GROWTH HORMONE CO-TREATMENT IN SUPEROVULATION PROGRAMMES

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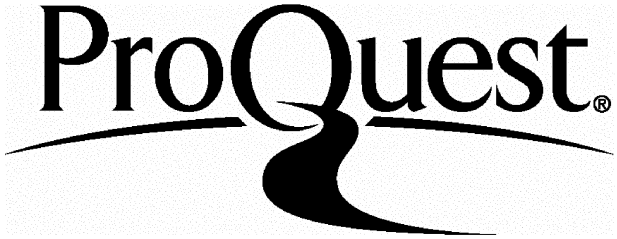


Abstract

The role of insulin-like growth factor I (IGF-I) in modulating the granulosa cell response to gonadotrophins is now well established and recent work has suggested that growth hormone (GH) administration increases intraovarian IGF-I production.

Following the successful use of GH administration in patients who had previously had a poor response to clomiphene citrate and human menopausal gonadotrophins (hMG) prior to in-vitro fertilisation and embryo transfer, twenty women were recruited into a prospective, double blind, placebo controlled trial of GH co-treatment. Women with PCO developed more follicles and had more oocytes collected but the women with normal ovaries showed no response. Circulating IGF-I concentrations rose during GH treatment but follicular fluid concentrations were significantly lower than serum. IGF-II concentrations did not alter with GH treatment.

Gonadotrophin releasing-hormone analogues are associated with increased requirements for hMG and twenty eight women were subsequently recruited into a randomised, placebo controlled study using GH in combination with buserelin and hMG. Women who received GH and who had ultrasound diagnosed PCO had an improved outcome, however there was a substantial placebo effect. Women with normal ovaries did not demonstrate any effect of GH treatment. IGF-I concentrations rose with GH administration but not with hMG alone. Follicular fluid concentrations of IGF-I were lower than serum concentrations, suggesting that IGF-I is not synthesised in the ovary.

As women with PCO formed the responsive group we wished to establish a biochemical marker for the condition. 11ß-hydroxyandrostenedione (11ß-OH-A_4) concentrations had been suggested to be abnormal in PCO. We therefore developed an in-house method for determining the concentrations of 11ß-OH-A_4 in serum and follicular fluid. We were, however, unable to define differences in concentrations between women with normal and polycystic ovaries and concluded that 11ß-OH-A_4 was not a suitable marker for PCO.

Contents

Chapter II. Methods

Chapter III. Results 1-CC and hMG studies

List of Tables

List of Figures

I. Introduction

Introduction

During the past four decades considerable advances have been made in our understanding of the pathophysiology of ovarian function and it is now appreciated that endocrine, paracrine and autocrine factors all play a part. It has more recently been established that, present within serum, there is a complexity of factors essential to cell growth and function.

Part of the work presented in this thesis reports a novel therapy for augmentation of ovarian stimulation, the founding idea for which was the demonstration of modulation of ovarian steroidogenesis by insulin-like growth factors (IGFs). The ovarian response to cotreatment with growth hormone (GH) was assessed in the context of super-ovulation prior to in-vitro fertilisation and embryo transfer (IVF-ET).

Access to follicular fluid, associated with oocyte collection prior to IVF-ET in the clinical studies, allowed investigation of several aspects of ovarian function. Analysis of the results of the clinical studies suggested that women with polycystic ovaries (PCO) had an improved response to the therapy. We therefore studied a potential marker for this condition: the androgen, 11-ß-hydroxyandrostenedione (11-ß-OHA_4).

The introductory chapter gives a summary of the physiology of ovarian function and provides details of the IGFs relevant to the studies subsequently described. An overview of the actions of growth hormone (GH) completes the background to the clinical studies. A review of PCO is included, with the details of studies relating to the use of 11ß-OH-A_4 as a marker for this condition.

The methodology of IVF-ET and descriptions of the clinical studies are outlined in Chapter *II* and the results of these studies presented in Chapters *III* and *IV*. The methodology and results of the 11-ß-OHA_4 studies are presented in Chapter *V*. Chapter *VI* contains a discussion of the details of the studies.

Physiology of ovarian function

Steroidogenesis

The classic model of ovarian function is gonadotrophin (Gn) stimulation of the granulosa and theca cells with the subsequent production of androgens, oestrogen and progesterone. The "two-gonadotrophin, two-cell" theory (Armstrong and Papkoff, 1976; Veldhuis *et al* 1982) forms the basis for the much of the work now pursued in the field of ovarian physiology.

Gonadotrophin releasing hormone (GnRH).

The hypothalamus produces GnRH, a decapeptide, whose pulsatile release is controlled by factors such as dopamine, noradrenaline, opiates and sex steroids. Oestradiol (E_2) and progesterone have both negative and positive feedback actions on the release of GnRH.

Pituitary.

After release into the portal system, GnRH interacts with specific receptors on the gonadotroph cells in the anterior pituitary. Activation of the receptors leads to secretion and increased synthesis of follicle stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary.

Gonadotrophins.

FSH and LH are glycoproteins which consist of alpha and beta subunits which are non covalently bound. The α subunit is common to all gonadotrophins. The ß subunit confers bio- and immuno-specificity on the assembled molecule.

The gonadotrophins are released into the circulation at various intervals, depending on the time of the cycle, and interact with specific receptors on the ovarian cell surface. Receptors for FSH are restricted to granulosa cells, whereas LH receptors are located on theca and

interstitial cells and, in addition, appear on granulosa cells in preovulatory follicles.

Serum concentrations of FSH rise in the late luteal phase and early follicular phase and subsequently decrease as the dominant follicle produces inhibin and E_2. LH increases in the mid follicular phase and then rises dramatically before ovulation to induce maturation and release of the oocyte.

Follicular cells contain gonadotrophin receptors within their plasma membrane which respond to FSH and LH binding by activation of adenylate cyclase and/or increases in diacylglycerol, production of intracellular messengers, alteration in steroid enzyme activities and a generalised enhancement of protein synthesis.

Theca cell steroidogenesis.

The second messenger in steroid secreting cells is cyclic adenosine monophosphate (cAMP). LH stimulation leads to increases in cAMP which precede steroid synthesis. In response to LH, theca cells metabolise cholesterol and produce androstenedione (A_4) and testosterone, the precursors for the synthesis of oestradiol by the granulosa cells (Fig *I*-1).

As well as LH, several growth factors such as IGFs, transforming growth factor ß (TGFß), inhibin and activin may also modulate thecal androgen synthesis (Caubo *et al* 1989, Hillier *et al* 1991).

Cholesterol side chain cleavage is the first process in steroid hormone synthesis and is the major rate determining step. The C_{21} steroid, pregnenolone, thus produced is converted to 17-hydroxypregnenolone by the enzyme P450c17 (17-hydroxylase/C-17-20 lyase). 17-hydroxypregnenolone is further metabolised to dehydroepiandrosterone (DHEA) by this enzyme (Fig *I*-1). The conversion of C_{21} to C_{19} steroids by P450c17 occurs in the thecal/interstitial cells and the theca-lutein cells and may be exclusively ovarian.

The action of 3ß-hydroxysteroid dehydrogenase/delta^5-delta^4 isomerase on pregnenolone

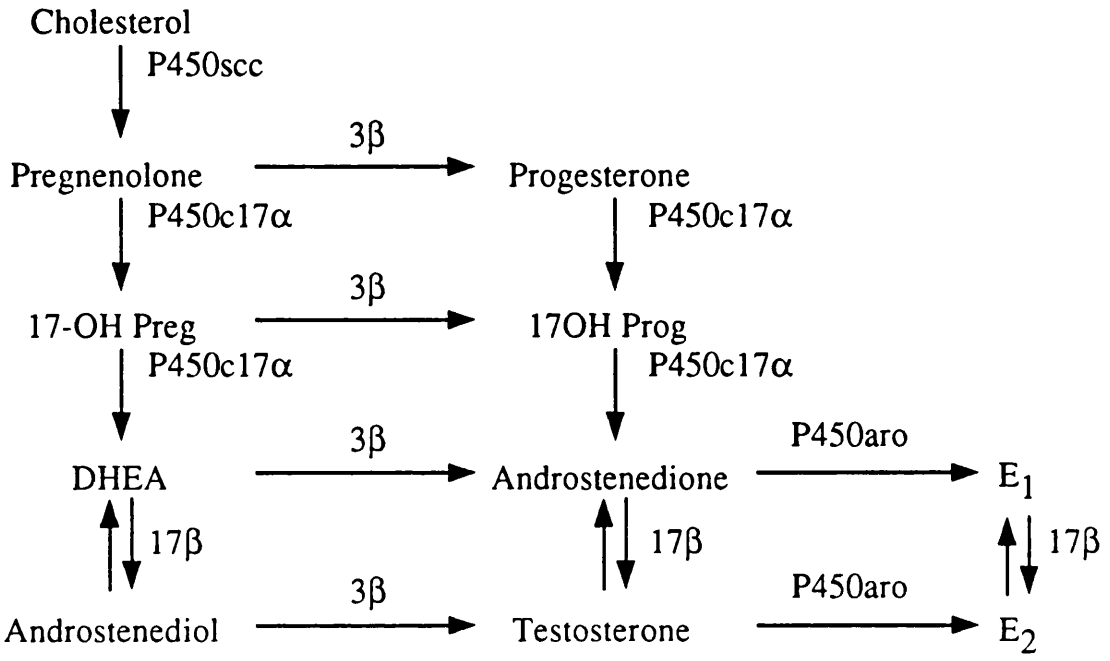


Fig. I.1 Steroidogenesis

P450scc	=	side chain cleavage enzyme
3β	=	3β-hydroxysteroid dehydrogenase/delta^5 - delta^4 isomerase
P450c17α	=	17α-hydroxylase/C-17,20-lyase
17β	=	17β-hydroxysteroid dehydrogenase (17-ketosteroid reductase)
P450aro	=	aromatase
17-OH Preg	=	17-hydroxypregnenolone
17-OH Prog	=	17-hydroxyprogesterone

results in the formation of progesterone and the enzyme's action on 17-hydroxypregnenolone results in the formation of 17-hydroxyprogesterone. P450c17 activity upon progesterone can also form 17- hydroxyprogesterone.

Androstenedione may be formed either by the action of P450c17 on 17-hydroxyprogesterone or the action of 3ß-hydroxysteroid dehydrogenase/delta^5-delta^4 isomerase on DHEA. DHEA and A_4 are C_{19} steroids.

Androstenediol and testosterone are formed from DHEA and A_4 respectively by the enzyme 17ß-hydroxysteroid dehydrogenase. The function of this enzyme is considered to be limited to thecal cells and is influenced by LH.

Granulosa cell steroidogenesis.

The steroid biosynthetic capability of granulosa cells differs from that of theca cells in two significant ways. First, in contrast to theca cells, granulosa cells lack the P450c17 enzyme necessary to produce androgens from C_{21} precursors. Second, granulosa cells have abundant aromatase activity which is induced by FSH. Aromatase is a key enzyme in the ovary (Erickson and Hsueh 1978, Daughaday *et al* 1982). The granulosa cells can thereby readily convert androgens to oestrogens, provided there has been some FSH stimulation of the cells.

Thus, under the influence of LH, the theca cells provide the C_{19} substrates for conversion to E_2 by the granulosa cells, a step which is under the influence of FSH (Leung and Armstrong 1980).

As the granulosa cells approach the preovulatory stage, they acquire the capacity to synthesise progesterone. This is regulated by two enzymatic steps; the first is the conversion of cholesterol to pregnenolone (P450scc) and the second the metabolism of pregnenolone to progesterone from the action of 3ß-hydroxysteroid dehydrogenase/delta^5-delta^4 isomerase. The genes for P450aro and P450scc enzyme proteins are both inducible by FSH.

Luteal phase steroidogenesis.

In the luteal phase the luteal cells produce large amounts of progesterone and oestradiol. The progesterone metabolism is under the influence of both FSH and LH which are thus essential for the maintenance of the corpus luteum. Negative feedback by progesterone on the hypothalamus leads to decreased circulating concentrations of the gonadotrophins but steroidogenesis continues because of increased number of receptors.

Follicular development and ovulation.

Primordial follicles.

During fetal life, non proliferating primordial follicles are formed. They consist of a relatively small oocyte surrounded by a single layer of squamous cells, destined to become the granulosa cell layer. These so called pre-antral follicles are approximately 0.2mm in diameter and are independent of gonadotrophin stimulation.

Antral follicles.

Antral follicles develop by mitotic divisions of the granulosa cells, followed by theca cell differentiation. These small antral follicles develop and function independently of the marked variations in Gn secretion occurring during the normal menstrual cycle. Throughout each menstrual cycle follicles are developing and undergoing atresia such that fewer than one percent of ovarian follicles which begin to grow actually ovulate. Granulosa cell proliferation at this stage is thought to be stimulated by non-steroidal, intrafollicular growth factors.

Up to ninety days may be taken to develop a follicle receptive to Gn (Gougen 1986). Although as many as 20 receptive antral follicles may be generated per month, only one usually responds optimally to FSH but the precise mechanism of this selection and dominance is poorly understood. The less responsive follicles become cohorts and usually undergo atresia.

Gonadotrophin stimulation.

FSH binds to the receptors on the granulosa cells of primary follicles to stimulate production of E_2 by the enhancement of P450aro activity. FSH also increases the granulosa cell sensitivity to its actions by, in part, the augmentation of expression of P450aro and P450scc enzyme complexes (Richards *et al* 1987, Hillier *et al* 1988).

Oestradiol leads to proliferation of granulosa cells and increased sensitivity of the follicle to further Gn stimulation (Richards *et al* 1987). In rats, E_2 can increase its own production by stimulating P450aro activity but the situation in human granulosa cells is less clear (Hsueh *et al* 1984, Richards *et al* 1987). Androgens have also been shown to augment FSH induced P450aro activity in rat granulosa cells, thereby enhancing E_2 synthesis (Armstrong and Papkoff 1979, Hillier *et al* 1982).

Within the developing dominant follicle there is an exponential rise in E_2 associated with increasing inhibin production. The high concentrations of E_2 ultimately lead to a decline in circulating FSH through negative feed back actions at the hypothalamus and pituitary. Pituitary FSH output is also selectively suppressed by circulating inhibin concentrations.

Dominance.

In the mid follicular phase the follicle destined to ovulate attains dominance over the other follicles and the remainder undergo atresia. This may be due to differential numbers or sensitivity of the FSH receptors, enabling the dominant follicle able to endure decreasing concentrations of FSH. Adashi *et al* have, however, suggested that there are other modulating factors within the ovary which selectively augment the follicular response to FSH (1985a). It is the role of these growth factors in follicular development which is pivotal to the work presented in this thesis.

Enlargement of the dominant follicle is caused partly by proliferation of the granulosa and the theca cells and partly by accumulation of follicular fluid (FFL) in the antrum. The

dominant follicle grows from 2mm to 25mm in approx 14 days with an increase in granulosa cells from approx $1x10^6$ to $50x10^6$ (McNatty *et al* 1979a, Kerin *et al* 1981). E_2 has been shown to stimulate mitosis of granulosa cells and also enhance the action of growth factors.

Ovulation.

In the late follicular phase, FSH rapidly induces formation of LH receptors in readiness for the LH surge. E_2 amplifies the stimulatory actions of FSH (Richards 1980, Rani *et al* 1981). The rise in E_2 leads to increased sensitivity of the pituitary to GnRH and through this positive feedback the preovulatory LH surge is produced. The LH surge induces in the dominant follicle a series of dramatic changes: follicular steroidogenesis shifts from predominantly E_2 synthesis to predominantly progesterone synthesis, granulosa cell proliferation ceases and oocyte meiosis is resumed. High concentrations of LH terminate FSH-induced follicular growth.

Approximately 36 to 40 hours following the initiation of the LH surge, rupture of the follicle occurs and the oocyte and accompanying FFL are released into the pouch of Douglas (Baird *et al* 1975).

Luteal phase.

The ruptured follicle collapses and vascularisation of the newly formed corpus luteum ensues. Progesterone production is under the influence of LH and increases with the rise in LH concentrations denoting the LH surge. E_2 continues to be produced by the luteinised granulosa cells but is under the influence of LH not FSH (Filicori *et al* 1984).

Corpus luteum function is dependent upon an intact hypothalamo-pituitary axis producing GnRH pulses through out the luteal phase, although feed back on the pituitary by progesterone slows the LH pulse rate from hourly to 4 hourly during this time (Knobil 1980). The concentrations of both gonadotrophins are low in the luteal phase due, not only to the negative feedback of E_2 and progesterone, but also to inhibin.

Regression.

Finally, in the absence of human chorionic gonadotrophin (hCG) produced by the implanting blastocyst, the corpus luteum begins to regress after 10-12 days. Progesterone production is inhibited and luteolytic agents such as prostaglandin $F_{2\alpha}$ play an important role in the demise of the corpus luteum.

Follicular fluid.

Production.

During the development of the follicle, the fluid therein increases from less than 1μL to 6,000μL or more. This fluid is formed both by transudation of plasma from the theca capillaries and by local secretion and metabolism. The formation of the follicular fluid is dependent upon the combined action of E_2 and FSH.

Although similar to ovarian venous plasma, the composition of FFL is unique to the mature dominant follicle and is rich in steroids and other non-steroidal ovarian regulators. The steroids produced in the follicle partly accumulate in the FFL and partly diffuse into the thecal vessels from where they circulate to extraovarian target organs. The steroids accumulating in the FFL create, in the individual antral follicle, a unique intrafollicular hormonal milieu which differs from that in adjacent follicles and that in plasma (Edwards 1974).

Contents.

Many substances circulating in the blood can enter the follicular fluid and reach the oocyte. No major concentration gradient exists across the follicle wall for electrolytes, although steroids and other substances may be in greater concentration than in plasma due to intrafollicular binding proteins, for example albumin (Gosden *et al* 1988). Most substances can diffuse freely into or out of the follicle according to their concentration gradient, but

because of continuous changes in volume and metabolism it is doubtful that equilibrium conditions exist.

Proteins:

Larger proteins penetrate more slowly than smaller ones and the concentrations present at any one stage can be expected to vary with the flux of water, the surface area to volume ratio of the follicle and the permeability of the theca blood vessels. Follicular fluid contains most of the plasma proteins, albumin being the most abundant. The follicular wall acts like a coarse molecular sieve and a blood-follicle barrier exists.

Steroids:

The concentrations of E_2 and progesterone in FFL increase during the follicular phase and are highest in the larger, more mature follicles (McNatty *et al* 1973, McNatty *et al* 1979a). The oestradiol concentration appears to reflect both the number of granulosa cells in the follicle and their steroidogenic productivity. Before ovulation the concentrations of both E_2 and progesterone are between 4,000 and 40,000 times greater than plasma.

E_2 concentrations in follicular fluid decline dramatically in the dominant follicle in the interval between the LH surge and ovulation. This decline is thought to be due to suppression of aromatisable androgen biosynthesis in the theca in direct or indirect response to the LH surge (Futz and Speroff 1982). Thus the fall in E_2 concentrations in FFL is paralleled by a similar drop in A_4, quantitatively the most important androgen in human FFL (Testart *et al* 1982).

Summary.

FSH is the primary stimulus for the growth and proliferation of granulosa cells. Together, granulosa and theca cells produce the steroids necessary for follicular growth and maturation but many other factors modify their action. Selective enlargement of one follicle (dominance)

is poorly understood but may involve a combination of acquisition of FSH receptors and local paracrine and autocrine growth factors.

As a result of the LH surge, a mature oocyte is released from the dominant follicle following which progesterone is produced from the corpus luteum. Failure of implantation of a fertilised oocyte results in demise of the corpus luteum and menstruation ensues.

Insulin-like growth factors

Introduction.

During the recruitment, selection and dominance of the leading follicle, many follicles are exposed to similar concentrations of gonadotrophins but the majority become atretic. The increasing response of the dominant follicle to stimulation by FSH may be due, in part at least, to alterations in intraovarian paracrine or autocrine factors. Paracrine factors are produced by various cells within the ovary and influence the function of other local cells, autocrine factors are those which modify a response within the cell of production.

Many steroidal and non-steroidal factors act as paracrine or autocrine modulators of the granulosa and thecal cell response to gonadotrophins. Androgens, oestrogens, insulin, IGF-I, TGFß, inhibins and activins, for example, alter granulosa cell function (Davoren and Hsueh 1984, Adashi and Resnick 1986, Hsueh 1986, Richards *et al* 1987, Adashi 1989a).

Androgens, epidermal growth factor, TGFα and many other substances influence the theca cells and there are several theca derived factors which exert paracrine control over FSH action (Kudlow *et al* 1987, Tonetta and DiZerega 1989). Granulosa cell derived factors may also stimulate thecal androgen synthesis.

Of all the potential factors modulating the ovarian response to gonadotrophins, the insulin-like growth factors appear to have the most diverse effects (Daughaday and Rotwein 1989). The IGFs have been shown to promote both replication and differentiation of cultured

granulosa cells in animals (Hammond *et al* 1988a, Adashi 1989). The growth factors enhance FSH-induced progesterone, E_2, cAMP and proteoglycan production as well as LH receptor induction (Adashi *et al* 1985a).

The clinical studies presented in this thesis were thus undertaken to investigate the influence of IGFs on the ovarian response to gonadotrophins.

History of insulin-like growth factors.

In 1940 it was postulated that GH stimulated growth by increasing insulin concentrations (Young 1940). Among the known hormones, only insulin could stimulate proliferation of cultured cells in vitro. In 1953, however, Ellis *et al* observed that GH administration to hypophysectomised rats in vivo led to a dramatic increase in uptake of radiolabelled sulphate by the cartilage of these animals.

Salmon and Daughaday (1957) were unable to replicate this effect in in-vitro experiments in which they assessed the stimulation by GH of sulphate uptake by rat cartilage. They observed that GH had no direct metabolic action in-vitro on cartilage and went on to describe a substance present in serum which appeared to be the intermediary factor for the effects of GH on skeletal tissue. They demonstrated that this intermediary factor was present in very low concentrations in the serum of rats which had undergone hypophysectomy, but was restored by treatment with GH.

In in-vitro experiments there was a significant increase in the incorporation of sulphate into cartilage using serum from hypophysectomised rats at least 6 hours post GH administration. Insulin in physiological concentrations did not stimulate sulphate incorporation in vitro, nor did GH. Salmon and Daughaday suggested that this "sulphation factor" was a mediator of GH action in promoting skeletal growth and, because of the persistence of its action after 24 hours of dialysis, they concluded that the active component was either a protein, or highly protein bound.

Over the next decade, Daughaday and coworkers demonstrated that this factor stimulated the incorporation of thymidine into deoxyribonucleic acid (DNA) (Daughaday and Reeder 1967), proline into the hydroxyproline of collagen (Daughaday and Mariz 1962) and uridine into ribonucleic acid (RNA) (Salmon and DuVall 1970a). They also established that the biological actions were not limited to cartilage and partially purified extracts of sulphation factor showed insulin-like activity on isolated rat diaphragm (incorporation of glucose into glycogen)(Salmon and DuVall 1970b) and in adipose tissue (glycogen synthesis and inhibition of lipolysis)(Hall and Uthne 1972).

Comparisons of insulin-like activity in plasma, as measured by bioassay, with the concentrations measurable by the specific radioimmunoassay (RIA) for insulin led to the discovery that only 10% of the bioactive insulin-like material in serum could be accounted for by immunoreactive insulin. This phenomenon was termed non-suppressible insulin-like activity (NSILA) because of the actions similar to insulin which were not suppressed by insulin antibodies (NSILA-S) (Froesch *et al* 1963, Froesch *et al* 1967, Jakob *et al* 1968). This serum factor appeared to have similar actions to the "sulphation factor" in cartilage (Zingg and Froesch 1973) and elsewhere (Morrell and Froesch 1973).

Because of the similarity of action, Daughaday *et al* (1972) suggested that the general term, somatomedin, be used to describe these GH dependent plasma factors that mediated the growth of responsive tissues. The prefix "somato" refers to the hormonal relationship to somatotrophin (GH) and the "medin" to indicate that it is an intermediary in somatotrophin action.

In 1973, Uthne *et al* reported the isolation of two other growth hormone dependent somatomedins (SM), SM-A and SM-B, differentiated by their responses in two bioassay systems (Van Wyk 1975). Somatomedin-A was described as a neutral peptide with a molecular weight of 7000 daltons (Da) and somatomedin-B, acidic, of about 4700 Da. A third more basic peptide, somatomedin-C, was described by Van Wyk *et al* in 1974. These somatomedins were noted to competitively bind to the insulin receptor on cell membrane preparations (Hintz *et al* 1972).

In an attempt to establish the site of production of these somatomedins, immature hypophysectomised rats were injected with labelled GH and whole body autoradiographs performed. Growth hormone was not taken up by the growing ends of the long bones but accumulated in the liver, kidneys, adrenals and to a lesser extent the submandibular glands (Mayberry *et al* 1971). Perfusion studies on isolated rat livers confirmed that somatomedin-like activity increased in response to stimulation by GH (McConaghey and Sledge 1970, Van Wyk *et al* 1974). Insulin was also considered to have a role in the regulation of somatomedin production in the liver (Daughaday *et al* 1976).

In 1976, Rinderknecht and Humbel reported the large scale purification of two polypeptides, NSILA-1 and -11, with insulin-like and cell-growth promoting activities (1976a). Using an acid ethanol extract of human plasma (precipitate B) obtained by a modified Cohn fractionation (Cohn fraction IV), they isolated two single chain peptides with three intra chain disulphide bridges each. They suggested that the amino acid composition of the two polypeptides differed slightly in their N-terminal amino acid sequences but subsequently noted that there was striking homology to the amino acid sequence of insulin B chain (Rinderknecht and Humbel 1976b). In in-vitro experiments they demonstrated that, for example, these insulin-like peptides had 50-100 times greater effect than insulin on [^{3}H]thymidine incorporation into DNA of chick embryo fibroblasts.

These two workers then went on to determine the complete amino acid sequence of the, newly named, insulin-like growth factor-I (IGF-I) (Rinderknecht and Humbel 1978) (Fig *I*-2). With a molecular weight of 7649 Da, containing 70 amino acids cross-linked by three disulphide bridges, this polypeptide displayed obvious homology with both the A and B chains of proinsulin.

In human proinsulin there is a connecting peptide 35 residues long between the A and the B chain, designated the connecting or C peptide. In IGF-I this intercalating peptide is only 12 amino acids long with no similarity with proinsulin connecting peptide. Whereas cleavage at each end of the connecting peptide of proinsulin produces the two chain structure of insulin linked by two inter chain disulphide bonds, no such cleavage occurs in IGF-I. In addition,

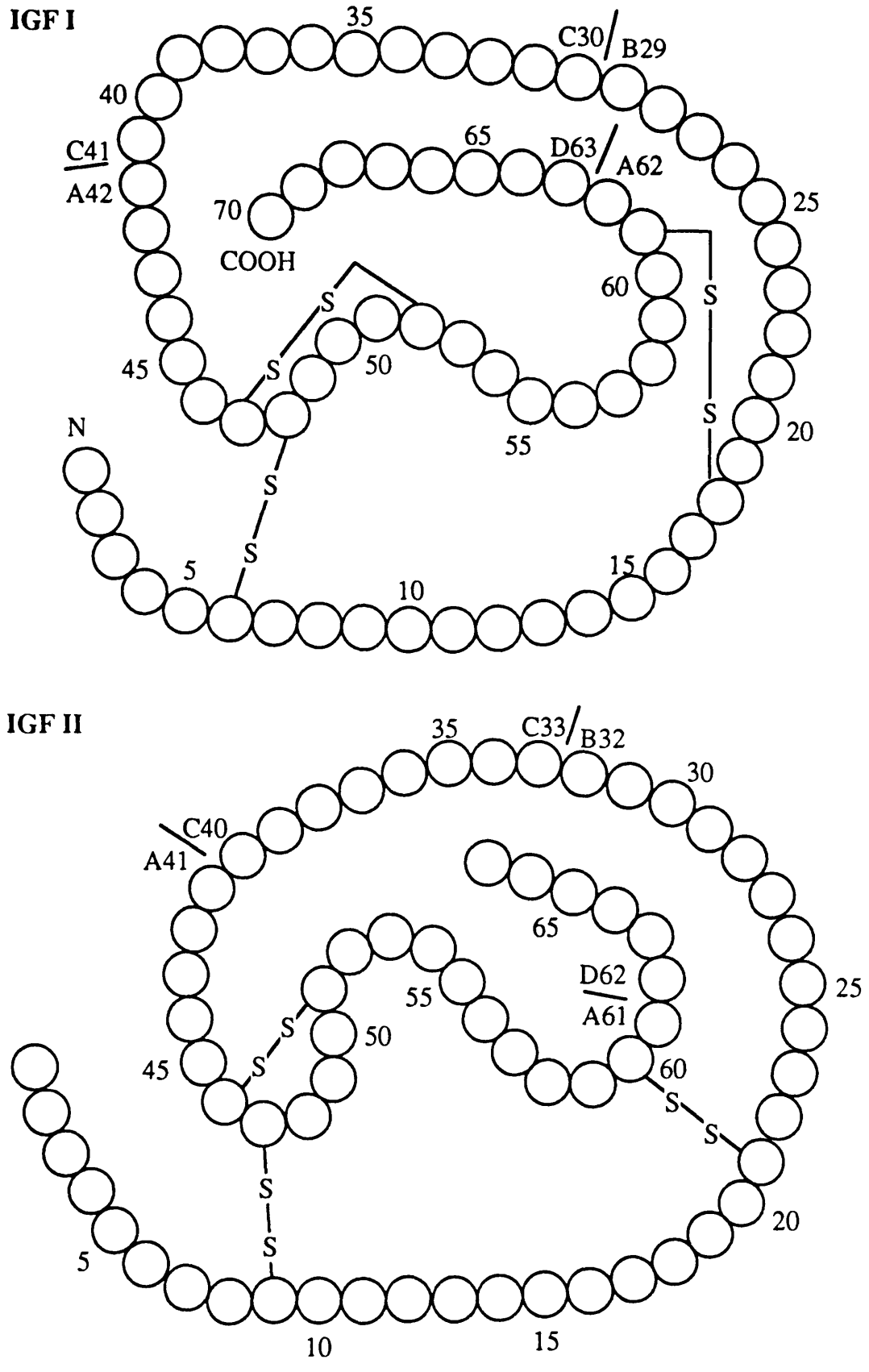


Fig *I.2* Structural formulae of IGF- I and IGF- II.

IGF-I has an extra 8 amino acid residues at the COOH-terminal end of its structure (D domain). Despite these differences however, the three-dimensional structure of IGF-I closely resembles that of insulin but possesses dissimilar amino acids on its surface (Blundell *et al* 1978). This may well explain the lack of reactivity of IGF-I with antibodies against insulin (Zapf *et al* 1978a). The sequences of IGF-I which are homologous to those portions of insulin which react with membrane receptors, however, are more exposed.

It is now recognised that somatomedin C is identical to IGF-I (Hintz *et al* 1980, Klapper 1983) and somatomedin A to deaminated IGF-I. The term somatomedin B is now considered a misnomer as it describes an antiprotease that lacks several important characteristics common to the other somatomedins.

The second polypeptide present in the NSILA of serum was designated IGF-II and found to be closely related to IGF-I both chemically and biologically (Rinderknecht and Humbel 1987). IGF-II is several times more potent as a stimulator of glucose oxidation by isolated fat cells but less active than IGF-I in promoting sulphate uptake by cartilage explants (Zapf *et al* 1978b).

Both growth factors circulate in blood tightly bound to a specific carrier protein, insulin-like growth factor binding protein (IGFBP), with total concentrations of IGF-II exceeding IGF-I by 4 fold in normal individuals (Zapf *et al* 1978b). Neither insulin nor proinsulin cross-react with the IGFs on the IGFBP.

There are only slight interspecies variation in the amino acid sequences between human, bovine, rat, swine and murine insulin like growth factors (Klapper 1983, Rinderknecht and Humbel 1987). It has been suggested that this reflects the importance of this growth factor as evolutionary changes in its structure have not occurred.

All the animal IGF-I's so far studied have 70 amino acids, but can vary by up to 4 amino acids substitutions. Bovine (Honnegar and Humbel 1986) and porcine (Tavakkol *et al* 1988) IGF-Is have the same amino acid sequence whereas rat has 3 amino acid differences (Rubin

et al 1982) and mouse the same 3 differences plus an additional amino acid substitution. IGF-II has a greater variation between species with rat having 4 substitutions (Dull *et al* 1984) and bovine and mouse several more (Honnegar and Humbel 1986, Steripen 1986).

Site and regulation of gene expression

The IGF-I and -II genes from the human and the rat have now been characterised. There are some similarities between the rat and the human gene (de Pagter-Holthuizen *et al* 1989) suggesting a common ancestral gene.

The gene for IGF-I is on the long arm of chromosome 12 (Hoppener 1985) and the gene for IGF-II is located on the short arm of chromosome 11 (Bell *et al* 1985). The genes for insulin and tyrosine hydroxylase are both found very close to the IGF-II gene although they are each expressed in different tissues and at different times of development. Indeed more recent work suggests that the genes for insulin and IGF-II are continuous with a maximal distance between them of 1.4 kilobase pairs (kb) (Dull *et al* 1984, Bell *et al* 1985, de Pagter-Holthuizen *et al* 1989). Both the IGF-I and -II genes produce several messenger RNA (mRNA) series. The IGF-II gene in the rat is predominantly expressed in fetal tissue and is therefore assumed to be important for fetal growth.

Storage.

Unlike most endocrine peptide hormones, IGF-I and -II are not synthesised in a single organ and are not stored in secretory granules before release (Clemmons and Van Wyk 1981). Tissue concentrations, therefore, tend to be lower than serum.

Site of production.

Messenger RNA for IGF-I and -II is found in most tissues studied in the rat, the mouse and the human (Murphy *et al* 1987, Hynes *et al* 1987). At some time in pre or post natal development most tissues express the codes for both growth factors. The cell lines in which

they are found are mainly of fibroblast or prechondrocyte origin.

IGF-I:

IGF-I displays marked GH dependence and is produced in large amounts by the liver (Takano *et al* 1977, Murphy *et al* 1987). IGF-I is also produced in a wide variety of other tissues where it may be active in a paracrine rather than an endocrine manner. The other organs containing the highest concentrations of IGF-I are the kidney, lung and testis.

Hypophysectomy leads to very low concentrations of IGF-I in the liver, but has a less marked effect in other tissues. If GH is given to hypophysectomised rats, tissue extractable IGF-I increases before serum IGF-I. This implies that autocrine and paracrine functions are of great importance.

The lack of a reservoir organ for this growth factor is supported by the demonstration of a lag period after GH administration, before there is a detectable increase in serum concentrations (Copeland *et al* 1980). As almost all of the IGF-I is, however, bound to IGFBPs the binding protein acts as a form of reservoir.

