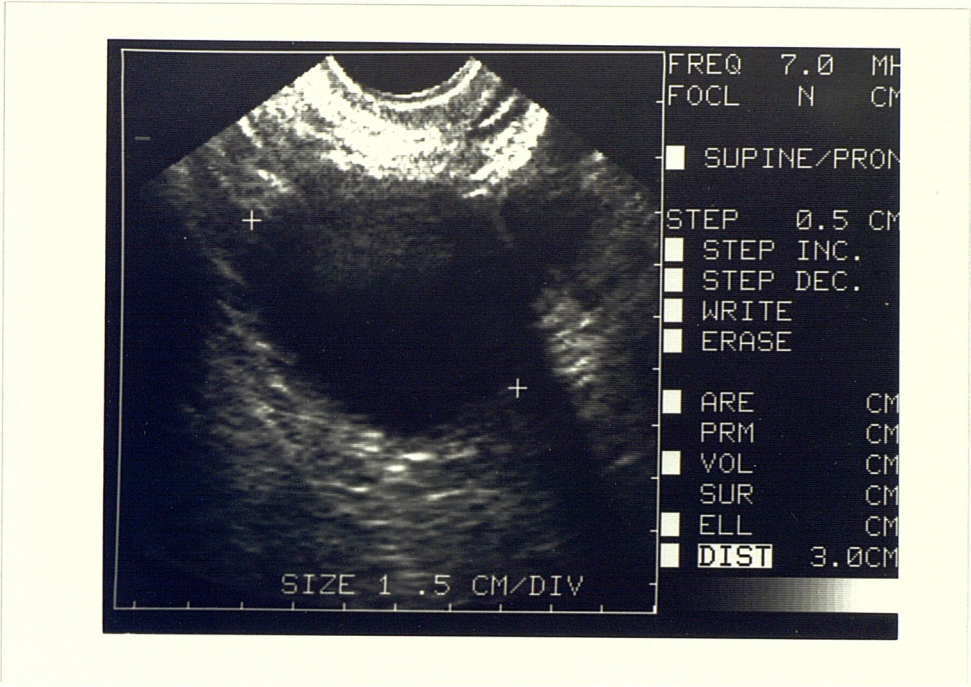


Figure 1.1 Transvaginal ultrasound of functional ovarian cyst

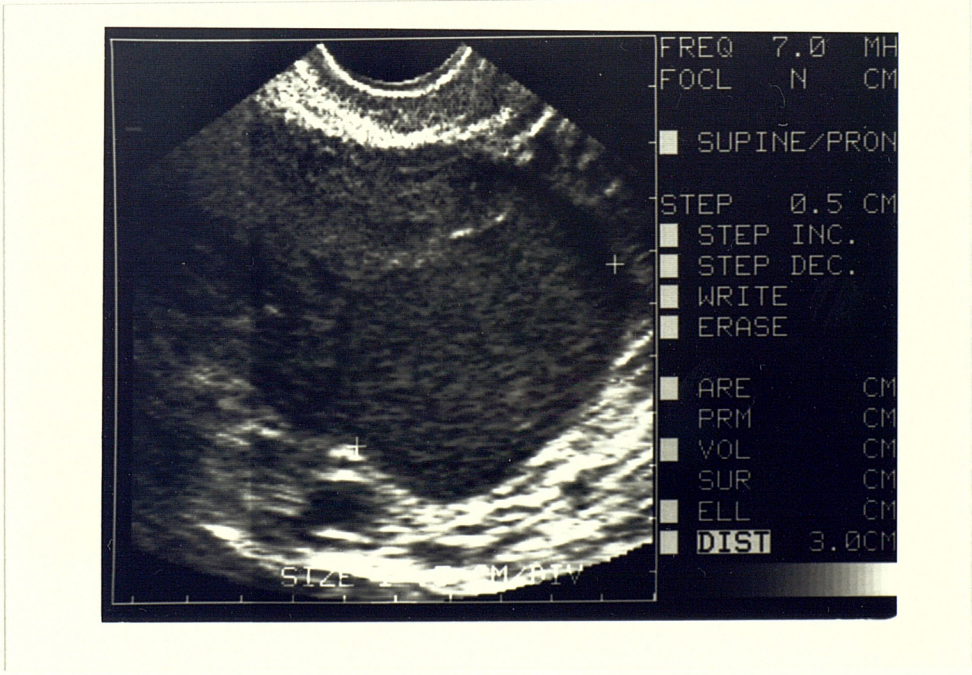


Figure 1.2 Transvaginal ultrasound of endometrioma

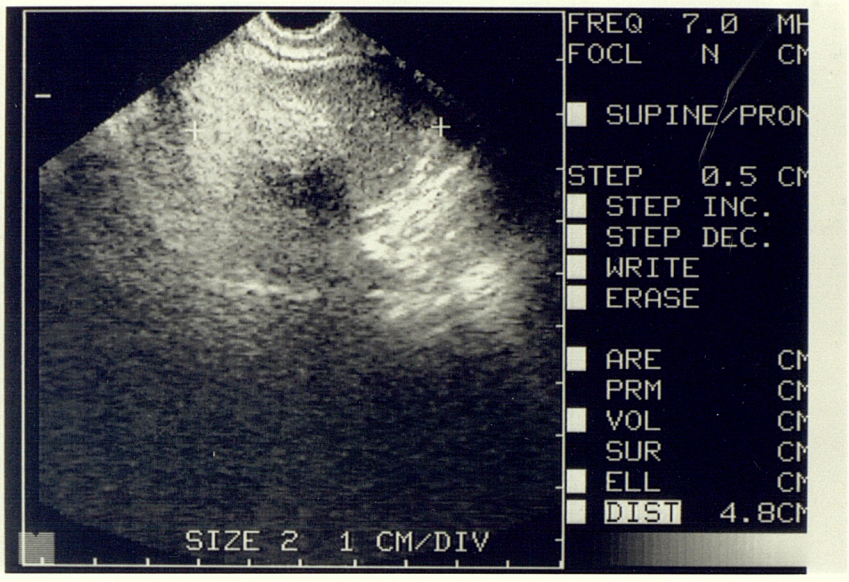


Figure 1.3 Transvaginal ultrasound of cystic teratoma

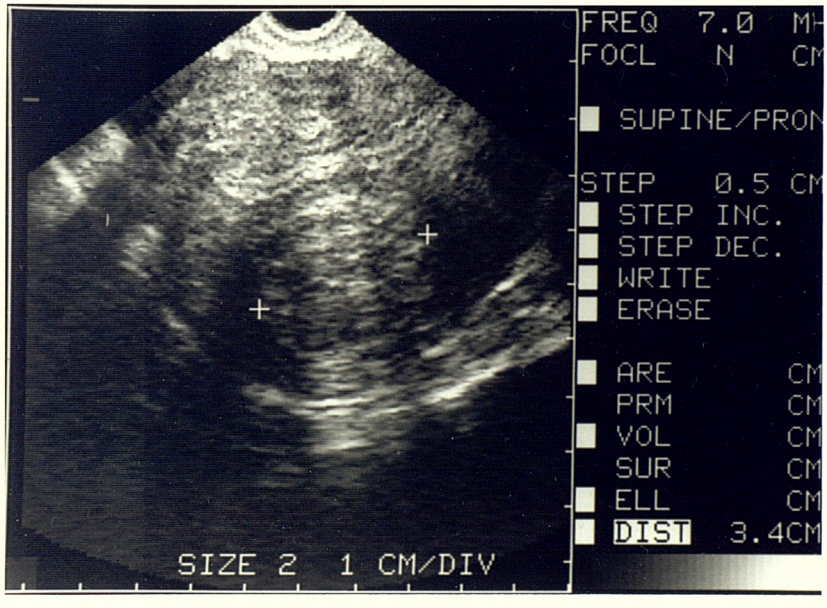


Figure 1.4 Transvaginal ultrasound of fibroid

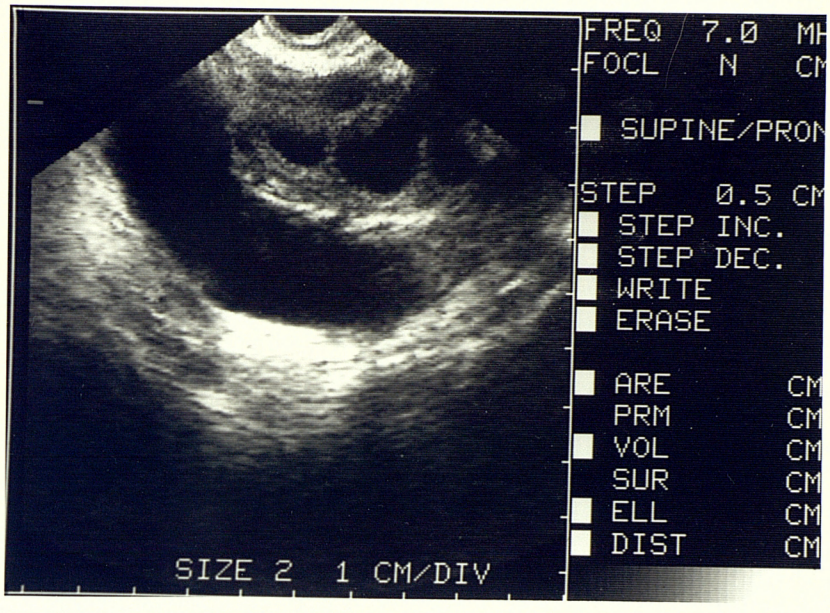


Figure 1.5 Transvaginal ultrasound of hydrosalpinx

The internal echo appearance on ultrasound may distinguish functional ovarian cysts from certain other intraovarian structures such as blood filled cysts from endometriosis (Reynolds *et al.*, 1986) or cystic teratomas (Quinn *et al.*, 1985) illustrated in figures 1.2 and 1.3 respectively. As can be seen in the accompanying figures using transvaginal ultrasonography it is usually possible to differentiate between a functional ovarian cyst (figure 1.1) and a non-ovarian structure such as a fibroid (figure 1.4) or a hydrosalpinx (figure 1.5).

To distinguish between a functional ovarian cyst and an ovarian follicle with a single ultrasound scan can prove difficult or even impossible. In the late follicular phase of the menstrual cycle it is not possible to distinguish by ultrasound between an 18 mm cyst and an 18 mm follicle. Measurement of the size of an ovoid follicle or an ovoid ovarian cyst presents a further problem as the diameters of an

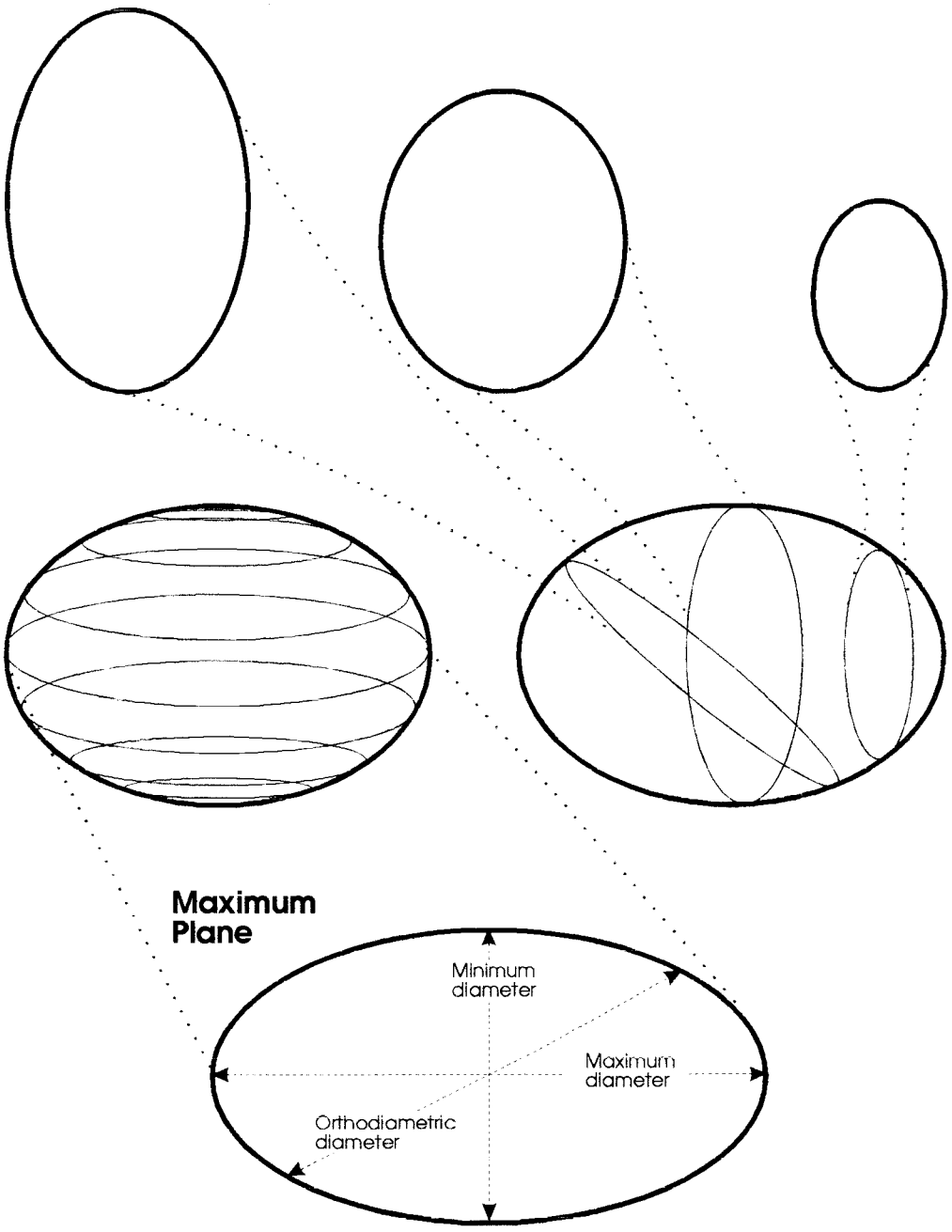


Figure 1.6 The diameters of an ovoid are dependent on the plane of measurement. (Measurements of a cross section of an ovoid include a minimum diameter, a maximum diameter and an orthodiametric diameter).

ovoid are dependent on the plane of measurement (see figure 1.6). One simple method of representing the size of a cyst is to measure the orthodiametric diameter in the largest plane of the cyst. The volume of an ovary can be calculated from the prolate epillipsoid formula (Fleischer, 1991):

length x height x width x 0.5 = volume

By measuring a cyst in 3 perpendicular diameters it is thus possible to estimate the volume of the cyst, or alternatively the size of the cyst may be represented as a mean diameter. A cyst may be distinguished from a follicle either because it is an inappropriately large size for the stage of the menstrual cycle in which it is identified or because the cyst does not change in size in relation to the menstrual cycle.

Classification of functional ovarian cysts into the types described by Robboy and his colleagues (1988) is not possible by ultrasound alone, although the clinical history may suggest a particular histological type.

1.2 Development of Functional Ovarian Cysts.

Functional ovarian cysts may result from disordered follicular development or delayed corpus luteal regression. After outlining the development of ovarian follicles the development of the various types of functional ovarian cysts will be considered. With particular reference to IVF cycles the literature regarding the development of functional ovarian cysts during pituitary downregulation will be reviewed.

1.2.1 Development of Ovarian Follicles

Gougeon (1982) has shown that the time from when the follicle first differentiates its theca interna until it reaches ovulatory size takes approximately 2 months. Independent of gonadotrophin stimulation primordial follicles within the cortex of the ovary develop to reach the antral stage, with a fluid filled antral cavity surrounded by an inner granulosa cell layer and an outer thecal cell layer separated by the lamina basalis (Gougeon, 1982). At the time of luteal regression there are up to 20 small antral follicles (2-5 mm diameter) in the ovaries (McNatty, 1982, Baird, 1988).

As the corpus luteum regresses pre-ovulatory follicular development is stimulated by increasing pituitary FSH secretion resulting from decreasing inhibition of pituitary FSH release by steroids and inhibin produced by the corpus luteum (Hsueh *et al.*, 1989). The only ovarian cells to possess significant FSH receptors are granulosa cells, and following binding of FSH to its receptor there is activation of adenyl cyclase (Richards *et al.*, 1987). In response to sustained stimulation from FSH over 10-12 days the granulosa cell content of a 5 mm antral follicle doubles 5 or 6 times to reach over 50 million, and attains a diameter of >20 mm (McNatty, 1981). Follicular fluid is

increased in part by FSH induced increased secretion of glycosaminoglycans (Ax and Ryan, 1979, Yanagashita *et al.*, 1981).

Although it is not completely understood why in human folliculogenesis usually only one follicle develops fully and ovulates each menstrual cycle, the threshold hypothesis has been proposed as a possible explanation for the selection of the dominant follicle (Brown, 1978). Brown (1978) treated patients with initially a low daily dose of a human pituitary gonadotrophin extract and increased the dose at 4-9 daily intervals. He found that as little as a 10-30% increase from the penultimate dose achieved a threshold level sufficient to induce a single follicle to undergo complete preovulatory maturation with ovulation induced by HCG, whereas a further increase beyond this threshold level resulted in excessive stimulation. Hillier (1981) has suggested that this threshold for follicular development may be due to several factors including:

a) the blood supply may differ between follicles varying the amount of gonadotrophin supplied to the individual follicles

b) there may be a difference in the number of cells in a follicle and/or the number of receptors per cell thereby altering the uptake and response to stimulation of gonadotrophins.

c) follicles may contain differing amounts of substances which modulate gonadotrophin stimulation thus resulting in differing responses of follicles to a fixed amount of gonadotrophin.

Once the threshold for a follicle has been achieved FSH induced aromatase will increase oestrogen secretion from this follicle exerting a negative feedback on the pituitary reducing FSH secretion thereby preventing the growth of other follicles (Hillier, 1981). Further the selected follicle will have increased sensitivity to gonadotrophins through the induction of LH receptors and the presence of gonadotrophin modulating factors (Hillier, 1981). The FSH dependent

development of follicles with a higher FSH threshold is thus prevented, and the selected follicle continues to develop due to increased sensitivity to FSH and LH.

Ovulation is initiated by a surge of LH released from the pituitary gland (Yen *et al.*, 1975). Although the exact mechanism of the pre-ovulatory discharge of LH is unknown, it seems likely that it is due to positive feedback of oestradiol on the pituitary and hypothalamus (Baird, 1990).

Rupture of the graafian follicle is initiated by LH acting through diverse second-messenger systems in granulosa and thecal cells. The cellular layers which must be broken down to allow rupture to take place are illustrated in figure 1.7. The effects of LH on the preovulatory follicle are mediated by LH binding to specific high affinity receptors on the surface of thecal and mural granulosa cells (Amsterdam *et al.*, 1975). Although thecal cells express LH receptors throughout antral follicular development, granulosa cells only develop LH receptors following cell differentiation induced by FSH (Channing *et al.*, 1977). The main post receptor signalling systems involve adenyl cyclase and arachidonic acid metabolism, which will be outlined in turn.

Following binding of LH to its receptor there is activation of membrane associated adenyl cyclase, which converts ATP to cAMP (Marsh, 1976). The signal is further amplified by cAMP binding to the regulatory subunits of protein kinase A, which thereafter phosphorylates specific proteins, which then either increase steroidogenesis directly, or exert a longer term influence by stimulating the transcription of cAMP regulated genes (Kurten and Richards, 1989).

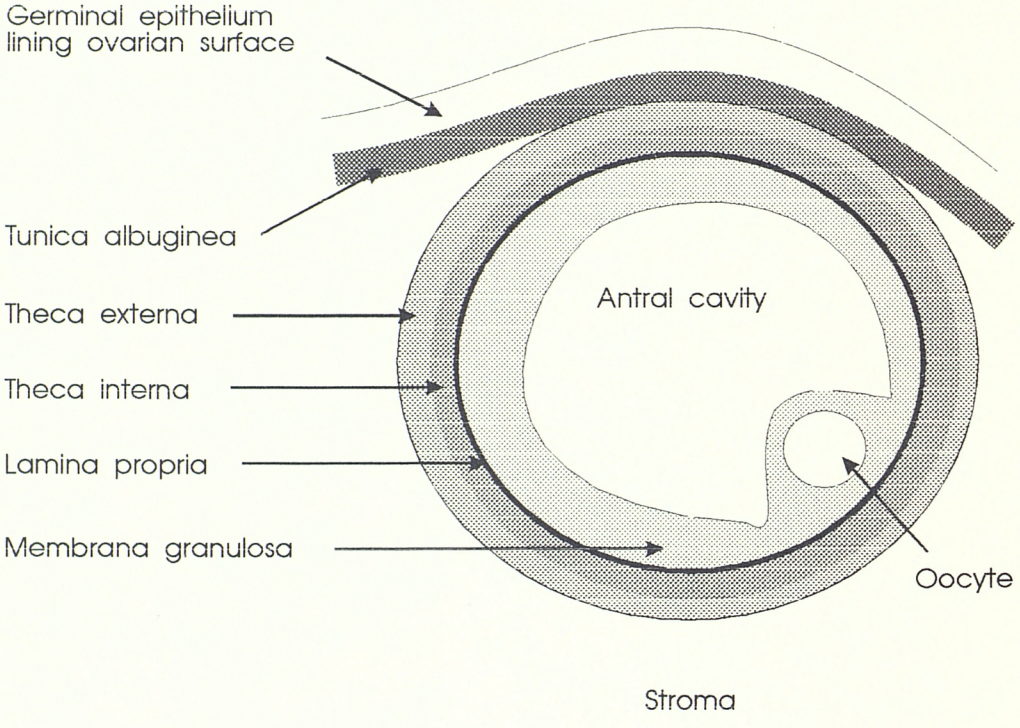


Figure 1.7 Diagram of pre-ovulatory follicle illustrating the cellular layers, which must be broken down to allow follicular rupture to take place.

LH once bound to its receptor activates phospholipase C and/or A_2 which act on phospholipids in follicular cell-surface membranes releasing arachidonic acid, which is either metabolised via the cyclooxygenase pathway to prostaglandins, prostacyclins and thromboxanes, or alternatively may be metabolised via the lipoxygenase pathway to leukotrienes (Brannstrom and Janson, 1991).

Prostaglandins have an important role in ovulation, and the concentration of prostaglandins increase significantly in the follicles

of most mammalian species in the preovulatory period (Lipner, 1988). Preventing prostaglandin synthesis with aspirin or indomethacin has been shown to block ovulation in primates (Armstrong, 1981), and results in luteinised unruptured follicles in women (Killich and Elstein, 1987). Prostaglandins are involved in the pituitary release of gonadotrophins (Harms *et al.*, 1974), but a direct action of prostaglandins on the ovary is supported by prostaglandin inhibitors preventing ovulation even following the onset of the LH surge (Tsafriri *et al.*, 1973).

Follicular rupture is promoted by prostaglandins which lead to weakening of the follicular wall, and the subsequent expulsion of the oocyte is aided by the generation of a hydrostatic force due to prostaglandin action. Prostaglandin E_2 stimulates granulosa cells in vitro to produce plasminogen activator (Strickland and Beers, 1976), which leads to the conversion of an inactive proenzyme to active collagenase thereby promoting the breakdown of collagen and the weakening of the follicular wall (Curry *et al.*, 1986). By chemotaxis prostaglandins attract leucocytes (Kaley and Weiner, 1971), which secrete proteases (Weissman *et al.*, 1973) further weakening the follicle wall leading to eventual follicle rupture. Prostaglandins E_2 and I_2 increase capillary permeability leading to the extravasation of blood cells and the efflux of plasma proteins including plasminogen, and the resultant communication between the antral cavity and the vasculature produces a hydrostatic force within the follicle to aid the expulsion of the oocyte (Espey, 1980).

1.2.2 Development of Follicular cysts

From the above it could be speculated that a follicular cyst will develop if a preovulatory follicle develops but fails to rupture, which may be due to the absence of a LH surge. Whereas the combined oral

contraceptive pill suppresses follicular development, the progestogen only pill may not suppress follicular development in some patients but it may nevertheless interfere with the midcycle LH surge (Guillebaud, 1983).

Vessey (1987) reviewed 17,000 patients taking part in the Oxford Family Planning Association contraceptive study. He found that follicular ovarian cysts occurred less frequently in patients taking combined oral contraceptive pills than patients not using oral contraceptives. However, the progestogen only pill was associated with a higher frequency of follicular cysts.

Tayob (1985) studied 21 progestogen only pill users with regular menstrual cycles. Ultrasound scans of the ovaries following menstruation revealed ovarian cysts between 30 mm and 58 mm diameter in 8 patients. Of the 13 patients without ovarian cysts 4 patients were found by ultrasound to develop cysts during the menstrual cycle.

Follicle cyst formation may thus occur as a result of disordered folliculogenesis without follicle rupture.

1.2.3 Development of Theca Lutein cysts

Theca lutein cysts develop in situations of excessive gonadotrophin stimulation such as in choriocarcinoma, hydatidiform mole, pregnancy, or during ovulation induction with exogenous gonadotrophin therapy (Robboy *et al.*, 1988).

Tummon (1988) found ovarian cysts (15-60 mm) after the onset of menses in 40 of 71 nonconception cycles following ovulation induction with HMG and HCG. Coulam (1983) performed serial ultrasound

scans on 8 patients, who had failed to conceive despite apparent ovulation on treatment with clomiphene ±HCG or gonadotrophins and HCG, and found failure of follicular rupture with the formation of a persistent cyst in 10 of 21 cycles studied. In 1 of the latter 10 cycles stimulation with clomiphene and HCG had resulted in the development of a 4.8 cms cyst, which was biopsied revealing histological evidence of luteinisation.

Ovarian hyperstimulation syndrome with theca lutein cyst formation does not develop if HCG is withheld even with high oestradiol levels (Hancock *et al.,* 1970). This illustrates the importance of LH/HCG stimulation in the pathogenesis of theca lutein cyst formation.

Further support of the role of LH/HCG stimulation in the development of theca lutein cysts comes from studies of hydatidiform moles, which may be associated with prolonged high serum levels of HCG. Theca Lutein cysts have been reported in association with hydatidiform moles in 14-30% of cases (Montz *et al.,* 1988, Curry *et al.,* 1975, Morrow *et al.,* 1977, Hobson, 1958, Coppleson, 1958).

1.2.4 Development of Corpora Lutea cysts

Corpora lutea cysts may result from a delayed resolution of the cavity of corpora lutea (Robboy *et al.,* 1988). Luteinized unruptured follicles have also been noted to form when follicles fail to rupture following a LH surge (Marik and Hulka, 1978). The aetiology of the latter is unknown, although experimentally the condition may be mimicked by giving prostaglandin synthesis inhibitors prior to ovulation (Killick and Elstein, 1987).

1.2.5 Development of Functional Ovarian Cysts during Pituitary Downregulation with LHRH agonists

To improve ovarian response during controlled ovarian hyperstimulation preceding IVF LHRH agonists were introduced to induce a reversible hypogonadotrophic, hypogonadal state (Porter *et al.*, 1984). Although premature LH surges may be largely avoided by using LHRHa (Flemming and Coutts, 1986), LHRHa may be less useful in improving the number and quality of oocytes developing during ovarian stimulation (Mckenna *et al.*, 1989, Jenkins *et al.*, 1991). It has been noted that LHRHa may cause the development of ovarian cysts (Felberg *et al.*, 1989), and the literature regarding this will now be reviewed.

Feldberg (1989) noted the development of ovarian cysts when both a depot LHRH agonist (decapeptyl) was injected subcutaneously between the first and third day of the menstrual period and also when a short half life LHRH agonist (buserelin) was administered nasally starting on day 1 to 3 of the cycle. Of 24 patients receiving decapeptyl 5 patients developed solitary ovarian cysts. Of 22 patients receiving buserelin 7 patients developed solitary ovarian cysts.

Meldrum (1988) found that cysts could form whether the LHRH agonist leuprolide was commenced in the early follicular phase or the mid luteal phase of the menstrual cycle. Herman (1990) compared the development of cysts following menstrual versus midluteal administration of a depot LHRH agonist. Of 198 IVF cycles with a depot LHRH agonist given during menstruation 27 (13.6%) developed cysts, and of 123 IVF cycles with a depot LHRH agonist given in the midluteal phase 19 (15.4%) developed cysts (Herman *et al.*, 1990). Ovarian cysts thus may develop not infrequently with both depot and

short half life LHRH agonist preparations, whether commenced in the follicular or luteal phase of the menstrual cycle.

Mode of action of LHRH agonists The modification of the decapeptide LHRH in positions 6, 9, and 10, especially the substitution of D-amino acids which are resistant to degradation, results in a range of powerful agonists with potencies at least 50 times greater than that of the natural decapeptide (Karten and Rivier, 1986). The significant increase in biological activity can be attributed to an increased resistance to enzymatic degradation illustrated in figure 1.8 (Koch *et al.*, 1977) and to increased affinity for the LHRH receptors (Clayton and Catt, 1980).

The pituitary response to LHRH agonists shows a biphasic pattern composed of an initial stimulatory phase, the so called 'flare-up effect', followed by an inhibitory phase (Sandow *et al.*, 1978). This phenomenon, common to a number of peptide receptor systems, is known as cell desensitization or pituitary downregulation (Catt *et al.*, 1979).