Granulosa cells:
Oliver *et al* (1989) demonstrated that the rat granulosa cell was a site of expression of IGF-I mRNA. The finding by Hammond (1981) of higher concentrations of immunoreactive IGF-I in porcine follicular fluid compared with serum suggested that the peptide may be produced in the pig ovary.

Davoren and Hsueh (1986) demonstrated that GH administration to immature rats lead to an increase in IGF-I concentrations in the ovary. Further studies support the hypothesis that, at least in animal models, granulosa cells secrete IGF-I and biosynthesis has been demonstrated to be GH, FSH and E_2 dependent ((Hammond *et al* 1985, Hsu and Hammond 1987a, Hsu and Hammond 1987b, Murphy *et al* 1987, Mondschein and Hammond 1988, Hernandez *et al* 1989).

Concentrations of IGF-I in human FFL are, however, less than in serum (Geisthoevel *et al* 1991). Adashi *et al* (1991a) demonstrated that the concentrations of IGF-I in follicular fluid were 30-80% those of serum and other workers suggested that human granulosa cells may be a site of IGF-II rather than IGF-I expression (Voutilainen and Miller 1987). In luteinised granulosa cells, obtained from women receiving human menopausal gonadotrophin (hMG) prior to IVF-ET, neither IGF-I nor its mRNA was found (Geisthoevel *et al* 1989).

Theca cells:
Studies in rats were unable to demonstrate IGF-I gene expression in thecal cells but IGF-I receptors were present on these cells (Hernandez *et al* 1989, Oliver *et al* 1989, Poretsky *et al* 1985, Cara and Rosenfield 1988). Possibly, in rats, IGF-I derived from granulosa cells acts in a paracrine manner on theca cells.

IGF-II:

Concentrations of IGF-II are high in the brain particularly the anterior pituitary. The tissue distribution otherwise is less well known. Gonadotrophin stimulation of human granulosa cells in-vitro induced expression of IGF-II mRNA (Ramasharma and Li 1987, Miller 1988). Production of IGF-II in human granulosa cell cultures has been demonstrated (Ramasharma and Li 1987), as have significant amounts in human follicular fluid (Ramasharma *et al* 1986).

Factors regulating production

IGF-I.

GH:

The predominant regulator for IGF-I is GH (Van Wyk *et al* 1974). GH enhances IGF-I transcription and increases the abundance of IGF-I mRNA in most rodent tissue (Mathews *et al* 1986). It is during the first years of life that GH receptors develop and growth begins to be GH dependent. IGF-I concentrations increase in parallel with the increase in GH.

Similarly, as GH concentrations decrease with age so do IGF-I concentrations.

After a single injection of GH there is a rapid (3-9 hrs) increase in IGF-I mRNA in GH deficient and normal animals, primary cell cultures and in cell lines (Mathews *et al* 1986, Doglio *et al* 1987, Hynes *et al* 1987, Norstedt and Moller 1987, Murphy *et al* 1987). The degree of increase of the mRNA depends on the tissue and the subclass of mRNA.

Although GH itself does not have a direct action on isolated cartilage cells, its action being mediated through the IGFs (Salmon and Daughaday 1957), other cells do respond directly to GH. This response may be mediated by stimulation of IGF-I mRNA; for example, if GH is given to hypophysectomised rats there is an increase in growth and in liver and kidney concentrations of IGF-I, without a change in serum IGF-I.

In a healthy male volunteer, a single intramuscular (IM) dose of GH (20 U) led to a dramatic increase in serum IGF-I concentration (Copeland *et al* 1980)(Fig *I*-3). Despite GH concentrations having increased 100 fold at 6 hrs post injection, no detectable rise in IGF-I was seen before 18 hrs, with the peak value occurring at 36 hrs. This suggests that GH stimulates de novo synthesis rather than release from storage.

In 3 out of 4 hypopituitary children given 0.1 U GH/kg IM, a rise in IGF-I was evident by 8 hours. The maximal response occurred between 16-28 hrs. Long term GH treatment of hypopituitary children was associated with a wide range of responses to the daily GH injections (Copeland *et al* 1980).

Several authors have suggested that the serum IGF-I concentrations do not necessarily reflect the growth promoting effects of GH because of stimulation of local tissue production of IGF-I which does not contribute to the serum levels (Clemmons *et al* 1981, Schlechter *et al* 1984, Jorgensen *et al* 1988).

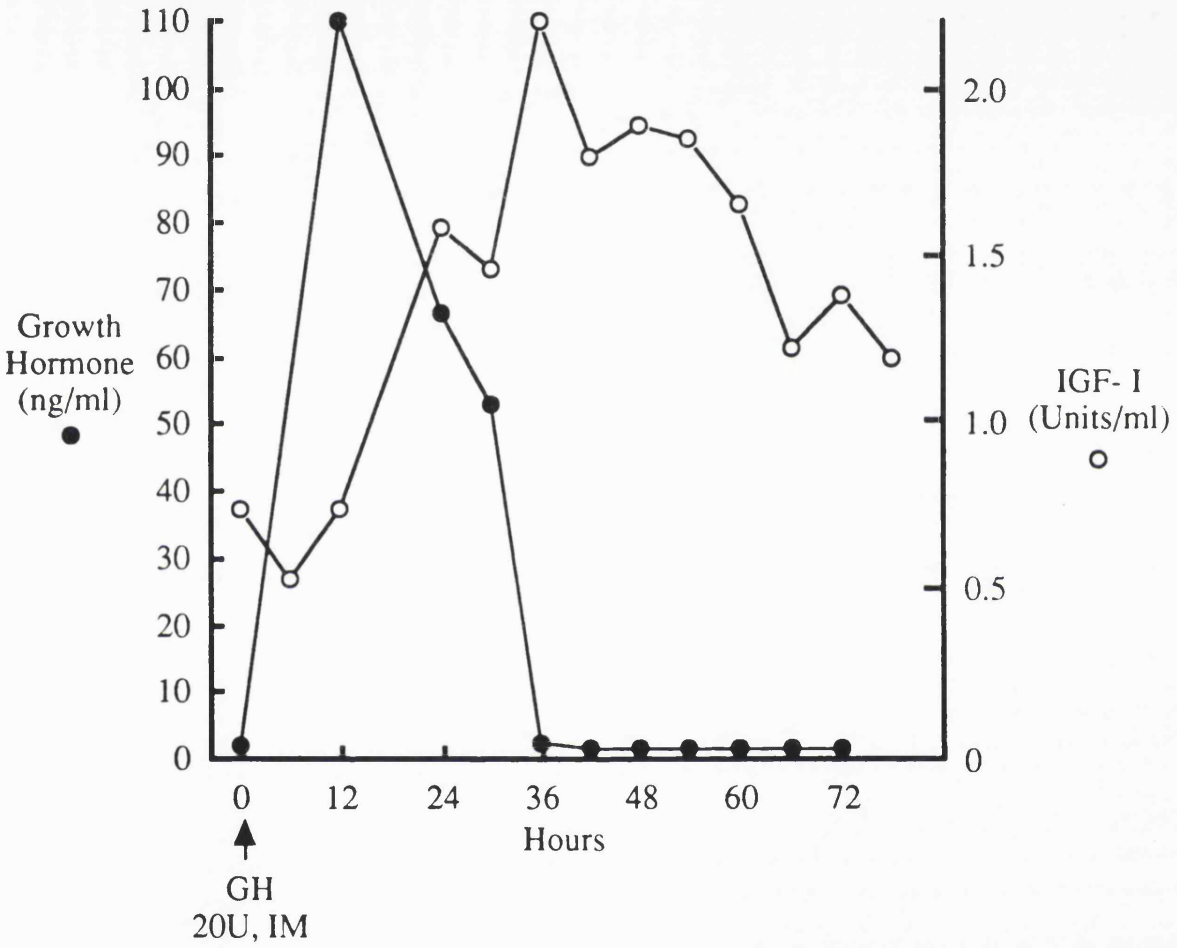


Fig *I*.3 Serum concentrations of GH (●) and IGF- I (○) in response to a single IM injection of 20U GH to a normal adult male.

Apart from GH, other factors can influence IGF-I gene expression:

Gonadotrophins:

In rat ovary, both adult and immature, gene expression may be gonadotrophin dependent (Hernandez *et al* 1987).

Oestrogens:

At puberty there is a 2 to 3 fold rise in IGF-I which correlates with the Tanner staging and sex hormone concentrations (Rosenfield *et al* 1983, Rosenfield and Furlanetto 1985). Raised concentrations of IGF-I are also found in children with precocious puberty, which can be reversed by the administration of GnRH analogues (GnRHa) (Harris *et al* 1985). In girls with ovarian dysgenesis there is no increase in serum IGF-I concentrations at the expected age of puberty. If the girls are treated with low doses of E_2, however, their concentrations of serum IGF-I rise (Cuttler *et al* 1985).

Conversely, large doses of oestrogens can inhibit growth despite normal or increased GH secretion (Frantz and Rabkin 1965a, Spellacy *et al* 1967, Whitelaw 1967, Berntsen 1968) and both epiphyseal closure and interference with GH action are involved (Schwartz *et al* 1969a, Strickland and Sprinz 1973, Daughaday *et al* 1975). Chronic E_2 administration leads to a decrease in serum IGF-I concentrations and a decrease in the magnitude of GH stimulation of hepatic IGF-I mRNA (Murphy and Friesen 1988).

If E_2 is given to prepubertal female Rhesus monkeys there is an increase in the IGF-I concentration, not associated with any change in the basal GH concentration (Wilson 1986). There was, however, a probable effect on the total GH secretion. In the rat the prepubertal increase in IGF-I concentration in serum is thought to be independent of sex hormones as the levels are unaffected by castration (Handelsman *et al* 1987).

Girls with excessive predicted height given pharmacological doses of E_2 have a lowering of

their IGF-I concentrations (Whitelaw 1967, Hall and Tally 1989) as do acromegalics, even though levels of GH remain high (Schwartz *et al* 1969b). Many studies demonstrate that the production, but not the action, of the growth factors is altered by oestrogens. (Weiderman and Schwartz 1972, "Iden" 1974, Phillips *et al* 1975, Weiderman *et al* 1976). If large doses of E_2 are given to post menopausal women decreases in their serum concentrations of IGF-I result (Duursma *et al* 1984).

In summary, during puberty there appears to be increased IGF-I production in response to oestrogens. After puberty high concentrations of oestrogens suppress the amount of circulating IGF-I.

Androgens:

Androgens may also have an effect on IGF-I concentrations as there is some evidence that giving testosterone to hypogonadal males can lead to an increase in IGF-I concentrations. This is probably not a direct effect, however, rather that the androgens are acting via GH.

Puberty is associated with an increase in testosterone production and also a rise in IGF-I concentrations. The increase in serum IGF-I is most likely, however, to be due to increased GH production at this time and not the rise in androgens (Utiger 1984, Jasper 1985, Zadik *et al* 1985). Androgens induce a rise in GH secretion (Hall and Tally 1989) which, at puberty results in a secondary increase in IGF-I. Androgens given to hypophysectomised rats potentiate the growth promoting action of GH (Simpson *et al* 1944, Salmon *et al* 1963) but androgens have little direct effect on cartilage in vitro and do not appear to potentiate IGF-I action (Salmon *et al* 1963, Phillips *et al* 1975).

Glucocorticoids:

Glucocorticoids are recognised to impair growth. This may be due to direct inhibitory effect on cartilage (Daughaday and Mariz 1962, Barrett *et al* 1966), some decrease in release of GH (Peale and Miller 1966, Stempfel *et al* 1968) or blunting of growth stimulation by GH

(Morris *et al* 1968, Sturge *et al* 1970). IGF production may also be affected. Children given steroids (prednisone or prednisolone) have a 50-70 % fall in somatomedin activity after 4 to 6 hours with a gradual return over the next 18 hours (Green *et al* 1978).

Thyroid hormones:

Thyroid hormones have been shown to interact with IGF-I in multiple target tissues to promote growth (Saenger *et al* 1974a, Froesch *et al* 1976). Data in clinical hypothyroidism is, however, less clear. Reduced concentrations of IGF-I in patients with hypothyroidism have been reported, with restoration to normal after thyroxine (T4) replacement (Van den Brande and DuCajin 1974, Burstein *et al* 1978).

GH related peptides:

Human prolactin has a similar structure to GH and competes weakly for GH receptors. Extreme hyperprolactinaemia has been associated with raised IGF concentrations (Guyda *et al* 1977).

Nutrition/systemic illness:

Although IGF-I is primarily GH dependent, insulin and nutrition also play a part in the regulation. IGF-I concentrations are low in patients with malnutrition and in chronic disease states.

Hepatic production of IGF-I needs both adequate proteins and calories. In patients with anorexia, IGF-I concentrations decrease despite increases in their GH concentrations (Emler and Schalch 1987).

Children with protein-calorie malnutrition (Kwashiorkor) have poor growth and low somatomedin activity despite normal or elevated GH (Grant *et al* 1973, Hintz *et al* 1978). Possibly, the low insulin and poor nutrition may override the effect of GH and result in low

IGF-I activity.

In patients with chronic disease states IGF-I concentrations are also low (Schwalbe *et al* 1977). This may be multifactorial as these patients are often malnourished, and may be taking drugs which affect IGF-I or GH, for example glucocorticoids. Children with uraemia and renal insufficiency have poor growth despite the presence of insulin and GH. Low IGF-I concentrations have been demonstrated (Saenger *et al* 1974b, Lewy and Van Wyk 1978).

Insulin also has some role in IGF-I activity. Streptozocin induced diabetic rats have a marked fall in IGF-I following the development of hyperglycaemia, which is not prevented by GH (Phillips and Ovranski 1977). Insulin treatment, however, does return IGF-I activity to normal.

IGF-II.

The highest concentrations of IGF-II occur during fetal development, declining during post natal life. Although levels remain high in the brain (Murphy 1987), the main site of production of IGF-II post natally is the liver, with high concentrations of mRNA found in tissue studies (Hall and Tally 1989).

IGF-II concentrations in normal children are independent of age and pubertal stage beyond the first year of life (Zapf and Froesch 1981). Concentrations of this growth factor do not change significantly during puberty, pregnancy or acromegaly (Zapf and Froesch 1981, Clemmons and Van Wyk 1981) and there is no decrease with age. In patients with complete GH deficiency, however, there is some lowering of the concentrations (Zapf and Froesch 1981) but this may be due to a decrease in the 150 kDa IGFBP complex (Hall and Tally 1989).

IGF-II production appears to be already maximally stimulated at normal GH levels. In contrast to IGF-I, therefore, its GH dependence becomes apparent only when GH concentrations fall below normal (Zapf and Froesch 1981).

Certain malignant tissues have been shown to have high concentrations of IGF-II including Wilms tumours, neuroblastomas, hepatoblastomas and leiomyomas and sarcomas (Scott *et al* 1985).

Insulin, itself a growth factor, may play an important part in the regulation of IGF-II expression. The genes for insulin and IGF-II are in close proximity on chromosome 11. It has been suggested that glucose may be involved in the regulation of both of these genes. Children with poorly controlled diabetes mellitus have low IGF-II concentrations despite high GH secretion. Insulin may induce GH receptors in the liver and may also increase IGF-II (Hall and Tally 1989).

In human granulosa cells IGF-II is thought to be regulated by FSH, hCG and cAMP (Voutilainen and Miller 1987, Ramasharma and Li 1987). Any GH control of IGF-II production by granulosa cells is less clear (Hynes *et al* 1987, Ramasharma and Li 1987).

Binding proteins.

In contrast to other polypeptide hormones, IGFs circulate bound to specific high affinity binding proteins, and it is thought that less than 1% of the growth factors are circulating free Hintz and Liu 1977, Hall and Tally 1989). There are two widely recognised groups of BPs, the large IGF binding complex involving IGFBP-3 and the smaller binding proteins IGFBP-1 and -2, -4,-5 and -6 (Ooi and Herrington 1988, Hintz 1991).

150kDa IGFBP complex.

The majority (75-90%) of the IGFs circulate bound as a large 150 kDa BP complex (Copeland *et al* 1980) which may act as a large circulating store, ensuring a constant source of growth factors for target tissues. The BP in this complex is BP-3 which exists in two different glycosylated forms with molecular weights of approximately 40.5 and 36.5 kDa.

The complex is largely retained in the circulation and concentrations are low at birth, increase in childhood and decrease in old age. In the adult the large IGFBP complex concentrations tend not to alter acutely, responding slowly to GH (Baxter and Martin 1986). Low concentrations are found in GH deficiency and high in acromegaly (Copeland *et al* 1980). The major production site of this BP is the liver (Ooi and Herrington 1988).

Smaller binding proteins.

Those smaller BPs which have been well characterised have molecular weights varying between 33 kDa and 24 kDa. They carry a small proportion of the growth factors in the circulation and are also present in many of the tissues where IGFs are active.

IGFBP-1 is identical to placental protein 12 (PP12) and has a molecular weight of 29 kda (Koistinin *et al* 1986). It is produced in the liver, decidua and secretory endometrium. The production of this BP is not related to GH secretion but insulin may be important in its regulation (Holly *et al* 1988, Suikkari *et al* 1988).

The majority of IGF-I within the follicle is bound to the IGFBPs and human granulosa cells have been shown to produce binding proteins (Suikkari *et al* 1989a, Koistinen *et al* 1990).

Functions:

Although the exact role of the binding proteins are uncertain several functions have been suggested. The BPs probably provide a reservoir to counteract fluctuations in production and may prolong the half life by decreasing clearance from circulation. The plasma half life of free IGF-I and -II in rats is 15 mins and 7 mins respectively, whereas the half life of the total (bound plus free) ligands was 4 hours in intact animals. (Cohen and Nissley 1976, Zapf 1986).

Holly *et al* have suggested that binding proteins reduce the rate of tissue delivery or decrease the biological activity of the IGFs (Holly *et al* 1989a). Other workers have demonstrated that

IGFBPs inhibit the mitogenic effects of the IGFs, possibly by competing with cell-surface receptors for IGFs (Rutanen *et al* 1988). Conversely Elgin *et al* have reported that IGFBPs potentiate the actions of IGF-I (1987). Thus the binding proteins appear to modulate the local actions of the IGFs but the effect may be dependent upon the relative expression in particular types of cells.

Measurements of IGF-I and -II.

Until the mid 1970s the measurement of IGF-I was exclusively by bioassay. One such assay was uptake of radiolabelled sulphate into the cartilage of hypophysectomised rats. Inherent variability in this methodology led to imprecise estimates of potency. The availability of purified IGFs now enables measurement by radioreceptor assay, competitive protein-binding assay and RIA.

Acidification.

The association of the IGFs with high affinity binding proteins make the interpretation of RIAs and immunoradiometric assays (IRMA) more difficult (Zapf *et al* 1975). This is particularly significant for the large binding protein as it is known to be GH dependent. Initially long incubation with antiserum was employed (Furlanetto *et al* 1977) but later acid ethanol extraction was found to remove most of the binding protein (Rinderknecht and Humbel 1987). A system whereby the serum is acidified to pH 3.5 with acetic acid and then passed through a gel filtration column is now commonly used (Zapf and Froesch 1981). The binding proteins are dissociated from the peptides and elute out at different points. RIA is then performed on each fraction.

During preparation for measurement by RIA, it is essential that acidification is performed to dissociate the IGF from the BP and particularly to decompose the 150 kDa BP complex (Furlanetto 1980).

Antisera.

IGF-I:

Over the last decade production of monoclonal and polyclonal antibodies has allowed development of the radioimmunoassays for IGF-I. The assays are now relatively specific, precise and simple. Initially a highly specific, high titre polyclonal antibody for IGF-I was raised in rabbits (Furlanetto *et al* 1977). The technique was further refined by separating IGF-I from its binding proteins by acidification and gel exclusion chromatography (Zapf and Froesch 1981). This assay did have, however, a considerable cross reactivity with IGF-II and wide species cross reactivity.

Monoclonal antibodies have now been produced (Baxter *et al* 1982, Scott *et al* 1987) and more recently an antibody to the amino acid sequences which differ between IGF-I and -II has been developed (Hintz *et al* 1980).

IGF-II:

An antiserum against the synthetic IGF-II was initially developed (Zapf, Walter and Froesch 1981) and later a polyclonal antiserum (Ramasharma *et al* 1986). A specific human IGF-II antibody using the synthetic peptide has now been produced (Hintz and Liu 1982).

Most of the assays use the double antibody, competitive binding technique but more recently an IRMA has been developed (Scott *et al* 1987).

Receptors.

Many, but not all, tissues possess separate receptors for insulin and the IGFs. Human granulosa and theca cells have been shown to have all three types of receptors (Poretsky *et al* 1985, Reanlez and Nissley 1985). The responsiveness of a given cell to insulin or IGFs is a function of the types of receptors present which may vary markedly among different

tissue types.

There are two types of receptors for the insulin-like growth factors, both membrane bound. They have different structures and peptide specificity. Hybrid or variant forms of the insulin and IGF receptors have recently been reported (Jacobs and Moxham 1991).

Type I.

The growth promoting actions of IGF-I are mediated through specific IGF receptors whereas the insulin-like actions are mediated through their cross reactivity with the insulin receptor (Clemmons and Van Wyk 1981). Many of the effects of IGF-II are also mediated through the type I receptor (Adashi *et al* 1989).

The type I receptor binds IGF-I and IGF-II with similar affinities and bears a close similarity to the insulin receptor (Steel-Perkins *et al* 1988). Insulin also cross reacts with the type I receptor (Sibley *et al* 1988).

Type II.

This receptor binds IGF-II with a 100 to 1000 fold higher affinity than IGF-I and only weakly recognises insulin (Rosenfeld *et al* 1987). The receptor has been shown to be identical to the mannose-6-phosphate receptor (MacDonald *et al* 1988, Tong *et al* 1988). It is unknown what biological effects are mediated through these receptors.

Insulin receptor.

IGF-I competes with insulin for insulin receptors in adipose tissue and liver which explains the insulin-like effect of IGFs (Hintz 1972). IGF-II binds with relatively high affinity to the insulin receptor. Insulin receptors have recently been demonstrated in the human ovary (Hernandez *et al* 1992).

Reception.

Ovary.

The ovary has been shown to possess receptors to IGFs which show the rank competition order characteristic of the type I IGF receptor (IGF-I greater than IGF-II which is greater than insulin).(Adashi *et al* 1985a, Adashi *et al* 1986a, Davoren *et al* 1986, Adashi *et al* 1988a, Adashi *et al* 1988b).

Using cultured rat granulosa cells it was demonstrated that IGF-I binding sites were enhanced by pretreatment with FSH (Adashi *et al* 1986a, 1988a). Recently it has been shown that in-vivo treatment with FSH produced a 2.5 to 3 fold increase in (^{125}I) iodo-IGF-I binding (Adashi *et al* 1988b). This effect appears to be due to an increase in binding capacity rather than apparent binding affinity.

In rat granulosa cell studies FSH induced augmentation of IGF-I binding was enhanced 1.5 fold by concomitant treatment with ovine GH. Ovine GH alone was without any effect (Adashi *et al* 1988b).

Specific IGF-I binding sites have also been demonstrated on theca interstitial cells from immature rat (Cara and Rosenfield 1988, Hernandez *et al* 1988).In rats, insulin has been shown to increase the number of IGF-I receptors while the insulin receptor number was reduced (Poretsky *et al* 1988). The porcine type I receptor appears to be similar to the rat receptor (Baranao and Hammond 1984, Veldhuis and Furlanetto 1985). IGF-I binding was enhanced by E_2 treatment due to increased maximal binding capacity.

In human granulosa cells, specific binding of IGF-I has been difficult to demonstrate (Poretsky *et al* 1985). More recently however the presence of IGF-I binding sites in thecal and granulosa cells were reported using immunostaining techniques (Balboni *et al* 1987). A minority of granulosa cells were positive to the staining although many thecal cells appeared to contain receptors.

Actions.

In general the growth factors are involved in initiation and maintenance of tissue growth. In vitro cell studies also show that these growth factors have metabolic actions similar to insulin.

Growth.

IGF-I and -II genes are expressed in many tissues in the fetus but relatively low serum concentrations suggest predominantly autocrine or paracrine actions for the growth factors. It is of interest to note that growth of the fetus is not GH dependent. Soon after birth, however, there is an increase in hepatic GH receptors and an increase in serum IGF-I.

The relative importance of endocrine, paracrine and autocrine function depends on the specific tissue. Autocrine action is probably important where there is high IGF-I gene expression and high IGF-I tissue concentrations, for example lung and kidney.

If IGF-I is administered to hypophysectomised rats they increase their weight (Schoenle *et al* 1982) which suggests the growth factor has an endocrine role. IGF-I does not, however, increase the 150 kDa BP complex (unlike GH) and therefore the half life of administered IGF-I is short and its action inferior to systemic GH.

IGF-II has one third of the affinity for the IGF-I receptor but may have some contribution towards growth. IGFs stimulate amino acid uptake and protein synthesis, thymidine incorporation into DNA and proliferation of cells (Hintz 1972).

Studies in man have shown that high dosages of partially purified NSILA produced prolonged lowering of the blood sugar and stimulation of ^{14}C glucose incorporation into glycogen by muscle. These were however none physiological doses and it is difficult to extrapolate to normal physiology.

Ovary.

Both in vivo and in vitro studies confirm that the ovary is one of the sites of action of IGF-I. There are many interspecies differences which make interpretation and application of the data more difficult; however, IGF-I undoubtably influences a wide variety of granulosa and thecal cell functions.

Theca cells:

The most important role of the theca cells towards folliculogenesis is the provision of androgens as substrate for aromatization and thus oestradiol production. Selective amplification of steroidogenesis may, in part, contribute to the development of the dominant follicle. The paracrine action of the growth factors could play a part in this process.

In cultured rat thecal cells, hCG or LH induced androstenedione secretion was significantly amplified by addition of IGF-I in a dose dependent manner (Cara and Rosenfield 1988, Hernandez *et al* 1988). Insulin also induced a significant increase of LH dependent androstenedione secretion.

Granulosa cells:

Cell growth:

Granulosa cell replication is regulated by the synergistic actions of FSH and E_2. The role of growth factors in granulosa cell growth is uncertain. Mitogenic activity has been induced by IGF-I without Gn priming in bovine (Savion *et al* 1981, Li 1983) and porcine cell cultures (Baranao and Hammond 1984, Veldhuis and Furlanetto 1985). In contrast, Adashi did not demonstrate any effect of IGF-I on granulosa cell number or DNA synthesis in rat granulosa cell cultures (Adashi *et al* 1985b).

Cytodifferentiation:

- Oestradiol production.

FSH is regarded as the prime factor in stimulation of P450aro activity, and thus oestradiol production, from androgens. Work over the past few years has, however, suggested that growth factors may modulate the activity of P450aro.

Studies on rat granulosa cells have shown that IGF-I synergises with FSH to increase E_2 production (Adashi *et al* 1985b). IGF-I has been demonstrated to be the more potent stimulator of P450aro when comparing IGF-I, -II and insulin (Davoren *et al* 1986).

In granulosa cell cultures from intact stilboestrol-treated rats addition of IGF-I to pregnant mares serum gonadotrophin resulted in augmentation of both aromatase activity and progesterone synthesis (Hutchinson *et al* 1988). IGF-I alone did not influence oestradiol production. Continuous FSH was not required for IGF-I stimulation of P450aro activity; initial FSH stimulation of IGF-I receptors is necessary but oestradiol and progesterone production can continue in the absence of FSH (Steinkampf *et al* 1988, Erickson *et al* 1989). Physiologically this suggests that IGF-I may play an important role in steroidogenesis at a time in the cycle when circulating FSH concentrations are diminished.

In human granulosa cells, however, IGF-I alone has been shown to stimulate P450aro activity to an equal or greater extent than FSH (Erickson *et al* 1989). IGF-I and FSH probably work synergistically to control P450aro activity.

- Inhibin secretion.

Inhibin secretion by cultured rat granulosa cells has recently been shown to be under the control of FSH and IGF-I in a dose and time dependent fashion (Bicsak *et al* 1986, Zhiwen *et al* 1987). This may also suggest a role for IGF in the selection of the dominant follicle.

- Progesterone production.

Basal progesterone secretion was enhanced by IGF-I in porcine granulosa cells (Veldhuis *et*

al 1986a). Studies in rat granulosa cells did not however, demonstrate increased basal progesterone biosynthesis but did show an increase in production with cotreatment with FSH (Adashi *et al* 1985b).

The enzymes important in the production of progesterone precursors are 3ß-hydroxysteroid dehydrogenase and P450scc. In both murine and porcine cells it has been demonstrated that the activity of these enzymes was activated by FSH and IGF-I, alone and synergistically (Davoren *et al* 1985a, Veldhuis *et al* 1986a).

Oestrogen has also been demonstrated to synergise with IGF-I in facilitating progesterone production by swine granulosa cells (Veldhuis *et al* 1982, 1984). Although IGF-I alone augmented progesterone production the addition of oestradiol amplified the granulosa cell stimulation.

- LH receptor binding.

FSH is important in the acquisition of LH receptors in the granulosa cells. Addition of IGF-I to rat granulosa cells in culture demonstrated increased binding capacity of the FSH-induced LH receptors (Adashi *et al* 1985c, Davoren *et al* 1986). As IGF-I also facilitates LH stimulation of progesterone synthesis, a role for this growth factor in luteinization has been suggested (Hutchinson *et al* 1988).

- Proteoglycan synthesis.

The viscosity of follicular fluid is primarily dependent upon the intrafollicular concentrations of proteoglycans and lipids. The production of glycosaminglycans (an inherent part of proteoglycans) is stimulated by FSH. Glycosaminglycans may play an indirect role in follicular selection and atresia and oocyte maturation by regulating the expansion of the cumulus oophorus. Co-incubation with IGF-I results in a doubling of proteoglycans synthesis in rat granulosa cells (Adashi *et al* 1986a).

- Adenylate cyclase.

Studies suggest that IGF-I synergises with FSH in stimulating adenylate cyclase activity

(Adashi *et al* 1988b) and extracellular cAMP accumulation (Adashi *et al* 1986c) in rat granulosa cells. IGF-I in the absence of FSH, however, failed to have any stimulative effects on the cAMP system.

Summary.

Thus, in summary, IGF-I has been shown to modulate many of the functions of ovarian cells important in folliculogenesis. Growth of the dominant follicle and augmentation of steroidogenesis may reflect an important endocrine, paracrine or autocrine role for this peptide.

The regulation of IGF-I production is principally under the control of GH. To investigate the role of IGF-I in a clinical setting, administration of GH was considered the most predictable method of ensuring a rise in serum concentrations.

Growth hormone

Physiology.

GH is a 191-amino acid single chain polypeptide with 2 intrachain disulphide bonds (Davidson 1987). GH secretion is controlled by two hypothalamic peptides: somatostatin (tonic inhibition) and GH releasing factor (episodic stimulation) (Tannenbaum and Ling 1984, Plotsky and Vale 1985). Somatostatin and GH releasing factor (GRF) bind to different receptors in the pituitary cells and thus effect secretion in a non-competitive fashion.

GH is released episodically from the somatotroph cells of the anterior pituitary during sleep, particularly during the first few hours of sleep. During waking hours the secretion is random. GH has a half life in serum of 20 minutes (Parker *et al* 1962, Hunter and Greenwood 1964, Frantz and Rabkin 1965a). There are rapid and unpredictable variations in concentrations throughout the day (Plotnick *et al* 1975).

Factors regulating production.

Exercise, physical and emotional stress, high protein intake and hypoglycaemia influence the release of GH (Roth *et al* 1963, Greenwood *et al* 1964, Hunter and Greenwood 1964, Knopf *et al* 1965, Rabinowitz *et al* 1966, Merimee *et al* 1969a). Secretion is enhanced by oestrogen, testosterone and thyroid hormones and is suppressed by high concentrations of glucocorticoids (Frantz and Rabkin 1964, 1965a,b, Martin *et al* 1966, Mintz *et al* 1967, Merimee *et al* 1969a).

Oestrogens.

GH secretion is influenced by a variety of factors including ovarian steroids. Basal GH concentrations do not vary between men and women, but concentrations of GH after activity are higher in women (Frantz and Rabkin 1965, Unger *et al* 1965). Oestrogens, in the form of diethyl stilboestrol, given to men for one week induced a similar pattern of high GH concentrations following activity, suggesting that oestrogens alter the sensitivity of the hypothalamus or pituitary. Higher GH concentrations are also seen in women taking the oral contraceptive pill (Garcia and Linfoot 1966, Spellacy *et al* 1967, 1969a,b).

Franz and Rabkin (1965a) demonstrated a rise in the ambulatory GH concentrations in women post ovulation and in the late luteal phase. Arginine-stimulated GH release has also been reported to be greater in women around the time of ovulation than during menstruation (Merimee *et al* 1969b). Other studies, however, suggest no difference in GH concentrations in follicular and luteal phases of the menstrual cycle (Spellacy *et al* 1969c, Zadik *et al* 1985).

Clomiphene citrate (CC), given to women at mid-cycle, blunted the arginine-induced rise in GH concentrations (Fiedler *et al* 1969). The concentrations of GH reached after CC were similar to those attained during menstruation and were significantly lower than during the midcycle peak without CC administration. Perlow *et al* (1973) described a reduction in both spontaneous sleeping and waking GH secretion in men following CC administration. They suggested that CC might affect GH secretion by a direct effect on pituitary GH release or as

a result of competitive inhibition with E_2 at hypothalamic receptor sites.

Progestogens.

Medroxyprogesterone acetate in pharmacological doses blunted GH secretion following arginine infusion or insulin-induced hypoglycaemia (Simon *et al* 1967), and during sleep (Lucke and Glick 1971).

Actions.

Although GH does not stimulate a particular endocrine target organ, many of the growth promoting peripheral actions of GH are mediated by somatomedins. IGFs may play a role in the negative feedback control of GH secretion either through stimulation of somatostatin release or by a direct inhibitory effect on GRF.

GH has two main actions: stimulation of general tissue growth and control of body metabolism. A putative role in puberty and ovarian function is discussed.

Growth.

The primary and most intensively studied action of GH is stimulation of growth of long bones. The effect of GH on skeletal growth is mediated through IGFs which act at the epiphyseal cartilage of the long bones, where they stimulate cell proliferation and incorporation of sulphur into chondroitin sulphate.

The effect of GH on the growth and maturation of bone is well recognised and is clinically useful for the treatment of short stature (Preece 1982). Numerous other uses are being proposed.

Although the growth promoting actions of GH are considered to be mediated by the IGFs there are data to suggest that GH might have a direct effect on cells and indeed high affinity binding sites have been demonstrated in various tissues (Hughes and Friesen 1985, Isakson

et al 1985). Recently Leung *et al* (1987) have identified the rabbit liver receptor as a 620 amino acid protein with a single, centrally located transmembrane domain and have cloned complementary DNA encoding GH receptors from both rabbit and human liver. The human and rabbit receptors are very similar, showing about 84% identity in amino-acid sequence. In the rabbit, the GH serum binding protein was established as being the extracellular, hormone binding domain of the membrane bound receptor.

Carbohydrate and fat metabolism.

In in-vitro studies GH has both insulin-like and antiinsulin-like effects but most clinical studies suggest the diabetogenic effect predominates (Lippe *et al* 1981, MacGorman *et al* 1981, Bratusch-Marrain *et al* 1982, Rosenfeld *et al* 1982, Davidson 1987, Ward *et al* 1987), even with the recombinant preparation (Rosenfeld *et al* 1982, Hart *et al* 1984, Koshyo *et al* 1985, Jorgensen *et al* 1987, 1988). Several studies, however, show no changes in basal or fasting glucose concentrations with GH administration (Fueberg and Merimee 1974, Metcalfe *et al* 1981, Rizza *et al* 1982, Sherwin *et al* 1983). Although insulin concentrations may be elevated (Lippe *et al* 1981, Metcalfe *et al* 1981, Bratusch-Marrain *et al* 1982, Rizza *et al* 1982, Rosenfeld *et al* 1982), GH is thought to diminish insulin action by altering hepatic glucose production and peripheral glucose uptake (Rosenfeld *et al* 1982, Bolinder *et al* 1986, Hansen *et al* 1986, Davidson 1987).

In both human and animal studies, GH has been shown to have some lipolytic effects with increased free fatty acid concentrations (Campbell *et al* 1978, Fueberg and Merimee 1974, Metcalfe *et al* 1981, Sherwin *et al* 1983, Jorgensen *et al* 1988). Untreated GH deficiency is associated with increased body fat stores, including increased subcutaneous adipose tissue.

Changes in nitrogen, calcium and creatine metabolism have also been reported. GH is considered to be important for maintenance of muscle bulk (Preece *et al* 1987). GH is an anabolic hormone which increases amino acid transport into cells and incorporation into proteins. The increased synthesis of proteins and growth are reflected by nitrogen retention which can be quantified by observing the decline in urinary nitrogen excretion and serum

urea nitrogen. Thus an effect of GH administration can be a decrease in the concentration of serum urea, evidently because of a diversion of amino acids into anabolic pathways.

No abnormalities in liver function have been noted with GH treatment (Manson *et al* 1986).

GH and puberty.

IGF-I may be concerned with the promotion of juvenile and early pubertal follicular development as it is during this time of low Gn that GH is rising. An association appears to exist between isolated GH deficiency and delayed puberty in both rodents and humans, a process reversed by systemic GH replacement therapy.

Both gonadotrophin secretion and changes in target tissue sensitivity are important factors involved in the onset of puberty. Growth, increasing body weight and the production of GH are intimately linked and are all associated with the onset of puberty. It is recognised that there is increased GH production during puberty but the exact role that this hormone plays in puberty is unclear.

Finkelstein *et al* (1972) found that the mean 24-hour secretion of GH in adolescent boys was higher than in prepubertal boys and adults. Zadik *et al* (1985) found higher GH concentrations in males and females at Tanner stage 5 compared with less mature individuals.

In rats, although gonadotrophin concentrations remain low before the first proestrus, there is a marked increase in ovarian steroid production. The only pituitary hormones which increase at this time are GH and PRL (Ojeda *et al* 1976, 1977, Eden *et al* 1978, Ogeda *et al* 1987). A study in weanling rats (Ramaley and Phares 1980), where suppression of circulating GH concentrations was induced by administration of pleroceroid growth factor (from tapeworm larvae), suggested that the maintenance of somatomedin-like activity, normal growth rates and body weights could not substitute for normal circulating GH levels. The authors suggested that some other action of GH may play a role in puberty onset, for example, a direct action on the ovary.

Suppression of endogenous GH secretion, using median eminence GH implants in rats, led to a delay in both the pubertal increase in ovarian steroidogenic responsiveness to gonadotrophins and the onset of puberty (Advis *et al* 1981). These authors demonstrated that GH deficient rats showed a delay in ovarian maturation and suggested that GH plays an important role in the process of female puberty by enhancing the steroidal response of the ovary to gonadotrophins.

It has also been noted that puberty is delayed and prolonged in children with Laron type dwarfism, that is resistance to the action of GH (Laron *et al* 1980). Puberty and gonadal maturation could be induced by the administration of GH to hypophysectomised animals (Sheikholislam and Stempfel 1972)

Ovarian steroids augment GH concentrations probably by enhancing the amplitude and frequency of GH pulses from the pituitary (Luk *et al* 1986, Moll *et al* 1986, Mauras *et al* 1987). This in turn leads to a rise in IGF-I concentrations. In children with precocious puberty, IGF-I concentrations increase to those levels usually seen during physiological puberty, but only in those children with normal GH concentrations (Cara *et al* 1989). Suppression of ovarian activity with GnRHa, in children with precocious puberty, results in decreased GH secretion associated with decreased IGF-I concentrations (Harris *et al* 1985, Mansfield *et al* 1988).

GH and sex steroid production.

In certain conditions GH deficiency, leading to dwarfism and delayed sexual maturation, are associated (Brasel *et al* 1965, Goodman *et al* 1968). Both the short stature and the delayed sexual maturation are corrected by the administration of GH. Sheikholislam and Stempfel (1972) describe administration of GH to an 18 year old with delayed puberty; within 6 months she had experienced menarche. Oestrogen treatment alone had not resulted in development of secondary sexual characteristics and it was postulated that the GH treatment altered peripheral end-organ tissue response to the action of both E_2 and androgen.

GH administration to experimental animals with deficiencies of GH and thyroid stimulating hormone (TSH) resulted in increased spermatogenesis in males and ovarian development in females (Bartke 1964).

Cell studies:

Pretreatment with GH of hypophysectomised oestrogen treated rats led to increased progesterone production in-vitro in response to hCG administration (Advis *et al* 1981). Increased in vitro production of progesterone from testosterone, in response to FSH stimulation, was also enhanced by pretreatment with GH. No increase in ovarian aromatase activity in response to GH was seen.

Other workers, however, demonstrated that GH, in the presence of pregnant mares serum gonadotropin enhanced aromatase activity in intact stilboestrol treated immature female rats (Hutchinson *et al* 1988). An augmentation in progesterone production was also described. Some of these actions were considered to be direct, non IGF-I mediated effects as coincubation with monoclonal antibodies to IGF-I only partially blocked the stimulatory effects of GH (Hutchinson *et al* 1987). The main action of GH on the ovary was still suggested to be mediated through autocrine or paracrine actions of IGF-I.

Jia *et al* (1986) confirmed the above findings in cultured rat granulosa cell studies. These workers found that GH augmented the LH receptor content of FSH treated cells but GH alone did not induce LH receptors. These LH receptors were functional as demonstrated by increased cAMP production following incubation with LH. Increasing doses of GH added to FSH treated cell cultures resulted in a dose dependent increase in progesterone production. Concomitant GH treatment did not, however, increase FSH stimulated aromatase activity. The authors concluded that GH had a direct effect on rat ovarian granulosa cell function. In vivo, however, they suggest that GH may influence ovarian function in two ways. GH may interact with putative GH receptors in the granulosa cells to modulate FSH action in the same cells. GH may also stimulate ovarian production of somatomedins which, in turn, could augment granulosa cell differentiation.

Work by the same group described increases in IGF-I concentrations in the ovaries of rats treated with GH (Davoren and Hsueh 1986). Immature female hypophysectomised rats were sacrificed 0, 8 and 12 hours after injection with GH. Homogenised extracts of the ovaries showed higher concentrations of immunoreactive IGF-I at 8 and 12 hours than at 0 hours. They conclude that GH may be influencing ovarian function through local increases in intraovarian IGF-I, as well as by the classically described effect on hepatic IGF-I production.

Therapeutic uses.

Before 1985, studies of the effects of GH, particularly on adults, were scant because of the limited availability of extracted human growth hormone from cadaver pituitary glands. Studies using bovine GH showed that GH had unpredictable anabolic activity in humans. In May 1985 the pituitary GH was withdrawn because of its association with Jacob-Creutzfeldt disease (Beardsley 1985, Preece 1986).

Biosynthetic human GH (hGH) produced by recombinant DNA technology from a non-pathogenic strain of Escherichia coli (E.coli) became commercially available in 1985 (Goeddal *et al* 1979). The first preparations of biosynthetic GH had the amino acid, methionine, at the N terminal end but since then the native 191 amino acid sequence of the 22K fraction has become available.

The natural sequence biosynthetic human growth hormone used in the studies presented in this thesis (Norditropin, Novo Nordisk, Denmark) is initially synthesized in E. coli with a short negatively charged sequence of extra amino acids added to the N terminal. This extension was designed to facilitate purification by ion exchange techniques. Multiple purification steps result in production of biosynthetic hGH with the same structure and conformation as pituitary hGH. Anti-GH antibodies have not been noted following treatment with the biosynthetic GH (Jorgensen *et al* 1987) and antibody concentrations to E.coli were not increased in a clinical trial of recombinant hGH (Ranussen 1988).

Therapeutically, GH can be administered by the subcutaneous (SC) or intramuscular (IM) routes. Jorgensen *et al* (1987) demonstrated that absorption of the GH by the SC route was slower than IM and resulted in smaller areas under the curves and suggested this was due to greater local degradation.

Currently the main therapeutic action of hGH is on stimulation of longitudinal bone growth. IGF-I concentrations in serum are increased and cell proliferation particularly in the epiphyseal cartilage is stimulated.

Naturally secreted GH has a short plasma half life (approximately 20 minutes) but its effect on growth is more prolonged owing to the longer half life of IGF-I (up to 20 hours). When given to growth hormone deficient patients, the response of serum concentrations of IGF-I to Norditropin and pituitary derived hGH is similar (Jorgensen *et al* 1987).

Polycystic ovaries and 11ß-OH-A_4

The results of the studies using GH therapy in IVF-ET (Chapter *III* and *IV*) demonstrated that the women with ultrasound diagnosed PCO formed the group who responded to treatment. No definitive marker for PCO has yet been developed and we therefore investigated the use of the androgen, 11ß-OH-A_4, in serum and FFL to fulfil this role.

History of PCOS.

In 1935 Stein and Leventhal described a syndrome complex of obesity, hirsutism and menstrual irregularity. Characteristically enlarged ovaries were found in this group of women at laparotomy and the ovaries were often subjected to wedge resection. In many cases this resulted in improvement in symptoms and even pregnancies (Stein *et al* 1949, Goldzieher and Axelrod 1963).

Biochemical abnormalities.

With the development of steroid and gonadotrophin assays in the 1960's and early 1970's raised serum and urinary LH concentrations were noted in women with PCOS (Keettel *et al* 1957, Yen *et al* 1970, Givens *et al* 1976, Baird *et al* 1977, Goldzieher 1981, Conway *et al* 1990). Many women with PCOS were also found to have raised circulating androgens (Deutsch *et al* 1978, Hughesdon 1982, Lobo 1985). A wider spectrum of clinical presentations was recognised, including male pattern baldness, seborrhoea, acne and menstrual disturbances, for example, late menarche, secondary amenorrhoea and oligomenorrhoea.

Although it is now generally accepted that the condition has a very variable presentation certain abnormalities of the hypothalamic/pituitary/ovarian axis are often demonstrated. The classic abnormalities are raised concentrations of LH and androgens in serum with normal concentrations of FSH and T_4 (Goldzieher 1981). Prolactin concentrations may be marginally raised.

In practice both the LH and the testosterone concentrations are variable (Conway *et al* 1989, Saxton *et al* 1990). Conway *et al* (1989) demonstrated a wide spectrum of LH concentrations in women with ultrasound diagnosed PCO and many women had normal concentrations of the gonadotrophin. Women with oligomenorrhoea or amenorrhoea did, however, have higher concentrations of LH than those women with hirsutism in the presence of regular menstrual cycles. Total testosterone concentrations were raised in only half of the women but there was a trend to increasing concentrations depending upon the androgenicity of the symptoms. It is now generally accepted that neither LH nor androgen concentrations can be used as specific diagnostic markers of the condition.

Ultrasound diagnosis.

In the 1980's, development of high resolution scanning of the pelvis demonstrated the characteristic ultrasound appearance of polycystic ovaries (Sample *et al* 1977, Schwalbe *et*

al 1977, Swanson *et al* 1981, Pansi *et al* 1982, Adams *et al* 1985, Adams *et al* 1986). The ovaries were noted to be enlarged with multiple peripherally distributed follicles, between 2 and 10mm in diameter, with a thickened central stroma. This appearance corresponded with the histological features of thecal hyperplasia and an increased number of cystic atretic follicles (Goldzieher and Green 1962, Hughesdon 1982, Saxton *et al* 1990).

The disadvantage of using the ultrasound appearance of the ovaries as a basis for the diagnosis of PCO is the need for an ultrasonographer with experience in recognising the condition. Scepticism about the validity of the ultrasound diagnosis of PCO persists but the different follicular response to ovarian stimulation regimens of women with PCO is now being recognised (Shoham *et al* 1992).

Further work has suggested that the polycystic appearance of ovaries may be found in up to 22% of women considering themselves to be menstrually normal (Polson *et al* 1988a). Seventy six percent of the women with ultrasound diagnosed PCO did, however, have an irregular cycle (a cycle length greater than 35 days) or amenorrhoea. Only one of the women with an irregular cycle had normal ovaries on ultrasound and three quarters of the women with ultrasound diagnosed PCO had an irregular cycle with or with out hirsutism. Of the women with a regular cycle, 7% had ultrasound diagnosed PCO but the majority of these women were hirsute.

In another study it was demonstrated that out of 556 women with ultrasound diagnosed PCO, 25% had a normal cycle length (Conway *et al* 1989). Other workers have demonstrated that 80% of women with recurrent miscarriage have ultrasound diagnosed PCO (Sagle *et al* 1988), that up to 60% of women with unexplained infertility undergoing IVF-ET have the PCO appearance of their ovaries (Owen *et al* 1989) and that a "high proportion" of women with tubal damage as their indication for IVF-ET have PCO (Rutherford *et al* unpublished data). It is now recognised that the vast majority of women with hirsutism have PCO on ultrasound (Franks 1989).

Androgens are considered to be important in the aetiology of the morphological appearance

of the polycystic ovaries as the typical PCO structure is also seen in women with Cushings syndrome, CAH and androgen secreting tumours (Goldzieher 1981).

PCO and PCOS.

Pivotal to much of the work presented in this thesis is the appreciation of the difference between the classical PCOS and women with incidentally diagnosed ultrasound PCO. The women who entered my studies had regular menstrual cycles and were ovulatory. The presence of hirsutism was not assessed nor were the women grouped according to biochemical findings. Although the use of ultrasound to classify women as having normal or polycystic ovaries remains controversial, the results presented herein suggest that differing follicular and biochemical responses do occur, depending upon ovarian morphology alone.

Inheritance.

Although the aetiology is unknown, a genetic component to the condition of PCO has long been implicated (Cooper *et al* 1968, Givens *et al* 1971, Hutton and Clark 1984, Givens 1988, Hague *et al* 1988). There are several descriptions of familial grouping of PCO but, with no distinct marker for this heterogeneous condition, clear patterns of inheritance are difficult to establish (Judd *et al* 1973, Simpson 1972, Ferriman and Purdie 1979, Givens 1988). Simple autosomal dominance or X-linked association of the condition is now considered to be unlikely (Hague *et al* 1988). It is thus unclear whether this heterogeneous condition is due to a single gene defect with variable expression, to polygenic inheritance or to multiple genetic or non genetic factors (Simpson 1991).

An aberrant gene may encode an enzyme or group of enzymes with altered function contributing to the abnormal steroidogenesis demonstrated by many workers. Dysfunction of several enzymes important in androgen metabolism in the ovary have been proposed in PCOS including 21 hydroxylase (Mahesh and Greenblatt 1964), 11ß -hydroxylase acting on C21 hydroxysteroids (Finkelstein 1968, Dehennin *et al* 1987a), 11ß-hydroxylase acting on C21 deoxysteroids (Maschler *et al* 1975, Goldzieher *et al* 1978), 3ß-hydroxysteroid

dehydrogenase/delta^5-delta^4 isomerase (Axelrod *et al* 1965), 17ß hydroxysteroid dehydrogenase (Pang *et al* 1987), 11ß-hydroxylase acting on C19 steroids (Polson *et al* 1988b) and P450c17α (Rosenfield *et al* 1990, Ehrmann and Rosenfield 1990).

If dysfunctional enzymes were present in the polycystic ovary abnormal concentrations of a product or products of androgen metabolism could be used to differentiate women with this condition.

Androgen metabolism.

As previously described, metabolism of cholesterol occurs in stromal and theca cells with the resultant production of the androgens, A_4 and testosterone (Tsang *et al* 1979, Erikson *et al* 1985). During the early follicular phase approximately 30% of the circulating A_4 is derived from the ovary, the remainder is produced in the adrenal. During the mid to late follicular phase up to 60% of the circulating A_4 is derived from the ovary as steroidogenesis increases (Baird 1977). High concentrations of A_4 and DHEA are found in the FFL as the follicle develops (McNatty 1981, Dehennin *et al* 1987b).

Depending on the stage of development of the follicle, granulosa cells metabolise A_4 and androgens to form products with varying functions. For example testosterone is produced by the activity of 17 ketoreductase, 5 α-reduced androgens by 5 α-reductase, E_2 by P450aro and catechol oestrogens by oestrogen hydroxylase.

As no negative feedback mechanism involving gonadotrophins exists for controlling androgen secretion by the ovary, modification of enzyme activity may be important in intraovarian regulation. Changes in the concentrations or type of androgens produced may thus reflect altered ovarian function in PCO.

11ß-OH-A_4.

The 11ß-hydroxylase complex is a cytochrome P450 enzyme located in mitochondria and responsible for 11ß hydroxylation of C21 steroids, for example 11ß-hydroxylase activity is essential for the biosynthesis of glucocorticoid and mineralocorticoid steroids (Klein *et al* 1976). 11ß-hydroxylation of C19 steroids, for example, A_4, also occurs in the adrenal gland (Jeanloz *et al* 1952, Cohn and Mulrow 1963, Goldzieher *et al* 1978, Fiet *et al* 1980, Schlaghecke *et al* 1986) and the metabolite, 11ß-OH-A_4, is a major adrenal secretory product. 11ß-OH-A_4 is a weak androgen (Goldzieher *et al* 1978, Franckson *et al* 1983) which may be important in the regulation of active androgen production (Goldzieher and Beering 1969, Goldzieher and Axelrod 1971, Rao *et al* 1974, Franckson *et al* 1983, Longcope 1986) as its production results in diversion away from testosterone (Rao *et al* 1974, Goldzieher *et al* 1978, Guthrie *et al* 1982). While 11ß-OH-A_4 may arise by side chain cleavage of cortisol, this is considered to be a minor pathway of production (Deshpande *et al* 1970, Axelrod *et al* 1973, Hudson *et al* 1974) (Fig *I*-4).

As no 11ß hydroxylase activity on C19 steroids had been demonstrated in the ovary, 11ß-OH-A_4 was thus, until recently, regarded as exclusively adrenal in origin and serum concentrations of 11ß-OH-A_4 were regarded as an indicator of adrenal androgenic function (Goldzieher *et al* 1978, Polson *et al* 1988b).

In the 1970's Finkelstein and coworkers demonstrated 11ß hydroxylase activity in the ovaries of women with PCO but only metabolising C21 steroids (Finkelstein 1968, Finkelstein 1973, Maaschler 1975). The urinary metabolite of 21 deoxycortisol, pregnanetriolone, was suggested as a marker, not only of congenital adrenal hyperplasia (CAH), but also of PCO. Suppression of serum concentrations of this metabolite was demonstrated in women with PCO following cortisol administration and in women undergoing wedge resection (Shearman and Cox 1966). Other workers subsequently confirmed the activity of 11ßhydroxylase in the human ovary but also only on C21 steroids (Dehennin *et al* 1987a).

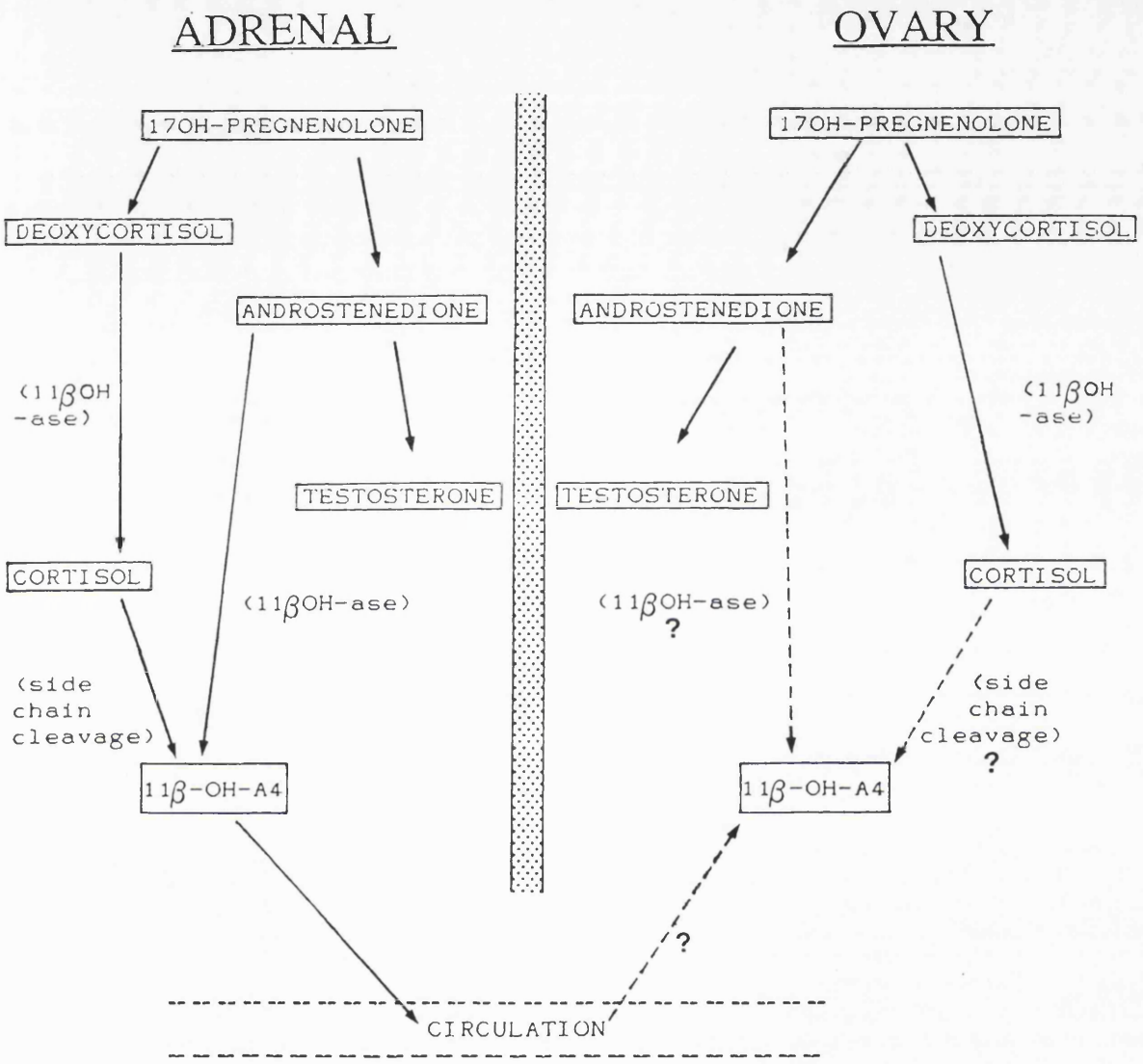


Figure *I*-4. Adrenal and ovarian biosynthesis of 11ß-OH-A_4

In 1985, Reed *et al*, using a commercially available assay to 11ß-OH-A_4, (Intersci diagnostics, USA) investigated 11ß-OH-A_4 concentrations as a indicator of excess androgen production in women with PCOS (Reed *et al* 1985, Polson *et al* 1988a). They did not detect any 11ß-OH-A_4 in the circulation of a woman who had an adrenalectomy nor in a woman with 11ß-hydroxylase deficiency. Concentrations of 11ß-OH-A_4 decreased after dexamethasone and increased after ACTH administration. They were unable to demonstrate any differences in the peripheral concentrations of 11ß-OH-A_4 between women with PCO and those with normal ovaries whether they were using the oral contraceptive pill or not, nor whether they responded to CC or not. They assumed that the 11ß-OH-A_4 concentrations were exclusively adrenal in origin and suggested that A_4 /11ß-OH-A_4 ratio could be used as a marker of abnormal androgen production in PCOS.

In 1987 Polson *et al* examined the 11ß-OH-A_4 concentrations in the serum of women with PCO and normal ovaries. There was no difference in 11ß-OH-A_4 concentrations between the peripheral and ovarian veins in a woman with normal ovaries but they reported high concentrations of 11ß-OH-A_4 in the ovarian veins of an adrenalectomised woman with PCO. They, therefore, suggested that this steroid may be being produced within the polycystic ovary (Polson *et al* 1987). High concentrations of both possible precursors, cortisol (F) and A_4, have been described in follicular fluid (Dehennin *et al* 1987a,b).

Summary.

11ß-hydroxylase activity on C19 steroids has not been demonstrated in the ovary and is considered an exclusively adrenal metabolite. High concentrations of 11ß-OH-A_4 have, however, been reported in the ovarian vein of a woman with PCOS. This finding suggested the possible expression of an aberrant gene in PCO and we wished to explore the opportunity of using 11ß-OH-A_4 concentrations as a marker for the condition.

II. Methods

In this chapter the methods used in the various studies are detailed. Initially the routine IVF-ET procedures, including some of the rationale behind them, are described. All additional procedures, the drugs used, the study design and the population studied are described for the GH trials.

History of IVF.

Methods for the in-vitro fertilisation of human oocytes were initially developed to assist infertile women who had had bilateral ectopic pregnancies and subsequent salpingectomies (Steptoe and Edwards 1970). These women had proven fertility but no longer had the ability to conceive. By obtaining oocytes during the follicular phase and inseminating them with the partners spermatozoa, fertilisation was achieved in the laboratory (Edwards 1965, Bavister *et al* 1969, Edwards *et al* 1969). Many technical problems had to be overcome before matured oocytes were able to be recovered with relative ease and fertilisation followed by growth of a high percentage of embryos ensured.

Safe, repeatable access to the woman's ovaries became available with the introduction into gynaecological practice of the laparoscope (Steptoe 1967, Steptoe 1969). Although general anaesthesia was required, the procedure had a low mortality and morbidity (RCOG Report 1978).

Initially oocytes were collected during spontaneous cycles but this had two main disadvantages; only one oocyte is produced in most non-stimulated cycles and the exact timing of ovulation is difficult to predict and thus women often ovulate before oocyte collection (Edwards 1981). If collected too early in the cycle oocytes are often immature and fail to fertilise; some maturation within the ovary under the influence of LH (or hCG) is required.

Stimulation of the ovaries with exogenous gonadotrophins increases the number of follicles which develop (Gemzell *et al* 1958, Lunenfeld 1963). The subsequent administration of hCG mimics the action of LH and induces completion of the first meiotic prophase

(metaphase II); ovulation occurs between 36 and 40 hours later (Edwards 1965, Jagiello *et al* 1968, Steptoe and Edwards 1970). If, however, high concentrations of circulating E_2 are present, spontaneous LH surges can occur before administration of hCG, with ovulation resulting earlier than anticipated.

In 1978 Steptoe and Edwards reported the birth of Louise Brown, the first child born after IVF-ET. Laparoscopic oocyte recovery had been performed in a spontaneous cycle with the resultant retrieval of a single oocyte. Reimplantation of an eight cell embryo was performed on the third day. Monitoring of the cycle was by measurement of 24 hour urinary oestrogen concentrations and by 3 hourly LH measurements (Edwards *et al* 1980).

Over the last decade the indications for treatment, the types of drug therapies, the follicular monitoring and the techniques for oocyte retrieval have expanded and been improved, resulting in better pregnancy rates (Edwards 1981). For example, indications now include endometriosis, male factor infertility, immune causes and unexplained infertility, as well as tubal damage (Lopata *et al* 1978, Steptoe and Edwards 1978, Trounson *et al* 1980, Cohen *et al* 1984, Edwards *et al* 1984, Trounson and Wood 1984, Yovich *et al* 1984, Jones *et al* 1985).

The range of drugs used to induce ovarian stimulation includes anti-oestrogens (clomiphene citrate and tamoxifen), hMG, purified FSH and GnRH analogues in combination with exogenous gonadotrophins (Edwards and Steptoe 1983, Edwards *et al* 1984). The use of GnRH agonist analogues in IVF-ET programmes became increasingly popular from 1985 onwards (Porter *et al* 1984, Wildt *et al* 1986, Smitz and Devroey 1987, Rutherford *et al* 1988). The analogues were initially used in women responding poorly to hMG, with or without CC, but their use is now standard for most patients in the majority of centres in the UK.

Monitoring has been much improved with the use of ultrasonic visualisation of the follicles for assessment of both the numbers and size of follicles (Rounberg *et al* 1978, Hackeloer *et al* 1979, Kerin *et al* 1981, Edwards *et al* 1984). Urinary oestrogen concentrations and serum

E_2 concentrations, and urinary and serum LH concentrations are now more readily available (Brown *et al* 1969, Edwards *et al* 1984).

Oocyte collection is frequently performed by the transabdominal or transvaginal route under ultrasound guidance avoiding general anaesthesia and laparoscopy (Lenz *et al* 1981, Lenz and Lauritsen 1982, Feichtinger and Kemeter 1984, Lewin *et al* 1986).

Routine IVF

Summary of procedures.

The IVF-ET procedures used in the studies are detailed below but are summarised thus: stimulation of the ovaries by exogenous gonadotrophin was monitored by ultrasound scanning and endocrinological assessment; the oocytes were collected using ultrasound guidance and inseminated with selected motile sperm and coincubated for 24 hours; a maximum of 4 embryos (currently 3 embryos - see below) were replaced after 2 days and if healthy embryos remained they were cryopreserved; luteal phase support was given by either progesterone or hCG (Profasi, Serono, Welwyn Garden City, UK) administration.

Pre treatment assessment.

Following referral for consideration of treatment with IVF-ET, all couples were interviewed by a nurse and a doctor independently. The cause(s) of infertility were assessed and the relative success rates explained. Although no formal counselling was performed, the couples were given the opportunity to express difficulties and fears about the treatment. The drug regimens and the nature of the monitoring of the ovarian response were explained. The technique of the oocyte collection was described and the couples made aware of risks and side effects.

All women were advised to have their rubella status checked and to be immunised if not protected. Hepatitis B antibodies and antigens and, if in a high risk group, human

immunodeficiency virus (HIV) antibody status were checked after discussion with the couple. No HIV antibody positive women have yet been identified.

Diagnostic indications for IVF-ET.

The following diagnostic categories were employed at the clinic and will be referred to in this thesis.

Tubal damage.

Tubal damage was considered to be the cause of infertility when there was a blockage of the fallopian tubes at any point along the length, demonstrated at hysteroscopy, laparoscopy or hysterosalpingography. If adhesions or fimbrial scarring were thought to have prevented proper functioning of the fimbriae this was also considered to have contributed to the failure to conceive.

Endometriosis.

Ectopic development of endometrium-like tissue, endometriosis, may be associated with infertility. Minimal endometriosis is a common finding in women who are investigated for failure to conceive, although whether the finding is causal is unclear (Thomas and Cooke 1987).

Severe endometriosis, associated with adhesions, ovarian cysts, or peritoneal scarring, was considered as a separate diagnostic category by the clinic.

Unexplained infertility.

Unexplained infertility was diagnosed when the couple had failed to conceive after twelve to twenty four months of unprotected intercourse and where no cause for the infertility could be demonstrated. For this diagnosis to be accepted the semen analysis had to be normal (see

below), the woman ovulating regularly and the fallopian tubes patent. An ovulatory cycle was defined as one in which there was a progesterone concentration of greater than 35 nmol/L in the mid-luteal phase, or ultrasound demonstration of follicular development and rupture. Normal tubal patency was confirmed by free passage of radioopaque fluid at hysterosalpingography, or methylene blue at laparoscopy and dye insufflation.

Oligospermia.

Men were considered to be oligospermic if the sperm density was less than 20 million per ml, with or without decreased motility (less than 40% motile; asthenospermia), with or without a high percentage (greater than 50%) of abnormal forms (teratozoospermia) (Sharma *et al* 1988). The number of motile sperm needed for insemination of each oocyte for in-vitro fertilisation was approximately 100,000 per oocyte; oligospermia was thus a common indication for IVF-ET as the few sperm there were could be concentrated and placed near the oocyte.

Antisperm antibodies.

Antibodies against sperm were occasionally detected in the cervical mucus of the woman, potentially causing immobilisation and agglutination of the sperm in the cervical canal. Antibodies were sometimes also found in the woman's serum. Antisperm antibodies in the male, leading to agglutination of the sperm and thus poor progression through the cervix, were diagnosed on the mixed antiglobulin reaction (MAR) test.

The diagnosis of antisperm antibodies was assigned to those couples where antibodies were the only recognised abnormality.

Failed donor insemination (DI).

If a woman had not conceived after at least six months of midcycle intracervical insemination with donor semen in ovulatory cycles, treatment with IVF-ET was offered.

Clinic results with different diagnoses.

Patients with tubal damage were originally considered the group most likely to become pregnant with IVF-ET. Data from the clinic in 1988 and 1989 demonstrated that patients with mild endometriosis or unexplained infertility had the same chance of conceiving as the tubal damage group (Sharma *et al* 1988)(Table *II*-1). Couples with a male factor cause for their infertility were the least successful. This group had the lowest overall fertilisation and cleavage rates but there was no significant difference in the number of oocytes collected.

Table *II*.1. Cumulative pregnancy rates related to diagnoses (%).

	1 cycle	3 cycles	6 cycles
Unexplained infertility	15.0	36.5	44.7
Mild endometriosis	13.0	34.9	36.9
Tubal damage	11.4	28.7	44.6
Male factor infertility	9.1	25.7	29.2

(With permission)

Drug regimens.

Clomiphene citrate and hMG.

Clomiphene citrate (Clomid, Merryl Dow Pharmaceuticals UK) 100mg orally, was administered from the second to the sixth day of the menstrual cycle in the first studies (Chapter *III*). The principal role of clomiphene citrate in ovarian stimulation regimens for IVF-ET was to stimulate endogenous FSH production. The circulating gonadotrophins were augmented by adding injections of hMG which led to the recruitment of multiple follicles.

In women with menstrual cycles greater than 26 days duration, treatment with hMG (Pergonal, Serono, Welwyn Garden City, UK) was given daily from day 4 of the cycle. If

the cycle was shorter than 27 days the exogenous gonadotrophin stimulation was given from day 2 concurrently with the clomiphene citrate. The initial dose of hMG was two ampoules IM (150 IU of FSH and 150 IU of LH) daily for at least four days. Because of the smaller ovarian response in older woman, women over 35 years received an initial starting dose of three ampoules per day (225 IU of gonadotrophin). Further dosages were individualised on the basis of the ultrasound examinations and E_2 concentrations after day 8, until an adequate ovarian response had been obtained.