Pituitary downregulation due to LHRH agonists is characterised by a number of features. There is a partial reduction in the number of plasma membrane receptors (Loumaye and Catt, 1983). However, in some circumstances there may be impaired response to LHRH in the presence of normal LHRH binding capacity suggesting the LHRH agonist is having an influence beyond the cell surface receptor (Loumaye and Catt, 1983). Of particular interest in the post receptor influence of LHRH agonists is a possible uncoupling between the LHRH receptor and the GTP binding protein (Conn *et al.*, 1987). The pituitary content of LH is reduced and there is an increased synthesis and release of LH free alpha subunit (Meldrum *et al.*,

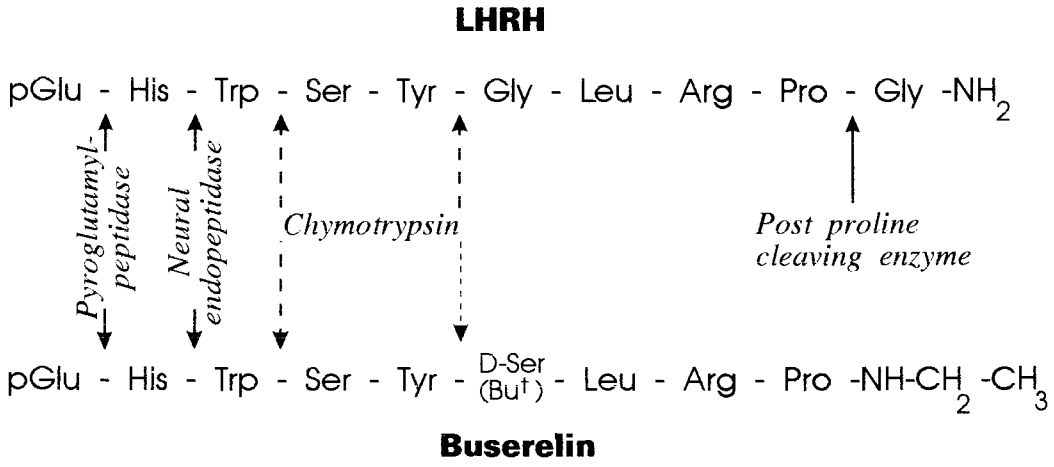


Figure 1.8 Chemical structure of LHRH in comparison to the LHRH analogue Buserelin with the enzymatic inactivation sites indicated by arrows refering to the relevant enzymes shown in italics.

1984). The bioactivity of LH is also reduced (Meldrum *et al.*, 1984), due to modification of gonadotrophin glycosylation which modifies the tertiary structure (Ryan *et al.*, 1987).

The initial administrations of LHRHa induce stimulation of LH and FSH secretion, the maximal effect being at 4-6 hours later (Sandow *et al.*, 1978). When the agonist administration is repeated the duration of response is dependent on the phase of the cycle. If LHRHa is started at the beginning of the cycle, LH stimulation lasts for a few days or up to 4 weeks, depending on dose and frequency of

administration (Loumaye, 1990). When the agonist administration is initiated during the luteal phase, the duration and amplitude of the stimulation is reduced and the 'flare-up' effect is over when menstruation begins (Loumaye, 1990). After this phase of stimulation, the LH concentrations measured by radioimmunoassay return to normal follicular phase values (Sandow, 1983). Interestingly, therefore, these treatments seem unable to completely suppress or even markedly reduce the circulating gonadotrophins measured with regular LH radioimmunoassays (Loumaye, 1990).

This apparent discrepancy between the persistence of normal levels of LH and inhibition of gonadal function led to the investigation of the bioactivity of the gonadotrophins during administration of agonists. When LH bioactivity was evaluated in the serum of women treated with LHRHa, it appeared more suppressed than immunoreactive LH, resulting in a marked reduction of the LH bioactivity to immunoreactivity ratio (Meldrum *et al.,* 1984). Additionally it was found that there was a significant difference in the LH concentrations when measured using an immunoradiometric assay as compared to those obtained using radioimmunoassay (Loumaye *et al.,* 1989). The significance of this discrepancy can be explained in the mechanisms of action of the assays. The RIA is based upon polyclonal antibodies and will cross-react with free alpha sub-units (Loumaye, 1990) and other elements of LH synthesis and breakdown which are not physiologically active. The IRMA on the other hand is based upon monoclonal antibodies which more specifically measures physiologically active LH. The modification of the LH immunoreactivity is consistent with the reduced bioactivity because of increased physiologically inactive sub-unit production observed after prolonged LHRHa administration (Loumaye, 1990).

Pathogenesis of cyst formation Three possible mechanisms for cyst formation during pituitary downregulation have been suggested. Feldberg (1989) suggested that the transient stimulatory phase induced by LHRH agonists may cause small foci of ovarian endometriosis to develop into endometriomata that appear identical to ovarian cysts. Hodgen (1988) has suggested that ovarian cysts may develop because of the presence on ovarian receptors of physiologically inactive heterogonadotropins secreted by the hypophysis depressed by a LHRH agonist. Ron-El (1989) hypothesised that the transient stimulatory phase caused by LHRH agonists stimulates primordial follicles to grow, but because of rapid pituitary downregulation follicular development is impaired, ovulation does not occur and follicular cysts are formed.

1.3 The influence of steroidogenesis in functional ovarian cysts on folliculogenesis.

The literature reveals that as ovarian follicles develop there are significant changes in the steroidogenesis within the follicles. The relationship between follicular development and ovarian steroidogenesis will be presented. The steroidogenic activity of functional ovarian cysts and the consequences this may have on follicular development will be considered.

1.3.1 Steroidogenesis in Ovarian Follicles

Steroids are a group of compounds sharing the cyclopentanoperhydrophenanthrene ring system of sterols and are interconverted by specific enzymes in different cells. The two cell two gonadotrophin model has been proposed to explained ovarian steroidogenic function (Armstrong and Dorrington, 1979), and the predominant pathways of steroidogenesis is governed by the enzymes expressed by the thecal and granulosa cells. The presence of both androstenedione and dehydroepiandrosterone in high concentrations in human follicular fluid (McNatty, 1981, Dehennin *et al.*, 1987) indicates that both the Δ4 pathway (progesterone to androstenedione) and the Δ5 pathway (pregnenolone to dehydroepiandrosterone) are operative in ovarian steroidogenesis (see figure 1.9). The key enzymes governing ovarian steroidogenesis fall into two groups: cytochrome P450 enzymes, which catalyse rate limiting reactions, and hydroxysteroid dehydrogenases, which do not appear to have important regulatory roles (Strauss and Miller, 1991).

Thecal and granulosa cells differ in the synthesis of cytochrome P450 enzymes. Further, the synthesis of the cytochome P450 enzymes changes with follicular development. The cytochrome $P450_{c17}$ is expressed by thecal cells at all stages of follicular development, but

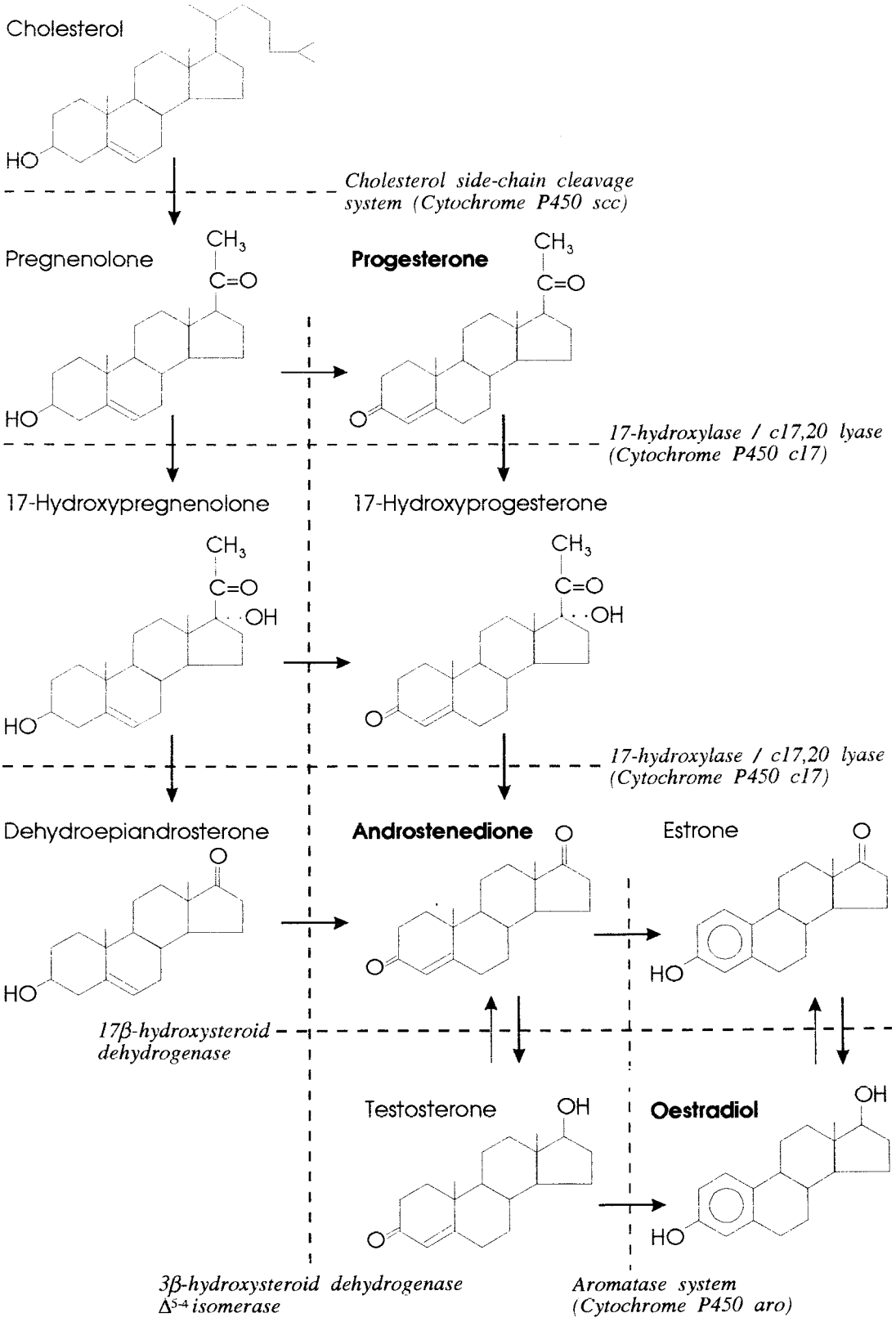
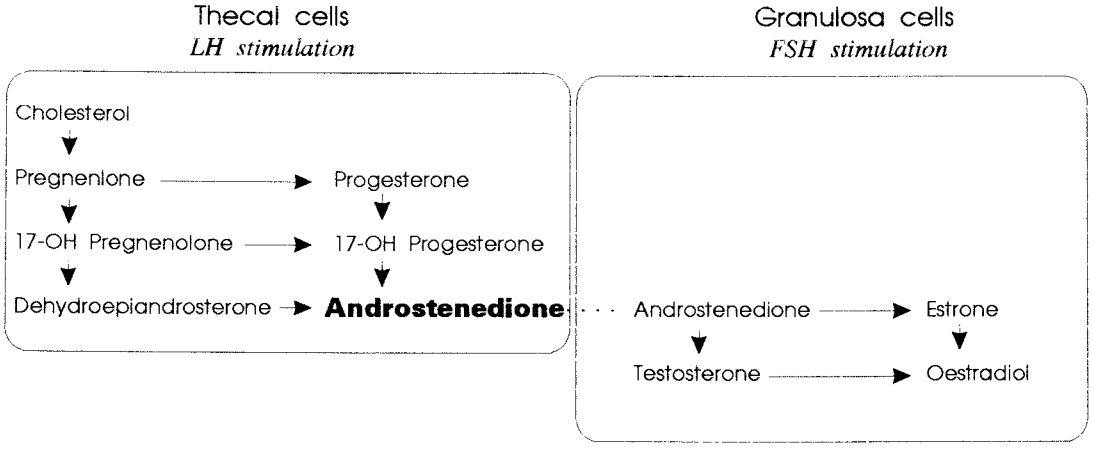
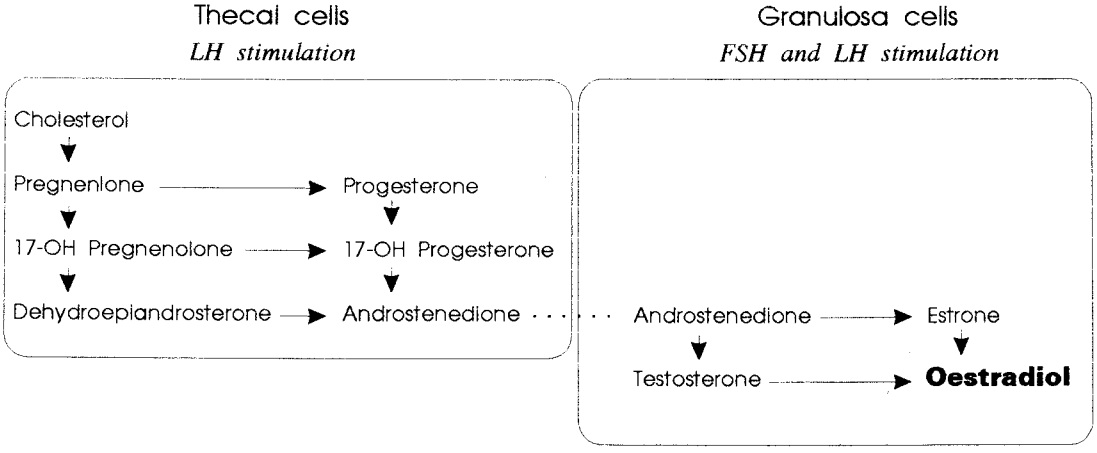


Figure 1.9 Major pathways of ovarian steroidogenesis. (Steroids are named in plain type alongside their chemical structure. Arrows denote the main steroid pathways with broken lines referring to the relevant enzymes, which are indicated in italics).

Predominant steroidogenesis in small (5 mm) unluteinised antral follicles.



Predominant steroidogenesis in mature ovarian follicles prior to luteinisation.



Predominant steroidogenesis in ovarian follicles following luteinisation.

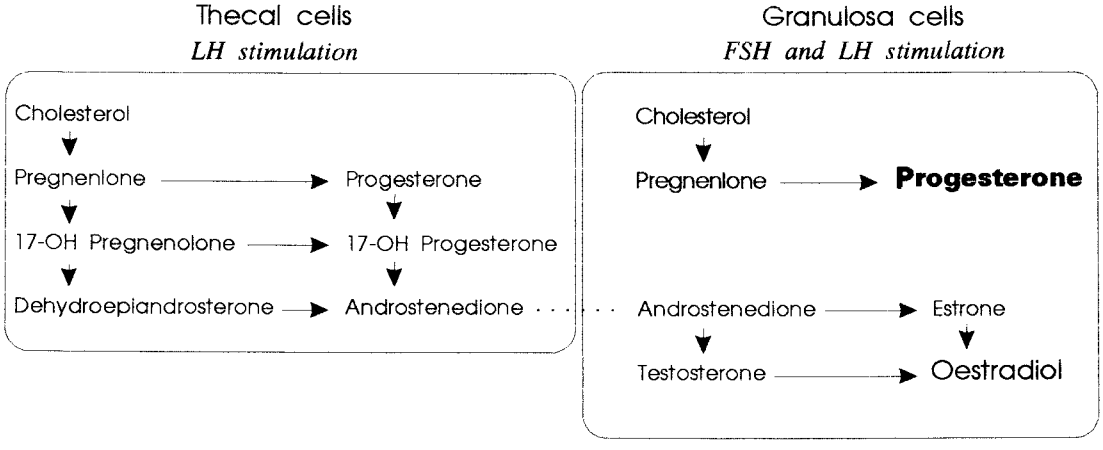


Figure 1.10 Diagramatic representation of predominant steroidogenesis at varying stages of follicular development according to the two cell two gonadotrophin hypothesis.

granulosa cells do not express cytochrome $P450_{c17}$ (Sasano *et al.*, 1989, Richards *et al.*, 1987). The cytochrome $P450_{aro}$ is expressed by granulosa cells and not by thecal cells, but this expression is low in small antral follicles and increases markedly with follicular growth (Hickey *et al.*, 1988, Sasano *et al.*, 1989, Steinkampf *et al.*, 1987). The cytochrome $P450_{scc}$ is expressed by thecal cells at all stages of follicular development, but cytochrome $P450_{scc}$ is only expressed by granulosa cells following luteinisation (Sasano *et al.*, 1989).

Granulosa cells and thecal cells function as a unit, and if one considers the expression of the above cytochrome P450 enzymes at different stages of follicular development with reference to figure 1.9 then the resultant steroidogenic activity will be as represented in figure 1.10. It can thus be seen that the steroid environment of small antral follicles will be androgenic. As the follicle develops the steroid environment will become progressively oestrogenic, and following luteinisation progesterone synthesis increases markedly (figure 1.10). At the time of the LH surge there is desensitisation of the LH receptor (Ekstrom and Hunzicker-Dunn, 1989), and suppression of 17-hydroxylase/C-17,20 lyase activity (Richards *et al.*, 1987) with a net decrease in oestrogen production.

1.3.2 Influence of steroids on folliculogenesis

Due to changes in steroid enzyme activity ovarian steroid production alters as the follicle develops. The implications of the changes in steroid synthesis on follicular development will be considered for oestrogens, androgens and progesterone in turn.

Androgens Although androstenedione is produced by the adrenal gland as well as the ovary, the ovarian contribution to the total serum androstenedione content in the human is ≈30% during the

early follicular phase rising to ≈60% at mid-cycle (Baird 1977). As illustrated in figure 1.5 in the early stage of follicular development there is dominance of androgen production. Androgens augment FSH induced differentiation of cultured granulosa cells (Hillier 1985, Hsueh 1986), and this may lower the threshold requirements for follicular stimulation by FSH. The response of granulosa cells to androgens is governed by the amount of simultaneous FSH stimulation: follicular atresia results if exogenous androgens are given alone (Hillier and Ross, 1979), or ovarian androgen production is stimulated with HCG/LH but no FSH (Louvet *et al.*, 1975). Excessive stimulation with LH inhibits steroid synthesis and promotes atresia possibly through supra-physiological androgen production (Armstrong *et al.*, 1989).

Androstenedione is converted to several potential regulatory steroids dependent on the stage of follicular development regulated by FSH. Androstenedione is converted to the more potent androgens testosterone by 17β-hydroxysteroid dehydrogenase (Bjersing, 1967), and 5α-reduced androgens by 5α-reductase (McNatty *et al.*, 1979). Aromatase activity is induced by FSH stimulation and results in conversion of androstenedione to oestrone and testosterone to oestradiol (McNatty *et al.*, 1979).

Oestrogens Regulatory actions of oestrogens have been demonstrated in several animals, and indirect evidence suggests a regulatory role in human folliculogenesis. Although there is no direct evidence of a role for oestrogens in human folliculogenesis, granulosa cell aromatase activity (Hillier *et al.*, 1981), and follicular fluid oestrogen levels (McNatty, 1981) increase during human folliculogenesis. In the rat oestrogens have been shown in vivo to increase the number of granulosa cell gonadotrophin receptors, to increase the

responsiveness to gonadotrophins, and to stimulate granulosa cell mitosis (Richards, 1980, Hsueh *et al.,* 1984, Goldenburg *et al.,* 1973). It is felt that oestrogen may help in the selection of the dominant follicle through a local positive feedback loop by stimulating granulosa cell proliferation and augmenting responsiveness to gonadotrophins increasing oestrogen production thus further increasing the development of the selected follicle (Hillier, 1981). Oestrogens produced by granulosa cells may also modify thecal cell function (Hillier, 1985), and suppress androgen synthesis during the mid-cycle LH surge (Erickson *et al.,* 1985).

Progesterone Serum progesterone levels rise in parallel to serum LH levels during the midcycle rise of LH (Hoff *et al.,* 1983), and progesterone probably enhances the positive feedback effect of oestradiol at the time of the LH surge (Liu and Yen, 1983). There is evidence to suggest that the peri-ovulatory rise in progesterone contributes to follicular rupture through a direct action on proteolytic enzyme synthesis (Rondell, 1974). However, progesterone inhibitors have been shown to be able to prevent ovulation only if given before peak pre-ovulatory prostaglandin levels are achieved, thus progesterone may act via prostaglandins (Vijayakumar and Walters, 1987).

1.3.3 Steroidogenesis in functional ovarian cysts

The steroid production of functional ovarian cysts depends on the enzyme activity within the cyst which relates to the nature of the cysts. As described above, following luteinisation progesterone production increases and androstenedione production decreases in ovarian follicles. Follicular cysts arise from unluteinised follicles whereas corpora lutea cysts develop from corpora lutea which contain luteinised granulosa cells, thus the pattern of steroid production will

follow this. Theca lutein cysts contain luteinised granulosa and thecal cells, and the cyst fluid has a higher progesterone concentration, a lower androstenedione concentration and a lower oestradiol concentration compared to preovulatory follicles (Vanluchene *et al.*, 1983).

1.4 Studies on Functional Ovarian Cysts during In Vitro Fertilisation Cycles.

Studies regarding functional ovarian cysts during IVF cycles will now be reviewed. Each study will first be appraised with regard to the definition of functional ovarian cyst used, the ovarian stimulation regime employed, and the number of IVF cycles with cysts studied. In these studies the patients with ovarian cysts will be compared to the patients without ovarian cysts. The influence of functional ovarian cysts during the studies will be assessed and the possible mechanisms of influence will be considered. Finally an overview of the studies will be taken to determine the appropriate management of patients with functional ovarian cysts during IVF cycles.

1.4.1 Definitions and Ovarian Stimulation Regimes

Thatcher (1989) defined an ovarian cyst as a discrete, circumscribed echolucent pelvic structure 16 to 60 mm diameter seen on a transvaginal ultrasound scan on the third day of menses. Of 136 patients scanned 6 patients were excluded because they were found to have cysts >60 mm, multiple (>3) or complex, and 4 patients were excluded because the cystic change was outside the ovary. Forty patients were found to have to have cysts >16 mm diameter, and these were subdivided into 17 patients with cysts 16 to 29 mm and 23 patients with cysts >30 mm to <60 mm. The control group for the study was stated as consisting of patients who had no significant

ultrasound findings other than early follicular changes (≤10 mm diameter cystic structures). However, there were 86 patients in the control group thus this would mean there was no patient who had a cystic structure of between 10 to 16 mm diameter. Further Thatcher did not state whether the diameter referred to was the mean or maximum diameter of the cyst thus the definition used for smaller cysts must be treated with caution. Most patients received HMG alone for ovarian stimulation although some patients received FSH with or without HMG. Ovarian stimulation commenced on day 3 menses and HCG was administered when at least 2 follicles were greater than 15 mm and serum oestradiol was >400 pg/ml (>1468 pmol/l). Oocyte retrieval was performed transvaginally 36 hours following the HCG injection.

Hornstein (1989) defined an ovarian cyst as an ovarian structure of between 10 mm and 45 mm mean diameter seen by transabdominal ultrasound scan on menses day 1 or 2. Patients with ovarian cysts greater than 45 mm diameter were excluded from the study. Review was restricted to 97 patients who had had a laparoscopy or hysterosalpingogram which had excluded hydrosalpinges. The latter patients had been shown previously to ovulate spontaneously, by serum progesterone, endometrial biopsy or by a biphasic temperature chart. Of the 97 patients studied 21 patients were found to have ovarian cysts: 13 patients had ovarian cysts between 10 mm and 19 mm diameter; 5 patients had ovarian cysts between 20 mm and 29 mm diameter and 3 patients had ovarian cysts between 30 mm and 45 mm diameter. All patients received HMG for ovarian stimulation. When there were at least 2 follicles of 15 mm diameter and the serum oestradiol exceeded 500 pg/ml (1835 pmol/l), HCG was administered and laparoscopic or transvaginal oocyte retrieval was performed 34 hours later.

Karande (1990) defined an ovarian cyst as a fluid filled structure within the ovary of between 20 mm and 50 mm diameter identified by vaginal ultrasound on day 6 of the menstrual cycle during which IVF was proposed. Patients were excluded if any of the following applied: patient had only one ovary, presence of hydrosalpinx, ovarian cyst >50 mm diameter or basal (day 3 menses) serum oestradiol >50 pg/ml (184 pmol/l). Of 352 patients reviewed ovarian cysts were seen in 21 IVF cycles. The latter 21 IVF cycles were compared to 35 IVF cycles with pure tubal disease and no ovarian cysts. In 19 IVF cycles there was a single ovarian cyst, and the number of follicles and oocytes collected were compared between the ipsilateral and contralateral ovary to the cyst. Ovarian stimulation was with gonadotrophins and HCG, and no cycles with LHRH agonists were included in the study.

Goldberg (1991) defined an ovarian cyst by vaginal ultrasound as an intraovarian cystic structure with a mean diameter of ≥12 mm diameter on day 3 of the cycle. Of 212 IVF cycles studied cysts were identified in 62 cycles. All patients received HMG for ovarian stimulation but some patients in addition received clomiphene, FSH or the LHRH agonist leuprolide acetate.

Feldberg (1989) defined an ovarian cyst as an ovarian structure >25 mm maximum diameter found after LHRH agonist administration and before gonadotrophin stimulation. Feldberg studied 24 patients treated with decaptyl depot 500 µg within 3 days of the onset of a menses, and 22 patients who started nasal buserelin 900 µg daily on day 1 to 3 of the cycle. Ovarian stimulation with HMG or FSH commenced day 14 if serum oestradiol was <50 pg/ml (<184 pmol/l). Of the 24 patients treated with decaptyl 7 patients developed solitary ovarian cysts (27 mm - 36 mm in diameter) between days 9 to 13. Of

the 22 patients treated with buserelin 5 patients developed solitary ovarian cysts (29 mm - 37 mm in diameter) between days 9 to 13.

Ron-El (1989) defined an ovarian cyst as an ovarian structure of ≥15 mm mean diameter found after LHRH agonist administration and before gonadotrophin stimulation. In Ron-El's study a depot LHRH agonist (decapeptyl microcapsules) was administered intra-muscularly on day 1 or 2 of menses. Of 198 IVF cycles reviewed ovarian cysts (15 mm - 40 mm mean diameter) developed in 27 cases (13.6%). Ovarian stimulation with HMG commenced when serum oestradiol was ≤30 pg/ml (110 pmol/l). The cysts had regressed prior to ovarian stimulation in all but 6 patients where the cysts were between 16 mm and 25 mm diameter.

Rizk (1990) defined an ovarian cyst as a unilocular or bilocular, sonolucent cystic structure with a mean diameter of between 20 mm and 60 mm. Rizk distinguished two groups of cysts. The first group comprised of 14 patients who were noted to have cysts on their baseline ultrasound scan on day 2 menses. Six of the latter 14 patients were chosen at random to have the cyst aspirated, and all patients received clomiphene citrate (Serophene; Serono, Welwyn Garden City, United Kingdom) and HMG for ovarian stimulation. The second group consisted of 23 patients who did not have cysts on their baseline ultrasound scan but developed cysts following buserelin administration. Subcutaneous buserelin was commenced on day 2 menses and after 14 days 23 patients were found in each case to have a unilateral ovarian cyst with serum oestradiol concentrations ranging from 600 to 3,500 pmol/L. Twelve of the latter 23 patients were chosen at random to have the cyst aspirated, and the other 11 patients continued on buserelin until the serum

oestradiol was <100 pmol/l. All patients received HMG for ovarian stimulation.

Sampaio (1991) presented two separate definitions of ovarian cysts for patients treated by two different protocols. Two hundred and eighty five patients were treated with daily subcutaneous injections of buserelin commenced on day 1 of the menses. Ovarian stimulation with HMG either alone or combined with FSH was commenced when ovarian quiescence was achieved (serum oestradiol <60 pg/ml (<220 pmol/l) and no follicular growth). An ovarian cyst was defined in 28 of the latter 285 patients as an homogeneous anechoic structure ≥16 mm diameter absent at the beginning of buserelin administration and present on the subsequent ultrasound performed approximately 15 days after the onset of buserelin treatment. Curiously the definition stated that the serum oestradiol had to be <60 pg/ml (<220 pmol/l) at the time the cyst was identified, and no mention was made of patients with cysts and a serum oestradiol >60 pg/ml (>220 pmol/l). Seventy four patients were treated with subcutaneous injections of buserelin commenced on day 1 menses and HMG stimulation was used from days 3 to 7. In 17 of the latter 74 IVF cycles ovarian cysts were defined as homogenous anechoic structures absent at the beginning of buserelin treatment and present after 6 days of ovarian stimulation with diameters at least three times larger than the mean diameter of the rest of the follicular structures. Endometriotic cysts were excluded from both definitions of cysts, and in addition the diagnosis of a cyst was confirmed at the time of follicular aspiration by the presence of clear follicular fluid not containing an oocyte.

Two abstracts have been reported regarding ovarian cysts during IVF cycles with HMG stimulation (Hung *et al.*, 1988, Grazi *et al.*, 1988).

Hung (1988) reviewed 65 patients with ovarian cysts of 30-50 mm diameter present during 14 cycles. Grazi (1988) compared 21 cycles with cysts paired with 21 cycles without cysts.

It can be seen that there are significant differences in the definition of an ovarian cyst used in the studies regarding cysts during IVF cycles. The size of the cyst has been presented as a mean diameter, a maximum diameter, and some studies have not specified which diameter they are referring too. Even in the studies referring to a mean diameter it is not stated whether this is the mean diameter of a single section through the cyst or a mean of 3 perpendicular diameters. It is thus not possible to make direct comparisons with regard to the size of the cysts between studies. Differences in exclusion criteria between the studies introduces further differences in the possible nature of the cysts that are studied. By excluding patients with a day 3 serum oestradiol of >50 pg/ml (184 pmol/l) Karande (1990) may have excluded some patients who had ovarian cysts with steroidogenic activity. Failure to exclude endometriomata from the definition may complicate the matter further as endometriomata are distinct from functional ovarian cysts.

The ovarian stimulation regime used is also of great importance when assessing whether ovarian cysts may influence IVF cycles. If the ovarian stimulation regime uses a LHRH agonist to achieve pituitary downregulation prior to ovarian stimulation, it is possible to delay starting ovarian stimulation until the cyst has regressed completely or at least the serum oestradiol is suppressed. The management of patients has differed enormously in the latter studies. In order to establish whether ovarian cysts have an influence on IVF cycles a balanced view of the literature will be taken

considering the possible areas in which an ovarian cyst may influence IVF cycles.

Aware of the difference in definitions and ovarian stimulation regimes in the studies on ovarian cysts during IVF cycles it is possible to make meaningful comparisons of the studies. Before considering whether ovarian cysts influence IVF cycles it is necessary to compare the patients who develop ovarian cysts to the patients without ovarian cysts to ensure that these are comparable groups of patients.

1.4.2 Comparison of patients with ovarian cysts to patients without ovarian cysts

In all of the preceding studies comparison between patients with ovarian cysts and patients without ovarian cysts revealed no significant difference in age nor indication for IVF. Further Karande (1990) found no significant difference in basal FSH, height or weight between patients with ovarian cysts and patients without ovarian cysts. The presence of an ovarian cyst does not appear to relate to any poor prognostic factors, thus any differences seen in IVF cycles with ovarian cysts are most likely attributable to the ovarian cysts.

1.4.3 Acute cyst complications during IVF cycles

In vitro fertilisation cycles may be cancelled when an ovarian cyst is present because of concern that the cyst may grow further, rupture, or undergo torsion during controlled ovarian stimulation (Goldberg, 1991). However, there was not a single reported case of an acute cyst complication during the IVF cycle in any of the studies reported above. Moreover Goldberg (1991) found that in only 8 of 62 IVF cycles with ovarian cysts studied was there a slight increase in the

size of the cyst, whereas in the remainder an equal proportion were either unchanged or decreased in size during ovarian stimulation.

1.4.4 Influence of ovarian cysts on follicular development.

The influence of ovarian cysts on follicular development during ovarian stimulation preceding IVF may be assessed by several means. The peak serum oestradiol level is an objective measure, which relates to the number of follicles developing in response to ovarian stimulation (Jones *et al.*, 1983). The cancellation rate is a clinically important measure of ovarian response to ovarian stimulation. Although exact criteria vary between different centres, the main reasons for cancelling IVF cycles are a poor follicular response to ovarian stimulation or premature luteinisation. The number of oocytes collected at the time of oocyte retrieval provides a further objective measure of ovarian response to stimulation.

In several of the studies mentioned above the peak level of serum oestradiol achieved during controlled ovarian hyperstimulation has been compared between patients with cysts and patients without cysts. Most studies found significantly lower peak serum oestradiol levels in the patients with ovarian cysts than in the patients without ovarian cysts (Thatcher *et al.*, 1989, Goldberg *et al.*, 1991, Karande *et al.*, 1990, Ron-El *et al.*, 1989). However, Hornstein (1989) and Sampaio (1991) found no significant difference between peak serum oestradiol levels in patients with ovarian cysts and patients without ovarian cysts .

Thatcher (1989) found a significantly higher cancellation rate in 40 IVF cycles with ovarian cysts than in the 86 IVF cycles without ovarian cysts, 53% v 25% respectively. Similarly Goldberg (1991) noted a significantly ($p< 0.05$) higher cancellation rate in 62 IVF

cycles with ovarian cysts than 150 IVF cycles without cysts, 43.5% versus 28% respectively. However, several other studies have shown no significant difference in the cancellation rates between IVF cycles with cysts and IVF cycles without cysts (Hornstein *et al.*, 1989, Ron-El *et al.*, 1989, Sampaio *et al.*, 1991).

Considering IVF cycles which proceeded to oocyte retrieval Ron-El (1989) found there were significantly fewer oocytes collected in IVF cycles with cysts than IVF cycles without cysts. However, other studies reported no significant difference in the total number of oocytes collected per oocyte retrieval between patients with ovarian cysts and patients without ovarian cysts (Hornstein *et al.*, 1989, Karande *et al.*, 1990, Sampaio *et al.*, 1991).

1.4.5 Influence of ovarian cysts on oocyte quality

Determination of impairment of oocyte quality by ovarian cysts is difficult, and probably the most useful parameters to consider are fertilisation rates and pregnancy rates. Oocyte quality could be inferred from the quality of the resultant embryo, but none of the studies above have presented a comparison of the embryo quality between the patients with ovarian cysts and the patients without ovarian cysts.

Although the fertilisation rates of the studies of ovarian cysts during IVF cycles varied between studies, there was no difference within each study between the fertilisation rates of IVF cycles with cysts and the fertilisation rates of IVF cycles without cysts (Thatcher *et al.*, 1989, Hornstein *et al.*, 1989, Ron-El *et al.*, 1989, Karande *et al.*, 1990, Hung *et al.*, 1988).

The ultimate test of oocyte quality is the achievement of a successful pregnancy. Thatcher (1989) found only 1 pregnancy from the 40 IVF cycles with ovarian cysts, whereas there were 11 pregnancies from the 86 IVF cycles without ovarian cysts, and the respective pregnancy rates per embryo transfer were 5% and 16.9%. However, several other studies have shown no decrease in pregnancy rates in IVF cycles with ovarian cysts (Hornstein *et al.*, 1989, Ron-El *et al.*, 1989, Karande *et al.*, 1990, Hung *et al.*, 1988, Grazi *et al.*, 1988).

1.4.6 Mechanism of influence

The apparent conflict in the above studies regarding the influence of ovarian cysts on IVF cycles should be considered with regard to the possible mechanisms of influence. As illustrated in figure 1.11 there are several possible ways in which ovarian cysts may exert an influence during IVF cycles.

Rizk (1990) suggested that ovarian cysts may influence IVF cycles by a mechanical effect with the cyst crowding the ovary possibly disrupting the blood supply to the developing follicles. However, this hypothesis does not explain the influence observed in the contralateral ovary to the cyst by Thatcher (1989) and Goldberg (1991). Further this hypothesis does not explain why Sampaio (1991) and Feldberg (1989) found no evidence of any influence of ovarian cysts during IVF cycles.

The cysts may exert their influence at the hypothalamo-pituitary level leading to premature luteinisation. Thatcher (1989) found evidence of premature luteinisation in some IVF cycles with ovarian cysts, and this may explain part of the problem. The mechanism by

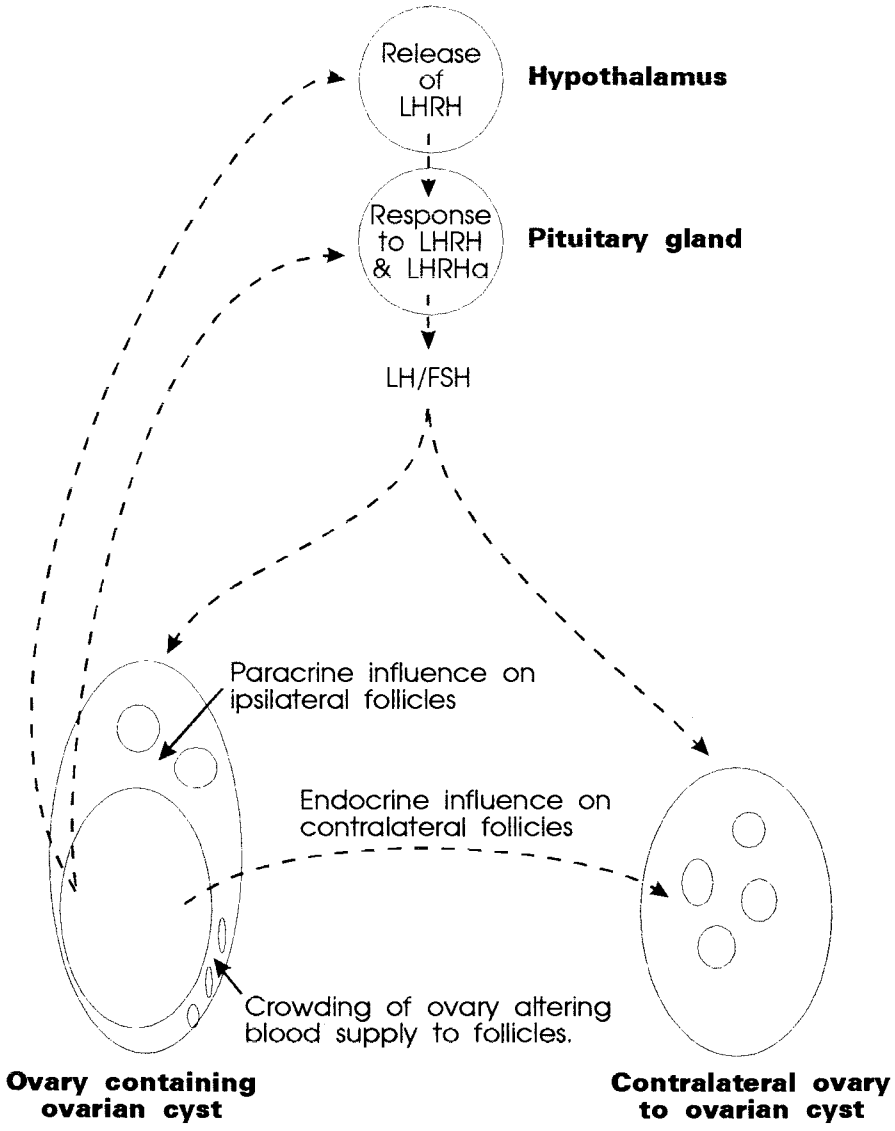


Figure 1.11 Diagramatic illustration of possible areas of influence of ovarian cysts during IVF cycles.

which functional ovarian cysts may influence hypathalmo-pituitary function could relate to oestradiol production in the ovarian cysts influencing pituitary function (Araki S. *et al.*, 1985).

Thatcher (1989) suggested that if cysts produced steroids this may alter the endocrine milieu leading to disturbance of normal follicular development. Differences in the influence of ovarian cysts during IVF cycles could thus relate to differences in the steroid production of the ovarian cysts. Ron-El (1989) found that ovarian cysts developing during pituitary downregulation were associated with elevation of serum oestradiol concentrations. Although Ron-El (1989) showed no difference in cancellation rates or pregnancy rates, he discovered there were significantly fewer oocytes collected in IVF cycles with ovarian cysts present than in IVF cycles without ovarian cysts present. Sampaio (1991) using a long buserelin regime confined his study to patients with ovarian cysts and serum oestradiol levels <60 pg/ml (<220 pmol/l) 15 days following the start of LHRH agonist therapy. Sampaio (1991) found no evidence of any influence of ovarian cysts on IVF cycles.

If steroid production by ovarian cysts had an influence on follicular development then one might expect more of an influence in the ipsilateral ovary to the cyst than in the contralateral ovary to the cyst. Rizk (1990) studied 14 patients with unilateral ovarian cysts on day 2 menses. In 6 patients the cysts were aspirated before stimulation with clomiphene /HMG, and the ipsilateral ovary to the cyst of these patients produced significantly more oocytes than the ipsilateral ovary of the 8 cycles where the cyst was not aspirated, although there was no significant difference in the number of oocytes collected from the contralateral ovaries (Rizk *et al.*, 1990). Karande (1990) studied 19 patients with unilateral ovarian cysts, and

compared the number of follicles and oocytes between the ipsilateral and contralateral ovary to the ovarian cyst. Karande found significantly ($P<0.005$) fewer follicles in the ipsilateral ovary to the ovarian cyst. There were fewer oocytes collected from the ipsilateral ovary to the ovarian cyst, but this did not reach statistical significance ($P=0.14$).

1.4.7 Management

It is difficult from the literature to formulate a definitive plan of clinical management for ovarian cysts encountered during IVF cycles, as there is considerable disagreement as to whether cysts present any problem. Cancellation of IVF cycles in the presence of ovarian cysts does not appear to be justified because of concern over acute cyst complications. However, there is evidence to suggest that cysts may exert a detrimental influence on IVF cycles, and this may relate to steroid production by the cysts. A conservative approach has been suggested continuing LHRHa until serum oestradiol levels fall and the cyst regresses before commencing ovarian stimulation (Ron-El *et al.*, 1989, Sampaio *et al.*, 1991). Alternatively ovarian cyst aspiration could be performed prior to initiating ovarian stimulation (Silverberg *et al.*, 1990). Aspiration of the cysts may reduce steroid production and remove a potentially detrimental influence on follicular development during IVF cycles. Further research is required to determine the appropriate management for patients with functional ovarian cysts encountered during IVF cycles. This thesis aims to contribute to the latter research by investigating the development and influence of functional ovarian cysts during IVF cycles.

1.5 Hypotheses

On the basis of the literature review presented above the thesis proposes three hypotheses.

It has been established that there is a relationship between follicular development and ovarian steroidogenesis. The studies on ovarian cysts during IVF cycles have revealed evidence suggesting a link between functional activity of ovarian cysts and an influence on IVF cycles. The first hypothesis proposes:

> **Functional ovarian cysts will adversely influence in vitro fertilisation cycles in relation to their steroidogenic activity but not by their physical presence.**

Meldrum (1988) has demonstrated ovarian cyst development during pituitary downregulation with LHRH agonists commenced both in the luteal and follicular phases of the cycle. Ron-El (1989) has found that the ovarian cysts, which develop during pituitary downregulation, are associated with a transient elevation in serum progesterone levels but a more marked and prolonged rise in serum oestradiol levels. The second hypothesis proposes:

> **Buserelin may cause the development of follicular ovarian cysts with significant steroidogenic activity.**

Steroidogenesis by ovarian cysts has been considered, and aspiration of ovarian cysts has been suggested as means of dealing with ovarian cysts with steroidogenic activity. The thesis thus proposes a third hypothesis:

> **Aspiration of functional ovarian cysts will reduce steroidogenic activity allowing the IVF cycle to proceed unhindered.**

1.6 Objectives

In order to test the above hypotheses four studies were performed, and these are presented in the subsequent chapters. The objectives of these studies were are follows:

Study 1

a. To establish whether there is a relationship between steroidogenic activity of functional ovarian cysts and a detrimental influence on IVF cycles.

Study 2

a. To establish whether buserelin commenced in the luteal phase of the cycle may result in the development of functional ovarian cysts with clinically significant steroidogenic activity.
b. To determine whether the latter functional ovarian cysts are corpora lutea cysts or follicular cysts through measurement of serum steroid levels.

Study 3

a. To establish if functional ovarian cysts, which develop during pituitary downregulation, may have a sufficiently high steroid concentration to influence the endocrine milieu of developing follicles.
b. To determine the nature of functional ovarian cysts, which develop during pituitary downregulation, by considering the relative steroid concentrations of fluid aspirated from the cysts with reference to follicular fluid collected at the time of oocyte retrieval.

Study 4

a. To determine whether the aspiration of functional ovarian cysts will remove steroidogenic activity allowing the IVF cycle to proceed unhindered.

METHODS

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CHAPTER 2

Southampton IVF Programme

2.1 Introduction

This thesis was based on work carried out in the Southampton IVF programme between 1988 and 1991. This chapter outlines the clinical and scientific protocols followed on the Southampton IVF programme. The chapter explains the methodology of the four studies presented in the thesis, and the alterations introduced into the management of patients on the Southampton IVF programme to investigate the development and influence of functional ovarian cysts during IVF cycles. The biochemical assays used during the studies are presented in detail in chapter 3.

2.2 History

Since its establishment the Southampton IVF programme has enjoyed a favourable pregnancy rate with a small but dedicated IVF team under the leadership of Mr G.M. Masson. The Southampton IVF programme was set up in 1987 as a joint project between the Department of Human Reproduction (University of Southampton) and the BUPA Chalybeate hospital. Initially a clomiphene / human menopausal gonadotrophin regime was used for controlled ovarian hyperstimulation. However, within 6 months of setting up the Southampton IVF programme the ovarian stimulation regime was changed to a long buserelin regime based on the work of Flemming and Coutts (1986). The regime was further modified in 1988 by the routine use of HCG for luteal support, when it was shown that this increased the pregnancy rate (Smith *et al.*, 1989). Since 1988 the latter regime exclusively has been used on the Southampton IVF programme, and this thesis considers only IVF cycles treated in this manner.

2.3 Patient referrals and Initial Assessment

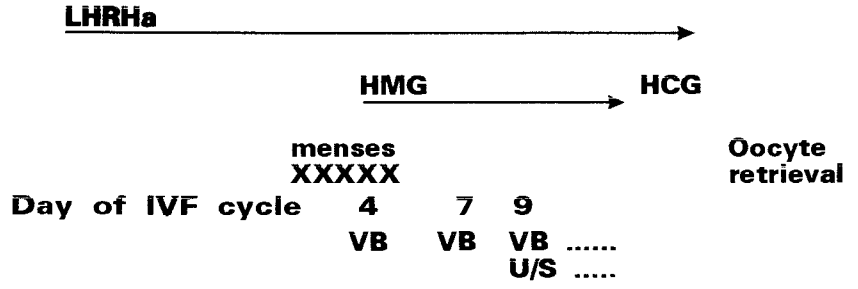
The Southampton IVF programme receives referrals mainly from within Wessex, but also has referrals from the Channel Islands and even a few patients from places abroad including France, Germany and Mexico. There is no absolute age bar to accepting patients, although patients are counselled appropriately regarding age related chances of achieving pregnancy. Patients are referred with a wide range of indications for in vitro fertilisation, and provided it is felt that IVF is appropriate they are accepted onto the IVF programme.

Prior to commencing an IVF cycle all patients had a vaginal ultrasound scan (Bruel and Kjaer 1815 ultrasound machine with a 7.5 Mhz vaginal probe). If an endometrioma, an ovarian cyst of >50 mm diameter or an ovarian cyst with solid elements was discovered, this was dealt with surgically before the patient commenced an IVF cycle. Patients with a simple sonolucent cyst of less than 50 mm diameter were allowed to have an IVF cycle, and the only difference in their management was that they had a further ultrasound scan before commencing HMG to see if the ovarian cyst was still present.

2.4 Controlled Ovarian Hyperstimulation regime 1988-1990

Pituitary Downregulation: Since 1988 all IVF cycles have been performed using a long buserelin controlled ovarian hyperstimulation regime, which is outlined in figure 2.1. Nasal buserelin (Suprefact, Hoechst, U.K.) 200 µg thrice daily is commenced one week prior to anticipated menstruation, or when convenient in the case of oligomenorrhoea or ammenorrhoea. It was assumed that by day 4 of the ensuing menses buserelin would have affected pituitary downregulation.

Controlled ovarian hyperstimulation: From day 4 menses a standard regime of 4 ampoules of HMG was administered daily (each



Key

LHRHa	Buserelin	**VB**	Venous blood sample
HMG	Human Menopausal Gonadotrophin	**U/S**	Ultrasound scan of ovaries
HCG	Human Chorionic Gonadotrophin		

Figure 2.1 Southampton IVF programme ovarian stimulation regime 1988-1990.

ampoule containing 75 iu FSH and 75 iu LH, Pergonal, Serono, UK). Patients who had previously had an abandoned IVF cycle on the standard regime were treated with 6 or 8 ampoules of HMG daily.

Monitoring of ovarian response to stimulation: Blood samples were taken for oestradiol analysis on day 4 menses, day 7 and daily from day 9 of the IVF cycle. Vaginal ultrasonography was performed daily from the ninth day following the onset of menstruation.

Decision to give luteinising injection of HCG: If there were less than 4 developing follicles as assessed by vaginal ultrasound following 6 days of HMG stimulation, the IVF cycle was abandoned. Otherwise when the leading 3 follicles had an orthodiametric diameter of ≥ 18 mm and the serum oestradiol level was >300 pmol/l for each follicle >14 mm in diameter 10,000 units of HCG was administered intramuscularly at 10.30 pm, 11.30 pm or 00.30 am for oocyte retrieval at 8.30 am, 9.30 am or 10.30 am respectively.

Luteal Phase Patients received luteal support with HCG 2,500 IU on day 3 and 6 following the initial HCG injection.

All serum samples taken during the IVF cycle were stored at -20°C for research purposes.

2.5 Oocyte retrieval

Thirty four hours following the HCG injection transvaginal oocyte retrieval was performed under ultrasound guidance in a purpose built theatre at the BUPA Chalybeate hospital, Southampton. One hour before the procedure patients were given 20 mg of temazepam oral premedication. Intravenous medazolam and pethidine were used routinely for sedation and analgesia. Patients oxygen saturation was monitored continuously throughout the procedure with a pulse oximeter, and oxygen was delivered by a controlled delivery face mask if necessary. General anaesthesia was available if requested. Patients were placed in lithotomy position, and sterile drapes applied. The vagina was cleaned with 0.015% chlorhexidene and then washed out with culture medium. Contact gel was applied to the vaginal transducer, which was then ensheathed in a sterile condom. A sterile needle guide was attached onto the transducer, and a sterile 15 gauge, double lumen needle (Casmed, Surrey, UK) was inserted through the guide.

Under ultrasound guidance the needle was inserted with a sharp motion through the vaginal wall directly into the ovary. Without withdrawing the needle from the ovary all the follicles in the ovary were aspirated in turn using mechanical suction controlled by a foot switch (Craft pump, Rocket, UK). The fluid aspirated from the follicles was examined immediately by an embryologist to identify oocytes.

2.6 Laboratory Procedures

Prior to the oocyte retrieval the embryologist prepared a medium for flushing the oocytes from the follicles and a medium for culture of the oocytes following oocyte retrieval.

2.6.1 Flushing medium

The flushing medium used was an Earle's balanced salt solution (Gibco, UK), 10x concentrate (Catalogue number 042-04050 H) made up with the following additions:

Bicarbonate: 4 mmol/l (Sigma, UK)

Pyruvate: 0.1 mmol/l (Sigma, UK)

HEPES: 21 mmol/l (Sigma, UK)

Penicillin: 60 μ/ml

Streptomycin: 50 μg/ml

Osmolality 284 ±2 mOsm/kg.

pH adjusted to 7.4 with 2 molar sodium hydroxide

Flushing medium was made up in batches, filtered using Sterivex Filter 0.22 μm filter unit (Millipore, UK) and stored up to a maximum of 14 days.

2.6.2 Culture medium

The culture medium used was an Earle's balanced salt solution (Gibco, UK), 10x concentrate made up with the following additions:

Bicarbonate: 25 mmol/l (Sigma, UK)

Pyruvate: 0.1 mmol/l

Penicillin: 60 μg/ml

Streptomycin: 50 μg/ml

Osmolality 284 ±2mOsm/kg.

pH adjusted to 7.4 with 2 molar sodium hydroxide

Albumin (10%) added immediately prior to use (Albuminar 5, Armour Pharmaceutical Company Ltd., UK)

Culture medium was made up in batches using Millex 0.22 μm filter unit and stored up to a maximum of 7 days.

2.6.3 Oocyte handling

The fluid aspirated from the ovarian follicles was poured into sterile Falcon tissue culture dishes and examined microscopically for an oocyte. The recovered oocyte was placed in a 4 ml Falcon tube containing 1.5 ml of culture medium, which had been equilibrated overnight under 5% CO_2 and placed in an incubator (Heraeus B5061 EK/CO_2). At the end of the oocyte retrieval the oocytes were taken in a transport incubator to the main laboratory in the Princess Anne Hospital. The oocytes were transferred into organ culture dishes containing 1 ml of culture medium and covered with 0.8 ml light liquid paraffin (Kirby Warrick, UK). The latter dishes had been equilibrated overnight with 5% CO_2.

The oocytes were pre-incubated for 6 hours prior to insemination.

2.6.4 Semen preparation

One of two different methods of semen preparation were used depending on the characteristics of the initial semen sample. The swim up method was used for satisfactory semen samples and the Percoll method was used in cases of oligospermia and/or astheniospermia.

Swim up method: Once the semen had liquefied (usually within 10 minutes) 3 volumes of flushing medium containing 10% albumin were added to the fresh semen sample. This was centrifuged at 200 g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 0.5 ml culture medium. 100 µl of the suspension was layered under 1.5 ml culture medium in Falcon tubes which had been pre-equilibrated. The spermatozoa were allowed to swim-up (approximately 30 minutes) and the top two thirds removed and used for insemination.

Percoll method (2 step density gradient centrifugation): The semen sample was diluted with 1:1 culture medium and layered over Percoll gradients of 40% and 80% Percoll. The suspension was spun for 20 minutes at 400 g to give a pellet, which is then washed in 15 ml culture medium at 200 g for 5 minutes. The resulting pellet is resuspended in 200 µl of culture medium for insemination.

Approximately 1 x 10^5 normal motile sperm were added to each oocyte containing dish. If the semen sample was felt to be of poorer quality then up to 1 x 10^6 motile sperm were added.

2.6.5 Embryo Culture

The morning following oocyte retrieval (approximately 18 hours later) oocytes were examined microscopically for the presence of pronuclei. The cumulus around the oocyte was stripped off using fine, hand drawn pipettes and the number of pronuclei were noted. The fertilised oocyte were each then transferred to a fresh culture dish, which had been pre-equilibrated with 5% carbon dioxide.

On the day of embryo transfer (approximately 42 hours post-insemination) the fertilised oocytes were examined microscopically to establish cleavage and to grade the embryos as explained below.

2.6.6 Morphometric Embryo grading

Grade 1	All blastomeres have equal size without anucleate fragments
Grade 2	Not all blastomeres have an equal size and/or anucleate fragments present in at most 20% of the embryo.
Grade 3	Not all blastomeres have an equal size and/or anucleate fragments are present in 20-50% of the embryo.
Grade 4	Totally fragmented embryo present.

2.7 Embryo transfer

Immediately prior to the embryo transfer, embryos selected for the transfer were placed in a Falcon tube, containing 0.6 ml albumin and 0.4 ml culture medium. The transfer catheter (Wallace, UK) was washed through with culture medium. The embryos were drawn up into the catheter in 20 µl medium with 10 µl air bubbles at either end. A 1 ml syringe was used on the end of the catheter. Routinely a maximum of 3 embryos were replaced. In exceptional circumstances 4 embryos might be replaced.

Embryo transfers were performed 42-46 hours following insemination. A warm bivalve speculum was inserted into the vagina and adjusted to obtain the optimal view of the cervix. The cervix was cleaned with culture medium. The end of the outer sheath of the catheter was placed at the external os and the inner catheter passed through the internal os into the uterus. The preset syringe was depressed and the embryo(s) released. The catheter was withdrawn and examined under the microscope to ensure that no embryo was retained in the catheter. If it was not possible to replace the embryos using the Wallace catheter even aided by gentle traction on the cervix with a sponge forceps, embryos were replaced with a TDT catheter (Laboratoires CCD, France), which is much more rigid than the Wallace catheter.

2.8 Study 1: The influence of functional ovarian cysts during IVF cycles related to serum steroid levels.

The objective of this study was:

a) to establish whether there was a relationship between steroidogenic activity of functional ovarian cysts and a detrimental influence on IVF cycles.

The study reviewed 780 completed IVF cycles, which had been managed as described above. The possible influence of the functional ovarian cysts during the IVF cycles was evaluated by reviewing the case records. Steroidogenic activity of the ovarian cysts was assessed by measuring steroid levels in serum that had been taked during the IVF cycles and stored at -20°C.

Before commencing buserelin in the 780 IVF cycles a vaginal ultrasound scan (Bruel and Kjaer 1846 scanner with a 7.5 Mhz 8538 probe) had been performed. The latter ultrasound scans had found no endometrioma, no ovarian cyst ≥50 mm maximum orthodiametric diameter nor any ovarian cyst with solid elements or any other suspicious characteristics. The day 9 ultrasound scan reports for the 780 IVF cycles were reviewed for the presence of an ovarian cyst, which was defined as a sonolucent structure within the ovary with an orthodiametric diameter in the largest plane of the cyst of at least 30 mm. By this definition 53 IVF cycles with cysts were identified amongst 49 patients. In 6 cycles the cyst had been present before commencing buserelin.

The serum samples which had been taken on day 4 of the IVF cycle were used to reflect steroidogenic activity of functional ovarian cysts. It was reasoned that by day 4 of the IVF cycle ovarian activity should have been suppressed by the action of buserelin thus serum steroid levels at this time could provide a useful marker of steroidogenic

activity within functional ovarian cysts. Oestradiol, progesterone and androstenedione were measured as these would shed light on the nature and steroidogenic activity of the functional ovarian cysts as explained in chapter 1.. The latter steroid levels were compared in the 53 IVF cycles with ovarian cysts according to 3 main outcomes:

1) the IVF cycle was abandoned before oocyte retrieval,

2) the IVF cycle proceeded to oocyte retrieval but failed to result in conception

3) the IVF cycle resulted in conception (conception was defined as a serum HCG >50 IU/l 12 days following embryo transfer).