GnRH analogues and hMG.

HMG stimulation preceded by the long regimen of GnRH analogues was used in the second study (Chapter *IV*). Buserelin (Suprefact, Hoechst, Hounslow, United Kingdom) was injected subcutaneously in a dose of 200μg daily from the first day of the menstrual cycle for a minimum of fourteen days. If ovarian suppression was confirmed at 14 days (see below), hMG was commenced and treatment with the same dose of buserelin continued until the day of hCG administration.

If, however, serum E_2 concentrations indicated lack of pituitary suppression (see below), the dose of buserelin was increased to 500μg SC daily for seven days. Serum E_2 concentrations were then remeasured. If pituitary desensitisation had still not occurred treatment with buserelin in a dose of 500μg SC was continued with weekly measurements of serum E_2 concentrations until hypoestrogenaemia was observed. Once ovarian suppression was achieved, the dose of buserelin was reduced to 200μg sc daily until the day of administration of hCG.

Women under 35 years old received hMG 3 ampoules (225 IU FSH and 225 IU LH) daily from the day following confirmation of ovarian suppression. Women over 35 years received 4 ampoules (300 IU FSH and 300 IU LH) per day. All women were scanned on day 6 of hMG administration to assess follicular growth. If the E_2 concentrations or the ultrasound examination of the ovaries indicated poor follicular development the dosage of hMG was increased by 1 to 3 ampoules per day every second day until follicular development occurred,

or the cycle was abandoned.

Monitoring of response.

The ovarian response was monitored by assessing follicular size and number on ultrasound scanning. In addition, 24hr urinary oestrogen excretion (during the first studies) and serum oestradiol (during the second study) were measured.

Ultrasound scanning of the pelvis.

Route:

The abdominal route of imaging was preferred for daily follicular measurements and oocyte collections during the period of the first studies (Chapter *III*). The vaginal approach subsequently became more prevalent, particularly for oocyte collections, and all the collections were performed with transvaginal imagining of the ovaries in the second studies (Chapter *IV*).

Abdominal imaging:

The ultrasound machines (Diasonics DRF-1, Bedford; Phillips SDR 1500, Hammersmith, London) were provided with two probes for abdominal scanning; 3.5 and 5 mHZ. To ensure good visualisation with the 3.5 mHZ probe, a full bladder was needed. The vagina, cervix, uterus, ovaries and pouch of Douglas (POD) were systematically examined. If the ovaries were found to be lying more superficially, the bladder was emptied and the 5 mHZ probe was used.

Vaginal imaging:

If the ovaries were positioned in the POD, clear definition of the ovarian morphology was invariably inadequate with the abdominal probe. A 5 mHz probe, designed for vaginal insertion, provided excellent images and obviated the need for a full bladder. Occasionally

ovaries positioned extremely laterally or high in the abdomen were not accessible with this probe and the abdominal approach was used.

Baseline morphology:

Ultrasound assessment of the pelvis was performed before administration of hMG in all women. If the women were to take CC and hMG, the baseline scan was arranged for the second day of the menstrual cycle. If buserelin and hMG were to be used, the scan was performed after at least 2 weeks of treatment with the analogue once hypo-oestrogenaemia had been confirmed (see below).

The pelvis was scanned to determine the presence or absence of cysts, ovarian or extraovarian, hydrosalpinges or uterine fibroids and other uterine abnormalities. The ovarian morphology was assessed for a polycystic pattern. The normal ovary in the early follicular phase was recognised as one which contained several small preantral follicles distributed throughout the stroma.

The polycystic ovary was defined as an ovary which contained, in any one plane, 10 or more peripherally distributed "cysts" or follicles 2 to 8mm in diameter, in the presence of stroma which appeared echodense and occupied a large proportion of the ovary (Adams *et al* 1985). The polycystic ovary was frequently larger than normal but this was not used as a diagnostic criterion. Clinical signs or symptoms were *not* part of the diagnosis.

Cycle monitoring:

The women were scanned each morning from the eighth day of their menstrual cycles if taking CC and hMG, and from the sixth day of hMG administration if treated with buserelin and hMG. The follicles were visualised individually in two planes; transverse and longitudinal, and the mean of the maximum measurements recorded. The number and size of the follicles were noted for each ovary. Cystic structures were also measured and recorded.

The scans were repeated daily until the criteria for hCG administration were reached (see below).

Hormonal monitoring.

Urinary oestrogen estimation.

During the period of the first studies, hormonal monitoring was performed by measurement of the daily urinary oestrogen excretion. The women collected their urine for 24 hours from the first to the second day of the menstrual cycle and daily from the seventh day until the morning after hCG administration. An aliquot was taken from the total amount of urine.

The results of the total 24hr urinary excretion of oestrogen were obtained on the same day. The urinary oestrogen was assayed by the fluorometric technique (Brown *et al* 1968). The assays were performed daily at Serono Laboratories (Welwyn Garden City, Herts, United Kingdom) except at weekends. The mean interassay variation during the period of the first studies was 6.2% (range 3.5% - 11.3%). The intra-assay coefficient of variation, taken from two assays at random, was 3.7% and 4.8%. The concentration of urinary oestrogens correlates with the number of large follicles (Edwards *et al* 1972).

Serum oestradiol monitoring.

During the period of the second study, measurements of serum E_2 concentrations were used to monitor the ovarian response. Blood was taken after two weeks of treatment with buserelin to detect pituitary desensitisation, indicated by low E_2 concentrations (see below). Samples were also taken daily from day 6 of the cycle until, and including, the day of hCG administration.

Serum was assayed for E_2 using the IDS gamma BCT oestradiol IVF assay kit (Usworth Hall, Tyne and Wear, UK). This kit employed a polyclonal antibody covalently bound to polypropylene tubes, together with iodinated oestradiol labelled ligand. The standard curve

was designed to cover the range 350-15,000 pmol/L. Hypoestrogenaemia was defined as less than 350 pmol/L. Between assay coefficient of variance (CV) ranged from 5.8 - 8.4% over low, medium and high quality controls. The within assay CV was 4.5 - 6.8%. The assay was under the surveillance of external quality control and performed within acceptable limits over the time period of the study.

The results from these analyses were used to make management decisions during the cycle but were not used in the evaluation of the GH studies.

Criteria for discontinuing treatment.

Oocyte collections were cancelled if there were fewer than 3 follicles greater than 14mm in diameter or urinary oestrogen concentrations were less than 250 nmol/24 hrs, after at least seven days of hMG administration. When serum concentrations of E_2 were measured, a concentration below 1000 pmol/L was considered an indication for abandoning the cycle.

HCG administration.

Human chorionic gonadotrophin, structurally related to LH and used to mimic the LH surge, has been demonstrated to cause release of an oocyte within 40 hours of administration in unstimulated cycles (Steptoe and Edwards 1970).

An IM injection of 5000u hCG was administered when three follicles of greater than 14mm in diameter were detected on ultrasound scanning of the ovaries, with at least one greater than 17mm in diameter, in the presence of adequate oestrogen concentrations (see above)(Edwards and Steptoe 1975, de Crespigny *et al* 1981, Edwards and Steptoe 1983). These criteria were used in an attempt to give hCG before a spontaneous LH surge occurred.

If the women were using buserelin and hMG, treatment was continued for one day after these criteria had been reached as it was considered that there was little risk of a spontaneous LH surge.

Ultrasound directed oocyte recovery was performed 35 hrs after hCG administration. This interval was chosen so that the mature cumulus mass was sufficiently loosely bound to the follicle wall to allow oocyte collection, but to be before the oocyte was spontaneously expelled (Edwards 1981).

Oocyte collection.

Analgesia.

All oocyte collections were performed as an outpatient procedure, under sedation. The women arrived 45 minutes before the planned starting time, having taken 1mg lorazepam (Ativan, Wyeth Laboratories, Taplow, UK) orally the previous night and 1mg lorazepam two hours before the oocyte collection. The women were advised to have nothing to eat for approximately twelve hours before the collection and to arrive with a full bladder, if undergoing a transabdominal collection.

Twenty minutes before the start of the collection an intramuscular injection was given of 12.5mg prochlorperazine (Stemetil, May and Baker, Dagenham, Kent) and, depending on the woman's weight, between 100 and 150 mg pethidine hydrochloride (Pethidine, Roche Products Ltd., Welwyn Garden City, UK).

Trans-abdomino-vesical collections.

During the period of the first studies the trans-abdomino-vesical route was the routine approach to the ovaries (Lenz *et al* 1981, Lenz and Lauritsen 1982, Riddle *et al* 1987). If the ovaries were lying just below the skin the direct transabdominal approach was used.

The woman's abdomen was cleaned with hibitane 0.5% in industrial methylated spirit and draped with sterile towels exposing the suprapubic region. Using the abdominal ultrasound probe, enclosed in a sterile bag, the ovaries were identified lying posteriorly to the full bladder. One percent lignocaine was used to infiltrate a small area of skin in the right iliac

ossa and then a stainless steel, single lumen, 17 gauge, 25 cm long needle (Casmed, London) was passed into the bladder.

One portal of the needle was connected to a syringe containing flushing solution (Earles culture medium with added heparin 11000u/ml (see below)). The other portal of the needle led, via a fine plastic and silicone tube and bung, into a sterile plastic tube which was connected, via suction tubing, to a pump. A maximum pressure of 100mm of mercury was obtained using a foot pedal.

Once the ovaries had been identified, the system was flushed with heparinised medium. The tip of the needle was then aligned over the midpoint of the first ovary and, using a single action, thrust through the posterior bladder wall, ovarian capsule, ovarian stroma and into the first follicle. The follicular fluid was aspirated and handed to the embryologist for microscopic examination. If this aspirate did not contain the oocyte and the follicle was then flushed with the medium. Flushing was continued until either the oocyte was found or until no more follicular cells were detected in the fluid.

The needle was then retracted into the ovarian stroma and the next follicle entered. If the needle were accidentally withdrawn into the bladder, the system was flushed with heparinised medium and the ovary reentered. Once all obvious follicles in one ovary had been aspirated, the second ovary was punctured in the same way. Ideally each ovary had only one site of entry, thus minimising trauma.

Occasionally urine extravasated into the periovarian space or pouch of Douglas. Discomfort so-caused rarely necessitated additional analgesia. At the end of the procedure the bladder was partially emptied and the needle withdrawn. The women rested for approximately one hour before discharge. They were encouraged to pass urine before leaving; occasionally catheterisation was required. Haematuria was a rare complication and when this occurred the woman's blood pressure was checked regularly for at least 4 hours before discharge. The women were encouraged to drink as much as possible and inform the clinic should the bleeding continue.

Transvaginal collections.

Using the transvaginal approach for oocyte recovery the women did not need to have a full bladder and many found the procedure less painful compared with the trans-abdomino-vesical approach (Gleicher *et al* 1983). A suppository of metronidazole (Flagyl, Rhone-Poulenc Rover Ltd., Dagenham, Essex, UK) was inserted at the same time as the premedication was administered.

Once in theatre, the woman was placed in the lithotomy position. The vault of the vagina was visualised using a speculum and the vagina was cleaned with 0.015% chlorhexidine gluconate plus 0.15% cetrimide (Savlodil, ICI, Macclesfield). A swab dipped in flushing solution was then used to wipe out any pool of disinfectant. The vaginal transducer and attached needle guide were placed in a sterile, non spermicidal condom. The needle was passed through the posterior fornix directly into the ovary and underlying follicle. Systematic puncturing of the follicles was performed as in the abdominal method of collection. The POD was occasionally drained if a follicle had ruptured spontaneously and the free fluid was thought to contain an oocyte. At the end of the procedure the vagina was wiped down to ensure there was no excessive bleeding.

Semen preparation.

Semen analysis.

Procedure:

Partners had a semen analysis performed before treatment was commenced, unless azoospermia had been confirmed elsewhere. The men were asked to complete a form asking about the number of days of abstinence, the amount of alcohol recently imbibed, and whether any drugs were being taken.

In the laboratory the volume of the semen was measured. Liquefaction was allowed to take

place over 30 minutes and the specimen then thoroughly mixed. A count of the total number of sperm per ml was made using a counting chamber and the number of progressively motile sperm estimated. The forward progression of the sperm was graded from 1 to 4, a normal progression score being 3 or 4, poor progression being 1 or 2. The morphology of the sperm was examined using a stained sample and the proportion of sperm with abnormal configuration (ie head, neck or tail anomalies) assessed. Comment was made on the number of white cells present and any other debris.

Any sperm agglutination was recorded and the viscosity of the sample assessed. All semen was tested for antisperm antibodies using the MAR test for IgG immunoglobulin. If the MAR test proved positive, further antibody tests were performed; the tray agglutination test (TAT) was used to measure antibody titres in the man's serum, and the specific immunoglobulin (Ig) classes (IgG, IgA, IgM), present on the sperm, were detected by immunoglobulin coated beads.

Normospermic semen parameters at the Hallam Medical Centre are shown in Table *II*-2.

Table *II*-2. Normal semen analysis values from Hallam Medical Centre

Count	$>20 \times 10^6$/ml
Motility	>40%
Progression	3-4
Abnormal forms	<60%
MAR test	-ve
Agglutination	-ve
White cells	$<3 \times 10^6$/ml

All partners had semen cryopreserved before the cycle began, in order to provide a reserve sample for the day of oocyte collection.

Oligospermia/donor sperm:

Partners with sperm parameters outside the above limits were advised that overall chances of fertilisation were reduced but depended on there being sufficient sperm with normal function present. Specific methods of preparation of the samples were performed to concentrate the most motile sperm for insemination of the oocytes.

Treatment with donor sperm was offered to couples where the partner was azoospermic. Treatment with donor sperm was also offered if failure of fertilisation was associated with poor semen characteristics. Fully informed signed consent was required from both partners. Donor semen was used for IVF, only after a pregnancy had resulted from its use in intracervical insemination.

The colour of hair and eyes, the height, the racial group and, if requested by the couple, the blood group and religion, of the donors were matched to the potential parents wherever possible. The donors were healthy volunteers who had no known physical or mental problems. A full family history was taken to exclude any recognisable hereditary diseases. The donors were screened for blood group, hepatitis B and HIV status, and karyotype.

A full semen analysis was performed and once normal results were confirmed, the donor was accepted on to the programme. Samples of semen were frozen following dilution with cryopreservation medium (egg-yolk buffer based) and stored in 0.5ml straws at -190° C in liquid nitrogen. After 3 months, if the donor's monthly HIV tests were negative, the samples were available for use.

Semen preparation for IVF.

The aim of the semen preparation prior to IVF was to produce a highly motile population of sperm in a seminal plasma and debris free environment. The methods used depended on the motile count and volume of the semen and the presence of antibodies, white cells or debris.

Layering:

The layering technique was used where there were more than $20x10^6$ motile sperm per ml present in the semen. Two ml of culture medium (Earles balanced salt solution supplemented with sodium pyruvate and sodium bicarbonate with Penicillin, plus 8% maternal serum or Albuminar serum substitute (human serum albumin, Armour Pharmaceuticals,UK) was layered over 1.5mls of semen and placed at 37°C for at least 30 mins. The interface layer was then collected and washed twice by centrifugation and resuspended in fresh medium. The sample was then counted and diluted as necessary.

"Swim up" technique:

The swim up technique was performed on cryopreserved sperm and on semen samples where the count was less than 20 $x10^6$/ml. Neat semen was spun and washed twice, with the final pellet resuspended in approximately 0.2ml of medium. Two ml of fresh medium was then pipetted onto the sample and left standing for up to one hour at 37°C. The interface layer was removed, counted and diluted as necessary.

Final preparation for insemination:

The final concentration of motile sperm required for in-vitro insemination of human oocytes was 100,000/oocyte.. If, however, the partner had a high (greater than 80%) proportion of abnormal sperm or was oligospermic, or if greater than 50% of the woman's oocytes needed to be reinseminated on Day 1, the final concentration of sperm was 200,000-500,000/oocyte.

Embryology.

Preparation.

A sterile oocyte incubation dish was prepared the day before for each woman undergoing an oocyte collection. The dishes contained nine droplets of 240 μl medium, under 100% paraffin oil, arranged around the circumference of the dish. Two "wash" droplets were placed in the centre. Culture of gametes and embryos was carried out under 5% CO_2 at 37°. Dishes were allowed to equilibrate in culture conditions for 18 - 24 hrs prior to use.

Oocyte recognition.

The aspirate, or flush, was handed to the embryologist as it was obtained. The fluid was examined on a water-heated stage at 120x magnification through a Wild binocular dissection microscope. The presence of granulosa cells and or cumulus cells was commented upon, as was the consistency and colour of the fluid.

The oocyte cumulus complex was graded from 1 to 5. Grade 1 oocytes were at the germinal vesicle stage i.e. the most immature. Grades 2 to 4 were increasingly mature and grade 5 was luteinised. The majority of oocytes were grades 4 to 5.

Incubation and fertilisation.

The oocyte was then pipetted into the wash drops and finally cultured in a droplet of medium. After 3 to 5 hours each oocyte was inseminated with 100,000 sperm and the gametes co-incubated overnight (Edwards 1981). If the oocyte was graded as very immature, insemination was delayed until 6 hours after recovery.

Seventeen to 24 hrs. following insemination, the oocytes were examined for the presence of pronuclei, following the removal by dissection of sufficient cumulus cells to allow visualisation of the cytoplasm. Two pronuclei visible in the oocyte indicated normal

fertilisation had occurred. If more than two were detected, polyspermic fertilisation was inferred and these embryos were not replaced. Any oocytes which had not fertilised were reinseminated.

Cleavage.

Two days following oocyte recovery, embryos were examined to see if cleavage had occurred. The embryos were scored for cell number and quality, as judged by morphological appearance. Grade 1 embryos showed evenly sized blastomeres of near spherical appearance with moderate refractility and intact zona. Grade 2 embryos had uneven blastomeres with mild variation in refractility. Grade 3 embryos showed fragmentation of no more than 50% of the blastomeres. The zona pellucida was intact. Grade 4 embryos show fragmentation of more than 50% of blastomeres, some of which were grossly variant in refractility.

Embryo transfer.

The couple was informed how many oocytes had fertilised and cleaved and of what grading they were. The number of embryos to be transferred was then discussed with the couple.

During the period of these studies, up to 4 embryos that had shown evidence of normal cleavage were transferred to the uterus after 48hrs of culture. Remaining embryos of sufficient quality were cryopreserved.

Procedure.

A speculum was inserted and the cervix visualised. The cervix was cleaned with a swab soaked in medium and any excess mucus wiped away. The embryos were placed in approximately 20 μl of medium, containing 25% Earles balanced salt solution and 75% serum or Albuminar, and the embryos loaded into a transfer catheter (Wallace Ltd, Colchester, UK) which was then handed to the operator. The tip of the catheter was passed through the cervical canal into the fundus of the uterus. The fluid containing the embryos, was then gently expelled into the uterus and the catheter withdrawn. The catheter was then

checked to ensure that the embryos had been expelled and the speculum was removed from the vagina.

Occasionally there were difficulties passing the catheter through the internal os and a tenaculum had to be applied to the anterior lip of the cervix. This aided the straightening of the cervical canal and often the embryo transfer then proceeded with ease. If, however, there were still problems of insertion, a catheter with a rigid outer sheath (Craft Catheter, Rocket of London) was used. The women were advised to rest for approximately 20 minutes after the procedure.

Number of embryos transferred.

Edwards and Steptoe (1983) reported that there was an increasing pregnancy rate with increasing numbers of embryos transferred. In 1988, analysis of the data on treatment outcome in the Hallam Medical Centre demonstrated that there was a trend towards increasing conception rates with increasing numbers of embryos transferred, to a maximum of four (Sharma *et al* 1988). The linear rise in pregnancy rate with the number of embryos transferred was similar to that reported by the World Collaborative report (Seppälä 1985).

Analysis of the data from the clinic showed that there were significantly more multiple pregnancies in the group of women who had 4 embryos transferred compared with those who had 2 transferred. There was, however, no significant difference in the multiple pregnancy rate between women who had 3 embryos transferred or 4 embryos transferred. During the period of the reported studies the couples were therefore informed that in this centre there was a greater chance of conceiving if 4 embryos rather than 3 were transferred, but that there was no significant difference in the multiple pregnancy rate (Table *II*-3).

Table *II*-3. Pregnancy rates per number of embryos transferred and multiple pregnancy rate (%).

Number of embryos transferred	n=	Pregnancy rate	Multiple pregnancy rate
1	245	8.6	
2	322	15.2	8.1
3	317	18.9	20.0
4	468	29.5	23.9
≥ 5	23	26.1	

(With permission)

Most women were advised to have 3 embryos transferred, particularly if the women had previously been pregnant, were young and had good quality of embryos. If, however, the woman had had several failed attempts at IVF-ET, had more than 10 oocyte collected, had fertilisation or cleavage in less than 60% of the recovered oocytes or was over 36 years old, transfer of 4 embryos was discussed (Hallam Clinic guidelines).

The work presented in this thesis followed the guidelines of the Interim Licensing Authority. Since August 1991 the Human Fertilisation and Embryo Bill became statutory and is enforced by the Human Fertilisation and Embryo Authority. The Act states that a maximum of 3 embryos (or oocytes) be transferred.

Luteal phase.

Given in the luteal phase, hCG stimulates LH receptors on the corpus luteum and increases progesterone production. An injection of 2000u hCG was given on the day of embryo transfer and again, three days later.

If the oestrogen concentrations two days before oocyte recovery were high (greater than 1000nmol/24hrs for urinary oestrogen excretion or greater than 10,000 pmol/l for serum E_2

concentration), hCG was avoided because of the fear of development of the hyperstimulation syndrome. Luteal phase support was, therefore, given as Cyclogest pessaries (Hoechst UK Ltd., Hounslow, Middlesex, UK), 200 mg twice daily for at least two weeks.

The women were recommended to continue their normal work and home lifestyles. They were advised that some lower abdominal discomfort could occur. Should this become severe, or associated with nausea or vomiting, they were told to contact the centre immediately. If no bleeding had occurred by 14 days after the embryo transfer, a pregnancy test was performed.

Pregnancy test.

The pregnancy test used was the Tandem Icon (Hybritech) beta hCG semi-quantitative enzymatic test. Development of a blue discolouration as dark or darker than the control spot, indicated a positive test, correlating with a result of greater than 25 iu/L of hCG.

Pregnancy scanning.

Once the pregnancy was confirmed, a scan was arranged. Ideally this was performed at 7 weeks gestation or later. The purpose of the scan was to determine whether the pregnancy was intrauterine, as a high ectopic rate with IVF-ET was recognised. Occasionally heterotopic pregnancies occurred, but they were rare (Rizk *et al* 1991). The pregnancy was considered viable if a heart beat was detected on ultrasound scanning of the fetal pole.

Suboptimal responders.

During ovarian stimulation prior to IVF-ET several forms of suboptimal response may occur. In 10 to 30% of cycles an unsatisfactory response leads to the treatment being discontinued (Vargyas *et al* 1984, Lyles *et al* 1985, Muasher *et al* 1985, Pellicer *et al* 1987).

Edwards *et al* (1984) categorised the women who were discharged without laparoscopy as follows; low urinary oestrogens with many small follicles when the LH surge began, "weak" follicular response to stimulation, high tonic LH or other technical reasons. Approximately 10% of cycles were abandoned in this way.

Lyles *et al* (1985) demonstrated ovarian cyst formation in 5% of treatment cycles, premature LH surge in 7% and poor follicular response to therapy in 18%. Results from the Hallam Medical Centre demonstrated a similar pattern. Of women subsequently offered buserelin because of previous suboptimal response, 21% had ovulated prior to planned oocyte recovery and 30% had a poor follicular response (Jones 1984).

The commonest reason for cancellation is inadequate follicular response. A paucity of follicles developing in response to the gonadotrophin stimulation is typically associated with low oestradiol output (Lopata 1983, Laufer *et al* 1986). Occasionally women have several follicles present in the ovary but the follicles do not continue to enlarge despite increasing doses of gonadotrophin.

A low response in oestrogen production in the presence of adequate numbers of follicles occurs in a small proportion of women and is interpreted as the development of dysfunctional follicles or a group of cysts containing few granulosa cells.

Following the initiation of the LH surge, circulating oestradiol concentrations usually drop for several days, before increasing again in the luteal phase. Thus if urinary or serum oestrogen concentrations fail to continue increasing, or indeed decrease, in association with adequate follicular size, a spontaneous LH surge is implied and treatment may be discontinued. Ten to 40% of women using CC and gonadotrophins have a spontaneous LH surge, or ovulate before the time of planned oocyte collection (Fishel *et al* 1984, Hillier *et al* 1985, Lyles *et al* 1985, Muasher *et al* 1985, Eibschitz *et al* 1987, Owen *et al* 1989)

If poor follicular or oestrogenic response is the indication for abandoning the treatment cycle this is likely to be a recurring phenomenon cycle after cycle (Jones 1984, Diamond *et al*

1985, Pellicer *et al* 1987). The introduction of GnRH analogues into treatment schedules has, however, reduced the occurrence of spontaneous LH surges.

The GnRH analogues are synthetic peptides which closely resemble GnRH and bind to the GnRH receptors with enhanced affinity. In the pituitary the acute effects of GnRH analogues are to enhance the numbers of GnRH receptors and stimulate the secretion of Gn. Due to structural changes in the analogues, however, enzyme degradation of the molecule occurs at a much slower rate resulting in biological potencies of over 100 times that of the native molecule (Sandow *et al* 1987). Chronic treatment with large doses results in desensitisation to GnRH as the occupied receptors are lost from the cell surface. Subsequent diminution of both FSH and LH production and secretion ensues. Hypogonadotrophic hypogonadism is induced and as oestradiol concentrations fall shedding of the endometrium may occur.

Treatment with exogenous gonadotrophins (hMG or FSH) stimulates the ovary and results in follicular development. With the use of GnRH analogues feedback on the pituitary is blocked, however, and, despite high oestradiol concentrations, the LH surge can not be initiated.

In women with high tonic LH concentrations, treatment with the analogues decreases the pituitary release of gonadotrophins. It has been suggested that suppression of the endogenous gonadotrophins and subsequent use of exogenous gonadotrophins results in more coordinated follicular development. The use of GnRH analogues has therefore become an important part of the treatment of suboptimal responders. An improved outcome in terms of follicular development, oocyte recovery and pregnancy rates has been reported (Wildt *et al* 1986, Awadalla *et al* 1987, Palermo *et al* 1988, Rutherford *et al* 1988, Owen *et al* 1989).

With the introduction of the analogues into clinical practice it was observed that increasing concentrations of hMG or FSH were required to induce multifollicular development. This was considered to be due not only to the omission of the clomiphene citrate but also to hyposecretion of the endogenous gonadotrophins. The period needed for recruitment of follicles is prolonged as the perimenstrual rise in FSH has been obliterated.

Growth hormone studies.

Pre treatment evaluation.

Inclusion/exclusion criteria.

To be eligible for participation in the study, women had to have undergone one or more IVF-ET cycle(s) in which ovarian stimulation had been carried out using CC and hMG (first studies) or the combined regimen of GnRHa and hMG (second study) and in which the response was considered suboptimal.

In the first studies this was defined as 3 or fewer oocytes collected or two or fewer embryos transferred, despite having received a starting daily dose of 300u (4amps) or more of hMG for ovarian stimulation. In the second study a suboptimal response was defined as a response in which fewer than 6 oocytes were collected from which fewer than 4 embryos developed, despite having received an initial starting daily dose of 225 u (3amps) or more of hMG.

Women with unilateral oophorectomies were excluded and, for the first studies (Chapter *III*) those women with partners who were oligospermic. The entry criteria were widened for the buserelin and hMG study and couples with oligospermia were included. Women over 40 years were excluded from the first placebo controlled study (CC and hMG). In the second study (buserelin and hMG) I was aiming to define a more responsive group and the age limit was decreased to 38 years or less.

Initial assessment:

When the women were seen and recruited for the various studies, a full pretreatment evaluation was undertaken. This consisted of a general medical history, physical examination and laboratory evaluation, including liver and kidney function tests. The blood pressure was noted and the body mass index (BMI kg/m^2) calculated. Blood was taken for estimation of liver function tests (LFTs; albumin, total bilirubin, alanine transferase (ALT) or aspartate

transaminase (AST) and alkaline phosphatase) and urea and electrolytes (U&Es; urea, sodium, potassium, bicarbonate), creatinine and calcium and phosphate. A fasting blood glucose concentration was performed and any family history of diabetes mellitus noted. Women with a fasting glucose above 5.6 mmol/L were excluded. All women were in good health without chronic illness.

A early-follicular phase ultrasound scan of the pelvis was performed in a non treatment cycle and gonadotrophin concentrations measured and serum saved for later analysis of insulin-like growth factors. Women with an early follicular phase FSH of greater than 15 IU/L were excluded from the study. Concentrations greater than 15IU/L were considered to be peri- or post-menopausal in our laboratory. This was supported by the clinical observation that the two women with concentrations above 15IU/L in the open study failed to have any follicular development in response to gonadotrophins with or without GH.

The women were fully informed about the study and particular attention was drawn to the double blind and placebo controlled nature of the study. All women gave their written informed consent to participate in the study, according to the Tokyo Amendment to the Declaration of Helsinki. The study protocols had been approved by the University College and Middlesex School of Medicine Ethical Committee.

Study design.

Initially eight women who were resistant to CC and gonadotrophin therapy were recruited into an open study to examine the effect of cotreatment with GH. The women had all shown a repeatedly poor follicular response to increasing doses of hMG. For this study each woman was maintained on the same daily dose of CC and hMG as in her previous cycle of treatment but received, in addition, GH (24IU IM) on alternate days concurrently with the hMG.

The two larger studies were designed as randomised, double blind trials of cotreatment with GH or placebo, in addition to the woman's standard treatment for IVF. An open arm of the study followed in which the women who received placebo were offered a cycle with GH

augmentation.

Sample size calculations were made using the number of follicles greater than 14mm on the day of hCG as the primary variable effect. The initial study had suggested that patients receiving GH developed approximately 2 more follicles than in prestudy cycles. For a T-test with 5% significance level (type I error = 5%) a mean difference of atleast 1.9 follicles between placebo and GH treatment would result in a rejection of the hypothesis that the two treatments are equal with a probability of 80% (type II error = 20%), if twenty patients completed the study.

In the first study, women were assigned a two digit patient number in sequential order. The patient-number encoded random assignment to one of two treatment groups; GH (24 units per injection given IM) or placebo presented in identical vials also reconstituted with 2mls of sterile water and given IM. In the second study, a pelvic ultrasound scan was performed prior to enrolment to establish the ovarian morphology. Two randomisation lists were made in blocks of four; one for women with PCO and the other for women with normal ovaries.

Standard ovarian stimulation was performed by administering a combination of CC (CC and hMG studies) or buserelin (buserelin and hMG study) as previously described, with the same daily starting dose of hMG as in the woman's previous cycle. The women were, in effect, acting as their own controls and comparisons with the prestudy cycles were thus included in the data analysis. The injections of GH or placebo were started on the first day of hMG treatment and were given on alternate days concurrently with the gonadotrophin treatment until the administration of hCG or for a maximum of 6 injections.

Upon completion of the cycle the assignment code was broken. Those women who had received GH were considered to have completed the study; women who had received placebo were then placed in an open study in which they received GH. In all cases, an interval of at least 2 months was allowed to elapse between cycles of treatment. The women in the open CC and hMG study were treated in the same manner except that there were no placebo

control cycles. Several of these women went on to receive GH cotreatment in further cycles.

Monitoring was undertaken in the standard fashion with ultrasound examinations and urinary oestrogen (CC and hMG studies) or serum E_2 concentrations (buserelin and hMG study) until an adequate ovarian response had been obtained. The criteria for hCG administration have been described above. Not only were the first arms of the studies double blind but the doctor making the decision for hCG administration was independent and not aware of the woman's recruitment into a study. Great care was taken to ensure that the study patients were not treated differently in any way.

Oocyte collection was by the trans-abdomino-vesical route for the CC and hMG studies and by the transvaginal route for the buserelin and hMG study. For the first studies (CC and hMG) the most experienced operator available on the day performed the recoveries. I was present at all the oocyte recoveries and all the collections of the follicular fluid samples were supervised personally. In the second study (buserelin and hMG) all of the oocyte recoveries were performed personally at 8.30am and the follicular fluids collected and processed immediately. The technique of IVF, culture of oocytes and embryos, fertilization and ET were performed routinely as described above.

End points.

The end points for comparison were:

- total number of ampoules of hMG used until the day of hCG administration or until treatment was discontinued.
- total number of follicles greater than 14mm in diameter on the day of administration of hCG or on the day the cycle was abandoned.
- total urinary oestrogen excretion in the 24 hours during which hCG was administered or on the day the cycle was abandoned (CC and hMG studies). Serum E_2 on the day of hCG administration or the day the cycle was abandoned (buserelin and hMG study).
- total number of oocytes collected

Additional parameters measured:

- number of oocytes fertilised
- number of polyspermic fertilisations
- number of embryos cleaved and their grades
- number of embryos replaced (to a maximum of 4)
- number of pregnancies
- number of fetuses which developed
- problems during pregnancy or delivery
- sex and birth weights of babies

Statistical analyses.

Methodology.

The data were entered onto an IBM compatible personal computer using either Lotus 123 or Easistat software packages. All data were analysed personally using the Easistat package.

Descriptive results.

The descriptive results are presented as medians and ranges as the data were not normally distributed and the numbers too small to describe percentiles (Gardiner and Altman 1989).

The first CC and hMG study was an open, pilot study and had no control group. The other studies were randomised, placebo controlled studies with an open arm for further GH treatment.
Comparisons of the number of oocytes collected were only made in those women who had had oocyte recoveries in both cycles.

Group analyses.

Analysis of the results from women randomised into the placebo group compared with those in GH group was performed by the Mann Whitney U test. Mann Whitney U tests were used for group analyses of the results from all of the GH cycles compared with the pretreatment cycles. Group analysis was also used to compare the results in women with PCO and those with normal ovaries.

In the buserelin and hMG study the degree of improvement with GH, or placebo, cotreatment compared with prestudy cycles was also analysed by the Mann Whitney U test.

Paired analyses.

Because of wide interpatient variation, paired analyses using the Wilcoxon signed rank tests (one tailed) were also performed. The results from the women's pretreatment cycles were compared with either the placebo cycle or the first GH cycle. Paired comparisons were also made between results from the cycles randomly allocated to placebo cotreatment and the subsequent GH cycles.

Serum and follicular fluid results.

Analysis of serum concentrations of steroids and peptides during the studies were made using analysis of variance and the Kruskal Wallace test. Analysis of variance was performed on the results throughout the cycles in each treatment group. Kruskal Wallace analyses were performed on the serum concentrations on day 2, days 8 to day of hCG administration and on the day of oocyte recovery in the CC and hMG studies and before buserelin administration, before hMG administration, from day 6 of hMG administration and on the day of oocyte collection in the buserelin and hMG study. Comparisons were made between results in the placebo and GH cycles and in women with normal ovaries and those with PCO.