As oestradiol is predominantly produced by steroidogenesis in ovarian tissue, oestradiol was used as an indicator of ovarian steroidogenic activity. The 95th centile of oestradiol (200 pmol/l) on day 4 of the 727 IVF cycles without apparent ovarian cysts was used to subdivide the IVF cycles with functional ovarian cysts into a steroidogenically active group and a steroidogenically inactive group. The latter two groups were compared to each other and to the IVF cycles without apparent ovarian cysts. The comparisons made were cancellation rates, pregnancy rates, number of oocytes collected per oocyte retrieval, number of fertilised oocytes per oocyte retrieval, number of cleaved embryos per oocyte retrieval, and number of morphometric grade 1 embryos per oocyte retrieval.

In chapter 1 it was reported that premature luteinisation was one mechanism by which functional ovarian cysts may influence IVF cycles (Thatcher *et al.,* 1990). For the 53 IVF cycles with ovarian cysts serum progesterone concentrations were measured on the day it was decided to give the HCG injection or abandon the IVF cycle. The latter progesterone concentration was used as an indicator of premature luteinisation, and this is discussed in more depth in chapter 3.

2.9 Alterations to patient management

When the Southampton IVF programme was set up in 1987, the development of functional cysts during pituitary downregulation was not documented. It is thus not surprising that the initial Southampton ovarian stimulation regime did not consider the latter as a potential problem. With the guidance of the literature review presented in chapter 1 and initial results from study 1 (presented in chapter 4) it was proposed to alter the routine ovarian stimulation regime with the dual aims of improving patient management, and furthering our understanding of the development and influence of functional ovarian cysts during IVF cycles. The alterations in management are first outlined and then the methodology of the three prospective studies are presented in turn.

The first alteration in patient management was to identify the functional ovarian cysts before commencing ovarian stimulation. On the day the patient attended clinic to be instructed how to use buserelin a vaginal ultrasound scan was performed and a blood sample was taken. A further vaginal ultrasound scan was performed on the fourth day of the IVF cycle and a blood sample was taken.

The treatment of patients was changed so that ovarian stimulation was not commenced if a functional ovarian cyst was present and the serum oestradiol was >200 pmol/l. If patients had a serum oestradiol on day 4 of the IVF cycle of >200 pmol/l, HMG stimulation was withheld and buserelin was continued. If the serum oestradiol fell to below 200 pmol/l after a further week, ovarian stimulation with HMG was commenced and patients received treatment according to the standard regime as described above. If the serum oestradiol remained >200 pmol/l and an ovarian cyst was present, patients were offered an ovarian cyst aspiration. Alternatively patients had the option of

continuing with buserelin until the serum oestradiol fell below 200 pmol/l or stopping buserelin and restarting the IVF cycle when the cyst had regressed spontaneously.

As a precautionary measure pituitary downregulation was confirmed prior to cyst aspiration by a LHRH stimulation test (see chapter 3). The patients having cyst aspirations all had a serum oestradiol >200 pmol/l, thus it was important to check that the serum oestradiol was not raised because buserelin had failed to downregulate the pituitary gland and ovarian activity had not been suppressed.

Transvaginal ovarian cyst aspiration prior to ovarian stimulation was introduced as a new treatment option and the technique is described below.

2.9.1 Transvaginal ovarian cyst aspiration

The technique of transvaginal ovarian cyst aspiration was very similar to the method used to collect oocytes in the routine IVF regime, and it is illustrated in figure 2.2. The only distinctions being that oocytes were not collected from cysts, and the greater volume of cyst fluid than follicular fluid meant that more tubes were required to drain the cyst completely. Intravenous Pethidine and Medazolam were given pre-operatively for sedation. The patient was then placed in lithotomy position and draped. The vagina was cleaned with savlon, and the bladder was catheterised if necessary. A sterile, spermicide-free condom was rolled over the ultrasound probe, and a sterile needle guide was attached onto the ensheathed ultrasound probe (Bruel and Kjaer 1815 ultrasound machine with a 7.5 MHz vaginal probe). The ultrasound probe was inserted in the vagina, and the ovarian cyst was identified through the lateral fornix of the vagina. The predicted path of the needle to be passed through the

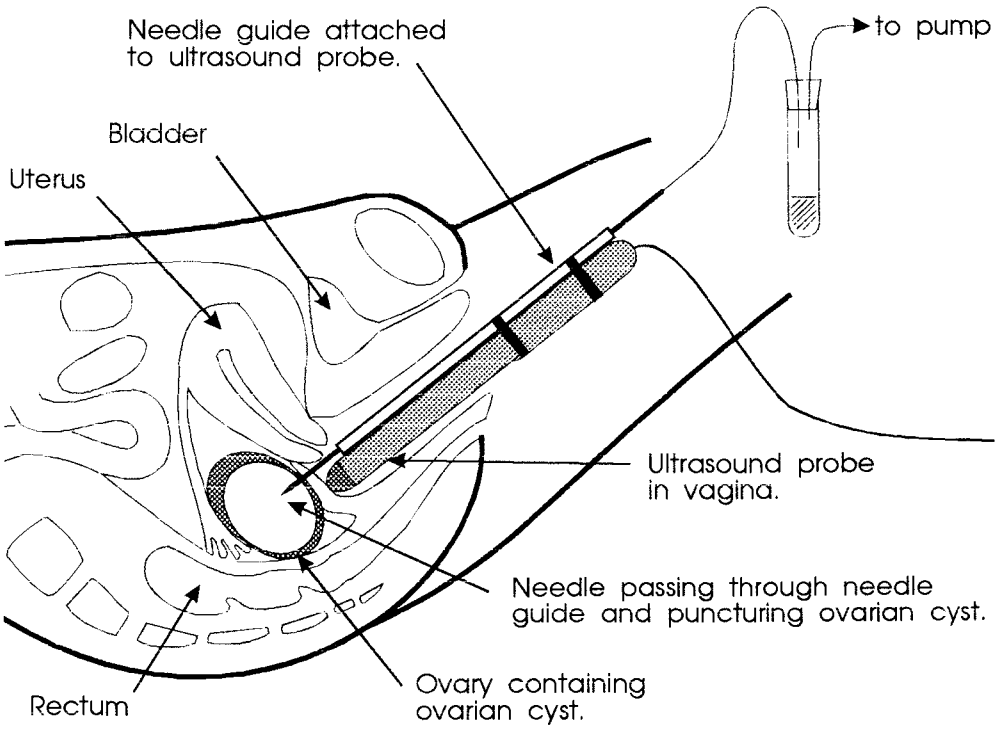


Figure 2.2: Diagram illustrating the technique of transvaginal ovarian cyst aspiration.

needle guide was superimposed on the ultrasound screen, and this was aligned with the cyst. A sterile needle (Casmed UK, Surrey, UK) was then inserted through the needle guide in a swift sharp movement to the centre of the ovarian cyst, as indicated by the ultrasound picture. Tubing attached to the needle conveyed the cyst fluid to a tube, which was connected via an air lock to a suction pump (Craft pump, Rocket, UK). The cyst fluid aspirated was collected in the latter tube, and the tube was changed as many times as was required to fully drain the cyst.

Serum oestradiol was measured 3-5 days following ovarian cyst aspiration. Patients returned for a repeat ultrasound scan 5 days following ovarian cyst aspiration. If at that time no ovarian cyst was present, ovarian stimulation with HMG was commenced and patients management proceeded as described above according to the standard regime. If patients started to menstruate as the oestradiol levels fell following the aspiration, ovarian stimulation was commenced on the fourth day of menstruation.

2.10 Study 2: The development of functional ovarian cysts during pituitary downregulation.

The objectives of this study were:

a) To establish whether buserelin commenced in the luteal phase of the cycle could result in the development of functional ovarian cysts with significant steroidogenic activity.

b) To determine whether the latter functional ovarian cysts are corpus luteal cysts or follicular cysts through measurement of serum steroid levels.

In 288 IVF cycles when patients attended the IVF clinic prior to commencing buserelin a vaginal ultrasound scan was performed and a venous blood sample was taken, spun down and the serum was stored at -20°C. In 264 cycles patients commenced buserelin in the afternoon of the day they attended the clinic, and all patients commenced buserelin within 2 days of the clinic visit. The serum samples were subsequently thawed for measurement of progesterone and oestradiol in batches. On the fourth day of the following menses a further vaginal ultrasound scan was performed, and a second blood sample was taken for estimation of serum oestradiol concentration.

On day 4 of the IVF cycle a functional ovarian cyst was defined as an intraovarian sonolucent structure with a mean diameter of >14 mm.

Significant steroidogenic activity was defined as a serum oestradiol on day 4 of the IVF cycle of greater than the 95th centile established for 727 IVF cycles without apparent cysts (200 pmol/l).

Of the 288 IVF cycles studied 16 patients by these definitions developed functional ovarian cysts with significant steroidogenic activity during pituitary downregulation with buserelin.

The origin and nature of the functional ovarian cysts was investigated by measuring serum progesterone and oestradiol levels. The steroid levels prior to commencing buserelin were used to investigate the aetiology of the functional ovarian cysts. The steroid levels on day 4 of the IVF cycle were used to investigate the nature of the functional ovarian cysts.

2.11 Study 3: Steroid concentrations in functional ovarian cyst fluid.

The objectives of this study were :

a) To establish if functional ovarian cysts which develop during pituitary downregulation contain a sufficiently high steroid concentration to influence the endocrine milieu of developing follicles.

b) To determine the nature of functional ovarian cysts, which develop during pituitary downregulation, by comparing the steroid concentrations of fluid aspirated from cysts with steroid concentrations of follicular fluid collected at the time of oocyte retrieval.

Of 288 IVF cycles studied in 16 patients functional ovarian cysts developed after commencing buserelin. Of the latter 16 patients in 10 cases the ovarian cysts remained steroidogenically active after 3 weeks buserelin treatment, and the patients opted for ovarian cyst

aspiration. Cyst fluid was collected from 9 of the 10 aspirations, and the fluid was frozen for subsequent analysis. It was not possible to collect cyst fluid from one patient, because of technical difficulties.

For the purpose of comparison follicular fluid was collected at the time of oocyte retrieval from 10 patients without functional ovarian cysts. To avoid confusion with small ovarian cysts only follicles of 18-22 mm in size were aspirated, and the sample was only used if an oocyte was found in the follicular fluid. Contamination from flushing medium, blood and other follicles was prevented by using only the follicular fluid obtained from the initial stab into the ovary before any flushing medium was introduced.

Ultrasound scans prior to commencing buserelin had revealed in a further 3 patients the presence of sonolucent ovarian cysts 7 to 10 cm in diameter without any features suggestive of endometriosis, haemorrhage or neoplasia. The latter 3 patients chose to have an ovarian cyst aspiration rather than an ovarian cystectomy prior to embarking on an IVF cycle. The patients were downregulated with buserelin, and the aspiration was performed following the onset of the subsequent menses. Although this group are too small in number to draw firm conclusions the results were felt sufficiently interesting for inclusion in the thesis.

Progesterone, androstenedione and oestradiol concentrations were measured in the fluid aspirated from ovarian cysts or ovarian follicles and paired serum samples taken at the time of aspiration (steroid concentrations were measured as described in chapter 3).

2.12 Study 4: IVF cycles following aspiration of functional ovarian cysts.

Ten patients with persistent functionally active ovarian cysts opted for ovarian cyst aspiration. Prior to the ovarian cyst aspiration a LHRH stimulation test was carried out to confirm pituitary suppression. Three days following cyst aspiration a serum oestradiol level was measured and 5 days following cyst aspiration a repeat ultrasound scan was performed. If patients menstruated following the cyst aspiration ovarian stimulation was commenced on the fourth day of menstruation, otherwise HMG stimulation was commenced 5 days following the cyst aspiration provided the serum oestradiol was <200 pmol/l. The treatment from the commencement of ovarian stimulation progressed as described above.

Three patients who prior to starting an IVF cycle were found to have simple ovarian cysts opted to have a cyst aspiration rather than cystectomy. Buserelin was commenced as described above, and the aspiration was performed following the ensuing menses. Cytology was performed on the ovarian cyst aspirate. The cytology was negative for the presence of malignant cells in all 3 cases. Five days following the aspiration a repeat ultrasound scan was performed. If no ovarian cyst was present, the patients commenced controlled ovarian hyperstimulation, and their management proceeded as described above.

2.13 Summary

It will be seen from this chapter that the management of IVF patients *following* the commencement of controlled ovarian hyperstimulation did not differ significantly between the four studies. However, there was a significant change in patient management *prior to* commencing controlled ovarian hyperstimulation introduced in studies 2, 3 and 4. Patient management was altered to identify functional ovarian cysts before commencing ovarian hyperstimulation and ovarian stimulation did not start until the serum oestradiol fell below 200 pmol/l. In some cases the latter was achieved by performing a transvaginal ovarian cyst aspiration.

The following chapter will describe the assays used in the studies, and the following section will present and discuss the results of the studies.

CHAPTER 3

Assays

Chapter 3 Assays

This chapter describes the assays employed in the measurement of steroid and gonadotrophin levels during the 4 studies presented in this thesis. The principle, method, precision, range and specificity of each assay will be given. The chapter presents the validation for use of the assays in measuring compounds in cyst and follicular fluid. The application of the progesterone assay for determination of luteinisation, and the use of the gonadotrophin assays for assessment of pituitary function will be explained.

3.1 Steroid assays

Commercially available radioimmunoassay kits, which have been validated for measurement in human serum, were used to assay progesterone levels (Amersham International plc, UK, code 3221) and oestradiol levels (Serono diagnostics Ltd., UK, code 12264). Androstenedione was extracted into hexane/ethane and measured with an in house radioimmunoassay validated for use in human serum (Egan *et al.*, 1989). The above assays were further validated to measure steroids in ovarian cyst and follicular fluid following dilution.

3.1.1 Progesterone

Principle This radioimmunoassay uses ^{125}I-labelled progesterone as a tracer, and a progesterone specific antibody bound to magnetic polymer particles. The progesterone in the specimen is placed in competition with the ^{125}I-labelled progesterone for a limited number of antibody binding sites. The radioactive progesterone bound to the antibody is thus inversely proportional to the concentration of progesterone in the specimen. The progesterone bound to the magnetic polymer particles is separated using a magnetic field, and the supernatant is discarded. By measuring the radioactivity of ^{125}I-

labelled progesterone bound in the presence of standard reference sera, unknown progesterone concentrations can be determined.

Method All samples, controls and standards are assayed in duplicate, and results are rejected if there is more than a 10% difference between duplicate values. The steps of the assay are outlined in figure 3.1, and the assays are performed using LP3 tubes. ^{125}I-labelled progesterone solution is added to samples, standards and controls. The progesterone antibody suspension is then added and the tubes are vortex mixed. It is important at intervals to vortex mix the progesterone antibody maintaining it in suspension to ensure equal amounts of antibody are pipetted into each tube. After 2 hours incubation at room temperature the tubes are placed in a magnetic separator for 15 minutes. While maintaining contact between the base of the tubes and the magnetic separator the tubes are inverted draining off the supernatant. It is important to blot the rims of the tubes to remove any residual supernatant. The supernatant contains excess ^{125}I-labelled progesterone, and this would produce an artifactually high radioactive count. The radioactivity of the tubes is measured with a gamma counter (NE1608, Nuclear enterprises), and the RIAcalc software programme (L.K.B. Wallac, Finland) is used to produce a standard curve and determine assay results.

Precision The interassay variation was determined over 10 assays using 3 control samples. The coefficient of variation was 4.7%, 4.8% and 5.9% at mean values of 4.1 nmol/l, 19 nmol/l and 63 nmol/l.

Range The sensitivity of this assay defined as two standard deviations from the zero standard had a mean value of 0.25 nmol/l. The top standard sample had a concentration of 127 nmol/l.

Specificity The specificity of the assay for the measurement of progesterone has been determined by measuring the interference from a variety of compounds using Abraham's method (see table 3.1).

Add 50 µl of specimen/control/standard to tubes

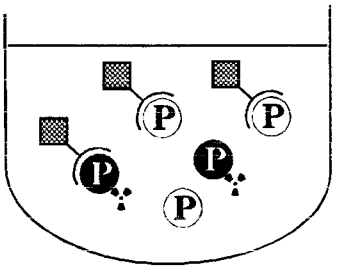
Add 500 µl of tracer

Add 500 µl Progesterone antibody coupled to magnetic particles

Vortex mix all tubes

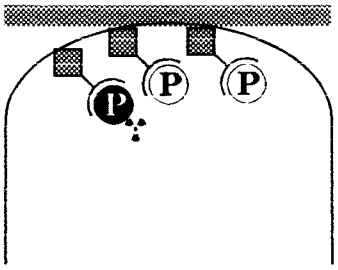
Cover tubes with parafilm

Incubate 2 hours at 20-25°C



Place tubes in contact with magnetic separator for 15 minutes

Invert tubes and decant supernatant blot rims of tubes



Measure radioactivity of residue with gamma counter

Figure 3.1 Outline of procedure for progesterone assay.

Abraham's method: The weight of progesterone is divided by the weight of interfering compound that causes a 50% decrease in binding capacity and expressed as a percentage.

Table 3.1: Specificity of progesterone antiserum (source Amersham International plc., Amersham, UK)

Antigen	Cross-reactivity
Progesterone	100.00%
17 -hydroxyprogesterone	0.05%
Pregnenolone	0.19%
Oestradiol	<0.01%
Oestriol	<0.01%
Oestrone	<0.01%
Testosterone	0.11%
Cortisol	<0.01%
Corticosterone	1.12%

3.1.2 Oestradiol

Principle This radioimmunoassay uses ^{125}I-labelled oestradiol as a tracer, one antibody to oestradiol raised in rabbits, and a second antibody to rabbit gammaglobulins. The second antibody is covalently bound to a magnetic particle. The oestradiol in the specimen is placed in competition with the ^{125}I-labelled oestradiol for a limited number of oestradiol binding sites on the first antibody. The radioactive oestradiol bound to the first antibody is thus inversely proportional to the concentration of oestradiol in the specimen. The second antibody is added and this binds to the first antibody enabling separation of the oestradiol bound complex in a magnetic field, and the supernatant is discarded. By measuring the radioactivity of ^{125}I-

labelled oestradiol bound in the presence of standard reference sera, unknown oestradiol concentrations can be determined.

Method All samples, controls and standards are assayed in duplicate, and results are rejected if there is more than a 10% difference between duplicate values. The steps of the assay are outlined in figure 3.2, and the assays are performed using LP3 tubes. ^{125}I-labelled oestradiol solution is added to samples, standards and controls. The oestradiol antibody is then added and the tubes are vortex mixed. The tubes are incubated for 1 hour at 37°C in a water bath. The second antibody is then added and the tubes are incubated for a further 10 minutes. It is important at intervals to vortex mix the second antibody maintaining it in suspension to ensure equal amounts of antibody are pipetted into each tube. The tubes are placed in a magnetic separator for 10 minutes. While maintaining contact between the base of the tubes and the magnetic separator the tubes are inverted draining off the supernatant. It is important to blot the rims of the tubes to remove any residual supernatant. The supernatant contains excess ^{125}I-labelled oestradiol, and this would produce an artifactually high radioactive count. The radioactivity of the tubes is measured with a gamma counter (NE1608, Nuclear enterprises) and the RIAcalc software programme (L.K.B. Wallac, Finland) is used to produce a standard curve and determine assay results.

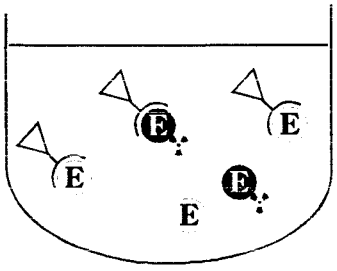
Precision The interassay variation was determined over 10 assays using 2 control samples. The coefficient of variation was 7.7% and 6.8% at mean values of 156 pmol/l and 989 pmol/l.

Range The sensitivity of this assay defined as two standard deviations from the zero standard had a mean value of 55 pmol/l. The top standard had a concentration of 18,350 pmol/l.

Add 50 µl of specimen/control/standard to tubes

Add 100 µl of tracer

Add 100 µl antibody to oestradiol



Vortex mix all tubes

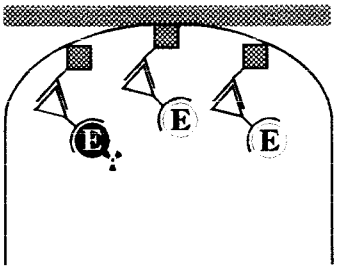
Cover tubes with parafilm

Incubate 1 hour at 37°C

Add 100 µl second antibody

Incubate 10 minutes

Place tubes in contact with magnetic separator for 10 minutes



Invert tubes and
decant supernatant

Measure radioactivity of residue with gamma counter

Figure 3.2 Outline of procedure for oestradiol assay.

Specificity This was determined by Abraham's method as described above, and the interference from a variety of compounds is shown in table 3.2.

Table 3.2: Specificity of oestradiol antiserum (source Serono Diagnostics Ltd., Woking, U.K.)

Antigen	Cross-reactivity
Oestradiol	100%
Oestrone	1.775
Oestriol	.47%
Androstenedione	.0001%
Progesterone	absent up to 100 µg/ml
Testosterone	.0033%
Dehydroepiandrosterone	absent up to 100 µg/ml
Cortisol	absent up to 50 µg/ml
Aldosterone	.007%

3.1.3 Androstenedione

Principle This radioimmunoassay uses ^{125}I-androstenedione-3-carboxymethyloxime-histamine as a tracer, and an antiserum raised to androstenedione-3-carboxymethyloxime-bovine serum albumin conjugate. Androstenedione is extracted from the specimen using a hexane:ether mixture, and the androstenedione in the dried residue is placed in competition with the ^{125}I-labelled androstenedione for a limited number of antibody binding sites. The radioactive androstenedione bound to the antibody is thus inversely proportional to the concentration of androstenedione in the specimen. The antibody/antigen complex is precipitated by adding polyethylene glycol/gamma globulin, and following centrifugation the supernatant

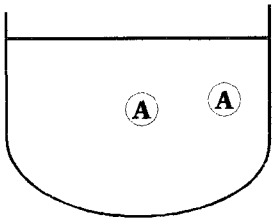
is discarded. By measuring the radioactivity of ^{125}I-labelled androstenedione bound in the presence of standard reference sera, unknown androstenedione concentrations can be determined.

Method All samples, controls and standards are assayed in duplicate, and results are rejected if there is more than a 10% difference between duplicate values. The steps of the assay are outlined in figure 3.3, and the assays are performed in glass tubes as the solvents used for extraction would react with polystyrene or polypropylene tubes. An assay buffer pH 7.5 (containing Tris(hydroxymethyl)methylamine, bovine serum albumin 5 g/l, and sodium azide 1 g/l) is added to samples, standards and controls. A 4:1 mixture of hexane and ether is then added, and the tubes are capped tightly. The tubes are vortex mixed for 5 minutes. It is important to ensure an adequate vortex is formed so that the androstenedione is extracted from the samples. However, too vigorous shaking of the tubes will result in the formation of an emulsion at the interface between the sample and the hexane/ether mixture. Any emulsion formed during the vortex mixing is broken down by gently tapping on the side of the tubes, and the tubes are left to stand for 5 minutes. In a fume cupboard the samples are snap frozen in a bath of dry ice and methylated spirits. The solvent layer is decanted into glass tubes, which are then evaporated to dryness at 40°C using compressed air.

The tracer and antibody are added to the dry tubes, mixed and incubated at 4°C overnight. The following day a mixture of 12% polyethylene glycol and 0.2% bovine gamma globulin is added. The tubes are vortex mixed and incubated for 15 minutes at 4°C. Following centrifugation the supernatant is discarded. The radioactivity is measured with a gamma counter (NE1608, Nuclear

Add 10 µl of specimens/controls to glass tubes
Add 100 µl of standards to glass tubes

Add 200 µl of buffer to specimens/controls
Add 100 µl of buffer to standards



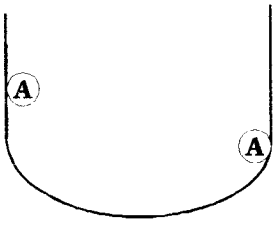
Add 2ml of hexane:ether (4:1) and cap tightly
Vortex mix for 5 minutes
Stand for 5 minutes

In fume cupboard:

Snap freeze using dry ice in methylated spirits

Decant solvent layer into glass tubes

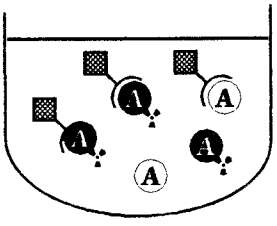
Evaporate to dryness at 40°C using compressed air



Add 100 µl of tracer to specimens/controls/standards

Add 100 µl of antibody to specimens/controls/standards

Mix and incubate at 4°C overnight.



Add 1 ml Polyethylene glycol/gamma globulin

Vortex mix

Incubate 15 minutes at 4°C.

Centrifuge for 15 minutes at 3000 r.p.m. at 4° C.

Discard supernatant

Read counts with gamma counter.

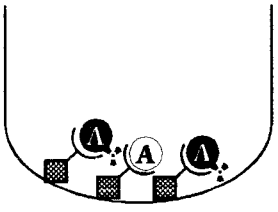


Figure 3.3 Outline of procedure for androstenedione assay.

enterprises), and the RIAcalc software programme (L.K.B. Wallac, Finland) is used to produce a standard curve and determine assay results.

Precision The interassay variation was determined over 10 assays using 2 control samples. The coefficient of variation was 9.7% and 6.8% at mean values of 2.1 nmol/l and 6.8 nmol/l.

Range The sensitivity of this assay defined as two standard deviations from the zero standard had a mean value of 0.8 nmol/l. The top standard has a concentration of 50 nmol/l.

Specificity

This was determined by Abraham's method as described above, and the interference from a variety of compounds is shown in table 3.3.

Table 3.3 Specificity of androstenedione antiserum (source Egan *et al.*, 1989).

Steroid	% Cross-reaction
Androstenedione	100.0
5α Androstanedione	21.6
5β Androstanedione	14.6
11 oxo Androstenedione	2.9
11β OH Androstenedione	2.9
Androsterone	2.6
Testosterone	2.6
Etiocholanolone	2.0
Dehydroepiandrostanedione	1.1
11β OH Androsterone	0.8
Epistestosterone	0.5
11α OH Androstenedione	0.3
5α (& 5β) Dihydrotestosterone	0.3
Progesterone	0.2

3.2 Steroid measurements in cyst and follicular fluid.

Measurement of steroids in cyst or follicular fluid presents two problems. Firstly the concentrations of many steroids is much higher in cyst or follicular fluid than in serum, thus cross reactivities with other steroids which may be insignificant in serum may present a problem in cyst or follicular fluid. Secondly to reduce the concentration of steroids into the range measurable by the steroid assays described above it is necessary to dilute the cyst or follicular fluid. The characteristics of the diluent used to dilute the cyst or follicular fluid could alter the binding of antibodies to antigens thus interfering with the validity of the steroid assays.

Gas chromatography-mass spectrometry of follicular fluid enables reliable identification of steroids which can be quantified by stable isotope dilution (Dehennin 1990). This technique has confirmed previous findings using radioimmunoassay for progesterone and oestradiol (Dehennin 1990). The latter suggests that there are no cross-reactive substances present in sufficiently high concentrations in follicular fluid to interfere with radioimmunoassays for oestradiol and progesterone. However, Dehennin (1990) felt that 19-norandrostenedione was present in sufficiently high concentration in follicular fluid to interfere with radioimmunoassay for androstenedione, if the androstenedione concentration was below 70 nmol/l.

To determine the appropriate diluent prior to steroid assay parallelism studies were performed. Serum influences the binding of antibody to antigen by a matrix effect, which is largely due to the 7% concentration of protein in serum. To mimic this matrix effect a cyst fluid sample was taken, and two separate double dilutions were made

one with 5% albumin and the other with 10% albumin. The dilutions were assayed for oestradiol as described above, and the radioactive counts were recorded. Within the same assay standard samples of known oestradiol concentrations were measured to provide a standard curve. The oestradiol concentration was plotted on a logarithmic X axis against the corresponding radioactive count expressed as a percentage of the count for the zero standard on a linear Y axis. On the same graph two further curves were plotted, one for each set of serial dilutions. At intervals equivalent to halving of values on the logarithmic scale of the X axis the radioactive counts of the dilutions in order were plotted, expressed as a percentage of the count for the zero standard on the Y axis. If antigen/antibody interaction was not influenced by the diluent, the curve of the serial dilutions should be parallel to the standard curve. It can be seen in figure 3.4 that serial dilutions of cyst aspirate in 10% albumin (but not 5% albumin) produced a curve parallel to the standard curve for oestradiol assay.

For progesterone assay a 5% albumin solution proved suitable for dilution (see figure 3.5). The difference in albumin concentrations used to measure progesterone or measure oestradiol was a reflection of the amounts of steroid measured and the avidity of the antisera to the steroid. Identical curves were seen for follicular fluid as cyst fluid. Similarly the assay buffer used for androstenedione dilution had been validated by the Wessex Regional Endocrine Laboratory.

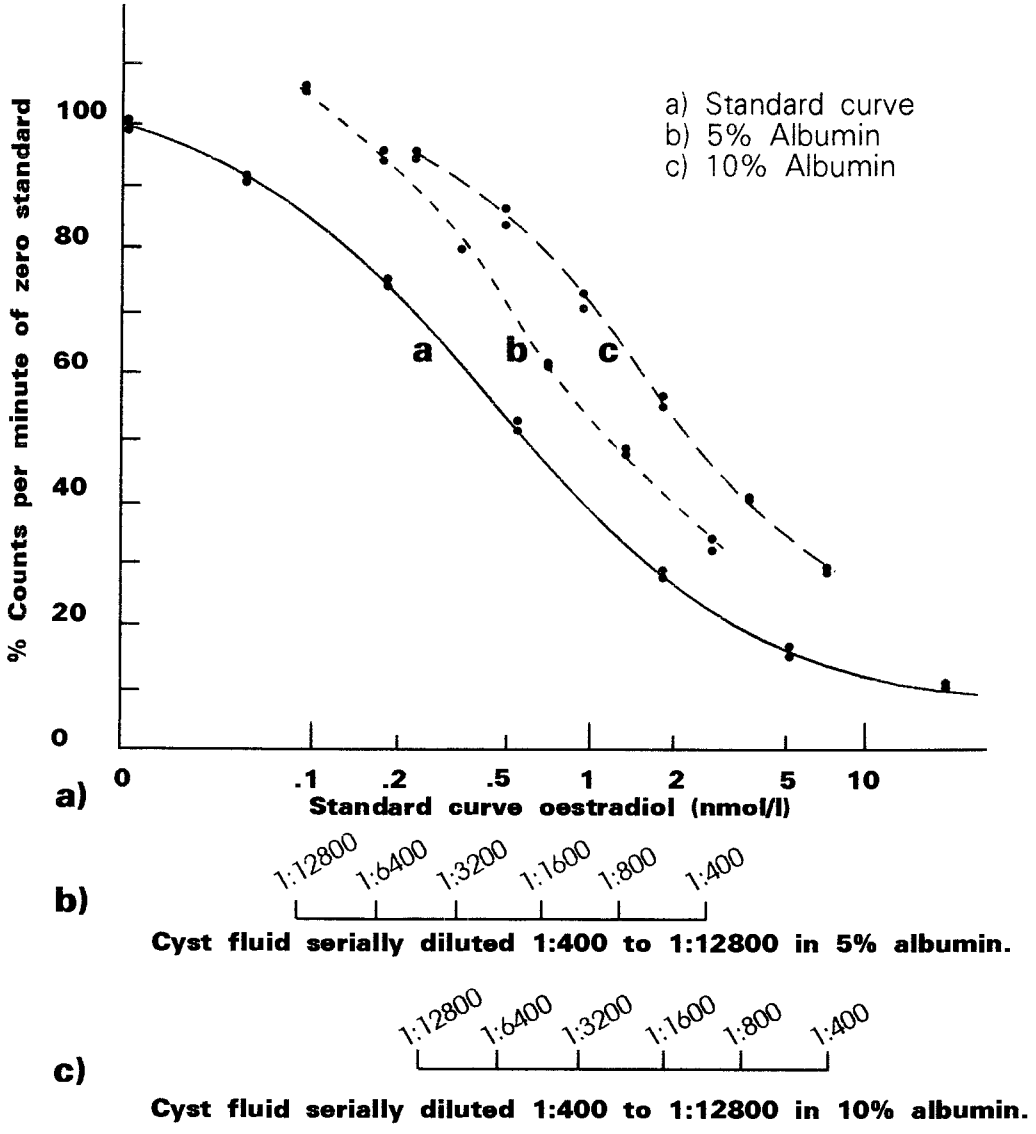


Figure 3.4 Demonstration by parallelism of the suitability of 10% albumin for dilution of ovarian cyst fluid prior to oestradiol measurement.

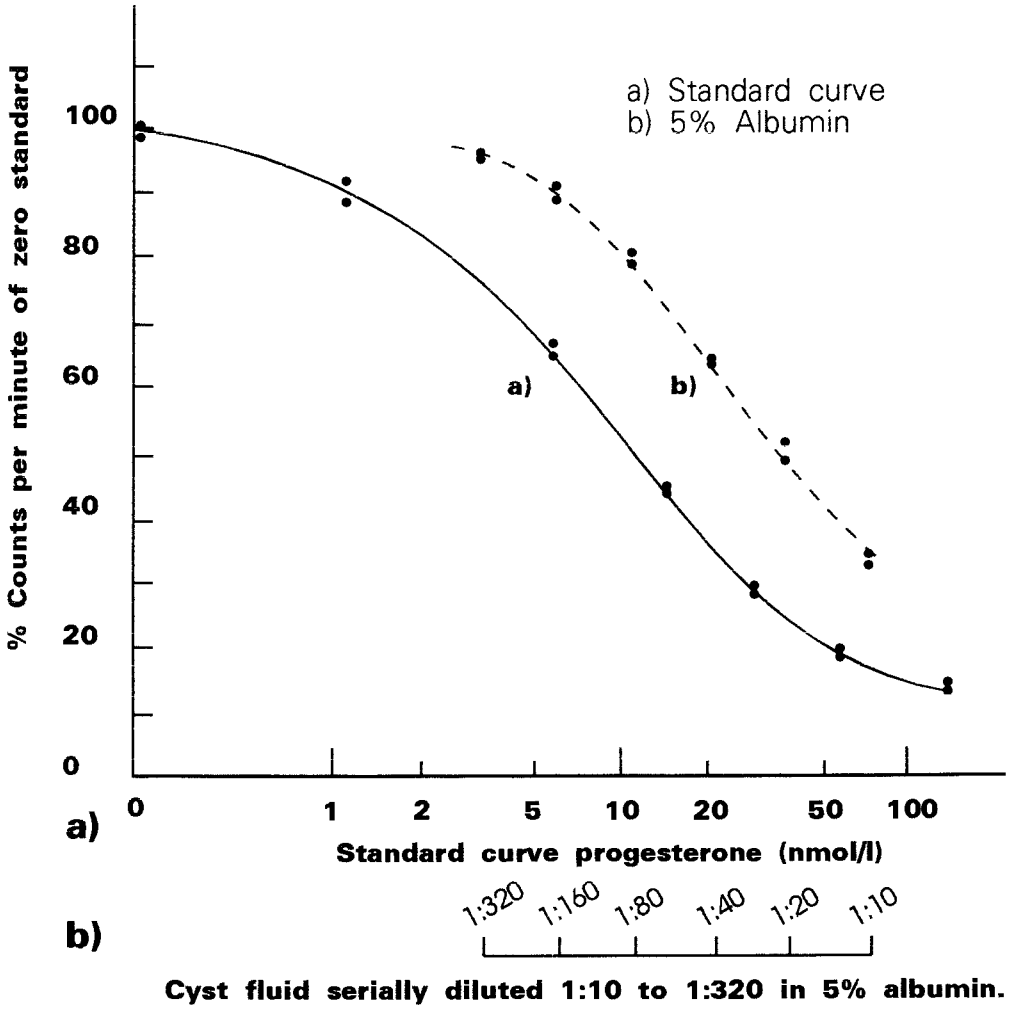


Figure 3.5 Demonstration by parallelism of the suitability of 5% albumin for dilution of ovarian cyst fluid prior to progesterone measurement.

3.3 Use of the serum progesterone assay as an indicator of luteinisation.

As was described in chapter 1 there is a shift of ovarian steroid output to progesterone production following luteinisation. It has been shown that the latter increase in serum progesterone can be used to predict spontaneous ovulation (Fleming and Coutts 1982). Thirty three cycles from 21 patients receiving donor insemination had serum progesterones the day prior to the LH peak, the day of the LH peak and the day following the LH peak of 1.37 ±0.45 nmol/l, 2.89 ±0.86 nmol/l and 5.79 ±2.13 nmol/l, mean and standard deviations respectively (Fleming and Coutts 1982).

Trounson (1984) investigated the relationship between serum progesterone levels and the onset of a spontaneous LH surge in 72 patients superovulated with clomiphene citrate and HMG for in vitro fertilisation. Trounson found the first significant rise in progesterone coincided with the onset of the LH surge, although this could be masked if the LH surge started at 8 am as there was a diurnal variation of progesterone with the nadir at 8 am. Blood samples taken at 08.00, 14.00 and 21.00 hours were assayed for LH and progesterone. Oocyte retrieval was performed 32 to 36 hours following the onset of the LH surge, which was defined as the first of three plasma samples in which the LH value was above the mean +2 standard deviations of the previous baseline samples. The plasma progesterone concentrations at the onset of the LH surge at 08.00, 14.00 and 21.00 hours were 1.2 ±1.7 nmol/l, 2.3 ±2.1 nmol/l and 2.7 ±4.9 nmol/l, mean and standard deviations respectively. Pregnancies resulted with progesterone levels at the onset of the LH surge at 08.00, 14.00 and 21.00 hours of up to 2.5 nmol/l, 8.3 nmol/l and 6.0 nmol/l respectively (Trounson and Calabrese 1984).

In view of the relationship between post luteinisation steroidogenesis and the serum progesterone level it was proposed to use the progesterone assay described above to establish the "maximum progesterone" level which was consistent with follicular steroidogenic activity prior to luteinisation. In order to establish a reference range for this purpose 70 non-conception and 35 conception IVF cycles proceeding to oocyte retrieval without apparent ovarian cysts were selected at random from the 569 IVF cycles in group C of study 2 described in chapter 2. Stored frozen serum samples taken between 9.30 am and 11.30 am the day it had been decided to administer the luteinising HCG injection in the specified 105 IVF cycles were thawed, and the serum progesterone and oestradiol levels were measured.

A significant correlation was obtained between serum oestradiol and serum progesterone on the day of HCG administration for conception IVF cycles ($p<0.01$, figure 3.6). However, progesterone levels were low and remained below 6 nmol/l. The correlation between serum oestradiol and serum progesterone on the day of HCG administration for non-conception IVF cycles failed to reach statistical significance (figure 3.7). The latter may be explained by an outlying result, which was most likely due to premature luteinisation. The patient with a progesterone of 11.8 nmol/l had 14 oocytes collected and 12 oocytes fertilised but only 1 embryo cleaved to produce a grade 3 embryo and 4 embryos fragmented completely. The same patient had had a previous IVF cycle when of 11 oocytes collected 8 fertilised and all 8 went on to form good quality cleaved embryos.

One case of premature luteinisation in the 105 IVF cycles studied is consistent with the report of 1 case of premature luteinisation in 87

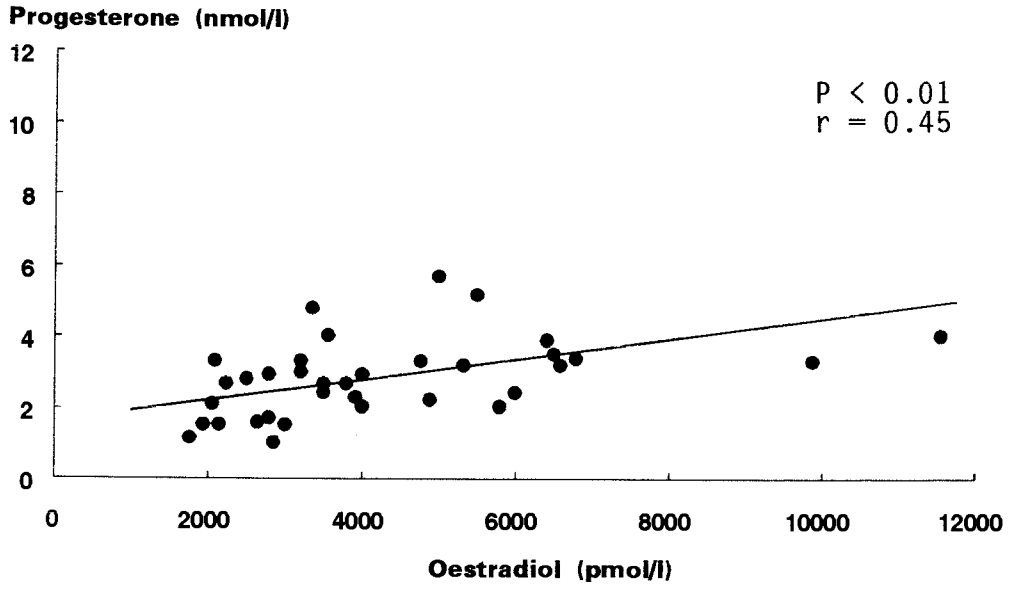


Figure 3.6 Serum progesterone versus serum oestradiol on day of HCG administration in 35 conception IVF cycles without apparent ovarian cysts.

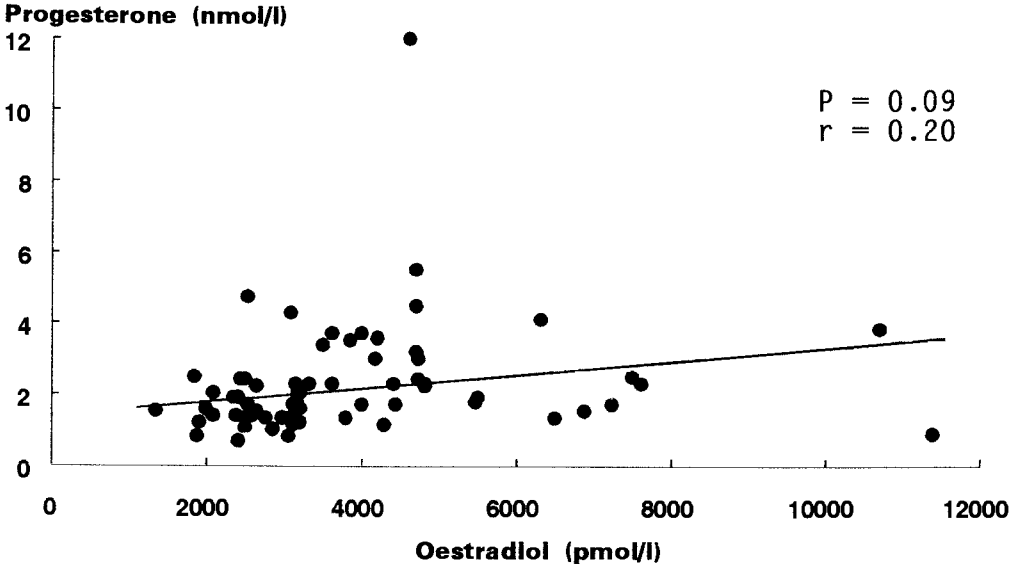


Figure 3.7 Serum progesterone versus serum oestradiol on the day of HCG administration in 70 non-conception IVF cycles without apparent ovarian cysts.

IVF cycles treated with a long buserelin regime (Fleming and Coutts 1986). Of the remaining 104 IVF cycles the range of serum progesterone was from 0.3 nmol/l to 5.7 nmol/l, and the range of serum oestradiol was 1365 pmol/l to 11570 pmol/l (see figures 3.6 and 3.7). From this data it is suggested that a serum progesterone greater than 5.7 nmol/l may be indicative of luteinised follicular activity even if the serum oestradiol is elevated as a consequence of controlled ovarian hyperstimulation.

3.4 Gonadotrophin Assays

Commercially available radioimmunoassay kits validated for measurement in human serum were used to assay LH (Serono, UK, code 13201) and FSH (Serono, UK, code 13101). These radioimmunoassays are referred to as immunoradiometric assays to distinguish them from other immunoassays. This is because these assays are not competitive binding assays and the amount of radioactivity (as labelled antibody) bound to the solid phase is directly proportional to the amount of antigen being measured.

3.4.1 Luteinising Hormone

Principle This radioimmunoassay uses a monoclonal antibody to LH labelled with ^{125}I as a tracer, a second monoclonal antibody to LH linked to fluorescein, and a third antibody raised to fluorescein and coupled to magnetic particles. The double monoclonal antibody produces a highly specific assay for the intact LH molecule. The antibody labelled with ^{125}I attaches to a unique site on the LH molecule, and the second monoclonal antibody linked to fluorescein binds at another discrete site on the LH molecule forming a sandwich. The antibody to fluorescein coupled to magnetic particles is added in excess producing a LH/antibody complex, which can be

separated in a magnetic field, and the supernatant discarded. The radioactivity of the sediment is proportional to the concentration of LH in the sample, and by measuring samples of known LH concentrations unknown LH values can be determined.

Method All samples, controls and standards are assayed in duplicate, and results are rejected if there is more than a 10% difference between duplicate values. The steps of the assay are outlined in figure 3.8 and the assays are performed using LP3 tubes. Both monoclonal antibodies to LH are added to samples, controls and standards. After a 1 hour incubation at room temperature, the antibody to fluorescein coupled to magnetic particles is added. After a further 5 minute incubation, the tubes are placed in a magnetic separator for 2 minutes. While maintaining contact between the base of the tubes and the magnetic separator the tubes are inverted draining off the supernatant. A wash buffer is added to all tubes and vortex mixed. The tubes are returned to the magnetic separator and after 2 minutes the supernatant is discarded as above. The radioactivity of the sediment is measured with a gamma counter (NE1608, Nuclear enterprises) and the RIAcalc software programme (L.K.B. Wallac, Finland) is used to produce a standard curve and determine assay results.

Precision

Intra assay variation: two serum samples were measured 10 times within one assay giving mean values of 7.3 IU/l and 195 IU/l with respective coefficients of variation of 4.7% and 1.9%.

Inter assay variation: 2 LH assays were performed with a commercially provided control sample with reported mean value of 6.8 IU/l and gave levels of 7.3 and 6.9 IU/l. This was within the expected range that was quoted for this control sample (5.2 - 8.6 IU/l 95% confidence range).

Add 40 µl of specimen/control/standard to tubes

Add 40 µl of mixture of antibody to LH (linked to ^{125}I)

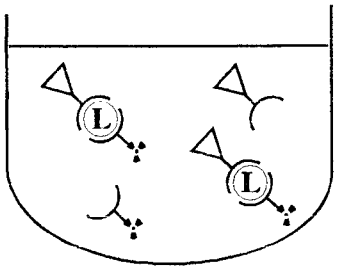
and

antibody to LH (linked to fluorescein)

Vortex mix all tubes

Cover tubes with parafilm

Incubate 1 hour at 20-25°C

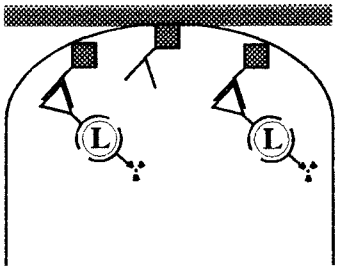


Add 80 µl antibody to fluorescein (linked to magnetic particle)

Incubate 5 minutes

Place tubes in contact with magnetic separator for 2 minutes

Invert tubes
Decant supernatant
Blot rims of tubes



Add 0.5 ml of wash buffer to all tubes

Vortex mix

Place in contact with magnetic separator for 2 minutes

Invert tubes and decant supernatant

Measure radioactivity of residue with gamma counter

Figure 3.8 Outline of procedure for LH assay.

Range The sensitivity of this assay defined as two standard deviations from the zero standard had a mean value of 0.28 IU/l. The top standard has a concentration of 200 IU/l.

Specificity The specificity of the LH assay has been determined by measuring the apparent LH level in samples containing high concentrations of potentially cross reactive substances (see table 3.4).

Table 3.4 Specificity of LH antiserum (Source- Serono Diagnostics Ltd., Surrey, UK)

Analyte	Concentration	Apparent LH value (IU/l.)
hFSH	800 IU/l (2nd IRP 78/549)	0.4
hTSH	100 mIU/l (2nd IRP 80/558)	1.6
hCG	100,000 IU/l (1st IRP 75/537)	3.0

3.4.2 Follicle Stimulating Hormone

Principle This radioimmunoassay uses a monoclonal antibody to FSH labelled with ^{125}I as a tracer, a second monoclonal antibody to FSH linked to fluorescein, and a third antibody raised to fluorescein and coupled to magnetic particles. The principle of this assay is the same as the LH assay described above.

Method This is the same as described for the assay of LH above except the antibodies used are directed to FSH instead of LH.

Precision

Intra assay variation: Two serum samples were measured 10 times in one assay giving mean values of 4.5 IU/L and 19.3 IU/l with coefficients of variation of 5.6% and 6.3% respectively.

Inter assay variation: 2 FSH assays were performed with a commercially provided control sample with reported mean value of 7.3 IU/l and gave levels of 7.6 and 6.7 IU/l. This was within the expected range that was quoted for this control sample (5.6 - 9.4 IU/l 95% confidence range).

Range The sensitivity of this assay defined as two standard deviations from the zero standard had a mean value of 0.27 IU/l. The top standard has a concentration of 150 IU/l.

Specificity The specificity of the FSH assay has been determined by measuring the apparent FSH level in samples containing high concentrations of potentially cross reactive substances (see table 3.5).

Table 3.5 Specificity of FSH antiserum (Source- Serono Diagnostics Ltd., Surrey, UK)

Analyte	Concentration	Apparent FSH value (IU/l.)
hLH	400 IU/l (1st IRP 68/40)	0.73
hTSH	1000 mIU/l (2nd IRP 80/558)	0.63
hCG	300 IU/l (1st IRP 75/537)	nil detected

3.5 LHRH stimulation test.

As was explained in chapter 1 buserelin following an initial stimulatory phase produces suppression of pituitary gonadotrophin secretion. To determine whether buserelin has made the pituitary become unresponsive to LHRH released from the hypothalamus a dynamic test of pituitary function can be performed. The LHRH stimulation test described by Mortimar in 1973 is a dynamic test of

pituitary function, specifically testing the ability of the pituitary to respond to LHRH.

The LHRH test was performed in a consistent manner for all patients. Between 9 am and 10.30 am a basal serum sample was taken, and the patient was given an intravenous bolus of 0.1 mg of LHRH (Gonaderelin, Hoechst, UK). Further serum samples were taken 30 minutes and 1 hour following the injection. The serum was separated and stored frozen at -20°C for subsequent batch assay. Serum LH and FSH concentrations were measured as described above. To avoid an acute response to buserelin confusing the test result the patient had her normal dose of buserelin the night prior to the test, but omitted the dose of buserelin on the morning of the test.

There is variation of the pituitary response to LHRH during the menstrual cycle with the peak response in the preovulatory period and the least response in the early follicular phase (Yen *et al.,* 1972). Mortimar (1973) defined an absent response as one in which following the intravenous bolus of LHRH the rise in gonadotrophins was less than three times the intra-assay coefficient of variation at basal gonadotrophin levels. For the assays described above this would give values of 14.1% for LH and 16.8% for FSH. Mortimar (1973) defined an impaired response as failure for LH and FSH levels to reach 4.3 IU/l and 9 IU/l respectively, and he defined a delayed response as a final value equal to or greater than the second value.

3.6 Statistical Analyses

The analysis of data in this thesis has often had to take the form of nonparametric analysis (usually the Mann-Whitney U test for comparison), because of skewness of data or non-normally distributed data. This has partly resulted from the type of populations studied but may also be inherent to some extent in the use of radioimmumoassays. The medians, interquartile ranges and absolute ranges therefore defined the data more appropriately. Where possible means, standard deviations and parametric tests have been used, if these have been the accepted methods of analysis.

Statistical analysis has been carried out using the University Statgraphics version 5.0. software package on an IBM compatible personal computer. This package has been used for the regression analyses and to plot the best lines of fit (regression lines) on the graphs. Logarithmic transformations or other models did not fit all data and were therefore not adopted.

3.7 Summary

This chapter has described the methodology of the assays used in this thesis to measure compounds in serum, cyst fluid and follicular fluid. The chapter has evaluated the reliability of the results obtained from the assays before applying the assays to clinical studies. Serum progesterone levels have been shown to be able to provide an indication of the onset of luteinisation, and thus the presence of a corpus luteum with steroidogenic activity. Dynamic testing of pituitary function assessed by gonadotrophin assays has been discussed. Finally the chapter has mentioned the statistical evaluation of assay results in the clinical studies which will be presented in the following section of the thesis.

RESULTS
and
DISCUSSIONS

Chapter 4

Results and Discussion of Study 1 :

The influence of functional ovarian cysts during IVF cycles related to serum steroid levels.

4.1 Introduction

This chapter presents and discusses the results of the first study reported in this thesis. Study 1 assessed the influence of functional ovarian cysts during IVF cycles in relation to serum steroid levels. The principal objective of this study was:

> *To establish whether there is a relationship between steroidogenic activity of functional ovarian cysts and a detrimental influence on IVF cycles.*

The methodology of study 1 was explained fully in chapter 2 with a detailed account of the assays used presented in chapter 3. The influence of functional ovarian cysts during 53 IVF cycles was assessed in relation to serum steroid levels and compared to 727 IVF cycles without apparent ovarian cysts. Serum steroid concentrations following pituitary downregulation and prior to ovarian stimulation were used to reflect steroidogenic activity of ovarian cysts present during ovarian stimulation..

4.2 Results

Comparing the 53 IVF cycles with ovarian cysts to the 727 IVF cycles without apparent ovarian cysts no significant difference was found in age or the incidence of the following infertility diagnoses: tubal factor, unexplained, male factor and endometriosis (see table 4.1). However, there was a significantly higher percentage of patients with ovarian cysts ($p<0.01$), where at least part of their infertility problem related to ovulatory dysfunction (see table 4.1).

Table 4.1: Age and indication for IVF in cycles with ovarian cysts compared to cycles without apparent ovarian cysts.

	Cyst (n=53)	No apparent cyst (n=727)	
Age	33.2 (SD 5.0)	32.9 (SD 4.1)	N.S.
Infertility diagnosis			
Tubal	50.9%	44.0%	N.S.
Unexplained	17.0%	17.2%	N.S.
Male Factor	28.3%	21.4%	N.S.
Endometriosis	3.8%	11.0%	N.S.
Ovulatory	26.4%	13.3%	**
** $p < 0.01$ χ^2 test NS non-significant Mann Whitney U Test or χ^2 test as appropriate			
(Where couples had more than one infertility diagnosis they were included in all relevant groups.)			

Of the 53 cycles with ovarian cysts the serum oestradiol on the day ovarian stimulation was commenced was significantly higher in the cycles which were abandoned than those which proceeded to oocyte retrieval (figure 4.1). Of the latter 53 cycles 23 had a day 4 serum oestradiol >200 pmol/l and these had a significantly higher cancellation rate for failure to develop adequate numbers of follicles than the 30 cycles with a day 4 serum oestradiol ≤200 pmol/l, 60.8% v 26.6% respectively ($p<0.05$). The cancellation rate for the 727 IVF cycles without apparent cysts was 21.7%, which did not differ significantly from the 30 cycles with ovarian cysts with day 4 oestradiol ≤200 pmol/l.

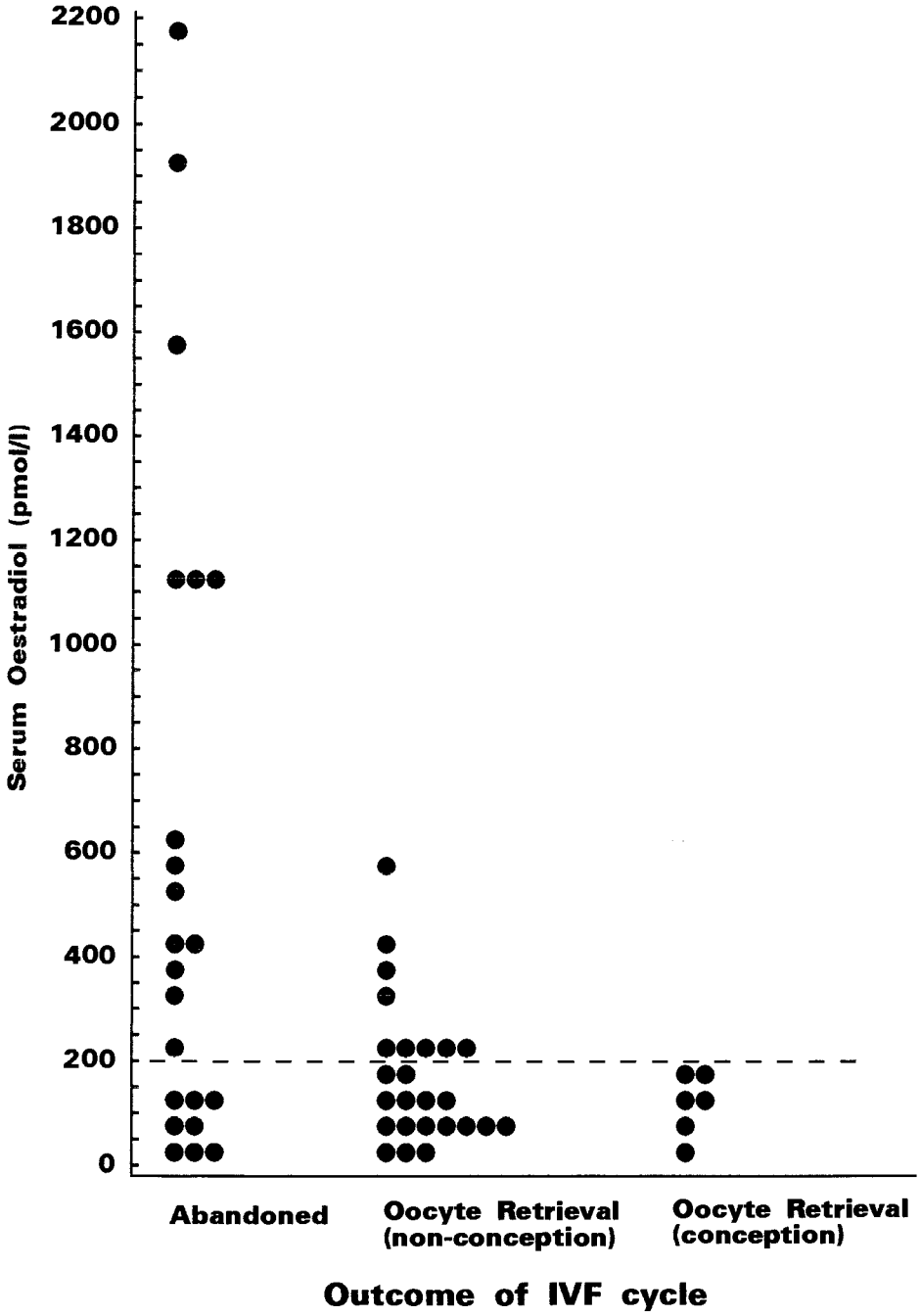


Figure 4.1 Serum oestradiol level on day 4 of IVF cycle in 53 cycles with ovarian cysts ● grouped according to outcome, with 95th centile- - for 727 IVF cycles without apparent ovarian cysts.