Mann Whitney analyses were performed to compare the results in the serum and follicular fluids in the various subgroups. Mann-Whitney analyses were also performed on the follicular fluid results between different subgroups. Where available, paired analyses (Wilcoxon's signed rank tests) were performed on serum and follicular fluid concentrations taken at the time of oocyte recovery from the same woman. Kendall's rank correlation coefficient (one tailed) was used to assess the relationship between follicular fluid and serum concentrations.

Significance was set at $p \leq 0.05$.

Sampling.

Blood.

All blood samples relevant to the studies (E_2, FSH, LH, IGF-I and -II, progesterone) were collected in the morning, spun, coded and the serum frozen at -20°C for analysis in batches at a later date. Results from the study samples taken during the treatment cycles were not available during that cycle and were thus not used to influence management.

Blood for LFTs, U&Es, creatinine, calcium and phosphate and glucose were analysed within 24 hours.

CC and hMG studies:

Blood was taken before the commencement of clomiphene citrate administration on day 2 of the treatment cycle and then daily from day 8 until the day of hCG injection. Blood was also taken immediately prior to the oocyte collection. Oestradiol, FSH, LH, IGF-I and -II, LFTs, U&Es, creatinine, calcium and phosphate analyses were performed on each sample.

Buserelin and hMG studies:

Blood was collected from the women on day one of the cycle before they had their first injection of buserelin. Further samples were then taken after 14 days of buserelin or weekly until pituitary desensitisation was achieved. Blood was taken on the day of starting hMG and daily from the sixth day of hMG administration until the day of hCG. Blood was taken immediately prior to the oocyte collection and on the seventh day after collection.

Oestradiol, FSH, LH and LFTs were measured on each sample except 7 days after oocyte collection. Progesterone was measured on the day of hCG administration, the day of oocyte collection and 7 days after oocyte collection. IGF-I, U&Es, creatinine, calcium, phosphate and random glucose were measured on day one of buserelin, on days one and 6 of hMG, on the day of hCG administration and on the day of oocyte recovery.

Follicular fluid.

Ultrasound directed oocyte collection was performed by the trans abdomino-vesical or trans-vaginal routes and clear follicular fluid collected as follows. Having positioned the needle in the ovary next to the follicle, the tubing system and needle were flushed with heparinised Earles solution and the follicle punctured. Immediately the first part of the aspirate entered the collection tube, the suction was released and the tube changed. Clear fluid then flowed into this second tube. Contamination with blood was avoided by diverting the final part of the aspirate into a third tube. Only clear fluid was conserved, which was subsequently spun and the supernatant frozen at -20°C.

Hormonal assays.

Serum.

Analyses of all serum samples for FSH, LH, E_2 and progesterone were performed at the Middlesex Hospital.

FSH:

Serum FSH concentrations were measured with a polyclonal antiserum (M931) by double antibody radioimmunoassay (RIA) (Chelsea method, Endocrine Department, Hammersmith Hospital, London, UK) using Medical Research Council 78/549 standard. Separation is by solid phase second antibody. The sensitivity of the assay was 0.2 U/L. Intraassay and interassay coefficient of variance (CV) were 3.0% and 10.3%, respectively. The assay bias varied from +5.1 to -6.8% (national external quality control assessment scheme NEQAS)

LH:

Serum LH concentrations were measured by RIA (Chelsea method, Endocrine Department, Hammersmith Hospital, London, UK) with a polyclonal antiserum (F87) raised against LH and standard LH International Reference Preparation 68/40. Separation is by solid phase second antibody. The sensitivity of the assay was 0.2 U/L. The assay bias varied from +2.1% to -6% (NEQAS) and the intraassay and interassay CV were 4.3% and 7.5% respectively.

Oestradiol:

Serum E_2 was determined using a direct RIA (Incstar Ltd, Wokingham, UK) with a polyclonal antibody raised against oestradiol-6-CMO-Bovine serum albumin and iodinated label. Separation is by solid phase second antibody. Cross reactivities of 0.6% and 0.7% were observed for oestrone and oestriol respectively. The intra-assay precision ranged from

7.2% at 36 pmol/L to 2.9% at 264 pmol/L and 4% at 918 pmol/L. The inter-assay precision ranged from 13.1% at 31pmol/L to 10.8% at 287pmol/L and 32.7% at 1345 pmol/L. The assay bias varied from 4.1% to 7.3% (NEQAS).

Progesterone:

Serum progesterone was determined by direct RIA (Gamma B kit from Immunodiagnostics, Tyne and Wear, UK) with double antibody separation. The cross reactivity with 17α OH-progesterone was 2.0%, with 11-deoxycorticosterone 1.7% and with Cortisol was 0.1%. The intra-assay precision was 10% at 1 nmol/L, 5% at 20 nmol/L and 11% at 100nmol/L. The inter-assay precision was 13% at 2 nmol/L, 8% at 20 nmol/L and 185 at 100nmol/L. The assay bias varied from 5.5% to -4.0% (NEQAS).

Serum and follicular fluid IGF-I and -II.

The analyses of serum and follicular fluid for IGF-I and -II were all performed by Dr T.Torresani, Kinderspital, Zürich.

IGF-I and IGF-II were measured in both serum and follicular fluids specimens by specific radioimmunoassays. Acidification of the samples was performed as described by Silbergeld *et al* (1986). The IGF's were then extracted by reverse phase chromatography prior to the RIA.

The reagents used for the IGF-I and IGF-II RIA's were kindly prepared and donated by Prof. R. Humbel, University of Zürich. Highly purified IGF-I and IGF-II (Rinderknecht and Humbel 1978) were used for both standard and labelling. Tracers were labelled by the chloramine-T method (Hunter and Greenwood 1962), followed by purification on a Sephadex column. Specific monoclonal antibodies, prepared according to Laubli *et al* (1982), were used in a dilution 1:10000, separation of bound and free fraction occurred by precipitation with PEG.Inter- and intra-assay variation coefficients of both RIA's were below 15%. Cross reactivities of less than 0.01% with insulin and IGF-II occurred.

Follicular Fluid.

All analyses of follicular fluid were performed by Dr T.Torresani, Kinderspital, Zürich.

E_2:

Follicular fluid concentrations of E_2 were measured using the Baxter direct ^{125}J-kit (Sorin Biomedica, Italy). The polyclonal antiserum was raised against oestradiol-6-CMO-Bovine serum albumin and an iodinated label was used. A solid-phase second antibody was used for separation. All samples were diluted 1:500. Cross reactivities of 0.6% and 0.7% were observed for oestrone and oestriol respectively. The intra-assay precision ranged from 7.2% at 36 pmol/L to 2.9% at 264 pmol/L and 4% at 918 pmol/L. The inter-assay precision ranged from 13.1% at 31pmol/L to 10.8% at 287pmol/L and 32.7% at 1345 pmol/L.

Progesterone:

Follicular fluid concentrations of progesterone were measured using a no-extraction, solid phase ^{125}I radioimmunoassay (Coat-a-count, Diagnostic Products Corporation, Los Angeles). The antibody-bound fraction of the radiolabelled progesterone remains fixed to the wall of the polypropylene tube. Samples were diluted 1:5000. Cross reactivities of 0.3% were detected for 17α-hydroxyprogesterone. The intra-assay precision was 8.4% at 5nmol/L, 7.5 at 10nmol/L and 5.8% at 60nmol/L. The interassay precision was 10.0, 6.6 and 7.2% respectively.

Testosterone:

Concentrations of testosterone in follicular fluid were measured using ^{3}H RIA kit (BioMerieux, France). Separation was with dextran-coated charcoal. Cross reactivities of 45% occurred with 5α-dihydrotestosterone, 22% with 5ß-dihydrotestosterone, 0.8% with A_4 and 0.05% with progesterone. The intraassay CV varied between 3.3 and 9.4% and the interasssay CV varied between 8 and 16%.

Growth hormone:

Concentrations of GH in follicular fluid were measured using the Tandem-R solid-phase, sandwich IRMA (Hybritech Europe, Liege, Belgium). Two mouse monoclonal IgGs recognising distinct antigenic sites on the GH molecule were used, one of which was labeled with ^{125}I. The standards were calibrated against the pituitary-derived reference preparation HS2243E and recalibrated against pituitary-derived International Reference preparation 66/217 to give results in mU/L. The intra-assay CVs ranged between 4.9-10.6% over a range of 1.4 to 99.4mU/L and the inter-assay CVs ranged from 5.4 to 10.5% over a range of 6 to 33.3 mU/L.

Insulin:

Follicular fluid concentrations of insulin were determined by a specific in-house RIA (Kinderspital, Zürich), using antiporcine insulin antibody and a rat insulin preparation (Novo Research Institute, Copenhagen) as standard. ^{125}I labelled insulin was prepared in-house and separation of bound and free insulin was achieved by dextran-coated charcoal. The intraassay CVs were 3.8 and 2.1% and the interassay CVs were 11.5 and 9.9%.

Other assays.

U&E, creatinine, LFT, calcium, phosphate, glucose:

All the analyses were performed on an automated system (Table *II*-4) - the American Monitor Perspective analyser in the Department of Chemical Pathology at the Middlesex Hospital. Calibrations were by commercial serum standards and the analyses were monitored by 2 external quality assurance schemes.

Table II-4. Details of U&E, LFT, creatinine, calcium, phosphate and glucose assays.

Analyte	Between batch CV (%)	Bias on EQAS (%)	Normal range
Albumin	3.6-4.1	<1	35-53 g/L
Alk Phos	2.3-3.5	2	100-280 IU/L
AST	2.3-3.4	1	11-55 IU/L
ALT	2.9-4.3	12	5-50 IU/L
Bilirubin	2.0-7.0	-3	3-17 μmol/L
Urea	1.9-3.5	4	3-8 mmol/L
Sodium	1.2-1.4	-0.6	137-145 mmol/L
Potassium	1.5-1.9	1	3.3-4.8 mmol/L
Bicarb	5.0-6.5	<5	20-30 mmol/L
Creatinine	1.5-2.3	4	50-125 μmol/L
Calcium	2.0-22.5	-1.5	2.2-2.6 mmol/L
Phosphate	1.5-1.8	-1	0.7-1.5 mmol/L
Glucose	1.1-1.5	0.8	3.3-5.6 mmol/L

III. Results 1-CC and hMG studies

Introduction to studies.

Background.

The cancellation rate of ovarian stimulation cycles prior to IVF-ET varies between 10 and 50% in different centres. Women who repeatedly have a poor follicular response to exogenous gonadotrophins pose a difficult problem. Conventional management includes measurement of FSH concentrations in the early follicular phase of a pretreatment cycle to exclude incipient ovarian failure.

Women with normal gonadotrophins are usually treated with increasing doses of hMG. Increasing the amount of exogenous gonadotrophins administered does not necessarily, however, lead to proportional increases in the number of follicles which develop. Indeed a plateau of ovarian response may be reached and no further follicular development is obtained whatever the dose of hMG used. The resistance to stimulation may reflect a degree of intraovarian dysfunction, whereby gonadotrophin stimulation cannot instigate or maintain granulosa cell growth and differentiation because of the absence or low concentrations of some intermediatory factors. Factors modulating the gonadotrophin response include the insulin-like growth factors.

Two observations were pivotal to the studies undertaken for this thesis: that insulin-like growth factors were capable of amplification of Gn action in granulosa and theca cells, and that systemic use of GH in rats was apparently able to increase intraovarian IGF-I production (Davoren and Hsueh 1986).

Objectives.

The aim of these studies was to determine whether, in patients who responded poorly to clomiphene citrate and hMG prior to IVF-ET, cotreatment with GH would improve the ovarian response. To investigate the possible mechanisms of action of GH, growth factors and steroids in serum and follicular fluid were also studied.

Open study - clinical results.

Initially, eight women who, despite having received large doses of gonadotrophins, had failed to have an adequate follicular response, were given GH in an uncontrolled, open study. Two patients were found to have raised early follicular phase gonadotrophins and their results will be discussed separately.

Perimenopausal Patients Case Reports.

Patient 1:

A thirty six year old nulliparous woman presented with seven years of primary infertility associated with bilateral hydrosalpinges. Her menstrual cycle varied between 21 and 61 days but was usually every 27 days. In her first treatment cycle she was given 100mg CC for 5 days with 3 amps of hMG daily for 6 days. One follicle developed on the right ovary and her urinary oestrogen excretion did not exceed 100nmol/24 hrs. The treatment was discontinued.

In a subsequent treatment cycle, 4 amps of hMG were given daily following the CC but there was no follicular development and no oestrogen rise. She was then treated with intranasal buserelin from the first day of her cycle with 6 amps of hMG for 3 days and then 10 amps of hMG for 3 days. One follicle developed on the right ovary and the treatment was again discontinued. She was finally given 6 amps, then 10 amps, of hMG daily in association with the CC but no follicles were detected on ultrasound scanning of her pelvis. The total dose of hMG administered was 70 amps.

The serum concentration of FSH in the early follicular phase of a non treatment cycle was 17.4iu/L. Her LH concentration was 18.5iu/L and serum oestradiol 149 pmol/L. Treatment with GH (24iu on alternate days concurrent with the hMG) was added to her CC and hMG treatment in the next treatment cycle. In total 64 amps of hMG were given with 96iu of GH. No follicles developed and her maximum urinary oestrogen excretion was 141nmol/24h. She was referred to the ovum donation programme.

Patient 2:

After 10 cycles of failed donor insemination this 39 year old woman requested treatment with IVF-ET. She had a regular cycle of 32 days and had been attempting to conceive for 16 years. In her first ovarian stimulation cycle, CC was administered for 5 days with 4 amps of hMG daily given for 7 days. Three follicles developed, in association with a urinary oestrogen excretion of 400 nmol/24 hrs but no oocytes were subsequently recovered. In her next treatment cycle, CC was given for 5 days with 6 amps of FSH (Metrodin, Serono, Welwyn Garden City, U.K.) for 5 days followed by 6 amps of hMG for 6 days. Two follicles developed but the treatment was abandoned.

Her FSH concentration in serum on day three of the cycle was 18.6iu/L, the LH concentration was 12.9iu/L and oestradiol 146pmol/L. Following CC therapy, six amps of hMG were given for 6 days with GH 24iu on alternate days until the 9th day of the cycle. Five thousand units of hCG were then administered as 4 follicles greater than 14mm in diameter had developed with a urinary oestrogen excretion of 476nmol/24hrs. No oocytes were collected and the relative paucity of granulosa cells within the follicular fluid suggested the structures were cysts. She was advised to discontinue treatment.

Open study of GH cotreatment in women with normal FSH.

Patients:

Six other women were recruited into the open study of GH cotreatment. The patients had a median age of 35 years (range 33 to 42) and all had serum concentrations of FSH less than 15iu/L. Three patients had tubal damage, two had unexplained infertility and one failed donor insemination as their reason for undergoing IVF-ET.

The group of women had a median of 4 previous cycles (range 3 to 7) and had required a median of 51 amps (range 36 to 80) of gonadotrophins per cycle to stimulate their ovaries in their previous treatment cycle (Table *III*-1). Despite these large doses of hMG, a median of only 1 follicle had developed in the prestudy cycles with a median excretion of 217 nmol/24h of oestrogen on the day of hCG administration or day on which the cycle was abandoned. Only two of the 6 women had had an oocyte collection in their prestudy cycle: one woman had 3 oocytes collected and the other woman had 4 oocytes collected but none of these fertilised.

Results:

One patient had one cycle, four patients had two and one patient had three cycles of cotreatment with GH. Thus 12 ovarian stimulation cycles were complemented with GH.

Comparing the results from the pre-study cycles with the first GH augmented cycles, there was no difference in the amount of hMG required but the follicular response was significantly improved (Table *III*-1). Larger amounts of oestrogens were excreted in the urine on the day of hCG administration. After GH treatment, all six women who had oocyte collections and had between 0 and 9 oocytes collected.

Table *III*-1. Open study of cotreatment with GH

	Previous cycles (n=6)	First GH cycles (n=6)	*p=	Second GH cycles (n=5)
Dose of hMG (amps)	51 (36-80)	48 (36-64)	0.14	70 (36-80)
Follicles ≥ 14mm	1 (0-6)	3 (2-5)	0.04	5 (2-6)
Urinary E day of hCG@	217 (124-663)	584.5 (227-3188)	0.03	521 (346-1183)
Oocytes collected	3.5 (3-4) (n=2)	3 (0-9) (n=6)		5 (1-6) (n=5)

Results presented as medians (range). @*=nmol/24hrs*
**p=Wilcoxon's signed rank test. (numbers of oocytes collected were not analysed as numbers too small)*

Forty oocytes were retrieved from five collections, 24 (60%) of which fertilised and 15 (38%) of which cleaved. Embryo replacements were performed in nine of the 12 cycles. Three patients had 1 embryo, three patients had 2, one had 3 and two patients had 4 embryos transferred.

In 3 of the GH complemented cycles of treatment, spontaneous LH surges occurred prior to oocyte collection but this was only diagnosed in retrospect as daily LH monitoring was not part of the routine management. Oocyte collections were undetaken in these women as spontaneous follicular rupture had not yet occurred. In one of the women no oocytes were collected, in a second woman two oocytes were retrieved one of which fertilised and cleaved and in the third woman one oocyte was collected which did not fertilise.

Randomised placebo controlled study of cotreatment with GH in patients receiving clomiphene citrate and hMG.

Following the encouraging results from the patients with normal serum gonadotrophin concentrations a randomised, double blind, placebo controlled trial of GH cotreatment in patients with suboptimal follicular development was designed. The study also included an open, cross over arm in which those patients who received placebo were offered GH in their subsequent treatment cycle.

Patients.

Twenty women who had responded suboptimally to clomiphene citrate and gonadotrophin stimulation were recruited for this study. They were all less than 40 years old (median age 36 years, range 29-39 years) and had serum FSH concentrations of less than 15iu/L in the early follicular phase. One patient withdrew prior to commencing the treatment due to marital problems.

In 9 women, the previous cycle of treatment had been abandoned because of inadequate follicular response (median number of follicles = 2, range 1-2) or poor oestrogenic response (median urinary oestrogen concentration = 242 nmol/24hrs, range 110-566 nmol/24hrs). Of the remaining ten patients, eight had 3 or fewer oocytes collected and 2 women had 2 or fewer embryos transferred. The suboptimal response in these previous cycles occurred despite the women receiving a starting dose of 300u (4 amps) or more of hMG daily for ovarian stimulation (median 4amps, range 4-6 amps). The total dosage of hMG used per cycle ranged between 20 and 44 amps (median 30 amps). The women had had a median of 2 previous cycles of treatment (range 1 - 4).

The indication for IVF-ET was tubal damage in 10 women, unexplained infertility in 6 and failed donor insemination in 3 women. The median duration of infertility was 5.5 years (range 2-11 years). In 12 women, all of whom had menstrual cycles of 6 weeks duration or less, a baseline ovarian ultrasound scan performed in a non treatment cycle had demonstrated a polycystic pattern (Adams *et al* 1985). The median LH concentrations, measured on day 2 of the cycle, were not different between patients with ultrasound diagnosed PCO (4.6 iu/L (range 3.5-9.9iu/L)) and those with ovaries that were normal on ultrasound scanning (4.1 iu/L (range 1.2 -10.4iu/L))($p=0.13$). There were no differences in the women's ages or duration of infertility in the two groups.

Eleven women were primigravid, 4 had had one miscarriage and 4 women had one child. The median body mass index for the whole group was 21.7 kg/m^2 (range 19.5-37), with no difference between patients with ultrasound diagnosed PCO and those with normal ovaries.

Results.

All women:

Nine women received placebo in their first cycle of treatment, 8 of whom went on to receive GH in their next treatment cycle. Ten patients received GH in their first study cycle.

Group comparisons:

Comparing the results from the group of nine women who received placebo with the group of ten women who received GH there was no difference in the number of follicles which developed, the amount of urinary oestrogen produced nor in the number of oocytes collected. Significantly less exogenous gonadotrophin was, however, used in the placebo group (Table *III*-2).

Table *III*-2: Group comparisons of placebo (n=9) and GH cycles (n=10)

	Placebo	GH	#p=
Dose of hMG (amps)	24 (20-32)	31 (20-52)	0.01
Follicles ≥ 14mm	3 (1-7)	3 (0-6)	0.42
Urinary E day of hCG@	328 (139-796)	441 (125-1013)	0.28
Serum E_2 day of hCG~	2030 (903-4185)	2250 (200-6360)	0.26
Oocytes collected	3 (1-6) (n=7)	3 (2-6) (n=8)	0.40

Results presented as medians (range). @=nmol/24hrs; ~=pmol/L.
#p=Mann-Whitney test.

Comparison with pretreatment cycles:

Comparing the results from the pretreatment cycles with the cycles in which placebo was given (n=9), significantly less hMG was administered (p=0.05) but there was no significant change in any other parameter (Table *III*-3).

Table *III*-3: Paired comparison of prestudy cycles with placebo cycles (n=9)

	Prestudy	Placebo	*p=
Dose of hMG (amps)	28 (20-36)	24 (20-32)	0.05
Follicles ≥14mm	2 (1-4)	3 (1-7)	0.08
Urinary E day of hCG@	279 (110-855)	328 (139-796)	0.48
Oocytes collected	2 (2-3) (n=3)	3 (1-6) (n=3)	0.50

Results presented as medians (range). @=nmol/24hrs.
**p=Wilcoxon's signed rank test.*

In the cycles in which GH was given in a randomised manner (n=10), there was no significant change in any parameter measured compared with the prestudy cycles (Table *III*-4).

Table *III*-4: Paired comparisons of prestudy cycles with first GH cycles (n=10)

	Prestudy	First GH	*p=
Dose of hMG (amps)	33 (24-44)	31 (20-52)	0.44
Follicles ≥14mm	3 (2-4)	3 (0-6)	0.36
Urinary E day of hCG@	362 (242-610)	441 (125-1013)	0.18
Oocytes collected	3 (1-5) (n=5)	3 (2-6) (n=5)	0.43

Results presented as medians (range). @=nmol/24hrs.
**p=Wilcoxon's signed rank test.*

Paired placebo and subsequent GH cycles:

Of the nine women who were randomised to receive placebo initially, eight went on to receive GH in a subsequent treatment cycle but there was no improvement in outcome (Table *III*-5).

Table *III*-5: Paired comparisons of placebo and GH cycles (n=8)

	Placebo	GH	*p=
Dose of hMG (amps)	24 (20-32)	31 (20-36)	0.09
Follicles ≥ 14mm	2.5 (1-7)	3 (1-7)	0.07
Urinary E day of hCG@	297 (139-796)	391 (328-810)	0.34
Serum E_2 day of hCG~	2107 (903-4185)	2280 (1301-3475)	0.42
Oocytes collected	3 (1-6) (n=6)	2 (1-5) (n=6)	0.35

Results presented as medians (range). @=nmol/24hrs; ~=pmol/L.
**p=Wilcoxon's signed rank test.*

Women with ultrasonically normal ovaries.

Three women with normal ovaries initially received placebo and four women received GH. All three women who were given placebo went on to receive GH in their next treatment cycles. There was no difference in the response in patients between the cycles in which they received GH and those in which they received placebo (Table *III*-6).

Table *III*-6: Group comparisons of placebo and GH cycles in women with normal ovaries

	Placebo (n=3)	GH (n=4)	#p=
Dose of hMG (amps)	24 (24-32)	40 (20-52)	0.18
Follicles $\geq$ 14mm	2 (1-3)	2.5 (0-3)	0.43
Urinary E day of hCG@	484 (139-708)	317 (125-603)	0.24
Serum E_2 day of hCG~	2616 (1084-3520)	2072 (200-2431)	0.08
Oocytes collected	4 (2-6) (n=2)	2.5 (2-3) (n=2)	0.34

Results presented as medians (range). @=nmol/24hrs; ~=pmol/L.
#p=Mann-Whitney test.

Pairing the study cycles with the pretreatment cycles there was no significant change with placebo or GH. Nor was there a significant difference when comparing the placebo cycles with the subsequent GH cycles.

Women with ultrasonically diagnosed PCO:

In contrast, when the results from patients with ultrasound diagnosed PCO (n=11) were analysed separately, significantly higher concentrations of serum E_2 were produced on the day of hCG administration during the cycles with GH cotreatment (Table *III*-7); significantly more hMG had, however, been given in the GH cycles.

Table *III*-7: Group comparisons of placebo (n=6) and GH cycles (n=6) in women with PCO

	Placebo	GH	#p=
Dose of hMG (amps)	24 (20-30)	30 (20-36)	0.01
Follicles ≥ 14mm	3 (2-7)	3.5 (2-6)	0.40
Urinary E day of hCG@	297 (168-796)	488 (354-1013)	0.07
Serum E_2 day of hCG~	1814 (903-4185)	2631 (1834-6360)	0.05
Oocytes collected	3 (1-6) (n=3)	3 (2-6) (n=4)	0.46

Results presented as medians (range). @=nmol/24hrs; ~=pmol/L.
#p=Mann-Whitney test.

In the women with PCO, analysis of the paired data showed that treatment with placebo (n=6) did not improve outcome compared with prestudy cycles (Table *III*-8).

Table *III*-8: Paired comparison of prestudy cycles with placebo cycles in women with PCO (n=9)

	Prestudy	Placebo	*p=
Dose of hMG (amps)	24 (20-36)	24 (20-32)	0.20
Follicles ≥14mm	2 (2-4)	3 (2-7)	0.13
Urinary E day of hCG@	314 (110-855)	297 (168-796)	0.29
Oocytes collected	2 (2-3) (n=3)	3 (1-6) (n=3)	0.10

Results presented as medians (range). @=nmol/24hrs.
**p=Wilcoxon's signed rank test.*

Analysis of paired data of GH cotreatment, received initially in the randomised manner (n=6), compared with prestudy cycles demonstrated that there was no improvement in outcome with GH treatment (Table *III*-9).

Table *III*-9: Paired comparison of prestudy cycles with first GH cycles (n=6) in women with PCO.

	Prestudy	First GH	*p=
Dose of hMG (amps)	36 (28-44)	30 (20-36)	0.17
Follicles ≥14mm	3 (2-4)	3.5 (2-6)	0.20
Urinary E@ day of hCG	481 (312-610)	488 (354-1013)	0.26
Oocytes collected	3 (2-5) (n=4)	3 (2-6) (n=4)	0.50

*Results presented as medians (range). @ =nmol/24hrs *p=Wilcoxon's signed rank test.*

Five of the six women with PCO who initially received placebo went on to receive GH. This additional treatment was not associated with any significant improvement in outcome (Table *III*-10).

Table *III*-10: Paired comparisons of placebo and GH cycles in women with PCO (n=5)

	Placebo	GH	*p=
Dose of hMG (amps)	24 (20-30)	24 (20-36)	0.50
Follicles ≥ 14mm	3 (2-7)	3 (2-7)	0.17
Urinary E day of hCG@	266 (168-796)	456 (354-810)	0.21
Serum E_2 day of hCG~	1598 (903-4185)	2210 (1723-2630)	0.50
Oocytes collected	3 (1-4) (n=4)	3 (1-5) (n=4)	0.39

Results presented as medians (range); @=nmol/24hrs, ~=pmol/L
**p=Wilcoxon's signed rank test.*

Comparison of results from women with normal and polycystic ovaries:

Comparing the results from the women with normal ovaries with those women with PCO there was no difference in their prestudy cycles nor in their response to placebo.

Analysing the results from the randomised GH cycles, however, the women with PCO responded with significantly higher urinary oestrogen excretion compared with the women with normal ovaries but there were no other significant differences in their cycles (Table *III*-11).

Table *III*-11: Group comparisons of first GH cycles in women with normal ovaries (n=4) and women with PCO (n=6).

	GH - normal ovaries	GH - PCO	#p=
Dose of hMG (amps)	40 (20-52)	30 (28-36)	0.19
Follicles $\geq$ 14mm	2.5 (0-3)	3.5 (2-6)	0.09
Urinary E day of hCG@	317 (125-603)	488 (354-1013)	0.04
Serum E_2 day of hCG~	2072 (200-2431)	2681 (1834-6360)	0.10
Oocytes collected	2.5 (2-3) (n=2)	3 (2-6) (n=6)	0.14

Results presented as medians (range); @=nmol/24hrs, ~=pmol/L
#p=Mann-Whitney test.

Comparing the results from all the GH cycles in women with PCO with those women with normal ovaries, more follicles developed and more oestrogens were excreted in the PCO group (Table *III*-12).

Table *III*-12: Group comparisons of all GH cycles in women with normal ovaries (n=7) and women with PCO (n=11).

	Normal ovaries	PCO	#p=
Dose of hMG (amps)	32 (20-52)	30 (20-36)	0.07
Follicles $\geq$ 14mm	3 (0-3)	3 (2-7)	0.04
Urinary E day of hCG@	328 (125-603)	475 (354-1013)	0.01
Serum E_2 day of hCG~	1852 (200-2560)	2210 (1723-6360)	0.08
Oocytes collected	2 (2-3) (n=4)	3 (1-6) (n=11)	0.07

Results presented as medians (range); @=nmol/24hrs, ~=pmol/L
#p=Mann-Whitney test.

Cycles abandoned.

Treatment was abandoned in five cycles because of poor follicular response. In the 2 placebo cycles which were abandoned, one patient with normal ovaries subsequently had a GH cycle which was also cancelled and the other patient had PCO and had a successful subsequent GH cycle. The 3 cycles of GH cotreatment which were abandoned were all in patients with normal ovaries, 2 of whom had GH in their first study cycle.

One patient had a spontaneous LH surge prior to hCG administration whilst receiving placebo. She had PCO and did not have a surge in her subsequent GH cycle. Three women had LH surges during GH cotreatment, all of whom had normal ovaries on ultrasound scanning of the pelvis. Thus 43% of patients with normal ovaries who received GH had a surge of LH prior to hCG administration. No patient with PCO had an LH surge during a GH augmented cycle.

Fertilisation and cleavage.

Of the seven women who had oocyte collection after placebo cycles, 5 had embryo replacements. Three women had 1 embryo replaced, one woman had two replaced and one woman had 4 embryos replaced.

Of the eight women who had oocyte collections in their first GH cycles, 5 had embryo transfers. One woman had 1 embryo replaced, 2 women had two replaced, one had three and one woman had 4 embryos replaced.

Of the 15 oocyte collection performed following all GH cycles, 9 women had embryo transfers. There were no pregnancies following any of the embryo replacements.

Biochemical and haematological parameters.

There was no alteration in the biochemical measurements throughout treatment; for example median plasma urea concentration was 4.0 mmol/L before GH administration (range 2.5-4.8 mmol/L) and 4.1 mmol/L after (range 2.7-5.0 mmol/L) ($p=0.3$) compared with 3.8 mmol/L before placebo administration (range 2.4-4.9 mmol/L) and 3.65 mmol/L after (range 1.9-4.9 mmol/L)($p=0.45$).

The only exceptions were the plasma alanine transferase concentrations which decreased during GH treatment (17 iu/L pre GH (range 5-46iu/L), 15 iu/L following treatment (range 5-43 iu/L), $p=0.05$) compared with placebo treatment (14 iu/L pre placebo (range 13-20 iu/L), 27 iu/L after (range 8-50 iu/L), $p=0.39$).

Serum FSH concentrations rose, as expected, with exogenous gonadotrophin administration (Fogel *et al* 1972) but with no difference between GH and non GH cycles nor between women with PCO and those with normal ovaries.

Serum and follicular fluid peptide and steroid concentrations.

Patients.

In the open study, 12 cycles were monitored, with oocyte recoveries performed in each cycle. A polycystic ovarian pattern on ovarian scanning was detected in 4 of the women who went on to have eight oocyte collections.

Clear follicular fluid (Table *III*-13) was obtained from 19 follicles aspirated during the oocyte recovery procedure of eleven cycles. In one woman the follicular fluid obtained was unsuitable for analysis. One patient had had follicular fluid saved prospectively from a prestudy cycle in which the same initial daily dose of CC and gonadotrophins as in the GH cycle had been used.

Table *III*-13. Samples collected for analysis of IGF-I and -II and E_2 concentrations.

	Patients	Cycles monitored	OC with FFL saved	Follicles aspirated
Open Study:				
GH cycles	6	12*	11	19
Prestudy cycles	(1)	0	1	2
PL controlled study:				
GH cycles	18	20#	17	31
PL cycles	9	9	7	14
Prestudy cycles	(4)	0	4	6
Total	25	41	40	72

OC=oocyte collection

**=1 patient had 3 cycles, 4 patients had 2 and 1 patient had one cycle.*
#=16 patients had 1 GH cycle and 2 patients had 2 cycles.

In the subsequent placebo controlled study, twelve patients had ultrasound diagnosed PCO and seven a normal appearance on ovarian scanning. Nine women received placebo initially, of whom 8 went onto receive GH. Ten women had GH in their first treatment cycle, 2 of whom had a second GH cycle.

Ultimately, sixteen patients had one GH cycle and two had two GH augmented cycles. Thus 20 GH and 9 PL cycles were studied. Five cycles were abandoned before hCG administration: 2 placebo and 3 GH cycles.

Follicular fluid from 45 follicles was collected from 24 cycles in this study: 14 follicular fluids from 7 placebo cycles and 31 follicular fluids from 17 GH cycles. Follicular fluid had also been collected prospectively in the pre study cycle in four patients. With the exception of the GH injections, the same type and dosage of drugs had been used as in the study cycles: 6 follicular fluids were subsequently analysed from these prestudy cycles.

In summary, in both the open and placebo controlled trials, a total of twenty four women received from one to three cycles of GH treatment and one woman had a placebo cycle only. Follicular fluid was collected from 28 GH cycles and 7 PL and 5 pretreatment cycles. Twenty two follicular fluids were subsequently analysed from 12 PL or prestudy cycles and 50 from 28 GH augmented cycles. A total of nine women had a normal appearance on ultrasound scanning of their ovaries and sixteen had ultrasound diagnosed polycystic ovaries.

Samples.

Serum was saved during all treatment cycles on the day of starting CC and daily from day 8 until the day of hCG administration. Blood was also collected immediately before the oocyte collection. In total, 41 cycles were monitored: 32 GH and 9 placebo cycles.

Ultrasound directed oocyte collection was performed by the trans abdomino-vesical route and clear follicular fluid collected as described in Chapter *11*. In total, 72 follicular fluids were analysed: 50 from GH augmented cycles and 22 from placebo or prestudy cycles.

The sera and follicular fluids were assayed for growth hormone, IGF-I and IGF-II concentrations as described in Chapter *II*. FFL was also assayed for oestradiol, progesterone, testosterone, insulin and GH. Two patients did not have serum saved at the time of oocyte recovery and one patient had only heavily blood stained follicular fluid collected which was discarded. Paired analyses of the results obtained on serum taken at the time of oocyte recovery, and follicular fluid were, therefore, performed on 26 GH and 7 PL cycles. A mean of the follicular fluid results was calculated for each patients' cycle, thereby giving results for analysis of 28 GH and 12 PL or previous cycles.

Results.

IGF-I concentrations.

Serum concentrations:

Pretreatment concentrations of IGF-I in serum were similar in those patients who received PL (32.7 nmol/L, range 15.3 - 44.8 nmol/L) and those who received GH (33.0 nmol/L, range 12.1 - 54.6 nmol/L) (p=0.38)(Fig *III*-1). There was no difference in concentrations in patients with ultrasound diagnosed PCO (35.1 nmol/L, range 15.3 - 54.6 nmol/L) compared with those in women with normal ovaries (32.7 nmol/L, range 12.1 - 53.4 nmol/L) (p=0.33).

IGF-I concentrations in serum did not change during administration of placebo (p=0.6), but rose significantly during GH treatment (p=0.05)(Fig *III*-1). Analysing the patients in subgroups dependent upon their ovarian ultrasound findings, the women with ultrasonically normal ovaries did not show a significant response in serum IGF-I concentrations to GH co-treatment (p=0.9) (Fig *III*-2). It was, however, in the subgroup of patients with PCO that this response occurred (p=0.008). Neither subgroup of patients showed a response in serum IGF-I concentrations during placebo treatment (p=0.9 for women with normal ovaries; p=0.2 for women with PCO). By the day of oocyte recovery, either three or four days after the final GH injection, depending on the day of hCG administration, the IGF-I concentrations

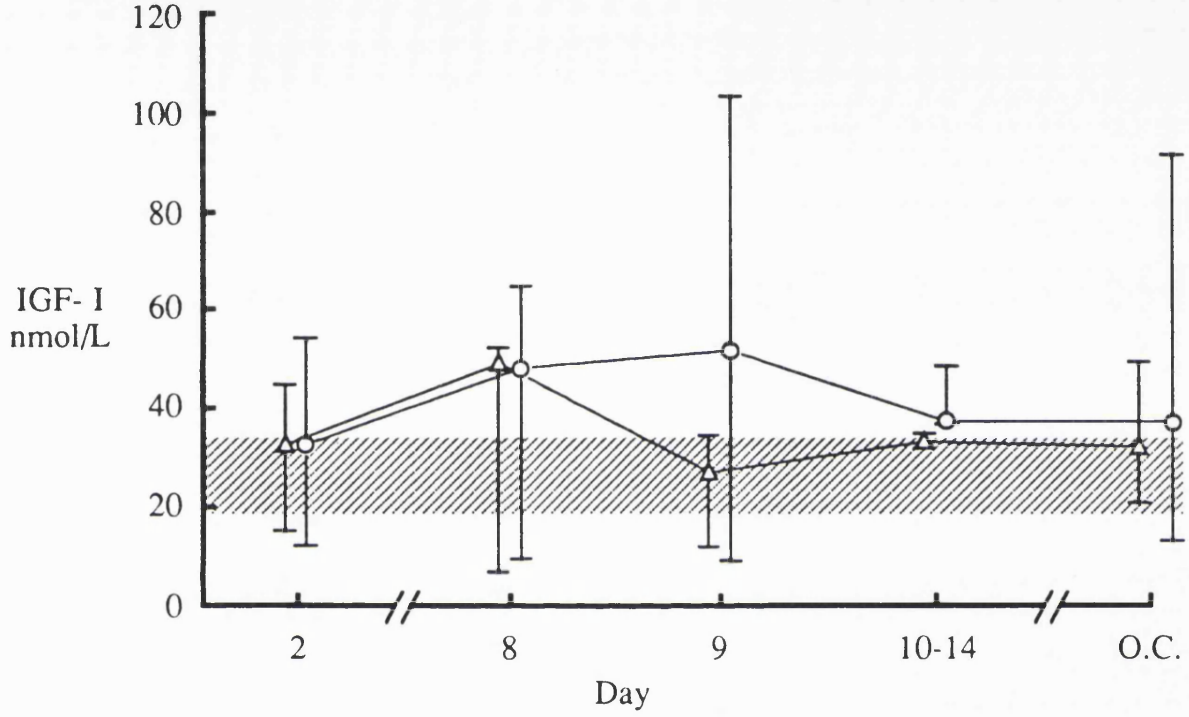


Fig *III*.1 Serum concentrations of IGF-I (medians and range) in PL (Δ) (n=9) and GH (o) (n=32) cycles, taken before initiation of treatment (day 2), on days 8, 9, 10-14 of the cycle and on the day of oocyte collection (O.C.)

The normal range for IGF-I is shaded.

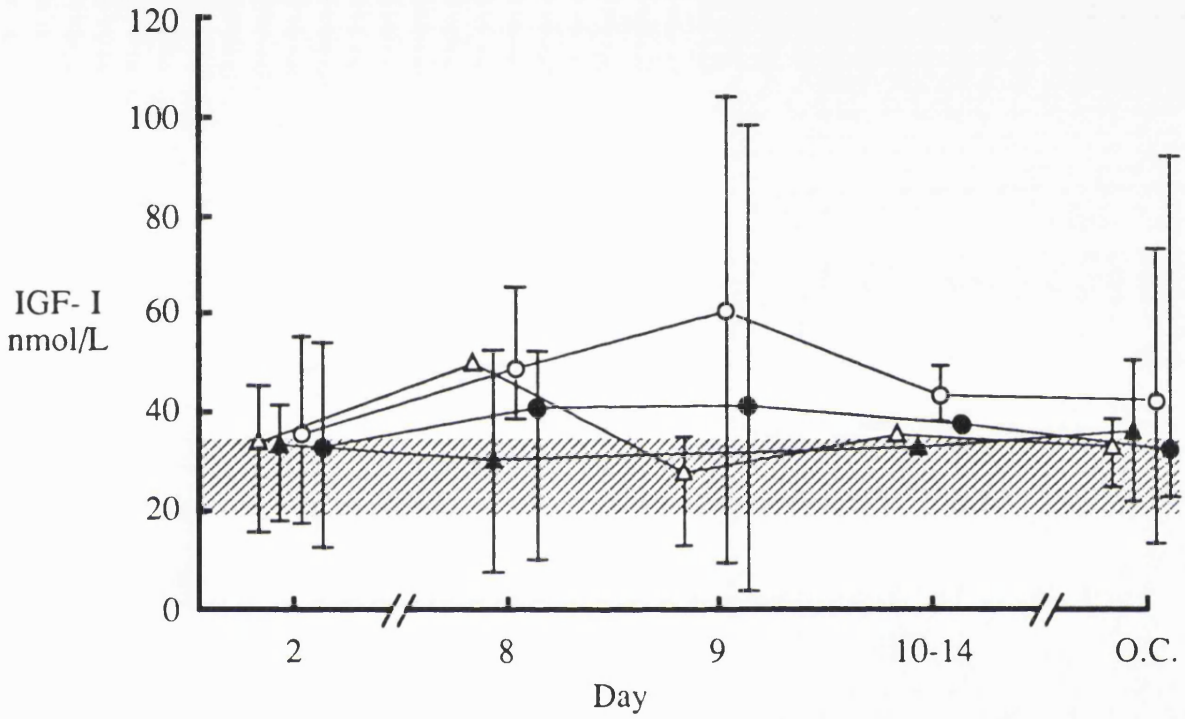


Fig *III*.2 Serum concentrations of IGF-I (medians and ranges) in women with normal ovaries who received PL (▲) (n=3) or GH (●) (n=13); and in women with PCO who received PL (△) (n=6) on GH (○) (n=19). Results are shown from before initiation of treatment (day 2), or days 8, 9, 10-14 of the cycle and on the day of oocyte collection (O.C.).

The normal range for IGF-I is shaded

had returned to pretreatment values. In the overall groups, there was no difference between those women who had received GH and those who had received PL (p=0.13), nor in the total group of patients with PCO or normal ovaries on the day of oocyte collection (p=0.17). The patients with PCO who received GH, however, had significantly higher concentrations of IGF-I on the day of oocyte recovery than before treatment with GH (p=0.04).

Serum IGF-I concentrations (36.6 nmol/L, range 14.0 - 92.1 nmol/L), at the time of oocyte recovery, were statistically higher than follicular fluid concentrations (29.9 nmol/L,range 14.3 - 61.5 nmol/L, p=0.02)(Fig *III*-3) in all groups, including those patients with PCO (p=0.03) and those patients who received GH (p=0.4).

Follicular fluid concentrations:

Follicular IGF-I concentrations, despite being lower than the serum concentrations, were higher in the GH cotreatment group than in the placebo or prestudy group (Table *III*-14) (p=0.05). The difference was attributable to the patients with PCO.

Table *III*-14. Group comparisons of concentrations of IGF-I in follicular fluid (nmol/L).

	GH cycles	PL/prestudy cycle	#p=
All women	32.2 (17.1-54.6) (n=28)	28.1 (14.3-61.5) (n=12)	0.05
Women with PCO	36.4 (18.1-51.0) (n=19)	29.1 (14.4-37.8) (n=8)	0.07
Women with normal ovaries	28.6 (17.1-54.6) (n=9)	20.1 (15.9-61.5) (n=4)	0.40

Results presented as medians (range).
#p=Mann-Whitney test.

There was no correlation between serum and follicular fluid IGF-I concentrations (correlation coefficient=0.14; p=0.15).

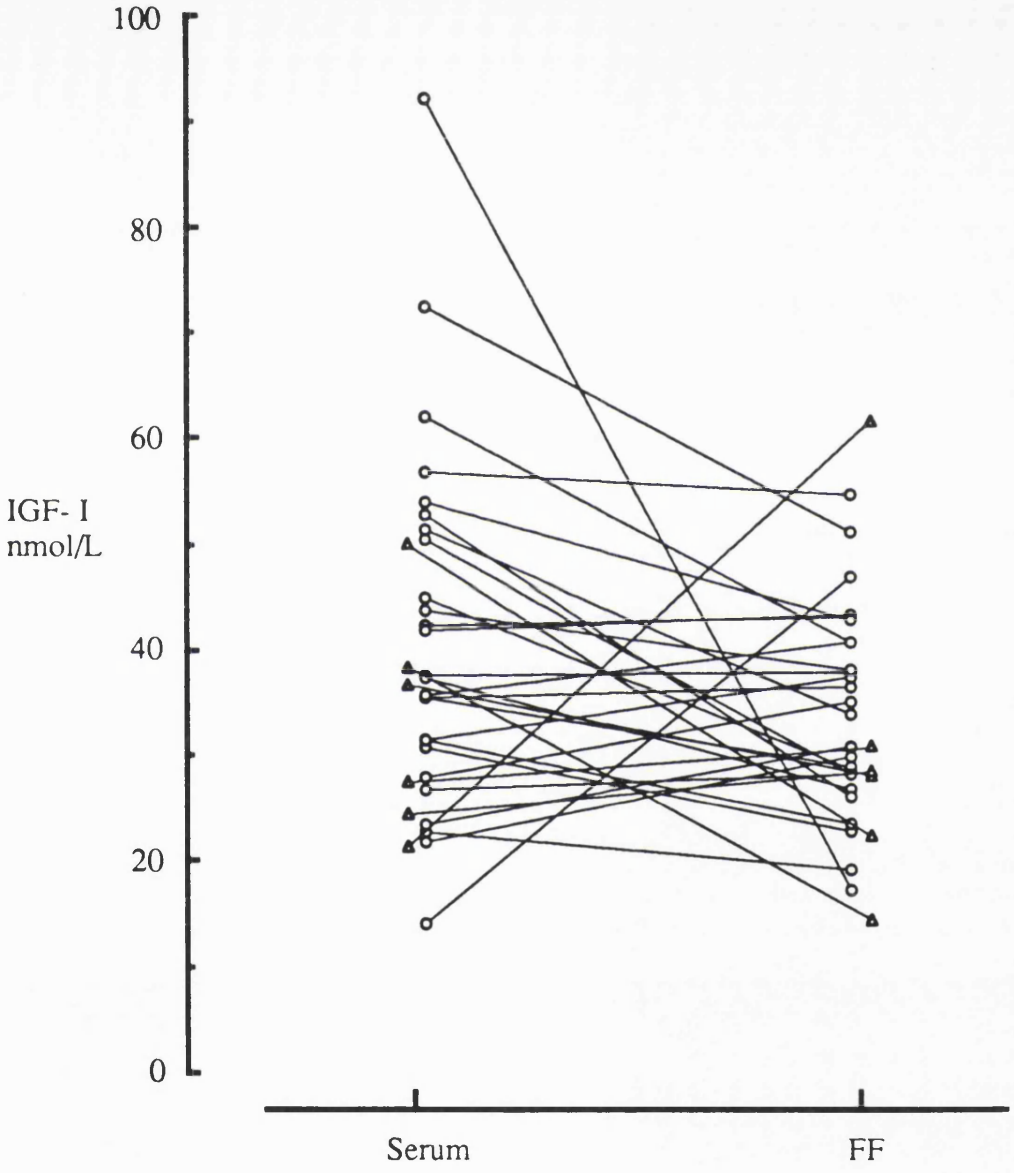


Fig *III*.3 Paired serum and FFL concentrations of IGF- I in women who received PL (Δ) (n=6) or GH (o) (n=26).

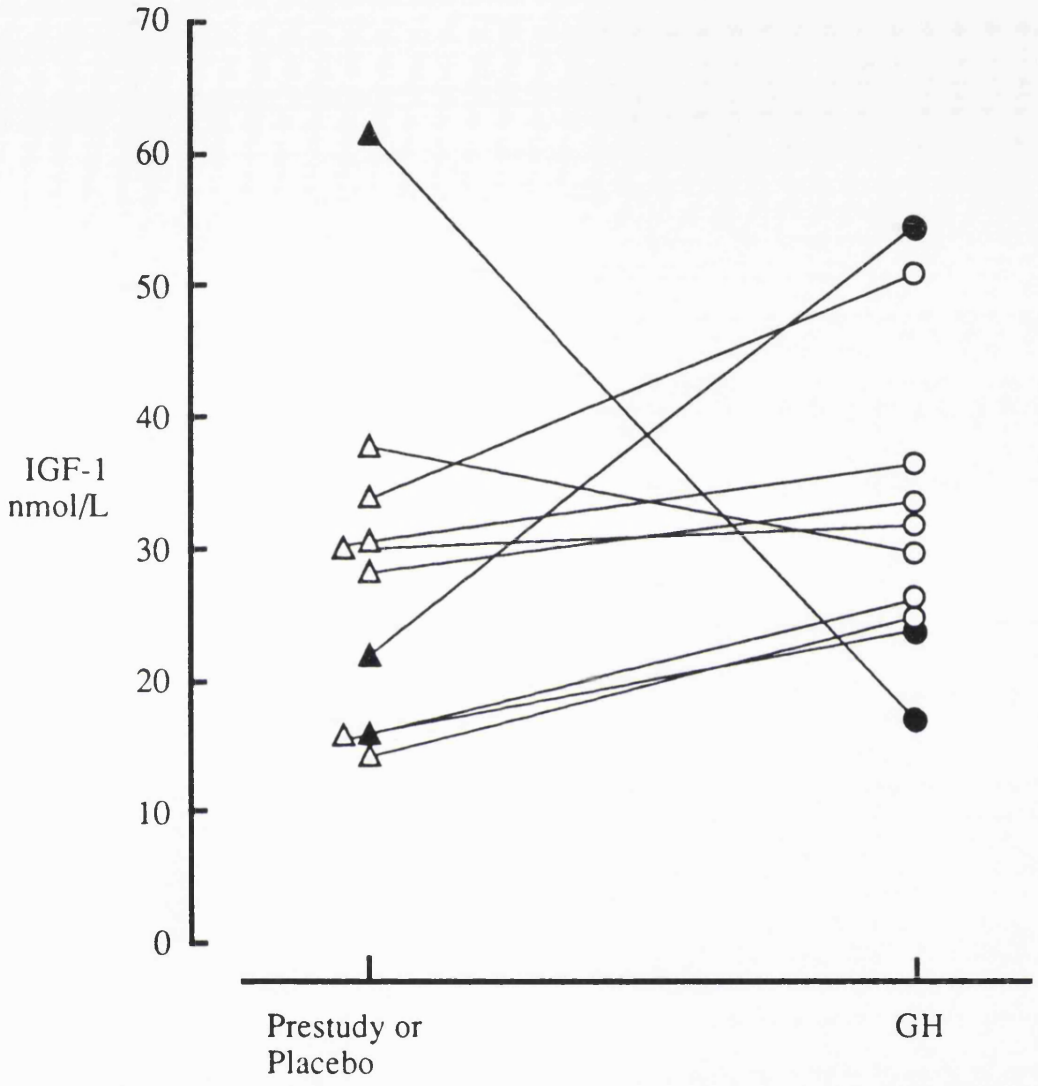


Fig *III*.4 Paired FFL concentrations of IGF-I from 3 women with normal ovaries in prestudy or placebo cycles (▲) and GH cycles (●); and 7 women with PCO in prestudy or placebo cycles (△) and GH cycles (○).

Ten patients had follicular fluid collected from both placebo or previous cycles and subsequently GH cycles; three had normal ovaries and seven had polycystic ovaries. Pairing the results in all ten patients, there was no difference in the concentrations of IGF-I in the PL or prestudy cycles (29.1 nmol/L, range 14.3 - 61.5 nmol/L) compared with the GH augmented cycles (30.8 nmol/L, range 17.1- 54.6 nmol/L) (p=0.11)(Fig *III*-4). In the subgroup of patients with PCO, however, there were significantly higher concentrations of IGF-I in GH treated cycles (31.8 nmol/L, range 24.7 - 51.0 nmol/L) compared with PL and prestudy cycles. (30.0, range 14.3 - 37.8 nmol/L) (p=0.05).

IGF-II concentrations.

Serum:

Baseline IGF-II concentrations in serum were not different between the groups: 80.1 nmol/L (range 35.2 - 219 nmol/L) in those patients who subsequently received GH and 82.3 nmol/L (range 57.7 - 118 nmol/L) in those women who received PL. Women who had ultrasound diagnosed PCO had median concentrations of 81.0 nmol/L (range 35.2 - 153 nmol/L) and those with normal ovaries 82.3 nmol/l (range 39.8-219 nmol/L).

During the ovarian stimulation cycle the concentration of serum IGF-II did not change significantly in any subgroup (p=0.36 in the GH group; p=0.08 in the PL group)(Fig *III*-5). There was no difference in serum IGF-II concentrations at the time of oocyte collection, either between GH treated patients and PL treated patients, or in comparison with baseline. The serum concentrations were, however, significantly higher than the follicular fluid concentrations (p=0.0001) (Fig *III*-6) both overall and in the subgroups.

Follicular fluid concentrations:

There was no difference in follicular fluid IGF-II concentrations between patients in the GH augmented cycles or in the PL cycles, nor between patients with PCO and those with normal ovaries (Table *III*-15).

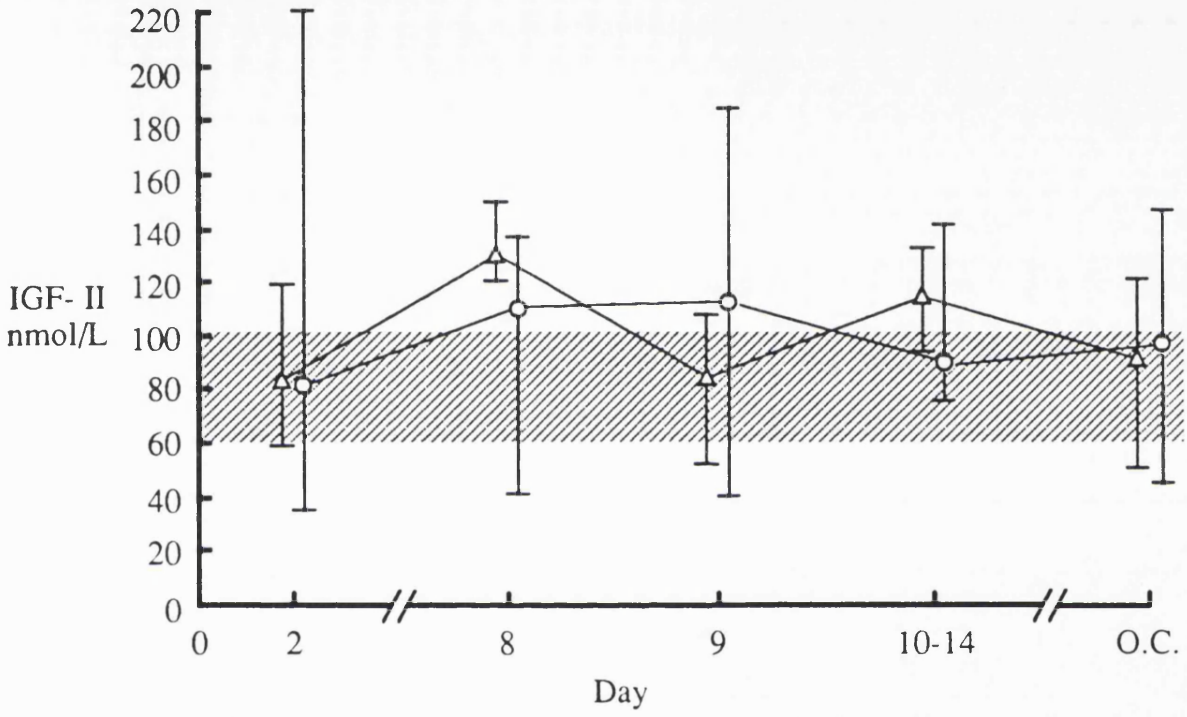


Fig *III*.5 Serum concentrations of IGF-II (medians and range) in PL (Δ) (n=9) and GH (O) (n=32) cycles, taken before initiation of treatment (day 2), on days 8, 9, 10-14 of the cycle and on the day of oocyte collection (O.C.).

The normal range for IGF-II is shaded.

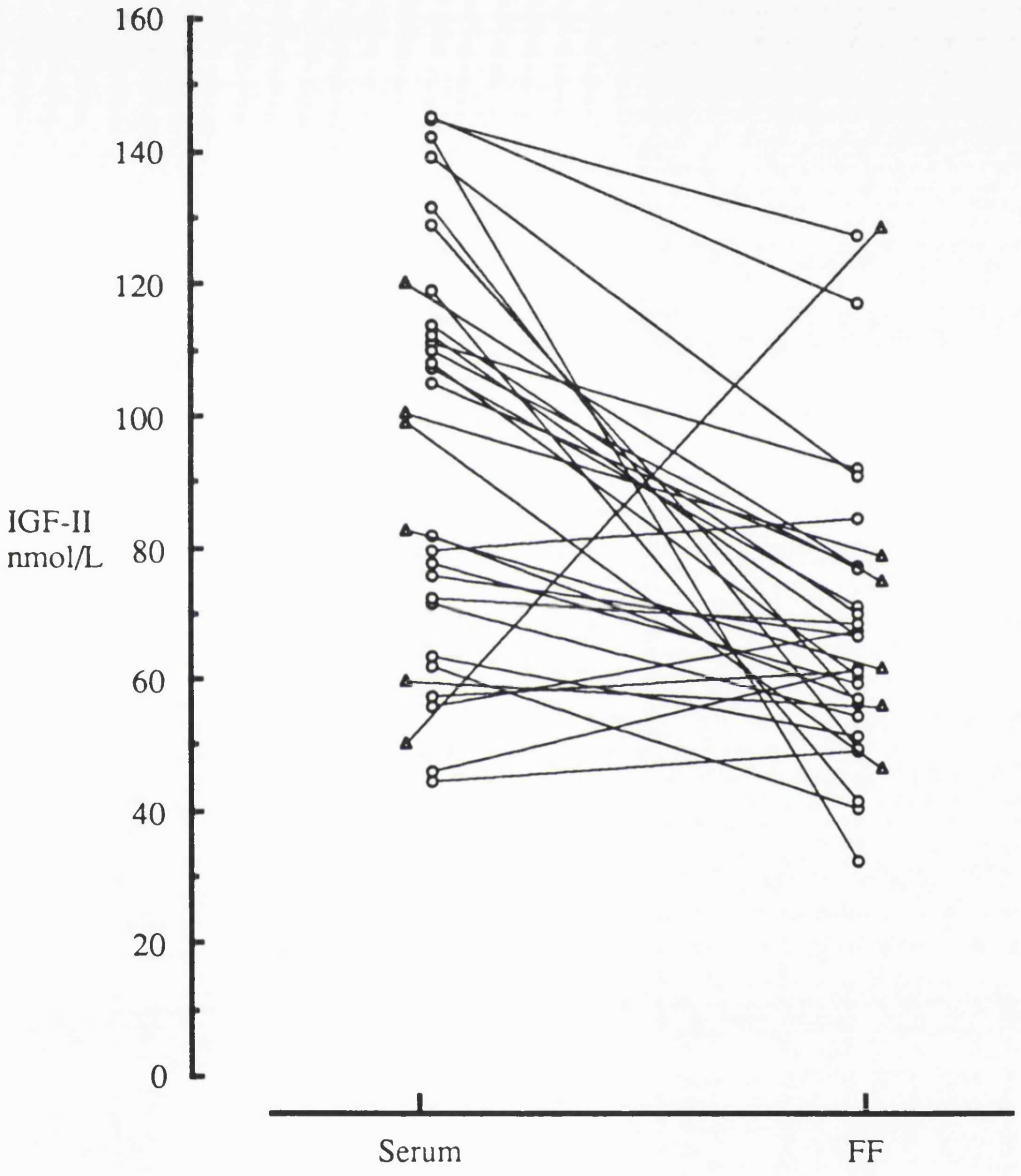


Fig *III*.6 Paired serum and FFL concentrations of IGF- II in women who received PL (Δ) (n=6) or GH (o) (n=26).

Table *III*-15. Group comparisons of concentrations of IGF-II in follicular fluid (nmol/L).

	GH cycles	PL/prestudy cycle	#p=
All women	61.7 (32.4-127.5) (n=28)	67.0 (30.0-128.5) (n=12)	0.49
Women with PCO	61.7 (40.4-127.5) (n=19)	60.7 (30.0-85.2) (n=8)	0.25
Women with normal ovaries	61.8 (32.4-84.3) (n=9)	61.6 (60.5-128.5) (n=4)	0.24

Results presented as medians (range).
#p=Mann-Whitney test.

Oestradiol.

Serum:

Oestradiol concentrations in serum rose significantly throughout treatment until the day of administration of hCG. There was no difference in E_2 concentrations in GH and PL treated groups at any stage of the cycle (Fig *III*-7) nor in the subgroup of women with normal or polycystic ovaries.

Relationship with IGF-I:

There was no correlation between serum E_2 concentrations and serum IGF-I concentrations on the day of oocyte recovery (correlation coefficient=-0.14; p=0.13) nor on the day of hCG administration (correlation coefficient=0.06; p=0.33).

There was no relationship between serum concentrations of E_2 on the day of hCG administration (correlation coefficient=-0.07; p=0.28), nor on the day of oocyte collection (correlation coefficient=0.12; p=0.16) with follicular fluid concentrations of IGF-I.

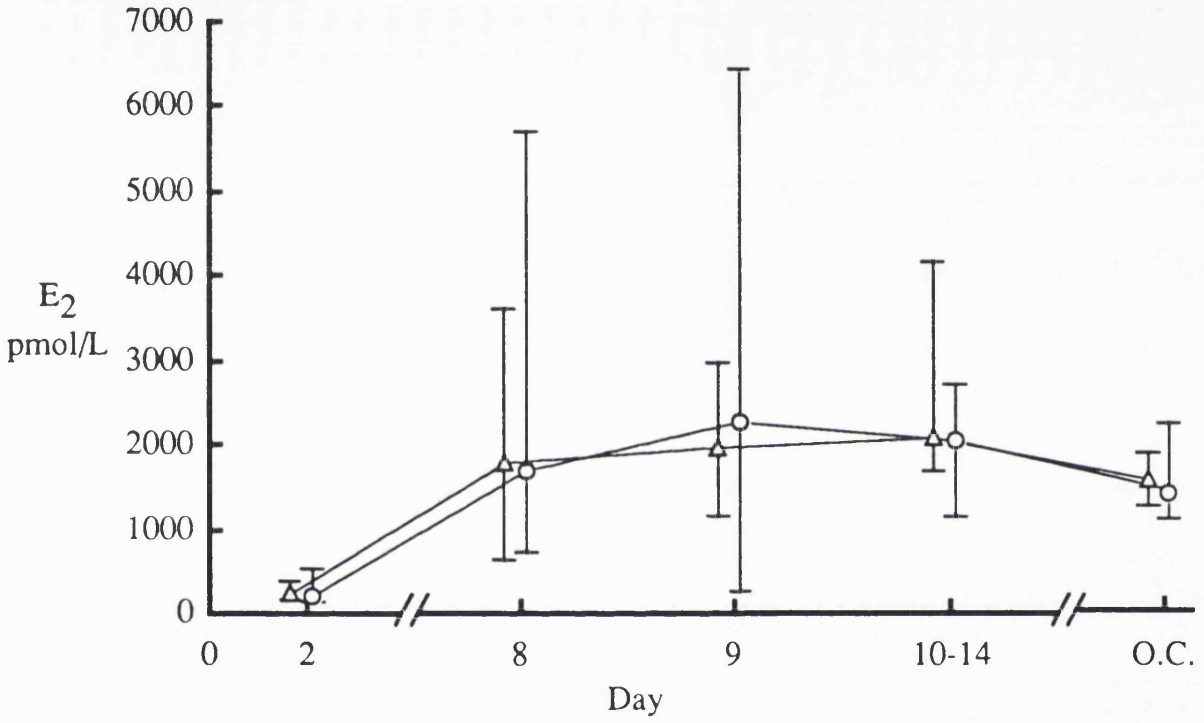


Fig *III*.7 Serum concentrations of E2 (medians and ranges) in PL (Δ) (n=9) and GH (o) (n=32) cycles, taken before initiation of treatment (day 2), on days 8, 9, 10-14 of the cycle and on the day of oocyte collection (O.C.).

Follicular fluid:

There were no significant differences in the follicular fluid concentrations of E_2 between those women who received GH and those who received placebo or were in prestudy cycles (p=0.17).

There were no differences in follicular fluid concentrations of E_2 within the GH (p=0.33) or placebo (p=0.29) groups whether the women had PCO or normal ovaries (Table *III*-16).

Table *III*-16. Concentrations of steroids in follicular fluid.

	E_2 (nmol/L)	Prog (μmol/L)	Testo (nmol/L)
All GH cycles	767 (513-1275) n=27	33.4 (12.2-90.6) n=29	10.4 (4.5-21.8) n=28
Women with PCO	758 (513-1275) n=19	26.6 (12.2-90.6) n=19	9.1 (4.5-12.6) n=18
Women with normal ovaries	778 (587-804) n=8	39.9 (31.8-85.9) n=10	11.8 (6.7-21.8) n=10
All PL cycles	850 (504-1652) n=11	41.8 (6.8-81.9) n=12	9.9 (5.0-15.2) n=12
Women with PCO	844 (504-1652) n=7	25.7 (6.8-81.9) n=8	10.6 (5.0-15.2) n=8
Women with normal ovaries	880 (752-954) n=4	49.9 (39.8-73.6) n=4	9.9 (7.4-12.4) n=4

Results presented as medians (range). Prog=progesterone; Testo=testosterone.

Serum concentrations of E_2, on the day of administration of hCG, correlated with follicular fluid concentrations of E_2 (correlation coefficient=0.25; p=0.02). There was however, no correlation between the serum E_2 concentrations on the day of oocyte collection and follicular fluid concentrations of E_2 (correlation coefficient=0.33; p=0.33).

Relationship with IGF-I:

Follicular fluid concentrations of E_2 did not correlate with serum concentrations of IGF-I (correlation coefficient=-0.13; p=0.16) nor with follicular fluid concentrations (correlation coefficient=-0.1; p=0.19)

Progesterone:

Follicular fluid:

There was no difference in follicular fluid concentrations of progesterone between those women receiving GH and those receiving placebo or being in the prestudy cycles. Concentrations of progesterone were, however, significantly lower in the women with PCO compared with those women with normal ovaries (p=0.001), particularly in the cycles with GH augmentation (Table *III*-16).

Testosterone.

Follicular fluid:

Analysis of the testosterone concentrations revealed a similar pattern to the progesterone concentrations. The concentration of testosterone in the follicular fluid of women receiving GH and in women receiving PL or in the pretreatment cycle was not significantly different. Surprisingly, however, women with normal ovaries who received GH had significantly higher concentrations of testosterone than women with PCO (p=0.01). (Table *III*-16).

Insulin.

Follicular fluid:

There was no significant difference in the intrafollicular concentrations of insulin between the GH cycles and the placebo or prestudy cycles; nor between women with PCO and normal ovaries (Table *III*-17).

Table *III*-17. Concentrations of insulin and GH in follicular fluid.

	Insulin (pmol/L)	GH (mU/L)
All GH cycles	50.5 (30.0-251.0) n=28	2.4 (0.4-11.2) n=29
Women with PCO	51.5 (30.0-251.0) n=18	2.3 (0.4- 8.7) n=19
Women with normal ovaries	49.3 (30.0-199.5) n=10	3.2 (1.3-11.2) n=10
All PL cycles	56.5 (36.5-192.5) n=11	1.8 (0.4- 6.3) n=11
Women with PCO	49.0 (36.5-192.5) n=7	1.7 (0.4- 4.8) n=8
Women with normal ovaries	94.5 (61.0-104.0) n=3	2.8 (1.4- 6.3) n=3

Results presented as medians (range).

Growth hormone.

Follicular fluid:

Concentrations of GH in follicular fluid were no different in those women who had received GH and those who had not whether they had PCO or normal ovaries (Table *III*-17).

Summary.

Eight women who were considered to be very poor responders to gonadotrophin stimulation were treated with GH 24iu on alternate days, given concurrently with hMG treatment. Of the 6 women with normal serum gonadotrophin concentrations only two women had had oocytes collected in their prestudy cycles, and none of the oocytes had fertilised. After cotreatment with GH, more follicles developed and more oestrogen was excreted on the day of hCG administration. In 3 cycles premature LH surges occurred but this was only diagnosed in retrospect.

The two women with raised serum gonadotrophins did not have an improved outcome with GH treatment and were dissuaded from continuing further therapy.

Nineteen women were then recruited into a randomised, double blind, placebo controlled trial. In this heterogeneous group of women who had previously responded suboptimally to CC and increasing doses of hMG, cotreatment with GH did not lead to a significant alteration in outcome. Significantly less gonadotrophin was, however, administered to the group of women receiving placebo compared with the group receiving GH. Comparison of paired results from prestudy cycles with those cycles with GH augmentation showed no improvement.

In a subgroup of patients with ultrasound diagnosed PCO those women who received GH initially produced more E_2 in serum on the day of hCG administration than the women receiving placebo but they were also given more hMG during the treatment cycle. Compared with women with normal ovaries, women with PCO who received GH in their first treatment cycle responded by producing significantly more urinary oestrogens. When comparing all of the GH cycles, more follicles developed in the women with PCO compared with the women with normal ovaries.