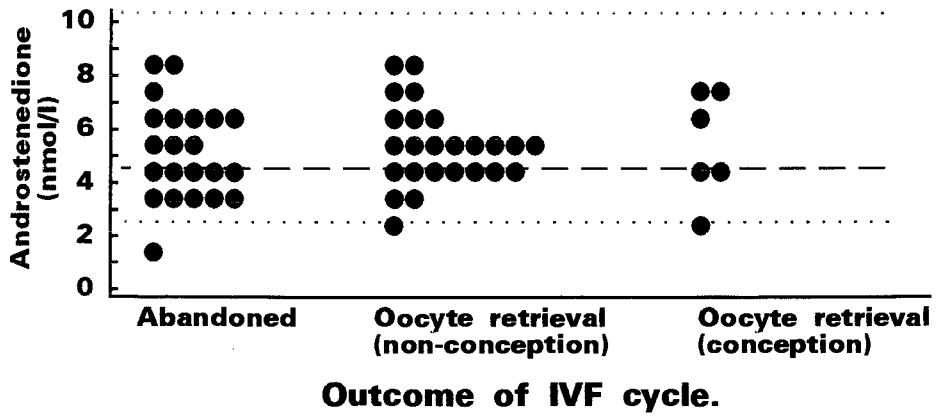


Figure 4.2 Serum Androstenedione level on day 4 of IVF cycle in 53 cycles with ovarian cysts ● grouped according to outcome with median — – and absolute range · · · · shown for 20 patients without apparent cysts.

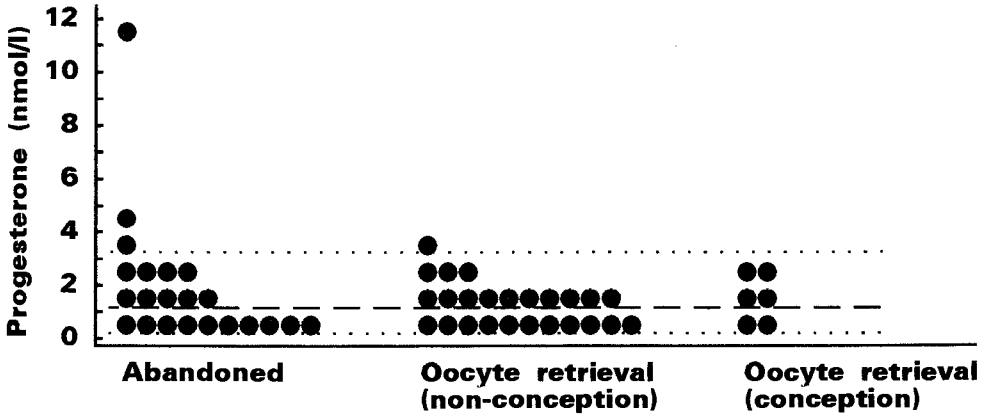


Figure 4.3 Serum progesterone level on day 4 of IVF cycle in 53 cycles with ovarian cysts ● grouped according to outcome with median — – and absolute range · · · · shown for 20 patients without apparent cysts.

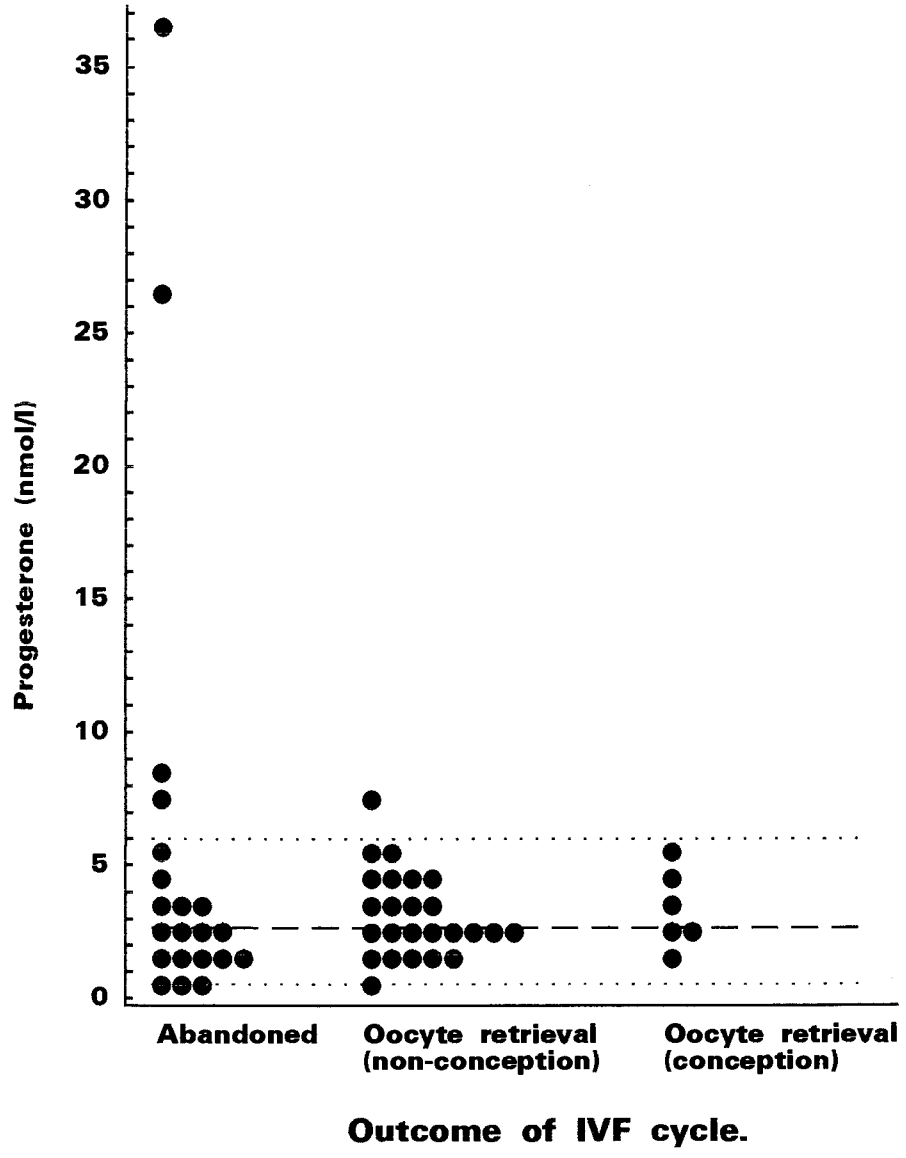


Figure 4.4 Serum progesterone level on day of HCG or abandonment in 53 cycles with ovarian cysts ● grouped according to outcome with median — – and absolute range ···· shown for 104 patients without apparent cysts on the day of HCG.

As illustrated in figure 4.2 the day 4 serum androstenedione levels of the IVF cycles with cysts did not differ according to IVF outcome (abandoned cycle, oocyte retrieval non-conception, oocyte retrieval conception). Further the latter results fell predominately within the range of day 4 serum androstenedione levels of 20 controls selected at random from the IVF cycles without apparent cysts.

Serum progesterones on day 4 of the IVF cycles with ovarian cysts were below 5.7 nmol/l in all but 1 case, and did not differ in relation to IVF outcome (see figure 4.3). One patient had a day 4 progesterone of 11.3 nmol/l, and the progesterone level was 8.9 nmol/l on day 10 of IVF cycle, when it was decided to abandon that cycle in view of an inadequate response to ovarian stimulation. Two patients had markedly elevated progesterone levels on the day it was decided to abandon their IVF cycle, and both of these patients had a day 4 serum oestradiol >1000 pmol/l. Serum progesterone >5.7 nmol/l was seen in a further abandoned cycle and 1 cycle which proceeded to oocyte retrieval (see figure 4.4).

The 31 IVF cycles with ovarian cysts, which proceeded to oocyte retrieval, were subdivided into 9 cycles with day 4 oestradiol >200 pmol/l (group A) and 22 cycles with day 4 oestradiol ≤200 pmol/l (group B), and compared to the 569 IVF cycles without apparent cysts, which proceeded to oocyte retrieval (group C). No significant difference was seen between these 3 groups in the number of oocytes collected, the number of fertilised oocytes, and the number of cleaved embryos, but there were significantly fewer grade 1 embryos in group A than in both group B and group C (see table 4.2).

Table 4.2: Comparisons of IVF cycles proceeding to oocyte retrieval related to presence of cysts and day 4 serum oestradiol level.

	Ovarian cyst		No apparent ovarian cyst (Group C, n=569)
	Day 4 serum oestradiol		
	>200 pmol/l (Group A, n=9)	≤200 pmol/l (Group B, n=22)	
Oocytes / OR Median (interquartile range)	9 (8-15)	12 (9-17)	10 (8-15)
Fertilised oocytes / OR Median (interquartile range)	5 (3-9)	8 (6-10)	6 (4-10)
Cleaved embryos / OR Median (interquartile range)	5 (2-6)	6 (3-8)	5 (3-8)
Grade 1 embryos / OR Median (interquartile range)	0 (0-2)	3 (1-5) *	3 (2-4) ***
Conception rate	0% (0/9)	27% (6/22)	26% (147/569)

* p <0.05 group A v group B
*** p <0.001 group A v group C
Mann Whitney U test
All other comparisons non-significant

Of the 31 IVF cycles with ovarian cysts proceeding to oocyte retrieval in 27 cases there was a unilateral cyst and two ovaries present. There was no significant difference in the number of oocytes collected from the ipsilateral ovaries to the cysts compared to the number of oocytes collected from the contralateral ovaries to the cysts. Of the 27 cases with unilateral cysts 7 cycles had a day 4 serum oestradiol >200 pmol/l, and 20 cycles had a day 4 serum ostradiol ≤200 pmol/l. Fewer oocytes were collected from the ipsilateral ovaries to the cysts in the former cycles than the latter cycles but this did not reach statistical significance (see table 4.3).

Table 4.3 Number of oocytes collected in 27 IVF cycles with unilateral ovarian cysts in relation to day 4 serum oestradiol and side of cyst.

	Oocytes collected from ipsilateral side to ovarian cyst. Median (interquartile range)	Oocytes collected from contralateral side to ovarian cyst. Median (interquartile range)
Day 4 serum oestradiol ≤200 pmol/l (n=20)	6.5[a] (4-9.5)	5.5 (4.5-8.5)
Day 4 serum oestradiol > 200 pmol (n=7)	2[a] (2-7)	6 (2-11)

a) p=0.2
All comparisons non-significant - Mann Whitney U test for unpaired data, Wilcoxon for paired data

4.3 Discussion

The review of the literature in chapter 1 revealed significant differences between studies regarding the influence of functional ovarian cysts during IVF cycles. It was hypothesised that the latter apparent conflict may be explained if functional ovarian cysts only influenced IVF cycles if the cyst had significant steroidogenic activity. This study has shown an influence on the IVF cycle exerted only by those ovarian cysts, which were functionally active as reflected in elevated serum oestradiol levels. In comparison to IVF cycles without ovarian cysts Karande (1990) found IVF cycles with ovarian cysts had a higher basal serum oestradiol level with a lower peak oestradiol, and more ampoules of HMG were required for stimulation. Thatcher (1989) found IVF cycles with ovarian cysts 16-30 mm diameter were associated with elevated basal oestradiol levels and a higher cancellation rate than IVF cycles without cysts. Sampaio 1991

found ovarian cysts developed in 28 of 285 IVF cycles with daily buserelin from day 1 of the cycle, but by continuing buserelin until oestradiol levels were suppressed there was no difference in the outcome of the IVF cycles with cysts compared to cycles without cysts.

Thatcher's group has published a more recent study on baseline ovarian cysts during IVF cycles using patients as their own controls (Penzias *et al.,* 1992). Thirty nine women had at least one IVF cycle with a cyst (14 to 53 mm diameter) and one cycle without a cyst. In total 82 IVF cycles were studied. Seven patients were given leuprolide acetate for pituitary downregulation prior to ovarian stimulation. There was no significant difference in baseline serum oestradiol levels between the IVF cycles with cysts and the IVF cycles without cysts. Further there was no significant difference in cycle cancellation rates, peak serum oestradiol and number of oocytes retrieved between the latter groups. Penzias (1992) concluded that if the baseline serum oestradiol level was low then baseline ovarian cysts do not affect the response to controlled ovarian hyperstimulation in IVF cycles.

In determining the nature of the functionally active ovarian cysts it is useful to note that theca lutein cysts have a high progesterone and a relatively low oestradiol content and atretic follicles have a high androstenedione and relatively low oestradiol content (Vanluchene *et al.,* 1983). The functionally active ovarian cysts had elevation of serum oestradiol, but not serum progesterone nor serum androstenedione suggesting these are follicular cysts.

There are 3 stages at which the ovarian cysts seen on the ultrasound scan on day 9 of the IVF cycle could have developed. Firstly the cysts

may have been present prior to commencement of LHRHa. The cysts could have developed spontaneously, or as a consequence of previous ovarian stimulation. Tummon (1988) found ovarian cysts following 40 of 71 non-conception cycles using HMG and HCG for ovulation induction, but repeated ovulation induction resulted in pregnancy in 6 of 15 cases despite the presence of cysts. In 6 cycles ovarian cysts were noted prior to LHRHa and all of these proceeded to oocyte retrieval resulting in 2 conceptions, thus these cysts do not appear to present a major problem.

The second occasion to consider when ovarian cysts may develop is during ovarian hyperstimulation following pituitary downregulation. Reviewing 74 IVF cycles with buserelin from day 1 of the cycle and HMG from day 3 Sampaio (1991) found that by day 9, ovarian cysts had developed in 17 cycles, but these cysts did not influence cancellation rates, number of oocytes nor pregnancy rates.

The third time when ovarian cysts may have developed is during pituitary downregulation prior to ovarian stimulation. It has been shown that LHRHa analogues may result in cyst formation whether commenced in the follicular (Ron-El *et al.,* 1989) or luteal (Meldrum *et al.,* 1988) phase of the menstrual cycle. It is of particular interest that Ron El (1989) found that of 198 IVF cycles where a depot LHRHa was administered at the start of menses 27 cases developed ovarian cysts before HMG was commenced, and in these cases the serum oestradiol between day 4 and day 16 of the IVF cycle was greater than in the cycles without cysts. It would seem likely that this is the explanation for many of the functional ovarian cysts found in study 1.

The finding of high oestradiol and low progesterone on day 4 of the IVF cycle suggests that most of the functional ovarian cysts were not persistent corpora lutea. Rather it is likely that most of the functional ovarian cysts were follicular cysts which had developed during pituitary downregulation. The one case of a progesterone of 11.3 nmol/l on day 4 of the IVF cycle may reflect a persistent corpus luteum.

This study would suggest that the mechanism by which ovarian cysts may influence IVF cycles relates to steroidogenic activity rather than a mechanical effect as was suggested by Rizk (1990). The outcome of the IVF cycles with functional ovarian cysts and serum oestradiol ≤200 pmol/l did not differ significantly from IVF cycles without functional ovarian cysts. However, the IVF cycles with functional ovarian cysts and serum oestradiol >200 pmol/l on day 4 had a significantly higher cancellation rate, and produced fewer grade 1 embryos than both IVF cycles without cysts and IVF cycles with cysts but a serum oestradiol ≤200 pmol/l on day 4.

An endocrine influence might be exerted through active synthesis or passive release of stored substances from the cyst fluid acting at pituitary or ovarian level. High oestradiol may feed back on the pituitary delaying pituitary downregulation (Araki S. *et al.*, 1985), and if the cysts are primed ready for luteinisation they may luteinise in response to a blunted endogenous LH surge or even in response to the LH in the HMG. Thatcher (1989) found premature luteinisation occurred more commonly in ovarian cysts 14-30 mm diameter associated with elevated pre-stimulatory oestradiol. Flemming (1986) has found that of 84 patients treated with 500 μg nasal buserelin per day only 1 case had premature luteinisation, which was similar to our finding of 1 case of premature luteinisation in 105 IVF cycles

without apparent cysts. In study 1 of the 53 cycles identified with cysts the progesterone on day of abandonment provides unequivocal evidence of premature luteinisation in 2 cases, which had a day 4 serum oestradiol >1000 pmol/l.

Ovarian cysts may also have a direct influence on folliculogenesis. Thatcher (1989) found a higher cancellation rate for poor response in IVF cycles with cysts >30 mm than in IVF cycles without ovarian cysts. Although Ron-El (1989) found no difference in cancellation rates or pregnancy rates between 27 IVF cycles which developed cysts compared to 171 which did not develop cysts, there were significantly fewer eggs collected in the cycles which had developed cysts.

Even if an endocrine influence is not apparent it could be speculated that functional ovarian cysts may exert a paracrine influence through local very high concentrations of steroids within the cyst fluid. In this study the difference between the number of oocytes collected from the ipsilateral ovary to the cyst compared to contralateral ovary to the cyst did not reach statistical significance (see table 4.3). However, the numbers studied were small and there was a trend towards lower oocyte numbers collected from the ipsilateral ovary to an ovarian cyst, associated with a day 4 serum oestradiol >200 pmol/l. Rizk (1990) studied 14 patients with unilateral ovarian cysts on day 2 menses. Rizk aspirated the ovarian cysts in 6 patients before stimulation with clomiphene /HMG, and the ipsilateral ovary to the cyst of these patients produced significantly more oocytes than the ipsilateral ovary of the 8 cycles where the cyst was not aspirated, although there was no significant difference in the number of oocytes collected from the contralateral ovaries.

In must be remembered that this study relied on case records identifying ovarian cysts >30 mm diameter on day 9 of the IVF cycle. Ultrasound scans had not been performed routinely for all patients prior to commencing HMG, and it is difficult to distinguish a small ovarian cyst from a large follicle following ovarian stimulation. Inevitably this study will underestimate the scale of the problem, and many of the patients with elevated day 4 oestradiols without apparent cysts may have had functionally active cysts <30 mm diameter.

This study has shown a relationship between the influence of functional ovarian cysts during IVF cycles and the serum oestradiol level immediately prior to commencing ovarian stimulation. The inference of the latter is that steroidogenically active ovarian cysts may adversely influence IVF cycles. Functional ovarian cysts with a serum oestradiol ≤200 pmol/l prior to ovarian stimulation were not shown to have any adverse influence on IVF cycles. The study suggested that the functional ovarian cysts with steroidogenic activity would have most likely been follicular cysts which had developed during pituitary downregulation. The following chapter will consider the development of functional ovarian cysts during pituitary downregulation to see if the latter assumption is correct.

Chapter 5

Results and Discussion of Study 2 :

The development of functional ovarian cysts during pituitary downregulation.

5.1 Introduction

This chapter presents and discusses the results of study 2 which investigated the development of functional ovarian cysts during pituitary downregulation with buserelin prior to commencement of controlled ovarian hyperstimulation. The objectives of this study were:

a. To establish whether buserelin commenced in the luteal phase of the cycle may result in the development of functional ovarian cysts with significant steroidogenic activity.

b. To investigate the aetiology and nature of the latter functional ovarian cysts through measurement of serum progesterone and oestradiol levels.

The methodology of study 2 is presented in detail in chapter 2, and the assays used in the study are explained in chapter 3. During pituitary downregulation functional ovarian cysts associated with a day 4 menses serum oestradiol >200 pmol/l developed in 16 of 288 IVF cycles studied. The nature of the latter functional ovarian cysts was investigated by comparing the serum oestradiol and progesterone levels immediately prior to commencing buserelin to the serum oestradiol and progesterone levels on day 4 of the ensuing menses. The aetiology of the latter functional ovarian cysts was investigated further by comparing the serum oestradiol and progesterone levels prior to commencing buserelin in the 16 patients who developed functional ovarian cysts to the other 272 IVF cycles studied.

5.2 Results

There was no significant difference in age between the 16 patients with functional ovarian cysts compared to the other 272 IVF cycles (245 patients). The 16 patients with functional ovarian cysts had a significantly higher proportion of patients with ovulatory dysfunction

than that seen in the other 272 IVF cycles, but no difference was seen for other infertility diagnoses (see table 5.1).

Table 5.1 Age and infertility diagnosis for patients who did develop functional ovarian cysts during pituitary downregulation compared to patients who did not develop functional ovarian cysts

	Cyst (n=16)	No cyst (n=272)	
Age	34.7 (SD 3.8)	33.5 (SD 4.6)	N.S.
Infertility diagnosis			
Tubal	37.5%	42.0%	N.S.
Unexplained	12.5%	17.9%	N.S.
Male Factor	25.0%	23.1%	N.S.
Endometriosis	12.5%	10.8%	N.S.
Ovulatory	43.4%	12.8%	**
NS non-significant Mann Whitney U Test or χ^2 test as appropriate ** $p<0.01$ χ^2 test with Yates correction			
(Where couples had more than one infertility diagnosis they were included in all relevant groups.)			

The number of days between commencement of buserelin and the onset of menstruation were significantly greater ($p<0.05$) for the 16 patients with functional ovarian cysts than for the other 272 IVF cycles (see table 5.2).

Table 5.2 Comparison of the interval between commencement of buserelin and the onset of menses between 16 patients with functional ovarian cysts and 272 patients without functional ovarian cysts.

	Steroidogenically active ovarian cyst (n=16)	No steroidogenically active ovarian cyst (n=272)	
Days - median (Interquartile range)	15 (10-27)	11 (9-14)	*
* P<0.05 (Mann Whitney U test)			

The 16 patients with functional ovarian cysts had a significantly higher ($p<0.01$) oestradiol level on day 4 menses following pituitary downregulation than their oestradiol level on the day buserelin was commenced. The latter 16 patients had a significantly lower ($p<0.01$) progesterone level on day 4 menses following pituitary downregulation than their progesterone level on the day buserelin was commenced (see figure 5.1).

All 16 patients with functional ovarian cysts had a serum progesterone level following pituitary downregulation of less than 5.7 nmol/l on day 4 menses (see figure 5.1). Eight of the 16 patients, who developed functional ovarian cysts, had a serum progesterone less than 5.7 nmol/l on the day buserelin was commenced (see figure 5.2). No patient with a serum progesterone of <5.7 nmol/l and a serum oestradiol of >330 pmol/l on the day buserelin was commenced developed a functional ovarian cyst (see figure 5.3).

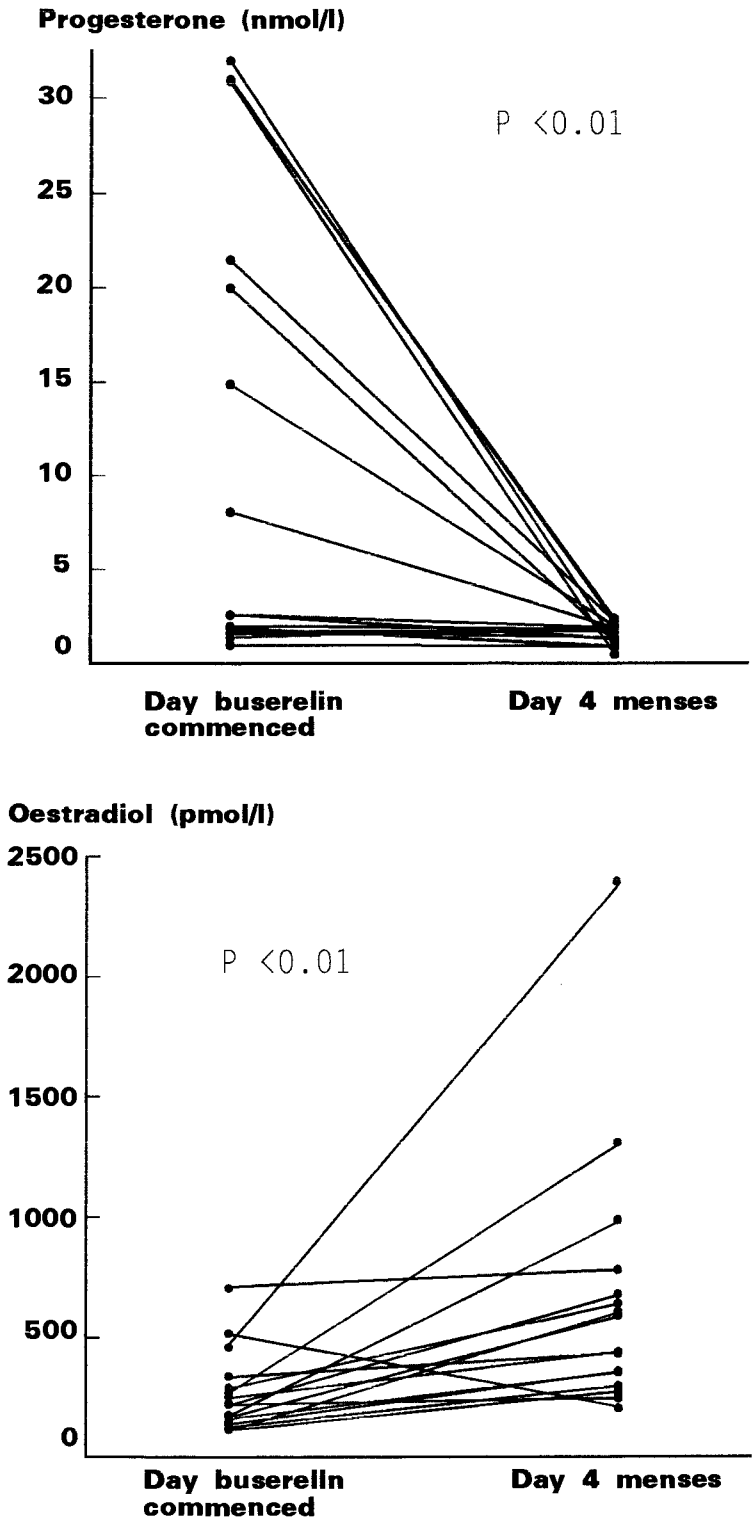
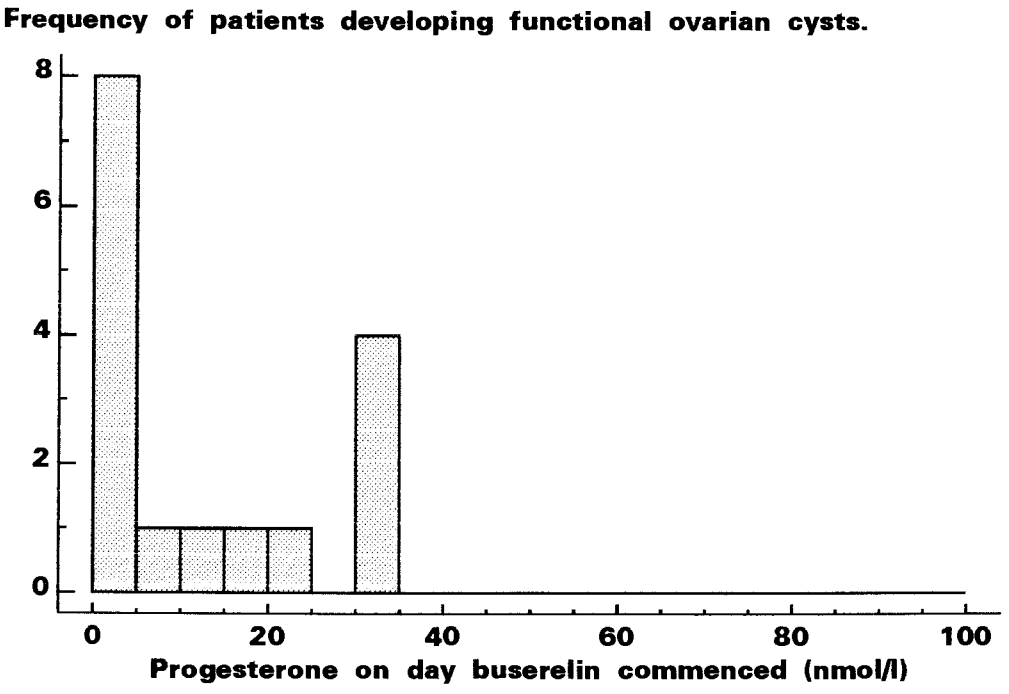


Figure 5.1 Comparison of serum oestradiol and serum progesterone between the day buserelin commenced and the fourth day of the subsequent menses in 16 patients, who developed functional ovarian cysts.