Treatment with growth hormone was not associated with any side effects and the only significant biochemical change was a decrease in serum alanine transferase concentrations.

Serum IGF-I concentrations were not altered by gonadotrophin treatment alone but did increase during treatment with GH. That this augmentation of circulating IGF-I concentration occurred to a greater degree in women with PCO is a novel observation. Women with normal ovaries showed no effect of GH cotreatment on their concentrations of IGF-I in serum. Both overall, and in the different subgroups, serum concentrations were consistently higher than follicular fluid concentrations.

Serum concentrations of IGF-II did not alter during cotreatment with GH. The concentrations in serum were significantly higher than in follicular fluid.

Serum E_2 concentrations rose throughout the treatment cycles with no differences between any of the groups. The concentrations of E_2 in follicular fluid showed no difference between the groups and showed no significant correlation with IGF-I concentrations in follicular fluid.

Follicular fluid concentrations of progesterone and testosterone and GH were lower in women with PCO compared to women with normal ovaries, particularly in GH cycles. There were no differences in the follicular fluid concentrations of insulin between different groups.

IV. Results 2-Buserelin and hMG study

Randomised double blind placebo controlled trial of cotreatment with GH in women receiving buserelin and hMG

Objectives.

The aim of the buserelin and hMG study was to determine whether, in women who were hypogonadotrophic in response to treatment with GnRH analogues, and subsequently received hMG for ovarian stimulation, treatment with GH was associated with an improved response.

Patients.

Twenty five women who had responded suboptimally to buserelin and hMG treatment were recruited for this study (see Chapter *II*). The aim of the slight change in entry criteria was to investigate a more responsive group than in the previous CC and hMG studies. None of the prestudy cycles had been abandoned.

Prior to treatement, there was no difference between those women who went on to receive GH and those who went on to receive PL in age, cycle length, number of previous cycles, parity or BMI (Table *IV*-1). The duration of infertility was, however, significantly longer in the women in the placebo group compared with those in the GH group ($p=0.014$).

Table *IV*-1. Patient data (medians and range)

	All women	GH cycles	PL cycles	Women with PCO	Women with normal ovaries
Age (yrs)	34 (27-37)	33 (27-37)	34 (29-37)	33.5 (29-37)	34 (27-37)
Length of infertility (years)	5 (2-12)	4 (2-9)	6.5 (3-12)	5 (3-12)	7 (2-12)
Cycle length (days)	28 (25-40)	28 (26-40)	28 (25-30)	28 (26-40)	28 (25-28)
BMI (kg/m_2)	21.5 (19.5-36.3)	21.1 (19.5-36.3)	21.9 (19.8-25.4)	21.5 (19.6-36.3)	20.1 (19.5-22.9)
Previous no. cycles	2 (1-3)	2 (1-3)	1.5 (1-3)	2 (1-3)	1 (1-2)

In the pre-recruitment cycles, the long regimen of buserelin had been used with hMG in all cases. Twelve women had had one previous attempt at IVF-ET, ten had had two and three women had had three previous attempts. Of the women who had had more than one previous attempt nine had had (an) addition cycle(s) with CC and hMG and two women had had a previous cycle using hMG alone.

There was no significant difference in the distribution of diagnoses between the groups ($chi^2 = 4.716$, $p=0.3176$) (Table *IV*-2).

Table *IV*-2. Diagnoses

	All women (n=25)	GH cycles (n=13)	PL cycles (n=12)	Women with PCO (n=18)	Women with normal ovaries (n=7)
Unexplained infertility	7	3	4	5	2
Failed D.I.	2	0	2	1	1
Male antibodies	2	1	1	2	0
Tubal damage	11	8	3	7	4
Oligospermia	3	1	2	3	0

The fasting glucose concentrations were all normal (median 4.4 mmol/L, range 3.7-5.3 mmol/L). The blood pressure of the women ranged from 90/60 to 135/90.

Ultrasound scanning of the ovaries.

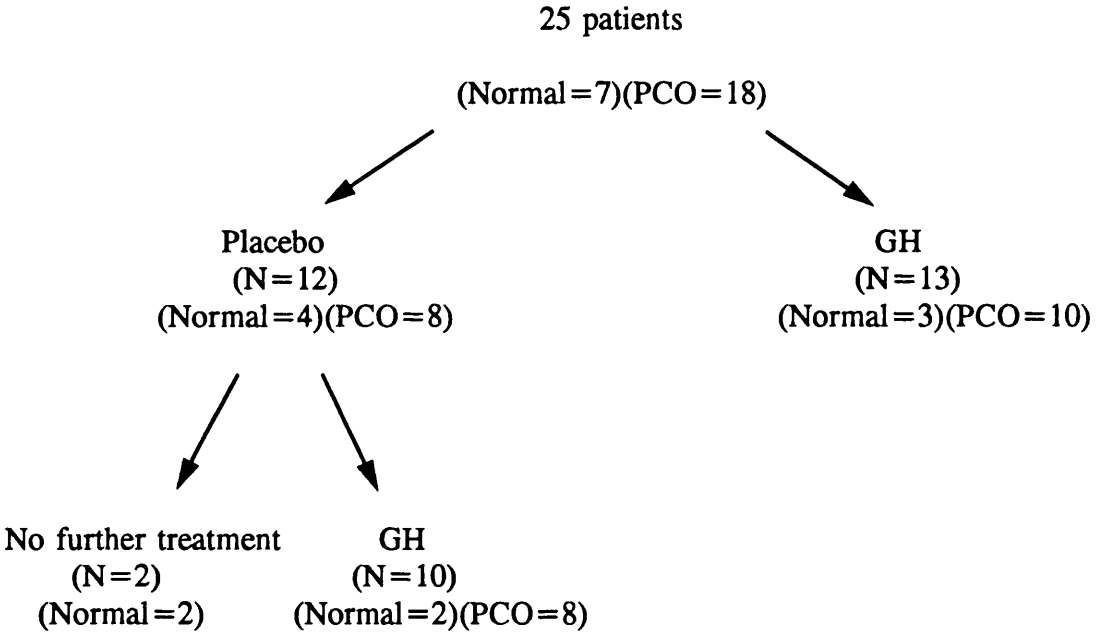
Eighteen of the women were diagnosed as having polycystic ovaries based on ultrasound scanning of the ovaries in the early follicular phase of a non treatment cycle. Ten of these women were randomised to receive GH and eight to receive placebo. Three women with normal ovaries initially received GH and four received placebo.

Serum LH concentrations were not significantly different between the patients with ultrasound diagnosed PCO (median 6.7 IU/L, range 1.0 to 14IU/L) and those with normal ovaries (5.1 IU/L, range 1.0 to 8.7IU/L) (p=0.19).

Open arm of study.

Ten women of the twelve women who had received placebo initially went on to have a further treatment cycle with GH (Fig *IV*-1).

Figure *IV*-1. Patient distribution



Total number of placebo cycles= 12
Total number of GH cycles = 23: 13 first cycles, 10 second cycles.

Results.

Clinical - all women.

Group comparisons of women randomised to receive GH or placebo:

In those women who were randomized to receive cotreatment with GH, the total dose of hMG used was significantly lower than in the group who received placebo (Table *IV*-3).

No significant difference was found when comparing the number of follicles greater than 14mm in diameter which were observed on the day of hCG administration between the two groups; yet, more oocytes were collected, more oocytes fertilised and more embryos cleaved from the patients receiving GH than those receiving placebo. The serum E_2 concentrations on the day of hCG administration were not significantly different between the groups.

Table *IV*-3: Group comparisons of placebo and first GH cycles

	Placebo cycles (n=12)	GH cycles (n=13)	#p=
Dose of hMG (amps)	36 (24-100)	28 (20-85)	0.03
Follicles $\geq$ 14mm	5 (2-9)	8 (3-19)	0.07
Serum E_2 day of hCG~	3139 (1495-8440)	3803 (1397-8960)	0.12
Oocytes collected	5 (2-13)	11 (22-16)	0.05
Oocytes fertilised	3 (0-6)	5 (0-11)	0.03
Embryos cleaved	2 (0-5)	4 (0-10)	0.03

Results presented as medians (range); ~ =pmol/L
#p=Mann-Whitney test.

Pre-study data:

When the results of the prestudy cycles in patients who went on to receive placebo or GH were compared, there was no difference between the groups in the dose of hMG used or in the number of follicles which had developed on the day of hCG (Table *IV*-4). There were, however, more oocytes collected in the prestudy cycles of the patients who went on to receive GH than those who went on to receive placebo.

There had been no significant differences in the number of oocytes which had fertilised or in the number of oocytes which had cleaved. All embryos had been transferred.

Table *IV*-4: Group comparisons of prestudy cycles

	Placebo group (n=12)	GH group (n=13)	#p=
Dose of hMG (amps)	33.5 (18-64)	28 (14-60)	0.07
Follicles $\geq$ 14mm	4 (2-6)	4 (2-7)	0.20
Oocytes collected	3.5 (2-6)	6 (0-8)	0.05
Oocytes fertilised	1.5 (0-3)	2 (0-4)	0.50
Embryos cleaved	1 (0-3)	1 (0-3)	0.40

Results presented as medians (range). #p=Mann-Whitney test.

Pairing results with prestudy cycles:

Comparing the results from the pretreatment cycles with the results from the randomly allocated treatment groups (placebo or GH), that is, using the patients' prestudy cycles as retrospective controls, several significant placebo as well as GH effects were observed (Tables *IV*-5 and -6).

Table *IV*-5: Paired comparison of prestudy cycles with placebo cycles (n=12)

	Prestudy	Placebo	*p=
Dose of hMG (amps)	33.5 (18-64)	36 (24-100)	0.378
Follicles ≥14mm	4 (2-6)	5 (2-9)	0.007
Oocytes collected	3.5 (2-6)	5 (2-13)	0.015
Oocytes fertilised	1.5 (0-3)	3 (0-6)	0.04
Embryos cleaved	1 (0-3)	2 (0-5)	0.056

*Results presented as medians (range). *p=Wilcoxon's signed rank test.*

Table *IV*-6: Paired comparison of prestudy cycles with GH cycles (n=13)

	Prestudy	GH	*p=
Dose of hMG (amps)	28 (14-60)	28 (20-85)	0.20
Follicles ≥14mm	4 (2-7)	8 (3-19)	0.005
Oocytes collected	6 (0-8)	11 (2-16)	0.003
Oocytes fertilised	2 (0-4)	5 (0-11)	0.005
Embryos cleaved	1 (0-3)	4 (0-10)	0.004

*Results presented as medians (range). *p=Wilcoxon's signed rank test.*

While there was no difference in the dose of hMG used during the treatment cycle compared with the prestudy cycle whether placebo or GH supplementation was given, more follicles developed in both the placebo group and the GH group compared with the pre treatment cycles. This enhanced response was also observed for the number of oocytes collected and the number which fertilised and cleaved. The size of the effect was, however, different.

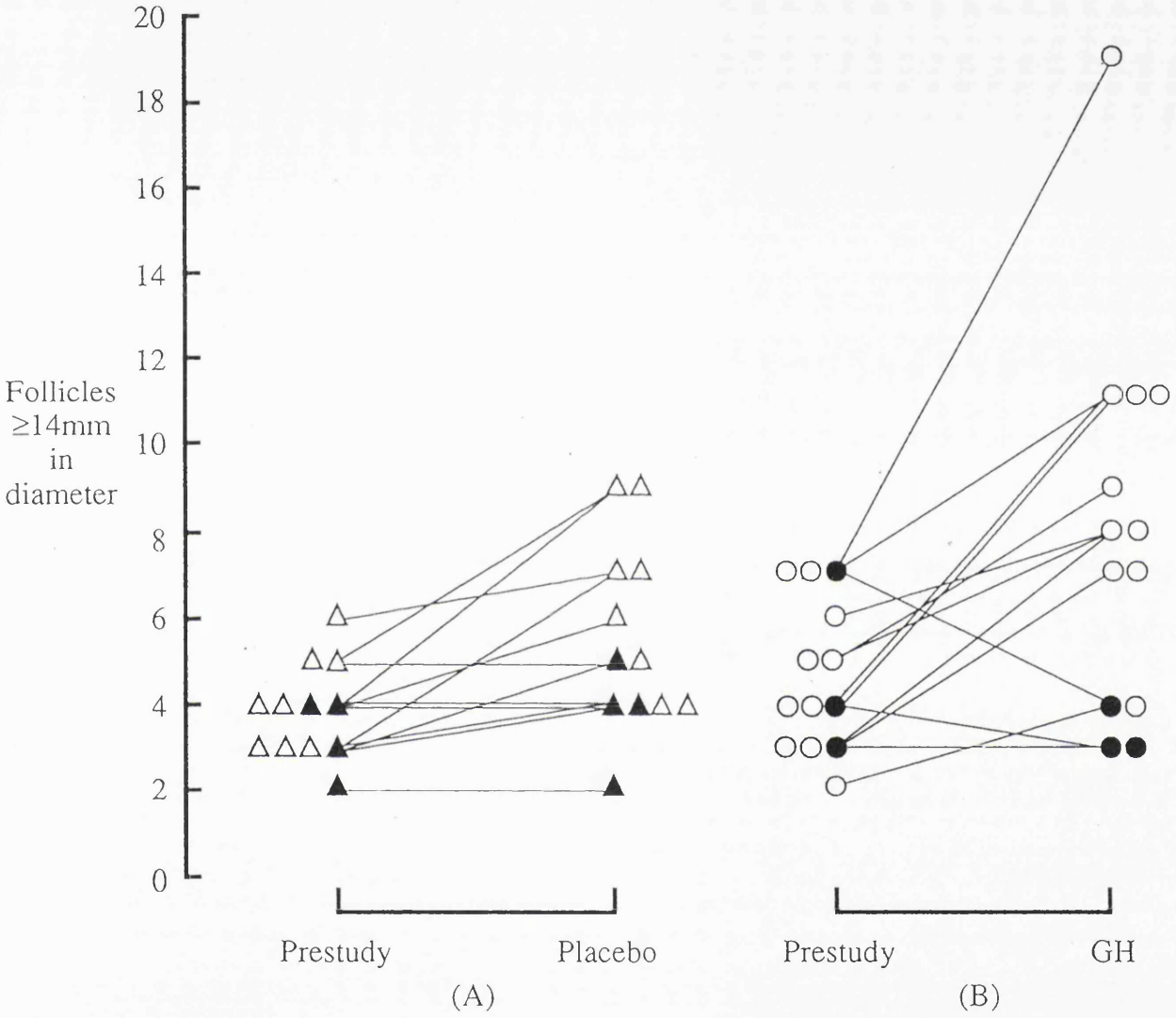


Fig *IV*.2 (A) The number of follicles ≥14mm in diameter on the day of hCG administration in prestudy cycles compared with placebo cycles in women with normal ovaries (▲) (n=4) and in women with PCO (△) (n=8).

(B) The number of follicles ≥14mm in diameter on the day of hCG administration in prestudy cycles compared with GH cycles in women with normal ovaries (●) (n=3) and in women with PCO (○) (n=10).

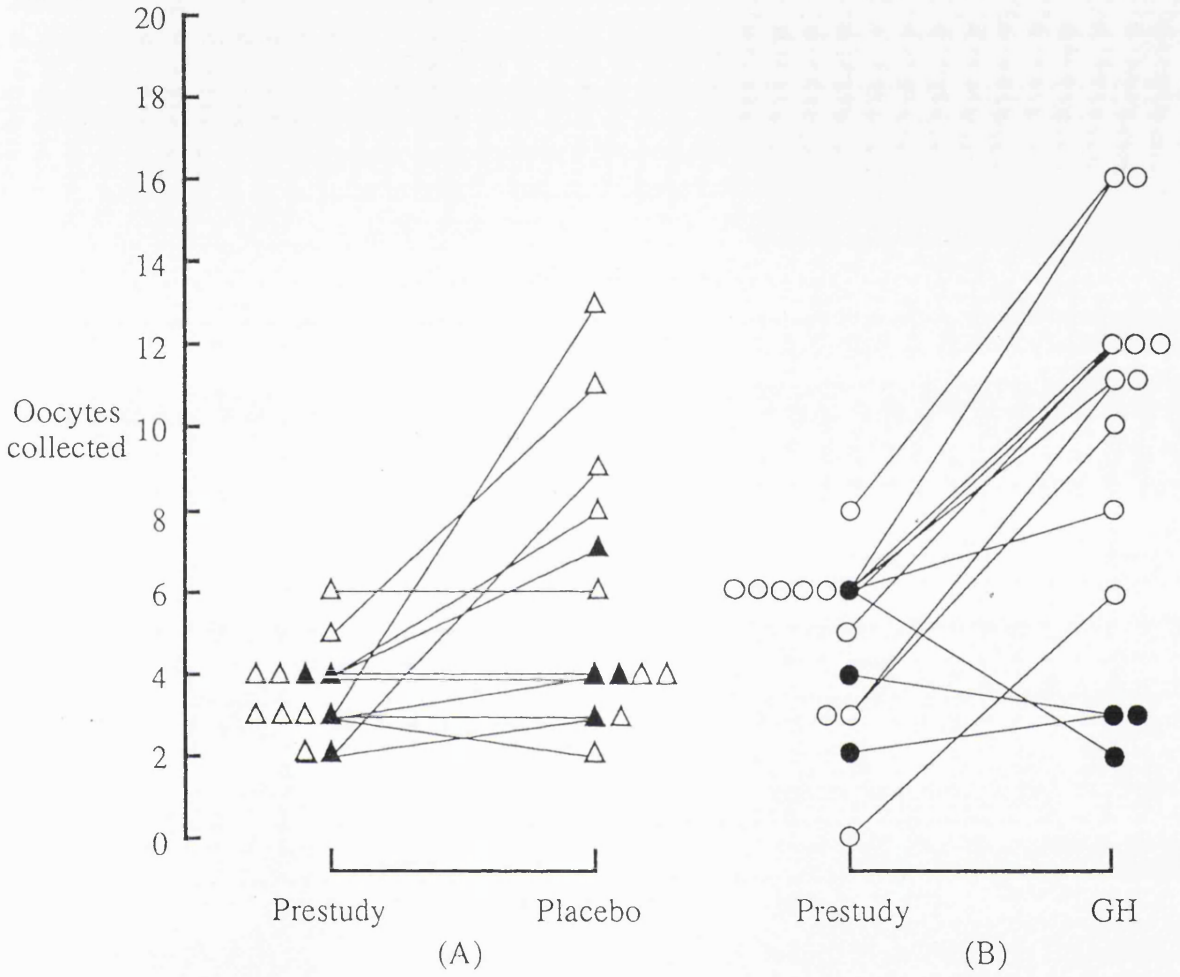


Fig *IV*.3 (A) The number of oocytes collected in prestudy cycles compared with placebo cycles in women with normal ovaries (▲) (n=4) and in women with PCO (△) (n=8).

(B) The number of oocytes collected in prestudy cycles compared with GH cycles in women with normal ovaries (○) (n=3) and in women with PCO (●) (n=10).

Comparison of degrees of improvement with placebo or GH:

When the degree of improvement in the two groups was compared, there was no significant difference between those patients who received GH treatment and those who received placebo compared with their prestudy cycles for dose of hMG used, follicles developed or oocytes collected (Table *IV*-7)(Figs *IV*-2 and -3). GH had, however, a significantly greater effect on the fertilisation and cleavage rate than placebo.

Table *IV*-7 : Comparison of degree of improvement between prestudy cycles and placebo cycles and prestudy cycles and GH cycles in all women.

	Prestudy and PL	Prestudy and GH	*p=
Dose of hMG (amps)	2.5 (-36-36)	0 (-9-25)	0.46
Follicles ≥14mm	1 (0-5)	4 (-2-12)	0.08
Oocytes collected	1 (-1-10)	6 (-4-10)	0.07
Oocytes fertilised	1.5 (-2-5)	5 (-3-10)	0.03
Embryos cleaved	1 (-2-5)	4 (-2-9)	0.03

Results presented as medians (range) of differences between cycles.
**p=Mann-Whitney test.*

Clinical - women with normal ovaries.

Group comparisons of women randomised to receive GH or placebo:

In the subgroup of women with normal ovaries, the women who received GH had fewer oocytes collected than the women who received placebo (Table *IV*-8). There were no other significant differences between the two treatment groups but the numbers were small.

Table *IV*-8: Group comparisons of placebo and first GH cycles in women with normal ovaries

	Placebo cycles (n=4)	GH cycles (n=3)	#p=
Dose of hMG (amps)	39 (27-48)	65 (24-85)	0.24
Follicles $\geq$ 14mm	4 (2-5)	3 (3-4)	0.23
Serum E_2 day of hCG~	2641 (1495-5060)	2222 (1397-3660)	0.24
Oocytes collected	4 (3-7)	3 (2-3)	0.03
Oocytes fertilised	2 (0-3)	1 (0-1)	0.07
Embryos cleaved	0.5 (0-2)	0 (0-1)	0.18

Results presented as medians (range); ~ =pmol/L
#p=Mann-Whitney test.

Pairing results with prestudy cycles:

In the women with normal ovaries there was no improvement in any measured parameter with either placebo or GH when comparing cotreatment cycles with the prestudy cycles (Tables *IV*-9 and 10).

Table *IV*-9: Paired comparison of prestudy cycles with placebo cycles in women with normal ovaries (n=4)

	Prestudy	Placebo	*p=
Dose of hMG (amps)	47 (24-63)	39 (27-48)	0.4
Follicles $\geq$14mm	3.5 (2-4)	4 (2-5)	0.5
Oocytes collected	3.5 (2-4)	4 (3-7)	0.09
Oocytes fertilised	1 (0-3)	2 (0-3)	0.3
Embryos cleaved	1 (0-3)	0.5 (0-2)	0.4

*Results presented as medians (range). *p=Wilcoxon's signed rank test.*

Table *IV*-10: Paired comparison of prestudy cycles with GH cycles in women with normal ovaries (n=3)

	Prestudy	GH	*p=
Dose of hMG (amps)	53 (24-60)	65 (20-85)	0.19
Follicles ≥14mm	4 (2-7)	3 (3-4)	0.19
Oocytes collected	4 (2-6)	3 (2-3)	0.29
Oocytes fertilised	2 (0-4)	1 (0-1)	0.21
Embryos cleaved	2 (0-3)	0 (0-1)	0.17

*Results presented as medians (range). *p=Wilcoxon's signed rank test.*

Comparison of degrees of improvement with placebo or GH:

In women with normal ovaries there was no greater improvement in outcome in the GH cycles compared with the placebo cycles when relating them to their prestudy cycles. In fact there were significantly fewer follicles on the day of hCG administration in the GH augmented cycles compared with their prestudy cycles when analysing the data of the placebo cycles compared with their prestudy cycles (Table *IV*-11)(Fig *IV*-2 and -3).

Table *IV*-11: Comparison of degree of improvement between prestudy cycles and placebo cycles and prestudy cycles and GH cycles in women with normal ovaries.

	Prestudy and PL	Prestudy and GH	*p=
Dose of hMG (amps)	4 (-16-36)	-12 (-25-0)	0.14
Follicles ≥14mm	0 (0-2)	-1 (-2-0)	0.04
Oocytes collected	1 (0-3)	-1 (-4-1)	0.07
Oocytes fertilised	0.5 (-1-2)	-2 (-3-1)	0.07
Embryos cleaved	-0.5 (-2-1)	-2 (-2-0)	0.18

*Results presented as medians (range) of differences between cycles. *p=Mann-Whitney test.*

Clinical - women with PCO.

Group comparisons of women randomised to receive GH or placebo:

Group analysis of the results from the 18 patients with ultrasound diagnosed PCO showed a different pattern (Table *IV*-12). Despite using significantly less hMG than in the women who received placebo, the women with PCO who received GH developed significantly more follicles and had more oocytes collected.

In addition, more of the oocytes from women with PCO who received GH fertilised and cleaved, compared with the oocytes from women who received placebo. There were no significant differences in the serum E_2 concentrations on the day of hCG, but a trend was observed of higher oestradiol concentrations in the GH group.

Table *IV*-12: Group comparisons of placebo and first GH cycles in women with polycystic ovaries

	Placebo cycles (n=8)	GH cycles (n=10)	#p=
Dose of hMG (amps)	36 (24-100)	27.5 (20-42)	0.02
Follicles $\geq$ 14mm	6.5 (4-9)	8 (4-19)	0.05
Serum E_2 day of hCG~	3991 (1613-8440)	5810 (2355-8960)	0.07
Oocytes collected	7 (2-13)	11.5 (6-16)	0.03
Oocytes fertilised	3.5 (0-6)	6 (5-11)	0.004
Embryos cleaved	3 (0-5)	5.5 (3-10)	0.02

Results presented as medians (range); ~ =pmol/L
#p=Mann-Whitney test.

Pairing results with prestudy cycles:

In the women with PCO the results from the study cycles showed improved results (Tables *IV*-13 and -14). There was no difference in the hMG usage when comparing the placebo or the GH cycles with their prestudy cycles but significantly more follicles developed and more oocytes were collected in the placebo cycles as well as the GH cycles.

Table *IV*-13: Paired comparison of prestudy cycles with placebo cycles in women with polycystic ovaries (n=8)

	Prestudy	Placebo	*p=
Dose of hMG (amps)	33.5 (18-64)	36 (24-100)	0.25
Follicles ≥14mm	4 (3-6)	6.5 (4-9)	0.01
Oocytes collected	3.5 (2-6)	7 (2-13)	0.05
Oocytes fertilised	2 (0-3)	3.5 (0-6)	0.06
Embryos cleaved	1 (0-3)	3 (0-5)	0.02

*Results presented as medians (range). *p=Wilcoxon's signed rank test.*

Table *IV*-14: Paired comparison of prestudy cycles with GH cycles in women with polycystic ovaries (n=10)

	Prestudy	GH	*p=
Dose of hMG (amps)	27.5 (14-42)	27.5 (20-42)	0.46
Follicles ≥14mm	4.5 (2-7)	8.5 (4-19)	0.003
Oocytes collected	6 (0-8)	11.5 (6-16)	0.003
Oocytes fertilised	1.5 (0-4)	6 (5-11)	0.003
Embryos cleaved	1 (0-3)	5.5 (3-10)	0.003

*Results presented as medians (range). *p=Wilcoxon's signed rank test.*

Comparison of degrees of improvement with placebo or GH:

In the women with PCO, the degree of improvement was greater in those women receiving GH compared with their prestudy cycles, compared with those women receiving placebo compared with their prestudy cycles for the number of follicles observed on the day of hCG administration, the number of oocytes collected and the fertilisation and cleavage rates (Table *IV*-15)(Figs *IV*-2 and -3).

Table *IV*-15: Comparison of degree of improvement between prestudy cycles and placebo cycles and prestudy cycles and GH cycles in women with polycystic ovaries.

	Prestudy and PL	Prestudy and GH	*p=
Dose of hMG (amps)	-2.5 (-36-14)	0 (-9-9)	0.28
Follicles $\geq$14mm	1.5 (0-5)	4 (2-12)	0.02
Oocytes collected	2 (-1-10)	6.5 (2-10)	0.04
Oocytes fertilised	1.5 (-2-5)	6 (1-10)	0.004
Embryos cleaved	1.5 (-2-5)	4 (2-9)	0.008

Results presented as medians (range) of differences between cycles.
**p=Mann-Whitney test.*

Paired placebo and GH cycles.

Ten of the women randomised to receive placebo had a subsequent GH augmented cycle. This was not a blind study but the women were acting as their own controls. Eight of the group had PCO. There was no difference in the amount of hMG used in the subsequent GH treated cycles compared with the placebo cycles. More follicles did, however, develop and the E_2 concentrations on the day of administration of hCG were higher in the GH group. There was no significant improvement in the number of oocytes collected, nor the number of oocytes which fertilised or cleaved (Table *IV*-16).

Table *IV*-16: Paired comparison of placebo and GH cycles (n=10)

	Placebo	GH	*p=
Dose of hMG (amps)	36 (24-100)	36 (20-90)	0.36
Follicles ≥14mm	5.5 (2-9)	7.5 (2-16)	0.03
Serum E_2 day of hCG~	3139 (1495-8440)	3880 (1749-10000)	0.04
Oocytes collected	5 (2-13)	6.5 (2-13)	0.34
Oocytes fertilised	3 (0-6)	3 (0-7)	0.50
Embryos cleaved	2 (0-5)	1.5 (0-7)	0.40

Results presented as medians (range); ~ =pmol/L
**p=Wilcoxon's signed rank test.*

Grading of embryos.

Eight of the nine patients who had more than one grade 1 embryo transferred were in the GH group. The single patient on placebo who had grade 1 embryos became pregnant with an ectopic gestation. In the ten patients who had both placebo and GH cycles there was, however, no improvement in the grading of the embryos (p=0.34).

Embryo transfers.

Embryo transfer was not performed in 7 of the 35 cycles. In three of the cycles placebo had been given and in 4 of the cycles GH had been administered. In four women normal ovaries had been diagnosed and in two women PCO had been diagnosed; one of the women with PCO had two cycles with failed fertilisation.

In two women with normal ovaries no fertilisation occurred; both were GH augmented cycles. One woman had unexplained infertility and one tubal damage. In the other two women with normal ovaries fertilisation occurred but not cleavage. One was in a placebo and one a GH cycle. They both had tubal damage as their indication for IVF-ET.

Of the two women with PCO, one women with failed DI as her indication for IVF-ET had no fertilisation and cleavage in her GH cycle. She had, however, had 4 embryos replaced in her placebo cycle and had conceived, but with an ectopic pregnancy. The other woman with PCO, and a partner with oligospermia, had no fertilisation in either her placebo or GH cycle.

Luteal phase support.

All the women with normal ovaries who had embryos transferred had hCG given in the luteal phase. There were 2 placebo and 3 GH cotreatment cycles. Twenty one cycles in women with PCO were supplemented in the luteal phase with hCG. In two women cyclogest pessaries were used; one woman had had 16 oocytes collected and the other 12 oocytes.

Pregnancies.

Five women became pregnant after GH treatment; four in their first treatment cycles and one who received GH following a placebo cycle (Table *IV*-17). One woman conceived an ectopic pregnancy in a placebo cycle and subsequently had a partial salpingectomy.

Table *IV*-17. Details of cycles in which pregnancies occurred.

Cycle	GH/PL	Ovaries	No. collect	No. fert	No. cleav	No. replac	Outcome
2	GH	Normal	6	1	1	1	Single
1	GH	PCO	12	11	7	4	Twins
1	GH	PCO	16	11	10	3	Single
1	GH	PCO	12	10	9	4	Twins
1	PL	PCO	6	5	5	4	Ectopic
1	GH	PCO	12	5	5	4	Single

No. collect=number of oocytes collected
No. fert=number of oocytes fertilised
No. cleav=number of embryos cleaved
No. replac=number of embryos replaced

One of the five women had normal ovaries and was complaining of unexplained infertility. She conceived in a GH cycle in which only one embryo was transferred and had a female infant weighing 7lb 2oz. In her placebo cycle she had had one cleaved and one pronucleate embryo replaced.

The other 4 women who had successful pregnancies had PCO and conceived on GH in their first treatment cycles; three had tubal damage and one had unexplained infertility. Three had had 4 embryos transferred and one had had 3 embryos transferred. Of the four pregnancies, two were twins and two were singletons.

One patient had 16 oocytes collected and the other three patients had 12 oocytes collected. Ten out of the 16 oocytes cleaved and 3 were replaced; she had a female infant weighing 7lb 9oz.

In another patient nine embryos cleaved and four grade 1 embryos were transferred. She had a male and a female infant weighing 5lb 14.5oz and 4lb 11oz. Seven embryos cleaved, and four embryos were transferred in another woman. She delivered healthy twins weighing 4lb 12.5oz and 4lb 5.5oz at 34 weeks. The final patient had 5 embryos cleaved and four replaced. A second sac was noted on ultrasound scanning of the uterus at 8 weeks but she went on to deliver a single female infant weighing 6lb 5.5oz.

There were thus two pregnancies in the 6 patients with unexplained infertility who received GH and who had embryo transfers. Three pregnancies occurred in the 8 women with tubal damage who received GH and who had embryo transfers. None of the patients with oligospermia, antisperm antibodies or failed donor insemination conceived following GH treatment. There were 5 embryo transfers following 7 GH cycles in these women. One woman with failed DI conceived on ectopic pregnancy in a placebo cycle.

Steroids and peptides in serum and follicular fluid.

FSH and LH concentrations.

There was no difference in the FSH concentrations at the beginning of the treatment cycles between those women who went on to receive GH and those who went on to receive placebo. Women with normal ovaries did, however, have significantly higher FSH concentrations (7.7 IU/L, range 5-11 IU/L) than women with PCO (6.25 IU/L, range 2-11IU/L) ($p=0.04$).

The LH concentrations were no different between either of the treatment groups nor between the patients with PCO and those with normal ovaries.

Nine women had FSH concentrations greater than 5 IU/L when their pituitaries were considered to be desensitised after a minimum of 14 days of buserelin, but only one patient had an oestradiol concentration over 150 pmol/L.

The LH concentrations were less than 10 IU/L in all but one patient on the day of starting hMG and remained low throughout the treatment cycle. On the day of hCG and also the day of oocyte collection there was no difference between any of the groups.

FSH concentrations were high throughout the hMG therapy but with no difference between patients receiving GH and those receiving placebo. There was no difference in FSH concentrations between the groups on the day of hCG or the day of oocyte collection.

IGF-I concentrations.

Serum:

There was no difference in IGF-I concentrations in serum between the various groups before treatment started; the baseline concentrations of IGF-I were no different between those patients who went on to receive GH and those who went on to receive placebo. Patients with PCO had concentrations no different from those with normal ovaries.

Desensitisation of the pituitary with buserelin did not alter the concentrations of IGF-I in the serum. There was, however, a significant rise in serum IGF-I concentrations during cotreatment with GH ($p=0.03$) but not during the placebo cycles ($p=0.23$)(Fig *IV*-4). The concentrations of IGF-I in serum declined into the normal range by the day of oocyte collection. Serum concentrations of IGF-I rose significantly in the group of women with normal ($p=0.04$), as well as polycystic ($p=0.02$), ovaries during GH treatment (Fig *IV*-5). There were no significant differences in the concentrations of IGF-I during placebo treatment in either subgroup.

On the day of hCG administration serum IGF-I concentrations were significantly higher in those patients receiving GH compared with those receiving placebo ($p=0.0065$). This was also true in the subgroup of women with PCO ($p=0.008$) and the subgroup with normal ovaries ($p=0.04$). The IGF-I concentrations in women receiving GH were higher in women with PCO than normal ovaries ($p=0.04$).

In the group of women receiving GH, serum IGF-I concentrations on the day of oocyte collection were, however, significantly lower than on the day of hCG administration. There were no differences between IGF-I concentrations on these days in the women receiving placebo. There was no difference in serum IGF-I concentrations on the day of oocyte collection between patients who had received GH and those who had received placebo.