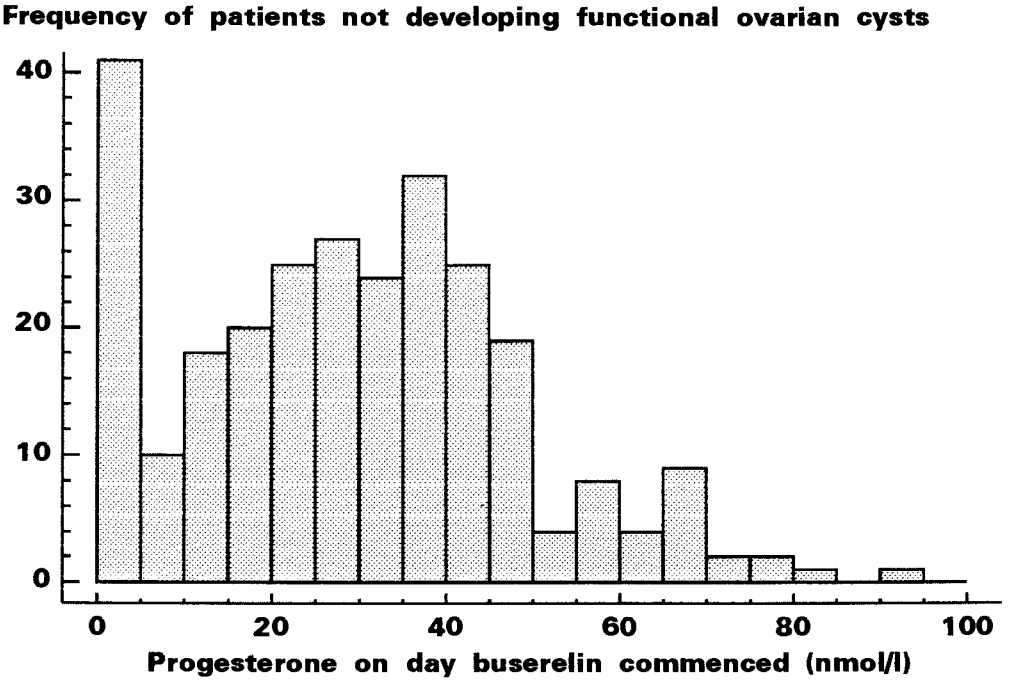


Figure 5.2 Frequency histograms of serum progesterone on the day buserelin was commenced in patients developing functional ovarian cysts and patients not developing functional ovarian cysts.

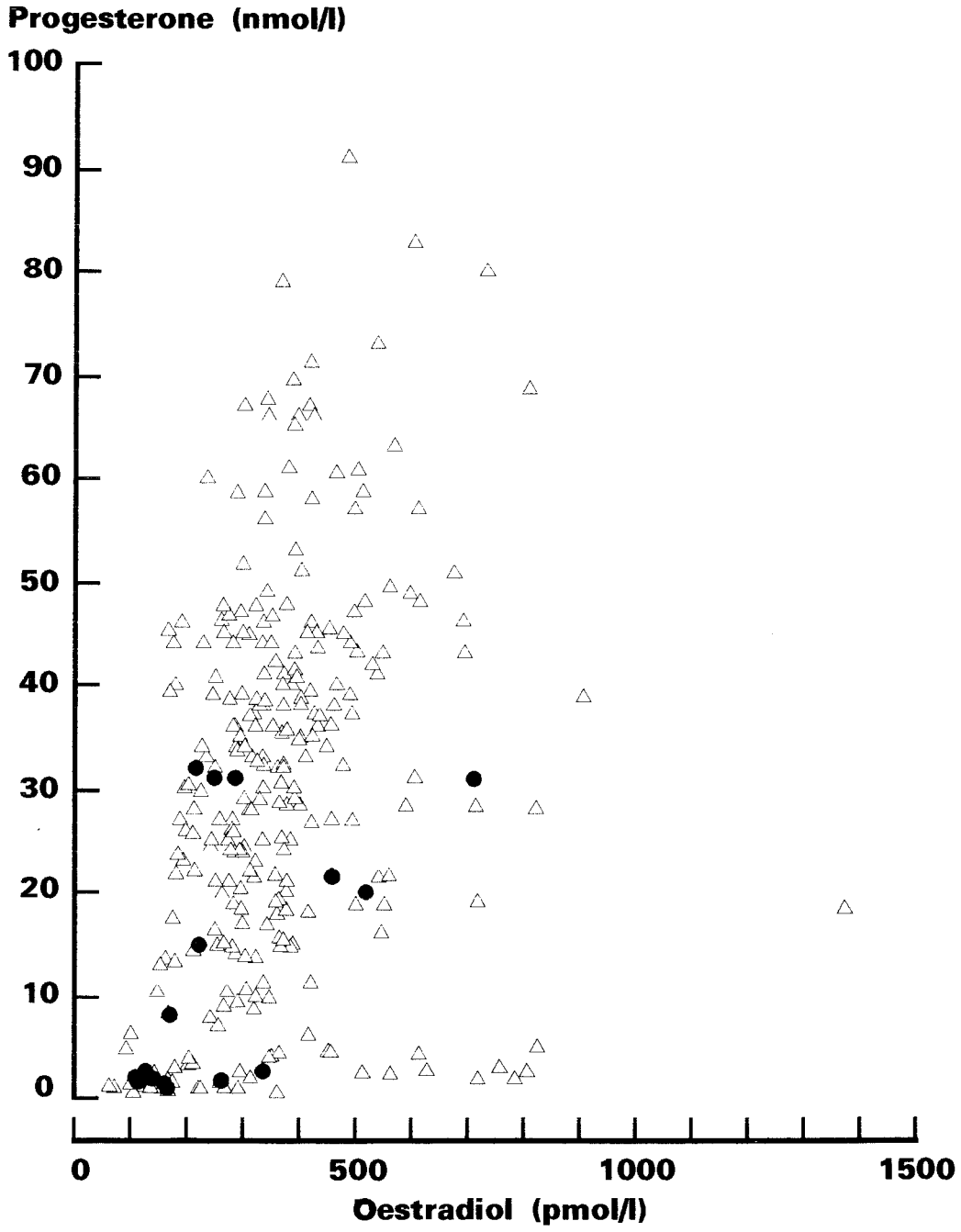


Figure 5.3 Scatterplot of serum progesterone against serum oestradiol on day buserelin commenced in 16 patients ● , who subsequently developed functional ovarian cysts, and 272 patients △ , who did not develop functional ovarian cysts.

There were significantly lower serum progesterone levels ($p<0.001$), lower serum oestradiol levels ($p<0.01$) and lower progesterone/oestradiol molar ratios ($p<0.01$) at the time buserelin was commenced in the 16 patients who subsequently went on to develop steroidogenically active ovarian cysts than in the other 272 IVF cycles (see table 5.3).

Table 5.3 Serum steroid concentrations on the day buserelin commenced in relation to subsequent development of functional ovarian cysts.

	Cyst developed (n=16)	No Cyst developed (n=272)	
Progesterone (nmol/l) Median (interquartile range)	5.3 (1.8.-26.2)	29.0 (15-42.0)	***
Oestradiol (pmol/l) Median (interquartile range)	218 (149.5-310.0)	338 (271.5-421.0)	**
Progesterone/Oestradiol (molar ratio) Median (interquartile range)	29.6 (11.2-57.6)	84.1 (43.0-118.7)	**
*** P< 0.001 (cyst versus no cyst) ** P< 0.01 (cyst versus no cyst)		Mann Whitney U test	

5.3 Discussion

The patients who developed functional ovarian cysts did not have a higher incidence of endometriosis than the patients without ovarian cysts. Further, when the cysts were aspirated they contained clear fluid without any blood making it very unlikely that they were endometriomata. It would appear that the ovarian cysts which develop during pituitary downregulation with LHRH agonists are not endometriomata, contrary to the suggestion of Feldberg (1989).

This study has confirmed a previous report stating that ovarian cysts could develop with LHRH agonists commenced in the luteal phase of the cycle (Meldrum *et al.,* 1988). Further, the study has confirmed Ron-El's (1989) study showing that the cysts developing during pituitary downregulation may be associated with elevation of serum oestradiol levels suggesting the cysts have steroidogenic activity. The first clinically apparent effect of the steroidogenic activity seen in this study was a delay in the onset of the subsequent menses.

The changes in serum progesterone and oestradiol concentrations from the time buserelin was commenced to day 4 of the subsequent menses suggests that the cysts which developed were not persistent corpora lutea. Although in the latter IVF cycles there was a significant rise in the serum oestradiol level from the day buserelin was commenced to day 4 of the subsequent menses, over the same time there was a significant fall in serum progesterone levels. On day 4 menses the progesterone level was <5.7 nmol/l in all 16 IVF cycles with functional ovarian cysts showing no evidence of persistent corpus luteal activity.

The origin of the functional ovarian cysts can be considered with regard to the serum steroid levels at the time buserelin was commenced. The functional ovarian cysts could have arisen from small antral follicles, mature ovarian follicles or corpora lutea. As was discussed earlier serum progesterone levels may be used to assess functional activity of a corpus luteum. In 8 of the 16 IVF cycles in which ovarian cysts developed serum progesterone levels were below 5.7 nmol/l at the time buserelin was commenced. In the absence of a functional corpus luteum the serum oestradiol provides an indicator of pre-ovulatory follicular development, and a pre-ovulatory ovarian follicle would be associated with a serum oestradiol

level of around 330-1211 pmol/l (using oestradiol kit code 12264, Serono Diagnostics Ltd., Woking, UK). In the 8 IVF cycles with ovarian cysts and serum progesterone <5.7 nmol/l at the time buserelin was commenced the serum oestradiol level was <300 pmol/l. This would suggest that the functional ovarian cysts in these patients originated from immature antral follicles.

Aware of the mode of action of buserelin and the probable origin of the functional ovarian cysts it is possible to speculate on the mechanism by which the functional ovarian cysts develop. During the initial phase of buserelin treatment there is a burst of FSH and LH released from the pituitary gland (Sandow *et al.,* 1978). Small antral follicles lack LH receptors on their granulosa cells (Channing *et al.,* 1977) and thus they will be unable to luteinise and initiate the chain of events leading to increased progesterone synthesis and follicle rupture. These antral follicles will possess LH receptors on their thecal cells, which will be stimulated to produce androstenedione (Channing *et al.,* 1977). Androstenedione will pass to the granulosa cells where under the influence of FSH follicle growth will be stimulated and the androstenedione will be converted to oestrogen. After the initial stimulatory phase buserelin will downregulate the pituitary gland reducing LH and FSH release, and blocking feedback from the developing follicle from causing a LH surge. Without a LH surge the follicle does not rupture. The follicle will be sensitive to gonadotrophins, and for a while may continue to grow to form a cyst actively producing steroids. Subsequently active steroid production may cease, but the cyst may continue to release the steroids stored within the cyst fluid. The absence of granulosa cells noted in the fluid aspirated from the cysts gives some support to the suggestion that the cysts may not be actively synthesising steroids by the time the cysts were aspirated.

If the above model is correct then it is interesting to consider the possible consequences on cyst formation of progesterone and oestradiol influencing the pituitary response to buserelin. Serum progesterone concentrations and progesterone/oestradiol molar ratios were significantly lower in the patients who developed ovarian cysts than in the patients who did not develop ovarian cysts. It has been shown that progesterone reduces the gonadotrophin release by LHRH, and this effect is reduced by oestradiol (Araki *et al.*, 1985). A relatively high serum progesterone may thus have a protective effect on cyst formation with buserelin by reducing the initial gonadotrophin release when buserelin is commenced. It has also been found that a 7 day course of a progestogen commenced 2 days before buserelin may prevent the development of ovarian cysts during pituitary downregulation (Wardle, P. personal communication).

It is important to consider the limitations of this study. It is possible that following the commencement of buserelin functional ovarian cysts with steroidogenic activity could develop, but then regress functionally and/or physically before menstruation. However, the latter cysts should not present a clinical problem other than possibly delayed menstruation.

Patients with a serum oestradiol >200 pmol/l and no functional ovarian cyst should be considered. These patients may represent a group of patients who are resistant to the action of buserelin or have persistent corpus luteal activity, which is not apparent on the ultrasound scans. Interestingly only 2 such patients were identified during this study. Oestradiol levels were suppressed in both cases following prolonged use of buserelin at the increased dose of 200 µg four times a day.

In summary buserelin commenced in the luteal phase may result in the development of functional ovarian cysts with clinically significant steroidogenic activity. Serum steroid levels would suggest that the latter cysts develop from immature ovarian follicles into follicular cysts, rather than develop from corpora lutea into corpora luteal cysts. Progesterone may have a protective role on cyst formation during pituitary downregulation, and it is possible that this may be used to prevent the development of functional ovarian cysts during pituitary downregulation.

Chapter 6

Results and Discussion of Study 3 :

Steroid concentrations in functional ovarian cyst fluid.

6.1 Introduction

This chapter presents and discusses the results of study 3. This study investigated the potential steroidogenic influence of functional ovarian cysts, which develop during pituitary downregulation. The objectives of the study were :

a. To establish if functional ovarian cysts, which develop during pituitary downregulation, may have a sufficiently high steroid concentration to influence the endocrine milieu of developing follicles.

b. To determine the nature of functional ovarian cysts, which develop during pituitary downregulation, by measuring steroid concentrations of fluid aspirated from the cysts with reference to follicular fluid aspirated at the time of oocyte retrieval.

The methodology of study 3 is detailed in chapter 2 and the assays employed are described fully in chapter 3. There were three groups in the study:

Group A: 9 patients, who had developed a functional ovarian cyst during pituitary downregulation and had had serum oestradiol >200 pmol/l despite at least 3 weeks of buserelin treatment. Fluid was aspirated from a solitary cyst in each of these patients.

Group B: 3 patients, who were noted to have a simple ovarian cyst >50 mm diameter before commencing buserelin. Two of these patients had two cysts thus a total of 5 cysts were aspirated.

Group C: 10 patients had fluid aspirated from a mature ovarian follicle at the time of oocyte retrieval.

As described in chapter 3 steroids were measured in the latter fluids and venous blood samples taken at the time of aspiration. These results are presented and discussed in this chapter.

Progesterone, oestradiol and androstenedione concentrations were measured in fluid aspirated in the above groups. Oestradiol and progesterone concentrations were measured in serum samples taken at the time of aspiration.

6.2 Results

Steroid concentrations were compared between serum samples taken at the time of aspiration and the concentrations of the steroids in the aspirated fluid. Although there was a significant difference in steroid concentrations between group A and group C (described later, see table 6.1), there was no significant correlation found for either progesterone or oestradiol between serum and aspirate concentrations within each group (see figure 6.1).

The aspirate concentrations of androstenedione, oestradiol and progesterone were compared against each other for groups A and C. As can be seen in figure 6.2 within each group no significant correlation was demonstrated for the following: progesterone versus oestradiol concentrations, androstenedione versus oestradiol concentrations, and androstenedione versus progesterone concentrations.

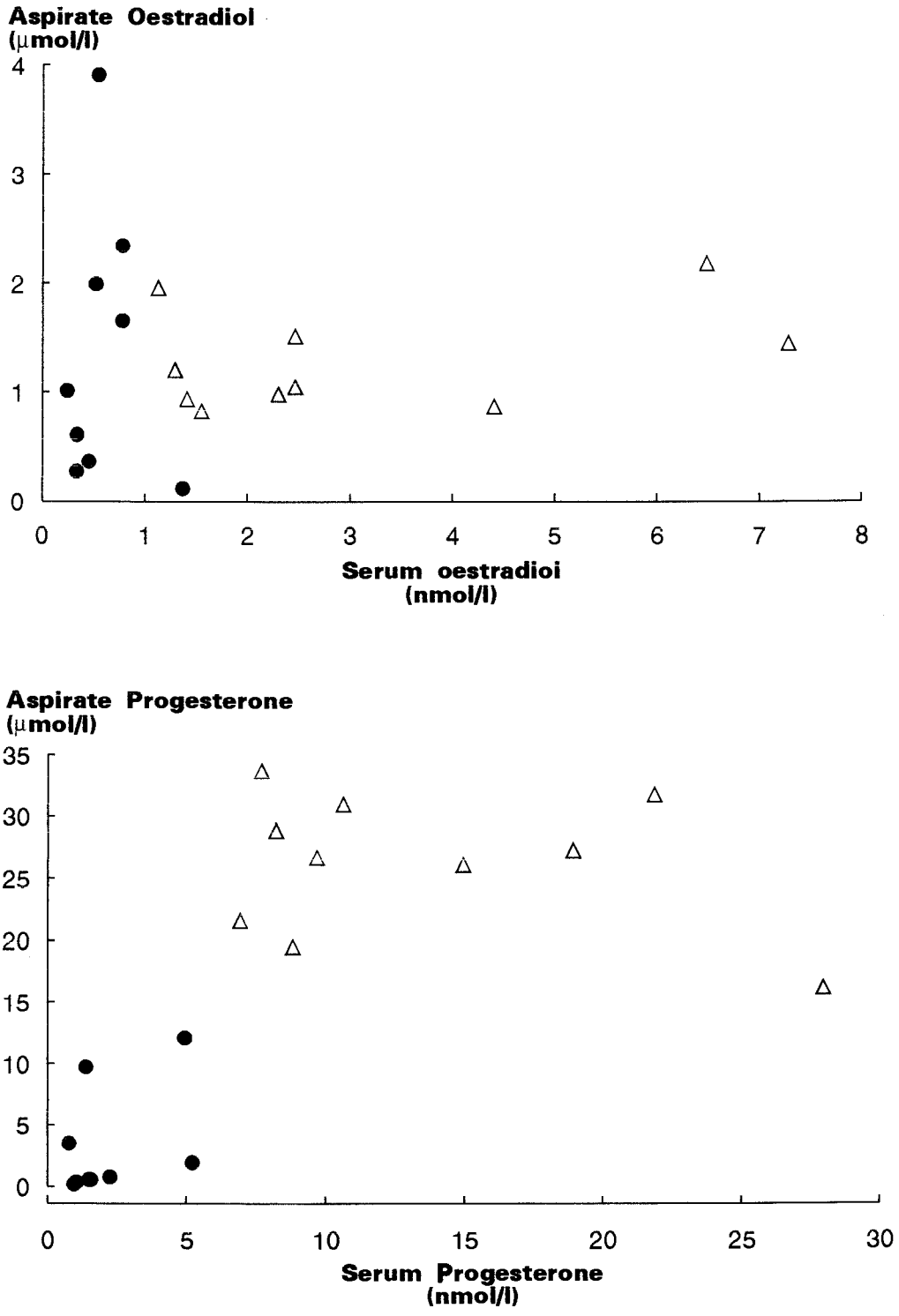


Figure 6.1 Comparison of oestradiol and progesterone levels between venous blood taken at the time of aspiration and the aspirate concentrations from functional ovarian cysts (●) and ovarian follicles (△).

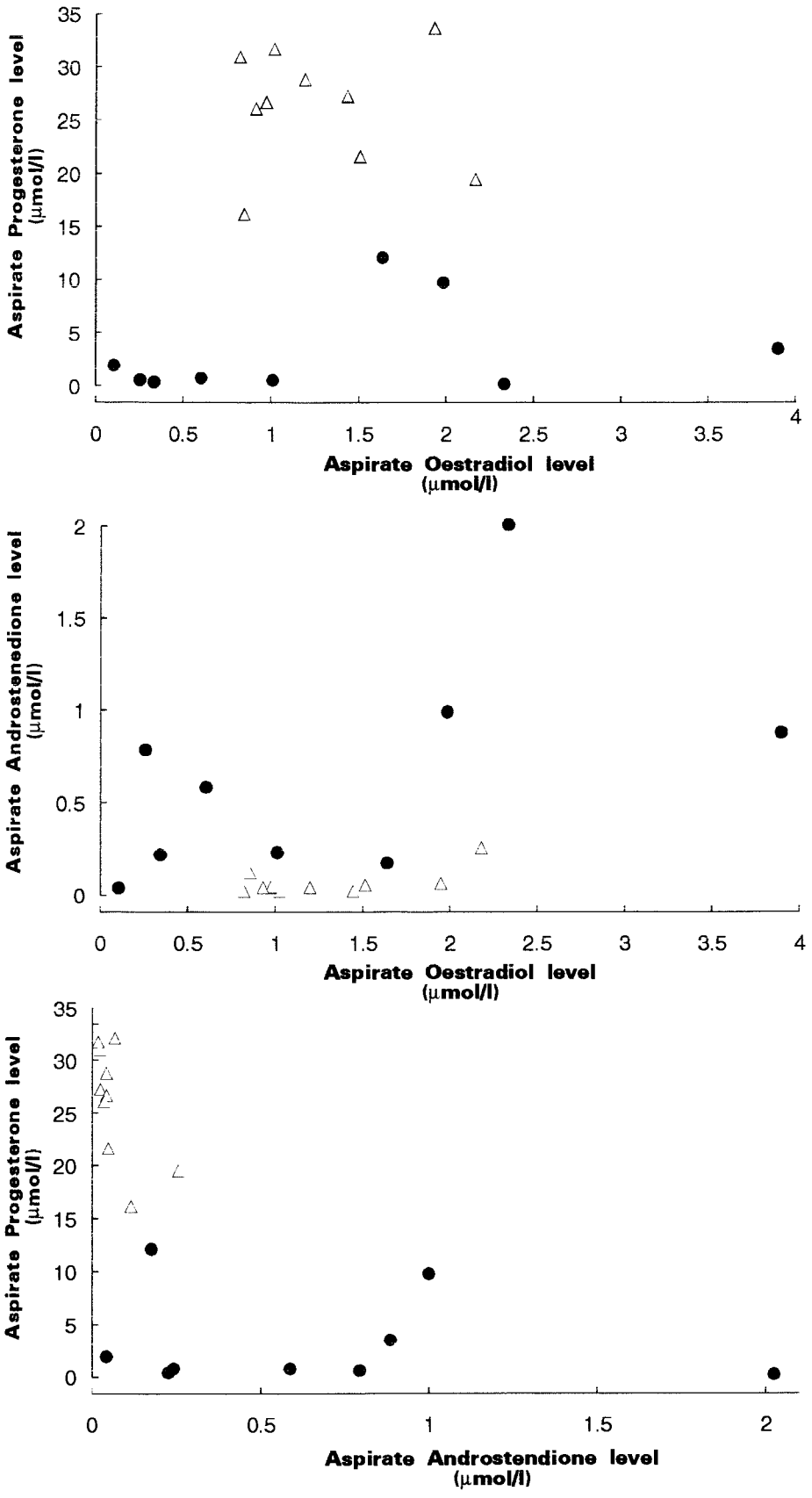


Figure 6.2 Scatterplots of aspirate concentrations of progesterone, oestradiol and androstenedione for 9 (●) functional ovarian cysts and 10 (△) ovarian follicles.

Androstenedione concentrations of the aspirated fluid were significantly higher ($P<0.01$) in group A than in group C. Progesterone concentrations of the aspirated fluid were significantly lower ($P<0.001$) in group A than group C. There was no overlap in androstenedione nor progesterone concentrations of aspirated fluid between group A and group C. Oestradiol concentrations of the aspirated fluid samples were not significantly different between group A and group C (see table 6.1).

At the time of cyst aspiration the 3 patients in group B had serum oestradiol concentrations <200 pmol/l and serum progesterone concentrations <5.7 nmol/l. All 5 cyst aspirates in group B were found to have oestradiol concentrations well below the lowest concentrations found in group A and group C. The cyst fluid aspirated from 2 cysts in the same ovary were found to contain very similar concentrations of oestradiol, progesterone and androstenedione in 2 patients. One patient in group B had an aspirate progesterone concentration above the median value and within the range of group A. The latter patient had an aspirate androstenedione concentration below the range for group A and just above the lower limit of the range for group C. The other cysts in group B had progesterone and androstenedione concentrations below the ranges for both group A and group C (see table 6.2).

Table 6.1 Steroid concentrations in the aspirates of functional ovarian cysts induced by buserelin (Group A) and the aspirates of follicles collected at the time of oocyte retrieval (Group C).

	Group A Cyst aspirate (n=9)	Group C Follicular fluid (n=10)	
Progesterone (µmol/l) Median (absolute range)	0.66 (0.09-12.00)	26.90 (16.20-33.60)	***
Androstenedione (µmol/l) Median (absolute range)	0.59 (0.05-2.03)	0.05 (0.02-0.25)	**
Oestradiol (µmol/l) Median (absolute range)	1.10 (0.26-3.90)	1.10 (0.83-2.17)	N.S.
N.S not significant ** P < 0.01 *** P < 0.001 Mann Whitney U test			

Table 6.2 Steroid concentrations in the aspirates of 5 functional ovarian cysts noted prior to commencing buserelin in 3 patients (group B).

Group B Patient	Progesterone (µmol/l)	Androstenedione (µmol/l)	Oestradiol (µmol/l)
11	0.021	0.006	0.0010
11	0.021	0.011	0.0014
12	0.001	0.003	0.0004
12	0.001	0.004	0.0002
13	1.2	0.023	0.0079

6.3 Discussion

It is important to consider the lack of correlation of steroid concentrations between paired aspirate and serum samples in group A and group C. The lack of correlation between serum and aspirate oestradiol concentrations is particularly important in the patients with functional ovarian cysts (group A). The serum oestradiol concentration may be governed by the rate of transfer of oestradiol from the cyst into the venous circulation, and the subsequent metabolism and excretion of oestradiol. The latter will only partly be related to the oestradiol concentration within the cyst. It is thus with caution that the serum oestradiol should be used to gauge the concentration of oestradiol within a functional ovarian cyst. In group C there is a further factor altering the relationship between aspirate and serum steroid concentrations. The steroid concentrations at the time of oocyte retrieval is a reflection of the total steroid output of all the ovarian follicles in the hyperstimulated ovary. The greater the number of follicles the higher the serum oestradiol concentration will be. One of the criteria for proceeding to oocyte retrieval explained in chapter 2 is a serum oestradiol >300 pmol/l for each follicle >14 mm.

The functional ovarian cysts (group A) had an oestradiol concentration in the cyst aspirate which was 1000 times that measured in the serum sample taken at the time of the aspiration. This concentration of oestradiol was similar to the oestradiol concentration found in the follicles at the time of oocyte retrieval. It was interesting to note that the cysts, which had been present prior to commencing buserelin, had aspirate oestradiol concentrations very much lower than in group A.

Measurement of steroid concentrations in the aspirates of the functional ovarian cysts which develop during pituitary

downregulation could be used to help determine whether the cysts are follicular cysts, theca lutein cysts or persistent corpora lutea. Following luteinisation there is suppression of androstenedione synthesis (Richards *et al.*, 1987) and increased progesterone synthesis (Sasano *et al.*, 1989). It has further been found that theca lutein cysts have a higher progesterone concentration, a lower androstenedione concentration and a lower oestradiol concentration than pre-ovulatory follicles (Vanluchene *et al.*, 1983). The functional ovarian cysts had a lower concentration of progesterone and a higher concentration of androstenedione than follicular fluid collected in the early stages of luteinisation. This suggests that the functional ovarian cysts which develop during pituitary downregulation are more likely to be follicular cysts than corpora luteal cysts or theca lutein cysts.

It is difficult to predict the consequences of the steroids present in functional ovarian cysts at the time ovarian stimulation is present. Referring back to the control of folliculogenesis as described in chapter 1 several potential problems could be seen to arise. Firstly the feedback of oestradiol on the pituitary may interfere with the mode of action of buserelin. It is known that oestradiol inhibits the gonadotrophin response to LHRH (Araki *et al.*, 1985), and this might result in a delay in the time required to achieve pituitary downregulation. Secondly in the absence of FSH androgens will induce follicular atresia (Hillier and Ross 1979). The high local androgen concentrations surrounding the functional ovarian cysts may cause atresia of the follicles, which would have been destined to develop when ovarian stimulation was commenced. Thirdly the steroid environment within the developing follicle shifts from androgen dominance to oestrogen dominance. The oestradiol released from the functional ovarian cysts may upset the endocrine milieu of

the developing follicles. The latter influence may even extend to the follicles in the contralateral ovary to the functional ovarian cysts.

Patients with functional ovarian cysts <50 mm diameter and serum oestradiol <200 pmol/l did not have the cyst aspirated prior to gonadotrophin stimulation. The steroid concentrations in the latter ovarian cysts at the time ovarian stimulation was commenced has thus not been measured. However, the results of study 1 presented in chapter 4 confirms the impression from the literature review that functional ovarian cysts without any significant steroidogenic activity may not have a significant influence on IVF cycles.

This study highlights the differences of steroid concentrations between ovarian cysts. The functional ovarian cysts which develop during pituitary downregulation contain fluid with steroid concentrations significantly higher than that found in serum. The steroid profile of the cyst aspirate suggests the latter functional ovarian cysts are follicular cysts. It only possible to speculate on the implications that the steroids in the functional ovarian cysts could have on the development of the surrounding follicles during controlled ovarian hyperstimulation in an IVF cycle. Considering the physiology of follicular development it does seem possible that the steroid content of the functional ovarian cysts could have a detrimental influence. The following chapter will evaluate whether aspirating the functional ovarian cysts will remove this suggested detrimental influence on the IVF cycle.

Chapter 7

Results and Discussion of Study 4 :

IVF cycles following aspiration of functional ovarian cysts.

Introduction 7.1

This chapter considers how patient management could be improved through an understanding of the influence of functional ovarian cysts during IVF cycles. Specifically this chapter presents study 4, the objective of which was:

> *a. To determine whether the aspiration of functional ovarian cysts will remove steroidogenic activity allowing the IVF cycle to proceed unhindered.*

Chapter 2 describes the aspiration of functional ovarian cysts in 13 patients. In 10 patients the ovarian cysts had developed during buserelin treatment (group A), and in 3 patients the ovarian cysts were present prior to the start of the IVF cycle (group B). The subsequent outcome of the IVF cycles of these patients is presented in this chapter.

7.2 Results

The median age of patients in group A was 36 years and ranged from 31 to 42 years. Two of the patients in group A had previously conceived following IVF, although one conception miscarried and the other resulted in an unexplained stillbirth at term. The duration of infertility in group A ranged from 2 to 14 years, and the infertility diagnoses are presented in table 7.1. Four patients had previously had an abandoned IVF cycle and accordingly these patients were treated with 8 ampoules of gonadotrophin daily.

The 3 patients in group B were aged 37 years and their details are presented in table 7.1. Patient 13 had previously on three occasions spontaneously developed functional ovarian cysts, which had been excised from the ovaries on 2 occasions, but on the third occasion it had been necessary to perform an oophorectomy.

Table 7.1 Clinical details of patients having aspiration of functional ovarian cysts.

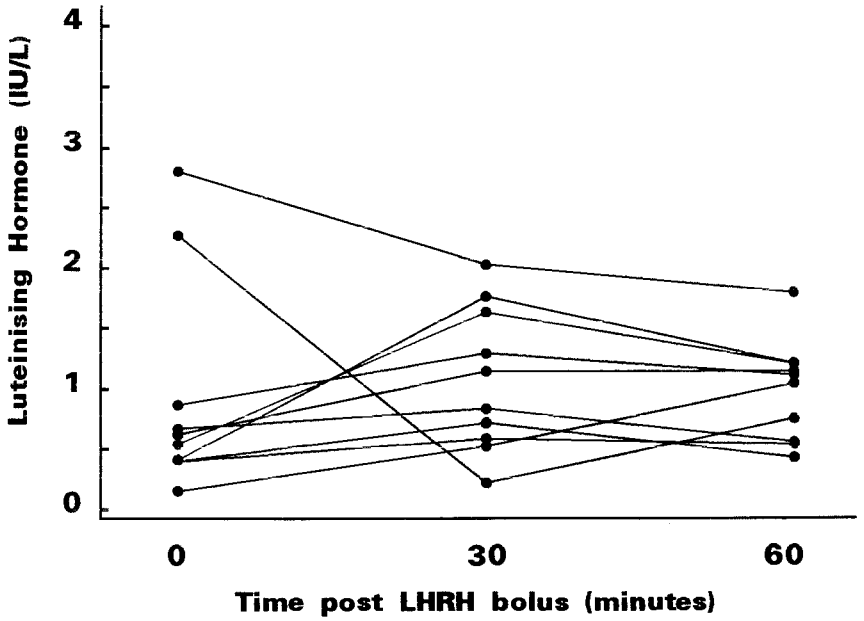
Patient	Age (years)	Infertility Diagnosis	Past pregnancy history	Duration of infertility (years)	Number of Previous IVF attempts
Group A (n=10)					
1	31	Ovulatory	0	3	0
2	31	Ovulatory	0	4	1
3	34	Male factor	0	2	0
4	35	Tubal	Miscarriage following IVF	9	1
5	35	Unexplained	0	11	0
6	36	Unexplained	Stillbirth following IVF	6	5
7	39	Male factor	0	8	2
8	40	Unexplained	0	14	2
9	41	Unexplained	0	11	1
10	42	Tubal	0	5.5	0
Group B (n=3)					
11	37	Tubal	Baby following IVF	Sterilised	1
12	37	Tubal	0	3	1
13	37	Tubal Right Oophorectomy	Baby	6	0

On the day of cyst aspiration 10 patients in group A had serum oestradiols ranging from 244 pmol/l to 1127 pmol/l, and their basal LH and FSH levels were below 3 IU/l and showed little if any rise over 1 hour following an intravenous bolus of 150 μg LHRH (figure 7.1). In group A following ovarian cyst aspiration there was a significant ($p<0.05$) fall in serum oestradiol levels over 3 days (figure 7.2).

When reviewed 5 days following the ovarian cyst aspiration neither group A nor group B showed any redevelopment of the ovarian cysts on vaginal ultrasound. There was no sign of ovarian cyst redevelopment during ovarian stimulation, and no ovarian cyst was noted at the time of oocyte retrieval. Subsequent vaginal ultrasound scans following the non-conception IVF cycles showed no reoccurrence of the ovarian cysts 2 months later.

Of 10 patients in group A 9 patients showed an adequate response to ovarian stimulation to proceed to oocyte retrieval. There was no significant difference in the number of oocytes collected from the ipsilateral ovary to the ovarian cyst compared to the contralateral ovary to the ovarian cyst. Patients showed a satisfactory fertilisation rate and 5 conception cycles resulted. Three pregnancies progressed to term resulting in the birth of 4 babies (see table 7.2).

The 3 patients in group B all demonstrated an adequate response to ovarian stimulation to proceed to oocyte retrieval. One patient conceived and progressed to a term delivery of a healthy baby (see table 7.2).



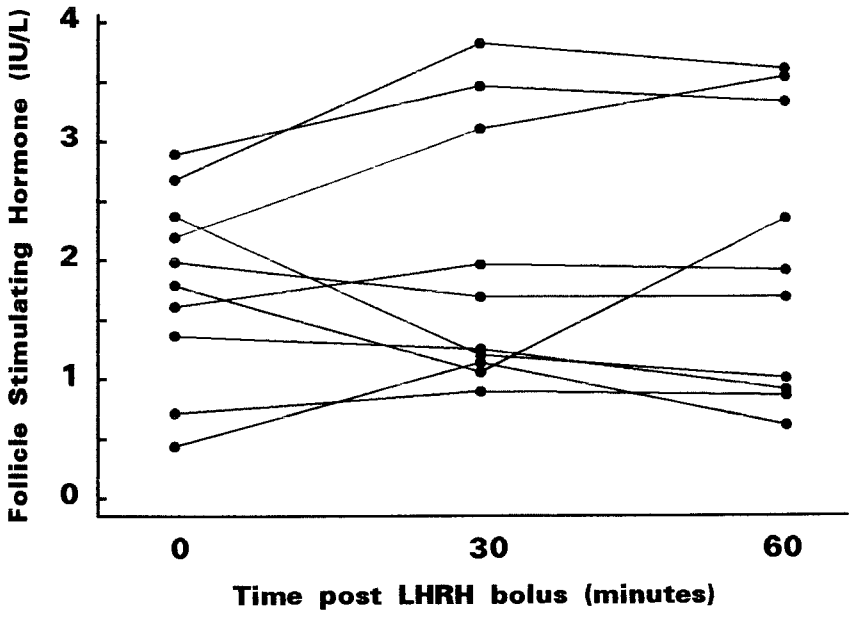


Figure 7.1 LH and FSH response to 0.1 mg intravenous bolus of LHRH in 10 patients with ovarian cysts and serum oestradiol >200 pmol/l on buserelin for at least 3 weeks.

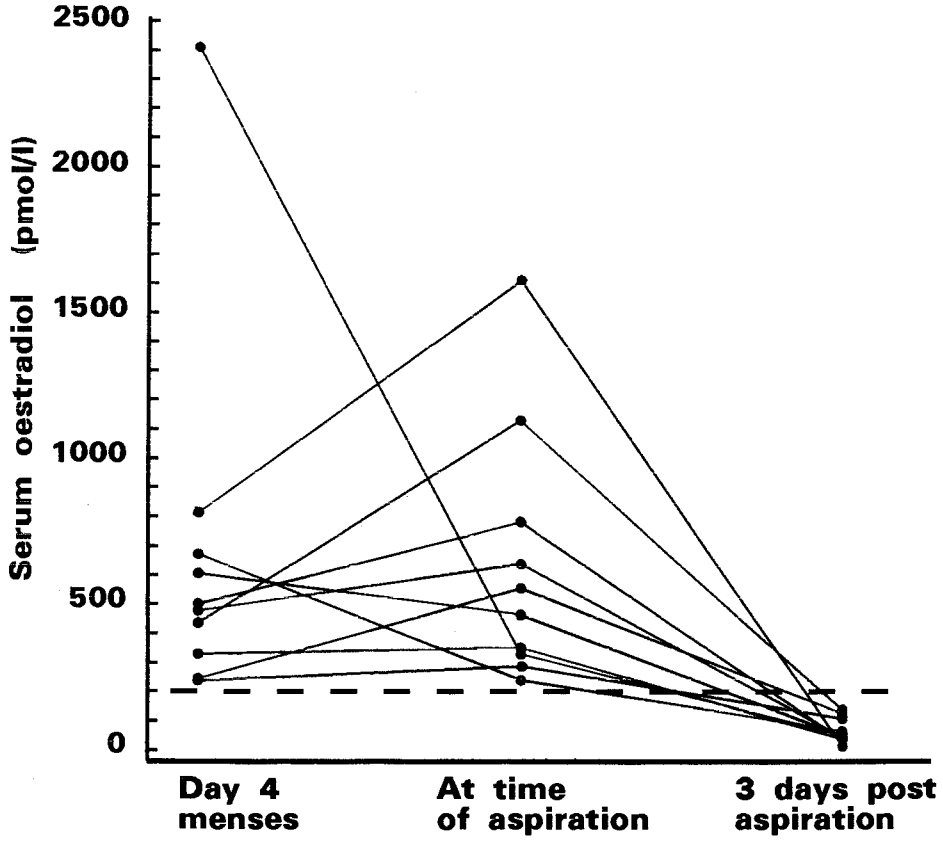


Figure 7.2 Fall in serum oestradiol following aspiration of functional ovarian cysts.

Table 7.2 Outcome of IVF cycles following aspiration of functional ovarian cysts.

Patient	Proceeded to oocyte retrieval	Number of oocytes collected		Number of oocytes fertilised	Final Outcome
		Ipsilateral ovary to cyst	Contralateral ovary to cyst		
Group A (n=10)					
1	yes	3	3	3	non-conception
2	yes	4	2	4	non-conception
3	yes	2	2	4	non-conception
4	yes	3	6	5	Twins (delivered)
5	yes	6	7	10	Singleton (delivered)
6	no	-	-	-	abandoned
7	yes	5	3	5	Miscarriage
8	yes	2	4	5	Miscarriage
9	yes	3	0	3	Singleton (delivered)
10	yes	2	0	1	non-conception
Group B (n=3)					
11	yes	8	8	10	Singleton (delivered)
12	yes	6	14	12	non-conception
13	yes	5	no ovary	4	non-conception

7.3 Discussion

This chapter illustrates that transvaginal ovarian cyst aspiration is an effective method of dealing with persistent functionally active ovarian cysts, and may offer an alternative to cystectomy in carefully selected cases. The place of ovarian cyst aspiration in the routine clinical management of ovarian cysts encountered during IVF cycles needs careful consideration, and it is important to realise the limitations of the technique. The possibility of ovarian malignancy or reoccurrence of the ovarian cysts following aspiration must not be ignored. In addition to the clinical benefit following ovarian cyst aspiration, the biochemical changes following ovarian cyst aspiration shed further light on the possible mechanism of action of functional ovarian cysts.

Study 1 discussed in Chapter 4 of this thesis suggested that only functionally active ovarian cysts had a detrimental influence on IVF cycles. This chapter suggests that functional activity and thus the detrimental influence of functional ovarian cysts may be removed by aspiration. As an alternative to cyst aspiration one can continue pituitary suppression with LHRHa until the cyst regresses or becomes functionally inactive. Rizk (1990) compared ovarian cyst aspiration with prolonged LHRHa. No differences were seen between the latter two groups in the number of ampoules of HMG required, number of oocytes collected or fertilisation rates. One pregnancy resulted from 12 patients who had a cyst aspiration, and 1 pregnancy resulted from 11 cycles where LHRHa was continued until serum oestradiol levels were suppressed (Rizk *et al.*, 1990). The main disadvantage with prolonged use of LHRHa until the cyst regresses is the unpredictable length of time it may take for regression to occur. One of the Southampton IVF patients with an ovarian cyst, who did not have a cyst aspiration, continued buserelin for 6 weeks

before she decided to abandon the IVF cycle. It took a further 2 months before the cyst regressed completely. It would thus seem reasonable to offer aspiration if there is no sign of regression of a functionally active ovarian cyst after 3 weeks of buserelin treatment.

It is important to consider the possibility that any ovarian cyst encountered during an IVF cycle may be neoplastic. If a functional ovarian cyst develops following buserelin then it is reasonable to consider conservative treatment options, but ovarian cysts discovered before buserelin is started need more careful attention. The use of high resolution vaginal ultrasonography is particularly helpful in determining whether aspiration of an ovarian cyst may be appropriate. On the Southampton IVF programme ovarian cystectomy was routinely performed in patients with clinically benign ovarian cysts greater than 5 cm in diameter discovered on ultrasound before embarking on an IVF cycle. In the latter cases cyst aspiration offers an alternative to ovarian cystectomy, but controversy exists as to whether this is a satisfactory option in view of the possibility of malignancy (Buckley, 1989). The use of high resolution, transvaginal ultrasonography in defining functional ovarian cysts distinguishing them from neoplastic cysts was reviewed in the introductory chapter of this thesis. Greenbaum (1992) studied 931 patients who underwent 1,544 ultrasound-guided oocyte retrievals. During the latter study 1 patient with cystic ovarian cancer was identified by transvaginal ultrasound and this cyst was not aspirated. A further 90 non-malignant ovarian cysts were identified by ultrasound and these cyst were aspirated with cytological confirmation of an absence of malignant cells in the aspirate (Greenbaum *et al.*, 1992). Trans-vaginal ultrasonography is very helpful in the selection patients with ovarian cysts suitable for ovarian cyst aspiration.

The need for cytological examination of the aspirate from the ovarian cysts is not clearly established but it would seem advisable to perform cytology if the cyst is large or if the aspirate is discoloured (Greenbaum *et al.,* 1992). It is important to provide the cytologist with an adequate volume of sample and to explain the route of sample collection to avoid confusion from sampling artifacts (Buckley, 1989). Hurwitz (1988) has recommended excision of cysts rather than aspiration, because even if the cyst is a benign neoplasm there is still the potential for malignant transformation. Certainly if a cyst aspiration is performed then it is important to follow up the patient to ensure the cyst does not reoccur.

Reoccurrence of ovarian cysts following aspiration is a potential problem, and may reflect in part inappropriate selection of patients for aspiration. Endometriomata are not suitable for treatment by aspiration alone. Hasson (1990) found 8 of 9 endometriomata reoccurred following a laparoscopic aspiration, but when the lining of the endometrioma was coagulated or removed only 2 of 18 endometriomata reoccurred. De Crespigny (1989) found that a heavily blood stained ovarian cyst aspirate was associated with a high likelihood of subsequent reoccurrence of the ovarian cyst. Of 28 patients with heavily blood stained cyst fluid 16 patients required subsequent surgery revealing 6 cases of endometriosis, 6 cases of benign cysts and in 4 cases there was no cyst but simply loculated fluid in pelvic adhesions (De Crespigny *et al.,* 1989).

Fortunately none of the 13 ovarian cysts aspirated in this study reoccurred when reviewed subsequently. This may partly be a reflection of appropriate selection of patients. The low gonadotrophin levels due to pituitary downregulation at the time of aspiration may

have also helped decrease the subsequent redevelopment of the ovarian cysts.

In group A despite elevated serum oestradiol levels there was little evidence of gonadotrophin stimulation with low basal gonadotrophin levels and little pituitary response during the LHRH stimulation test. In study 3 it was shown that the steroid concentration within the functional ovarian cysts was 1000 times that seen in the serum. Functional ovarian cysts may thus influence the serum oestradiol levels through passive diffusion of oestradiol from the cyst fluid into the venous circulation rather than by active synthesis. The fall in serum oestradiol 3 days following aspiration of the functional ovarian cysts is further support that the functional ovarian cyst fluid acts as a reservoir of oestradiol. If the granulosa and thecal cells comprising the wall of the cysts were engaged in active oestradiol synthesis then the serum oestradiol may not have fallen so quickly following the aspiration. Furthermore if the latter had been true then there may have been a disruptive influence on the subsequent response to ovarian stimulation.

Pituitary downregulation in the presence of elevated serum oestradiol levels at first would seem to contradict the finding of premature luteinisation in study 1 in patients with ovarian cysts and elevated serum oestradiol levels. There are two possible explanations for this apparent conflict. Firstly premature luteinisation noted in study 1 may have been triggered by the iatrogenic bolus of LH in the HMG given for ovarian stimulation. Alternatively pituitary suppression may have been delayed by the elevated oestradiol levels (Araki *et al.*, 1985) and a small endogenous surge of LH may have occurred. With either LH trigger luteinisation could only occur if LH receptors had developed on granulosa cells as explained in chapter 1. If a functional

ovarian cyst develops during the initial period of stimulation when buserelin is commenced then during this time the granulosa cells in the wall of the cyst may develop LH receptors. A LH surge would thus result in luteinisation of the functional ovarian cyst.

This study was limited by the absence of an appropriate control group, because it was felt unethical to allow a patient to commence ovarian stimulation in the presence of a functionally active ovarian cyst. However, the patients involved in the study did not form a good prognosis group as can be seen from table 7.1 and the treatment regime following the commencement of ovarian stimulation did not differ between study 1 and study 4. Although the numbers were small, the low cancellation rate and high pregnancy rate are encouraging that cyst aspiration may be an effective method of clinical management for patients with persistent functional ovarian cysts associated with steroidogenic activity

CONCLUSION

In this the concluding chapter the four studies presented in the thesis are brought together. The chapter summarises the studies, and the hypotheses formulated in the introduction section of the thesis are re-evaluated in the light of the results of the studies. The clinical benefits of the thesis are considered, and the chapter speculates on possible future developments of the thesis.

The first study assessed the influence of functional ovarian cysts during IVF cycles in relation to serum steroid levels. A review of 780 IVF cycles identified 53 cycles with cysts present during controlled ovarian hyperstimulation. Following pituitary downregulation on day 4 of the IVF cycle it was reasoned that ovarian steroid production should be minimal. Day 4 serum concentrations of oestradiol, progesterone and androstenedione were thus used to reflect steroid production by ovarian cysts present at that time. Of the 53 IVF cycles with cysts day 4 serum oestradiol concentrations were significantly ($P<0.05$) higher in the cycles which were cancelled than in the cycles which proceeded to oocyte retrieval. The 95th centile of serum oestradiol on day 4 of the IVF cycle in cycles without apparent ovarian cysts was used to divide the cycles with functional ovarian cysts into two groups. Comparing the latter groups the group with a higher day 4 serum oestradiol not only had significantly ($P<0.05$) more cycles abandoned for "poor" response to ovarian stimulation, but they also produced significantly ($P<0.01$) fewer grade 1 embryos per oocyte retrieval, although there was not a significant difference in the number of fertilised oocytes per oocyte retrieval between the two groups. Serum androstenedione concentrations did not differ according to IVF outcome, which suggests that hyperandrogenism may not be a mechanism of influence of ovarian cysts during IVF cycles. To establish a value of serum progesterone consistent with luteinised ovarian steroid activity the peak progesterone

concentration before administering the luteinising injection of HCG was measured for 105 IVF cycles. Excluding one case of premature luteinisation the peak progesterone level was 5.7 nmol/l, and progesterone concentrations above this value were taken to reflect luteinised ovarian steroid production. Only 1 of 53 cycles with ovarian cysts had a day 4 serum progesterone >5.7 nmol/l suggesting that only 1 of the cysts may have been a persistent functional corpus luteal cyst. A significant rise in serum progesterone concentrations prior to the luteinising HCG due to premature luteinisation was shown to occur in a few of the cycles with ovarian cysts despite the use of buserelin. Premature luteinisation may be one problem associated with steroid producing ovarian cysts, and this was suggested by Thatcher (1989). This study has shown an adverse influence of functional ovarian cysts during IVF cycles, but only when the basal oestradiol levels are elevated.

The second study concerned the development of functional ovarian cysts during pituitary downregulation. In 16 of 288 IVF cycles studied functional ovarian cysts with significant steroidogenic activity developed during pituitary downregulation. In agreement with Ron-El's (1989) study the cysts were associated with an elevation in serum oestradiol levels but not serum progesterone levels. The significant ($P<0.01$) fall in serum progesterone levels despite a significant ($P<0.01$) rise in serum oestradiol levels during the development of the functional ovarian cysts suggested that the functional ovarian cysts were follicular cysts rather than corpora lutea cysts. Serum oestradiol and progesterone levels prior to downregulation were used to reflect the likely state of follicular development at the time buserelin was commenced, and this suggested that the functional ovarian cysts may have arisen from immature ovarian follicles. Comparing the progesterone

concentrations between the IVF cycles which did develop ovarian cysts and the cycles which did not develop ovarian cysts there appeared to be a protective effect of progesterone decreasing the likelihood of cyst formation. It is possible that progesterone may decrease the "flare up" effect of buserelin (Araki *et al.,* 1985), and progestogens may be used to prevent cyst formation with buserelin (Wardle P., personnel communication).

The third study investigated the steroid content of functional ovarian cysts developing during pituitary downregulation. In 10 of the 16 patients with functional ovarian cysts identified in study 2 the serum oestradiol levels remained elevated despite at least 3 weeks of buserelin. Transvaginal cyst aspirations were performed in the latter cases, and the cysts aspirates were available for steroid measurement in 9 cases. The cyst aspirates had oestradiol concentrations 1000 times higher than the serum oestradiol concentrations at the time the cysts were aspirated suggesting that the cysts had significant steroidogenic activity. The cyst aspirates had significantly lower progesterone ($P<0.001$), higher androstenedione ($P<0.01$) and similar oestradiol concentrations to 10 follicular fluid samples collected at the time of oocyte retrieval. The cyst aspirates thus had a steroid content more consistent with follicular cysts rather than corpora lutea cysts or theca lutein cysts (Vanluchene and Vanderkerckhove, 1983).

The fourth study followed the outcome of IVF cycles following cyst aspiration in the 10 cases above. A prompt significant ($P<0.01$) fall in serum oestradiol was seen following cyst aspiration in all 10 cases suggesting that the source of elevation of serum oestradiol levels were the functional ovarian cysts. Although only 10 IVF cycles were managed in this manner, the outcomes of these cycles (with 9 cycles

proceeding to oocyte retrieval resulting in 5 conceptions and 4 babies) provides reassurance that this is an effective method of dealing with this problem. Rizk (1990) compared cyst aspiration with continuing LHRH agonist until serum oestradiol levels had fallen below 100 pmol/l, and found no difference in outcome between these treatment regimes. Although the numbers were small in Rizk's study, it would support the view that the influence of ovarian cysts on IVF cycles is not a consequence of the physical presence of the cyst but rather relates to the functional activity of the cyst, which may be removed by aspiration. The advantage of aspiration is that it quickly lowers steroid levels whereas continued pituitary downregulation may take several weeks.

The principal hypothesis of the thesis suggested that functional ovarian cysts adversely influence in vitro fertilisation cycles in relation to their steroidogenic activity but not by their physical presence. The latter hypothesis receives considerable support from the results of study 1. Some studies have shown no difference between IVF cycles with ovarian cysts present compared to IVF cycles without ovarian cysts, thus the presence of an ovarian cyst in itself may not be enough to influence IVF cycles (Feldberg *et al.,* 1989, Hornstein *et al.,* 1989, Sampaio *et al.,* 1991). Thatcher (1989) was the first to suggest that if functional ovarian cysts produced steroids then this may disrupt the endocrine milieu adversely influencing follicular development during controlled ovarian hyperstimulation, and this thesis would support this view. A more recent study reported by Thatcher's group concluded that baseline ovarian cysts in the setting of a low baseline oestradiol level do not affect the clinical response to controlled ovarian hyperstimulation in IVF cycles (Penzias *et al.,* 1992).

The hypothesis that buserelin could cause the development of follicular ovarian cysts with significant steroidogenic activity was supported by the results of studies 2, 3 and 4. Study 2 confirmed that functional ovarian cysts could develop during buserelin treatment. These cysts were associated with elevation of serum oestradiol levels suggesting the cysts were producing oestradiol. Study 3 discovered the cysts contained fluid with oestradiol concentrations many times higher than serum oestradiol concentrations. The final evidence of the steroid activity of these cysts came from the fall in serum oestradiol concentration observed following aspiration of the cysts in study 4. The oestradiol, progesterone and androstenedione concentrations of the cyst aspirates were consistent with follicular cysts.

The final hypothesis stating that aspiration of functional ovarian cysts could remove steroidogenic activity allowing the IVF cycle to proceed unhindered was supported by studies 3 and 4. It was shown in study 3 that the cyst aspirate contained very high concentrations of steroids. Study 4 found that following aspiration of functional ovarian cysts in 10 patients there was a significant fall in serum oestradiol levels. Nine of the latter 10 patients had a sufficient response to ovarian stimulation to proceed to oocyte retrieval, 5 patients conceived and 3 pregnancies progressed to term delivering a total of 4 babies. Although the number of patients treated by cyst aspiration were small the results of this study provide encouragement that the above hypothesis is correct.

The findings in this thesis have had a distinct clinical benefit in the management of patients on the Southampton IVF programme. The standard Southampton IVF regime has now been changed to identify functional ovarian cysts before commencing ovarian stimulation.

Patients with ovarian cysts and serum oestradiol levels >200 pmol/l do not commence ovarian stimulation on day 4 menses. Buserelin is continued until the serum oestradiol falls below 200 pmol/l, and then ovarian stimulation is commenced. If serum oestradiol levels do not fall over 2 weeks then cyst aspiration is offered as a proven method of reliably dealing with the problem. Patients with serum oestradiol >200 pmol/l and no ovarian cyst are uncommon, and their management simply consists of continuing buserelin at a dose of 200 µg four times a day until the serum oestradiol falls below 200 pmol/l. There are thus now clear guidelines based on scientific evidence to provide appropriate management of patients with ovarian cysts during IVF cycles.

The publication and the presentation at several scientific meetings of the work in this thesis (see page x) may aid clinical management decisions in IVF centres and infertility clinics other than Southampton. Outside of IVF clinics ovarian cysts present a clinical management dilemma. Considering anovulatory patients treated with gonadotrophin therapy Tummon (1988) suggested that ovarian stimulation may proceed without interference from an ovarian cyst. However, the results of this thesis would caution the latter view suggesting that the influence of functional ovarian cysts during ovulation induction should be reassessed with regard to the steroidogenic activity of the ovarian cysts.

One can speculate further on the possible mode of influence of functional ovarian cysts on folliculogenesis, considering possible avenues for future research. The thesis suggests that steroids released from functional ovarian cysts may adversely influence IVF cycles, and serum oestradiol is a marker of this happening. It may not be oestradiol that is the main steroid influencing follicular

development, and non-steroidal regulatory factors can not be dismissed. Catecholoestrogens have been shown to promote steroidogenesis (Spicer and Hammond, 1989a) while inhibiting cell proliferation in porcine granulosa cells (Spicer and Hammond, 1989b). Catecholoestrogen production increases with follicular maturity in the pig (Hammond *et al.,* 1986), and catecholoestrogens have been found in human follicular fluid (Dehennin *et al.,* 1984). Whether catecholoestrogens exert a paracrine or possibly even an endocrine influence in human ovaries is unknown. However, a positive correlation in human follicular fluid has been shown between the concentrations of oestradiol and the catecholoestrogens, 2-hydroxyoestradiol and 4-hydroxyoestradiol (Dehennin, 1990), and catecholoestrogen production by functional ovarian cysts merits further consideration. Follicular development appears to be influenced by a number of non-steroidal factors including insulin-like growth factors and their binding proteins (Geisthovel *et al.*, 1990, Hartshorne *et al.*, 1990), inhibin and activin (de Jongh, 1988), epidermal growth factor and transforming growth factor-α (Gospodadarowicz and Balecki, 1979), transforming growth factor-β (Skinner *et al.*, 1987) and fibroblast growth factor (Neufield *et al.*, 1987). Although a detailed appraisal of the latter is beyond the scope of this thesis, the production of non-steroidal regulatory factors by functional ovarian cysts is worthy of future research. It could be hypothesised that steroidal or non-steroidal regulatory factors may have autocrine influences stimulating steroid production within functional ovarian cysts and paracrine or possibly even endocrine influences inhibiting cell division and growth of ovarian follicles.

The thesis has addressed a common clinical problem: *"the development and influence of functional ovarian cysts during IVF cycles"*. The thesis has contributed to the medical literature showing

that the presence of a cyst in itself is not important, but functional activity of a cyst may have an adverse influence on IVF cycles. It has been confirmed that during pituitary downregulation functional ovarian cysts may develop with steroidogenic activity consistent with follicular cysts. Aspiration of the latter cysts has been demonstrated to provide effective clinical management. The thesis invites speculation regarding the mode of influence of functional ovarian cysts on follicular development during IVF cycles with possible relevance to the concept of follicular dominance. Further work in this area may aid both our understanding of the development and influence of functional ovarian cysts during IVF cycles and extend our knowledge of folliculogenesis.

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