There was a weak correlation between serum concentrations of IGF-I and E_2 on the day of hCG administration in the women receiving GH (correlation coefficient=-0.36; $p=0.057$)

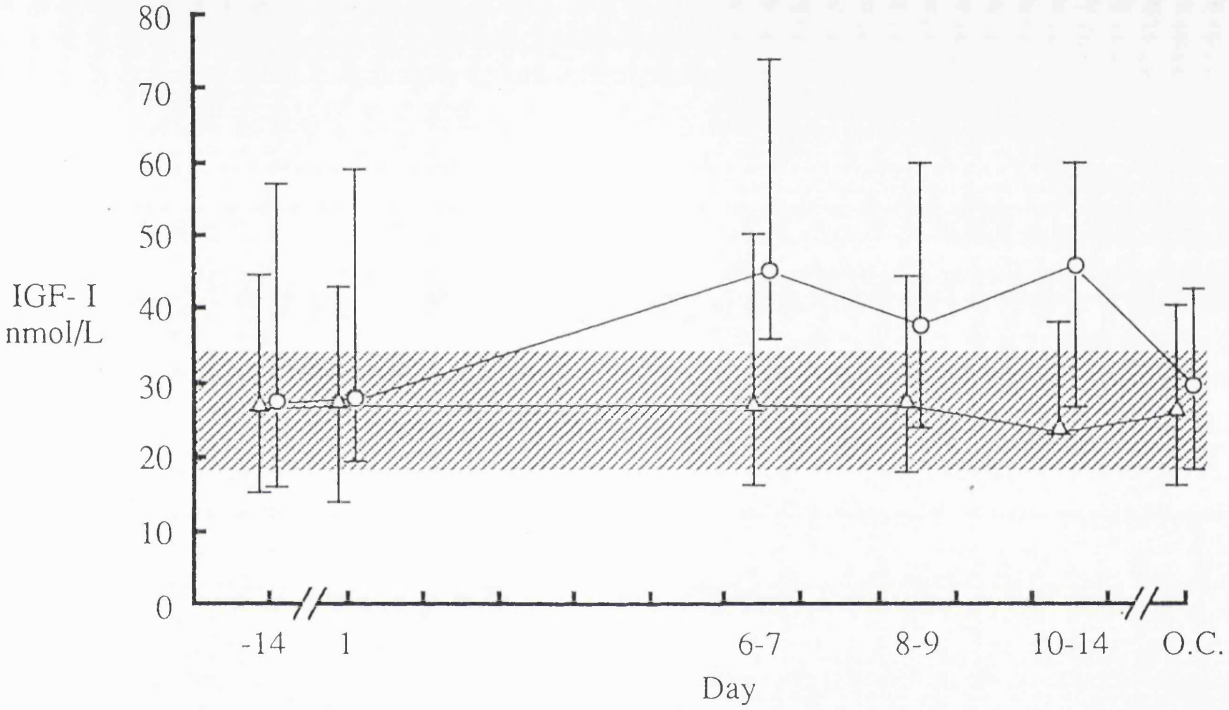


Fig *IV*.4 Serum concentrations of IGF-I (medians and ranges) in PL (Δ) (n=12) and GH (O) (n=13) cycles, taken before initiation of any treatment (day -14), before initiation of hMG (day 1), days 6-7, 8-9, 10-14 of treatment and on the day of oocyte collection (O.C.).

The normal range for IGF-I is shaded.

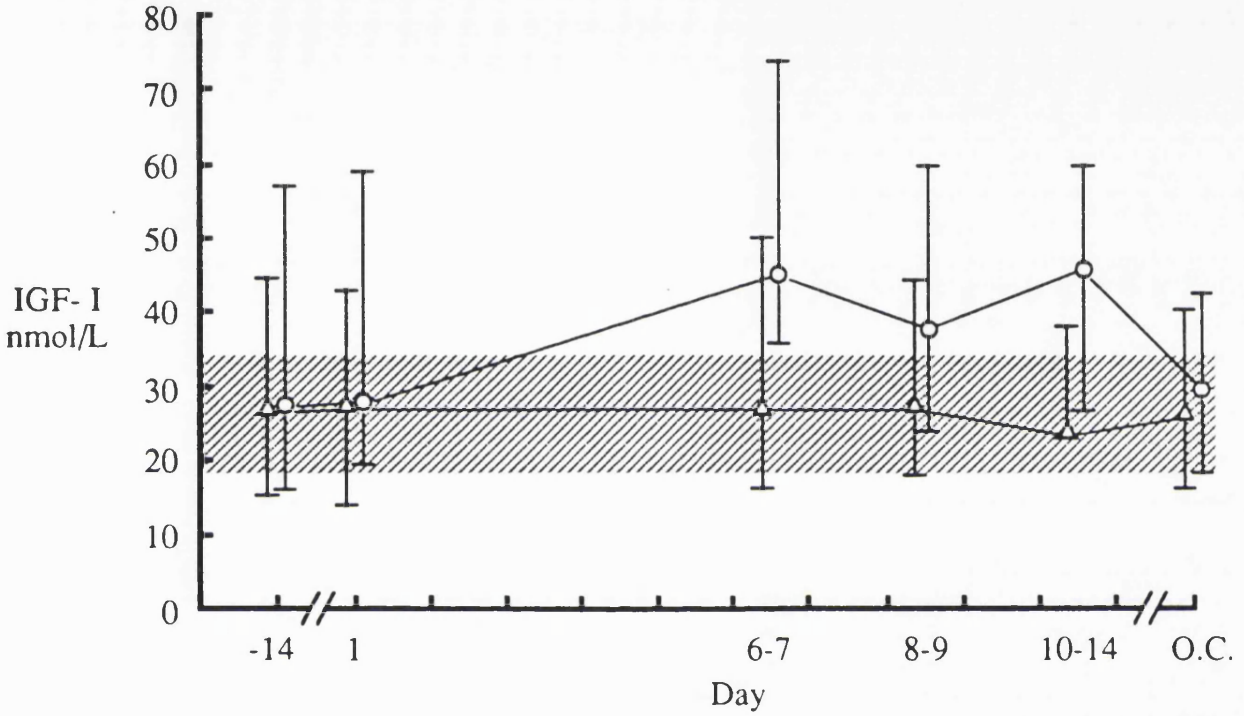


Fig *IV*.4 Serum concentrations of IGF-I (medians and ranges) in PL (Δ) (n=12) and GH (O) (n=13) cycles, taken before initiation of any treatment (day -14), before initiation of hMG (day 1), days 6-7, 8-9, 10-14 of treatment and on the day of oocyte collection (O.C.).

The normal range for IGF-I is shaded.

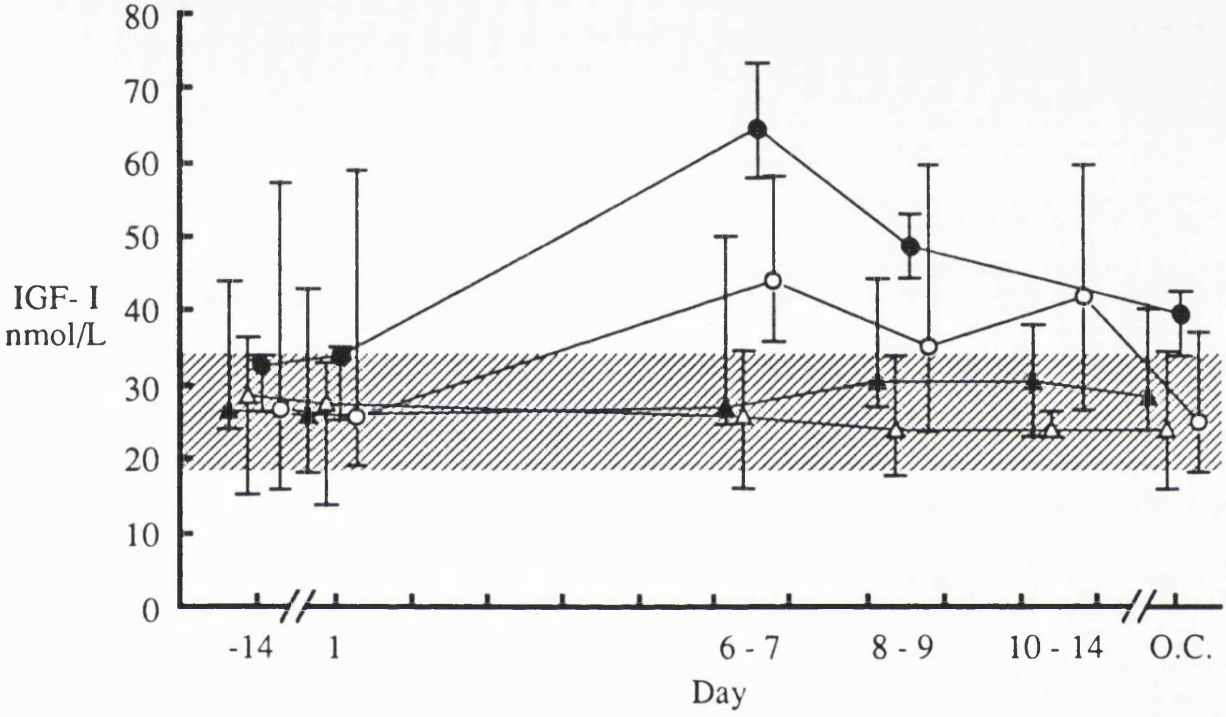


Fig *IV*.5 Serum concentrations of IGF-I (medians and ranges) in women with normal ovaries who received PL (▲) (n=4) or GH (●) (n=3); and in women with PCO who received PL (△) (n=8) or GH (○) (n=10). Results are shown from before initiation of any treatment (day -14), before initiation of hMG (day 1), days 6-7, 8-9, 10-14 of treatment and on the day of oocyte collection (O.C.).

The normal range for IGF-I is shaded.

but not in the women receiving placebo (correlation coefficient=-0.15; p=0.27). Analysis of the data from the women with PCO or normal ovaries did not show any correlation between IGF-I and E_2.

There was no correlation between serum concentrations of IGF-I and E_2 on the day of oocyte collection in any group.

Follicular fluid:

Clear follicular fluid samples were analysed from 23 women; 13 had GH and 10 had placebo cotreatment. Two samples were obtained from every woman who had GH; only one sample was suitable for analysis from two women on placebo. Thus twenty six samples of follicular fluid were analysed from 13 women after GH treatment and 18 samples from 10 placebo treated women. Fluid was analysed from follicles greater than 14mm in diameter on the day of oocyte collection which, wherever possible, contained a healthy oocyte.

Concentrations of IGF-I in follicular fluid were significantly lower than in serum (Table *IV-*18). This was observed both in women who received GH (p=0.01) and in women who received placebo (p=0.003). This was also demonstrated in the subgroup of women with PCO whether they received GH (p=0.05) or PL (p=0.01).

Group analysis demonstrated that the concentrations of IGF-I in follicular fluid were higher in women who had cotreatment with GH than those who had placebo (p=0.04). No significant difference was demonstrated betwee the subgroups of women with PCO or those with normal ovaries.

The concentrations of IGF-I in serum on the day of oocyte collection correlated with the concentrations of IGF-I in follicular fluid in GH (correlation coefficient=0.5; p=0.009) and placebo cycles (correlation coefficient=0.69; p=0.004).

Table *IV*-18. IGF-I concentrations: comparison of serum concentrations on day of oocyte collection with follicular fluid concentrations (nmol/L).

	Serum IGF-I	FFL IGF-I	*p=
All women			
GH cycles	29.1 (18.0-42.3) n=13	24.6 (15.7-37.6) n=13	0.01
PL cycles	26.0 (16.0-40.2) n=12	20.0 (14.3-25.2) n=10	0.003
Women with PCO			
GH cycles	24.5 (18.0-36.6) n=10	21.8 (15.2-37.6) n=10	0.05
PL cycles	23.6 (16.0-34.3) n=8	19.8 (14.3-23.1) n=7	0.01
Women with normal ovaries			
GH cycles	39.3 (34.1-42.3) n=3	29.4 (24.6-30.9) n=3	0.09
PL cycles	28.3 (23.8-40.2) n=4	20.9 (19.6-25.2) n=3	0.09

Results presented as medians (range). *p=Wilcoxon's signed rank test*

There was no correlation between mean follicular fluid concentrations of IGF-I and serum E_2 on either the day of hCG or the day of oocyte collection in either treatment group.

Oestradiol concentrations.

On the day of starting buserelin treatment the oestradiol concentrations were 262 pmol/L or lower in all women (median 154 pmol/L, range 91-262 pmol/L for women receiving GH and median 120 pmol/L, range 90-196 pmol/L for women receiving placebo) (Fig *IV*-6). There was no difference between women with PCO and women with normal ovaries. After a minimum of 14 days of treatment with buserelin, the oestradiol concentrations were in the menopausal range in the majority of patients. Three women did, however, have oestradiol concentrations between 415 and 437 pmol/L when the stored serum was later analysed. All the women had had low oestradiol results using the in-house IVF kit. The FSH concentrations in these three women were interestingly, however, all less than 3 IU/L.

The oestradiol concentrations rose steadily following the initiation of hMG treatment, with or without GH, until the day of hCG administration. There was no difference in the peak E_2 concentrations nor the E_2 concentrations on the day of hCG administration between those patients who received GH and those who received placebo. Women with PCO who received GH did, however, have E_2 concentrations on the day of hCG administration significantly higher than those with normal ovaries on GH (5810 pmol/L, range 2355-8960 pmol/L; 2222 pmol/L, range 1397-3660 pmol/L)($p=0.017$).

On the day of oocyte collection, the oestradiol concentrations were significantly lower than on the day of hCG administration in the placebo group. Oestradiol concentrations in women receiving GH were, however, significantly higher than those receiving placebo (3651 pmol/L, range 700-8110 pmol/L; 1737 pmol/L, range 908-5375 pmol/L)($p=0.028$). This was also demonstrated within the subgroup of women with PCO and the subgroup with normal ovaries. Women with PCO who were treated with GH had higher concentrations of oestradiol than those with PCO who were treated with placebo (4870 pmol/L, range 1582-8110 pmol/L; 2722 pmol/L, range 1100-5375 pmol/L)($p=0.017$).

There were, however, no differences between the various groups if the oestradiol concentration per follicle greater than 14mm in diameter on the day of hCG, or per oocyte collected, was analysed.

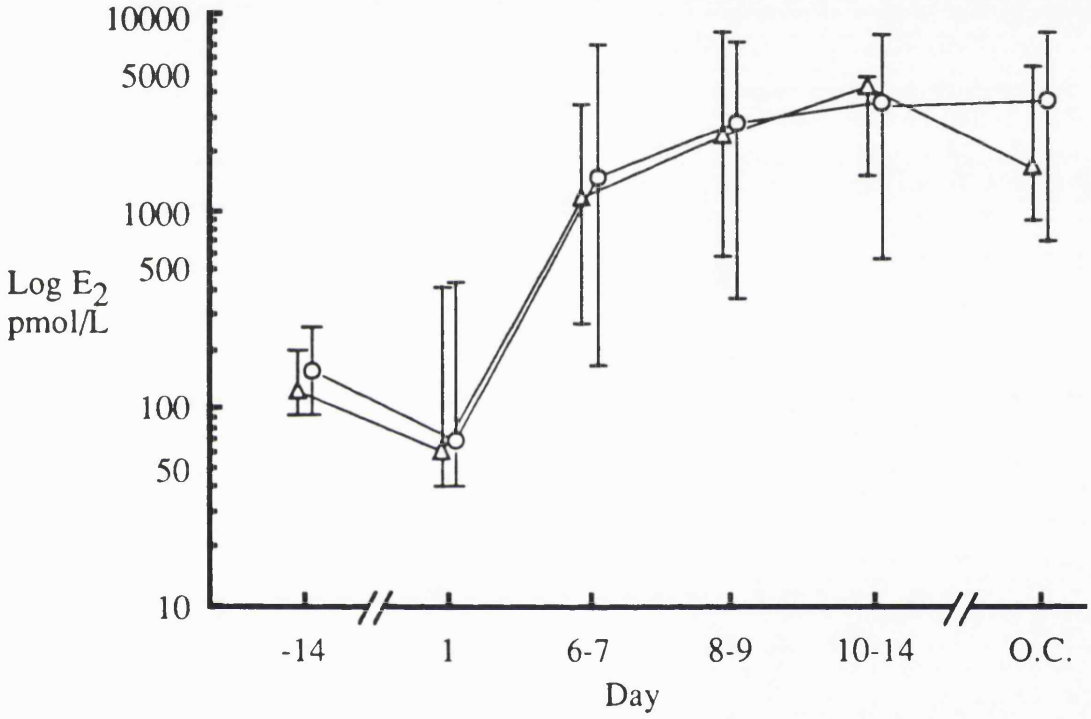


Fig *IV*.6 Serum concentrations of E2 (medians and ranges) in PL (Δ) (n=12) and GH (O) (n=13) cycles, taken before initiation of any treatment (day -14), before initiation of hMG (Day 1), days 6-7, 8-9, 10-14 of treatment and on the day of oocyte collection (O.C.).

Progesterone concentrations.

The concentrations of progesterone in serum on the day of administration of hCG was low in all groups (range <2-8 pmol/L). The progesterone concentrations on the day of oocyte collection were no different between those women receiving GH and those receiving placebo (p=0.44). Women with PCO, however, who received GH had significantly higher concentrations of progesterone than the women with normal ovaries (Table *IV*-19).

Table *IV*-19. Group comparisons of serum concentrations of Progesterone on day of oocyte collection (nmol/L).

	n=	Median (range)	#p=
GH cycles	12	24.5 (7.9-48.0)	
Women with PCO	9	27.0 (11.0-48.0)	0.12
Women with normal ovaries	3	9.0 (7.9-9.9)	
Placebo cycles	12	17.5 (6.9-52.0)	
Women with PCO	8	18.5 (14.0-52.0)	0.006
Women with normal ovaries	4	16.0 (6.9-32.0)	

Results presented as medians (range). #p=Mann-Whitney test

Summary.

Twenty five women who had responded suboptimally to buserelin and hMG were recriuted into a randomised, double blind, placebo controlled trial of cotreatment with GH. Despite requiring significantly less hMG, the women who were randomised to receive GH had more oocytes collected and fertilised and had more embryos which cleaved than the women receiving placebo. The subgroup of women with PCO formed the group most responsive to GH.

When the treatment cycles were paired with the pretreatment cycles significant effects were noted with both placebo and GH. The degree of improvement in response was, however, greater in those women receiving GH but only in those women with PCO.

Of the 12 women randomised to receive placebo 10 women went on to receive GH. More follicles developed and the serum E_2 on the day of hCG was higher in the women receiving GH.

Five women conceived in GH cycles and one in a placebo cycle. The latter pregnancy was an ectopic. Of the five women delivering after GH treatment two delivered twins and three delivered singletons.

Serum concentrations of IGF-I rose significantly during GH treatment but not during placebo treatment. On the day of oocyte collection there was no significant difference in serum IGF-I concentrations between those women who received GH and those who received placebo. Concentrations of IGF-I in follicular fluid were significantly lower than serum whether GH or placebo had been given. In women receiving GH the follicular fluid concentrations were, however, higher than in women receiving placebo.

Serum E_2 concentrations were higher on the day of hCG administration in the women with PCO who received GH than in women with normal ovaries who received GH. On the day of oocyte collection women who had received GH had higher E_2 concentrations than the women who received placebo.

V. Results 3-11β-hydroxyandrostenedione

Background

In the GH studies presented in Chapters *III* and *IV* all women had a routine ultrasound scan of the ovaries performed at the time of recruitment into the studies. Thirty four of the fifty women in the different studies were found to have a PCO pattern on scanning their ovaries. The women with PCO formed a subgroup who appeared to respond to the addition of GH, whereas those with ultrasonically normal ovaries failed to show an improved response with GH cotreatment. With this is mind we wished to establish if there were a biochemical marker for PCO to enable us to define a population potentially responsive to treatment with GH.

Hypothesis.

We hypothesised that, if present in the polycystic ovary, activity of the 11ß-hydroxylase complex would lead to raised concentrations of 11ß-OH-A_4 in FFL as a reflection of increased androgen metabolism. We therefore determined the relative amounts of 11ß-OH-A_4 within the ovary compared with the serum, under various circumstances.

Outline of studies.

Study 1: Establishment of reliable quantitative method for assessment of 11ß-OH-A_4 concentrations in biological fluids.

Previous studies used direct/extracted RIA methods which were unreliable due to the presence of high concentrations of other cross reacting steroids (Goldzieher *et al* 1978, Lejeune-Lenain 1980, Schlaghecke *et al* 1986,). This is a particular problem in the steroid rich environment of the ovary (McNatty *et al* 1979b, Khalil and Lawson 1983, Vanluchene *et al* 1990).

There was only one antibody preparation available commercially for use in a RIA for 11ß-OH-A_4 and the specificity of the assay had not been evaluated for determination of the hormone in follicular fluid. Androstenedione was known to be present in FFL in high

concentrations and it was considered likely that this steroid may displace labelled 11ß-OH-A_4 so giving a false value for the required analyte.

There are also in FFL a variety of steroids which have yet to be tested for cross reactivity in the RIA for 11ß-OH-A_4 and for A_4. These include 19-hydroxyandrostendione, 19-norandrostenedione and 19-nortestosterone (Short 1960, Dehennin *et al* 1986). To minimise potential cross reactants, a solvent extraction followed by a normal phase high performance liquid chromatography (HPLC) separation was suggested prior to the measurement of steroids by RIA (Schoneshofer 1981, Owen *et al* 1988). We also wished to measure steroid concentrations in follicular fluid by GC-MS and thus illustrate the very high concentrations of steroids present in FFL potentially interfering with the results of the RIA.

We needed to prepare a suitable radiolabel for the assay and planned to achieve this by chemical modification of radioactive cortisol and subsequent purification by HPLC.

Study 2: Comparison of serum and follicular fluid concentrations of 11ß-OH-A_4 and assorted steroids in women with normal and polycystic ovaries.

Having established a reliable and sensitive radioimmunoassay (Holownia *et al* 1991) for 11ß-OH-A4 we investigated whether 11ß-OH-A4 concentrations were associated with abnormal androgen metabolism in women with PCOS, as well as to determine the origin of 11ß-OH-A_4 in the ovary. We compared serum concentrations from patients with PCOS, with those in whom PCO was an incidental finding and with women with normal ovaries. Follicular fluid from women with normal and polycystic ovaries undergoing ovulation induction prior to IVF-ET were also analysed.

Study 3: Determination of the source of 11ß-OH-A_4 within the ovary.

The presence of 11ß-OH-A_4 in the ovary may be explained by ovarian metabolism (11ß-hydroxylation of androstenedione or side chain cleavage of cortisol)(Hudson *et al* 1974, Goldzieher *et al* 1978), or concentration of the steroid from the circulation (Ben-Rafael *et al* 1986). In an attempt to investigate the origin of 11ß-OH-A_4 in the ovary, incubations of

granulosa cells were also undertaken using the two potential substrates, cortisol and androstenedione.

Summary.

We developed and validated an in-house method for quantification of 11ß-OH-A_4 and investigated whether concentrations of this steroid in serum or follicular fluid might serve as a marker of abnormal androgen metabolism in women with PCO. In vitro studies were also performed by incubations of granulosa cells with radiolabelled substrates, in an attempt to determine the origin of 11ß-OH-A_4 in the ovary.

Materials

Instrumentation.

A Waters HPLC system was used (Waters Associates, Milford, Massachusetts 01757) with a solvent degasser, dual pumps (6000A), solvent programmer (660), autoinjector (WISP) and data module (M730). A Lichrosorb diol cartridge column (25cm x 4.6mm and 10um pore size) incorporating a guard column (Cat.No. 8749 obtained from Alltech Associates/Applied Science, Carnforth, Lancashire LA5 9XP) was maintained at 40°C by means of a column thermostat (Model 7960, Jones Chromatography Ltd. Mid Glamorgan, CF8 8AU). Column eluates were collected using a fraction collector (LKB 7000 Ultra Rac) and a home made column switching device (Stoks and Benraad 1983).

A quadrapole mass spectrometer (Hewlett Packard 5970 benchtop MSD) of ionisation energy 70eV coupled to a gas chromatograph (HP 5890 GC) was used. The voltage at the electron multiplier was 2800vg and digital signals were processed and stored on a 40 megabyte hard disc HP3000 data system. The GC was equipped with a fused silica column (Chrompack CP-Sil-5, Unit 4, Indescon Court, Millharbour, London EC1 9TN), 25m long and internal diameter of 0.32mm. The liquid scintillation counter was a three channel Packard 3330.

Chemicals and Reagents.

All steroids were obtained from the MRC Steroid Reference collection. Tritium labelled steroids 1,2-^{3}H Cortisol (45Ci/mmol), 1,2,6,7-^{3}H androstenedione (90Ci/mmol) and 1,2-^{3}H testosterone (47Ci/mmol) were obtained from Amersham International, Lincoln Place, Green End, Aylesbury, Bucks. HP20 2TP).

An antibody raised against a 11ß-hydroxy-androstenedione-6ß-hemisuccinyl-BSA conjugate was provided for our study by Dr R.Hampl (Putz *et al* 1987). A commercial kit for 11ß-OH-A_4 was purchased from Intersci Diagnostics, Los Angeles, USA. Testosterone kits were purchased from the Department of Chemical Pathology, (Dr M.Wheeler), St.Thomas' Hospital, London SE1 7EH, England. Bioclinical Services Ltd. (Cardiff) supplied the cortisol kits and Guildhay Antisera Ltd. (University of Surrey, Guildford, England) provided the antisera for androstenedione.

Solvents and buffer reagents (pyridine, cyclohexane, dichloromethane, isopropanol, hexane, ethanol, methanol, toluene, citric acid, sodium dihydrogen/hydrogen phosphate, sodium azide, Polyethylene glycol 7000 (PEG), gelatin, sodium metabisulphite and sodium chloride) were bought from BDH Ltd. Dagenham, Essex (analar grade or where applicable HPLC grade). Sodium bismuthate and bovine serum albumin (BSA) were obtained from Sigma Chemical Co.Ltd. (Poole, Dorset). Lipidex-Tm-5000 was supplied by United Technologies Packard (Caversham, Reading, Berks. England). Trimethylsilyl-imidazole (TMSI), Methoxyamine hydrochloride (MO-HCL) and Hexamethyldisilazane (HMDS) were obtained from Pierce (Luton, England). Sep-Pak cartridges (C18) were purchased from Waters. Thin layer chromatography (TLC) glass plates used were of dimensions 20x20cm square and 0.25mm (Merck, Kieselgel 60/Kieselgur F-245) supplied by BDH. Donkey anti-rabbit serum and normal rabbit serum were bought from Immunodiagnostics (Usworth Hall, Tyne and Wear, England) and quality control samples (Lyphochek) were purchased from Biorad Laboratories, Watford, Herts, England). Scintillation fluid used was the biodegradable 'Ecoscint' obtained from National Diagnostics (Manville, New Jersey, USA).

Methods.

Study 1: Development of 11ß-OH-A_4 assay.

Sample preparation - extraction and HPLC separation.

A procedure for steroid extraction and HPLC purification was developed which provided the means for simultaneous measurement of several steroids from a single sample. This required assessment and refinement of existing methodologies, the results of which are presented below. The successful performance of this method required close attention to detail by the operators.

Extraction:

Steroids were extracted from a 200μl aliquot of serum or follicular fluid into 2mls. of dichloromethane. The upper layer was removed by aspiration and a fixed volume (1.8 ml) of the solvent was transferred into a tube and then dried down under oxygen free nitrogen (OFN). The dried residue from the FFL sample was reconstituted in 300μl of initial mobile phase (5% isopropanol in hexane) prior to HPLC analysis.

HPLC Separation:

From the 300μl volume of extract in mobile phase, 200μl were injected onto the diol column maintained at a constant temperature of 40°C. The initial mobile phase consisted of 5% isopropanol in hexane followed by a linear gradient to 20% IPA over 50 minutes at a flow rate of 1ml/min. Equilibration time between runs was 30 minutes and the back pressure during operation never exceeded 1500 psi.

Recoveries were assessed after spiking parallel samples with the radioactively labelled (tritiated) steroids in question and repeating the extraction/HPLC procedure. Tritiated steroids, at approximately 20,000 counts/20mins/20μl, were added to tubes, evaporated to

dryness under OFN and redissolved in the follicular fluid by sonication. Radioactivity was determined after extraction and HPLC separation. To ensure equivalence of quenching in subsequent liquid scintillation counting, a constant aqueous matrix was used prior to counting before and after extraction/HPLC. 5ml of Ecoscint were added and after thorough mixing, vials were counted for 20mins.

Synthesis of tritium labelled 11ß-OH-A_4 from Cortisol.

Tritium labelled 11ß-OH-A_4 was synthesised from commercially available cortisol label (^{3}H-F) using the sodium bismuthate oxidation method (Brooks and Norymberski 1953). The synthesis was performed on 120μCi preparation of neat ^{3}H-F label (specific activity 45Ci/mmol). The final reaction products were dried down and resuspended in 0.5ml of dichloromethane. TLC separation was then performed on a Kieselgel F254 silica plate using a mobile phase of dichloromethane and methanol (97:3 v/v). Standards of cortisol and 11ß-OH-A_4 (100ng) were run alongside, facilitating the detection by ultra violet light of the required fraction. The area corresponding to 11ß-OH-A_4 (R_f=0.25) was eluted into ethanol overnight at 4°C and centrifuged for 10 mins. at 2000 rpm. The supernatant was then removed and evaporated in stages under OFN and resuspended in 2 x 300μl of initial HPLC mobile phase (5% IPA in hexane).

Two samples of 200μl were injected onto the HPLC under identical conditions and fractions corresponding to the retention time of 11ß-OH-A_4 were collected between 18.5-20 mins. These pooled fractions were dried down and resuspended in 5ml of toluene:ethanol (9:1) and stored at -25°C ready for use in subsequent RIA. When required, an aliquot (250μl) of this solution was removed, dried down and resuspended in 12 mls assay buffer ready for each 100 tube radioimmunoassay giving approximately a total of 10,000 counts/10 min/tube.

The synthesised label was verified after repeating the TLC separation using a small amount of reaction mixture remaining after the sodium bismuthate oxidation step. After TLC separation the plate was divided into 8 equal horizontal sections, each of which was scraped out, eluted in ethanol as before and then assessed for radio- and immunoactivity. 100μl from each TLC section was removed for liquid scintillation counting to check for radioactivity

whilst the remaining supernatant was used for the determination of immunoreactivity. This was accomplished by using a crude and unoptimised RIA, measuring the percent displacement between zero standard and a standard with 11ß-OH-A_4 present in excess (40nmol/L).

Radioimmunoassay.

Background binding:

For each sample, in addition to fraction collection for RIA and recoveries, a blank run was performed in parallel. Here an injection of initial mobile phase was made followed by collection of fractions with retention times corresponding to the steroids in question. These fractions were used to assess non-specific interference in each of the steroid RIA methods by any unknown material in the column eluates.

RIA:

Appropriate volumes of assay buffers (sufficient for duplication in subsequent RIA) were used to reconstitute the dried residues of collected HPLC fractions according to the steroid in question. Each of the steroid fractions were resuspended in assay buffer by vortexing, sonication followed by a 30 min incubation at room temperature to minimise any recovery losses. To the 11ß-OH-A_4 fraction was added 700μl of 50mM sodium phosphate/saline buffer (pH=7.4) containing 0.1% BSA and 0.1% sodium azide. A 300μl aliquots was used for each RIA duplicate. The testosterone, A_4 and cortisol fractions were assayed by commercially available RIA kits.

The 11ß-OH-A_4 fraction was assayed using a modification of our in-house RIA method (Owen *et al* 1988). Samples (300μl) or standards (in the range 0.2-15nM/L), obtained after extraction and HPLC chromatography, were mixed with 11ß-OH-A_4 label (100μl) and antisera (100μl) at a working dilution of 1 : 15,000. The reaction mixture was vortexed thoroughly and left to incubate overnight at room temperature. 500μl of separation reagent was added followed by vortexing and a further incubation for at least 30 mins.

The separation reagent consisted of a mixture of second antibody (DARS at a 1:1000 dilution) and normal rabbit serum (at 1:100 dilution) made up in 8% PEG assay buffer. The incubation mixture was centrifuged at 1,500 *g* for 30 min at 4°C after which 0.5ml aliquots of supernatant were removed into scintillation vials followed by the addition of 5ml of Ecoscint scintillant and vigourous mixing by vortex before counting. Sample results were calculated by using a four-parameter logistic curve fitting procedure incorporated in the computer programme (Mr P.Edwards, Middlesex Hospital). Final results were adjusted for known volume losses and measured recoveries.

GC/MS validation.

Derivation of follicular steroids:

Follicular fluid (25 ml) was pooled from several ovarian follicles. Steroids were initially extracted with 250 ml of dichloromethane. The solvent was dried down in stages under OFN in a 100 ml round bottomed glass flask. This ensured a concentration of extracted material into the bottom of the flask. All subsequent reactions were carried out in silanised glassware.

The dried residues were subjected to solid phase extraction by reconstitution in 25mls 11ß-OH-A_4 assay buffer and passage through a (C_{18}) Sep-pak cartridge which had been previously primed with 2mls ethanol followed by 5mls water. The steroids retained on the cartridge were then eluted with 5mls ethanol and dried in stages under OFN. A mixture of internal standards (androstenediol, stigmasterol and cholesteryl butyrate), at concentrations of 100 µg/ml, were added to the dried residue and also dried down as before. This dried mixture was now finally resuspended in 200µl 2% methoxyamine hydrochloride in pyridine. This was incubated at 60°C for 1 hour followed by an addition of 100µl of trimethyl-silyl imidazole and an overnight incubation at 100°C. The reaction mixture was then evaporated under OFN at 60°C and reconstituted by sonication in 1ml of a solvent mixture containing cyclohexane, pyridine & HMDS in 98:1:1 by volume. Meanwhile simple columns containing approximately 2g (wet weight) of Lipidex 5000 in solvent were prepared in long glass pasteur pipettes plugged through the columns. The reaction mixture was loaded onto the column and the eluents collected. A further 1ml of solvent was passed through the column and collected.

This was evaporated in stages under OFN at 60°C and reconstituted into a final volume of 20μl cyclohexane of which 1μl aliquots were used for injection onto the GC/MS. A similar procedure was adopted using the 11ß-OH-A_4 fraction after HPLC separation of 25ml follicular fluid samples.

Total ion chromatogram (TIC) analysis by GC/MS:

A total ion chromatogram was obtained from the GC/MS analysis of derivated steroids from follicular fluid. The GC was run at a temperature ramped programme as follows. After sample injection the column was held for 2 mins at 60°C. Thereafter the column was heated at 60°C per minute to 200°C and then at 3°C per minute to 280°C and held at 280°C for 9 minutes. The MS was set to monitor and record all fragment and molecular ions produced after electron impact and quadropole analysis of the gaseous GC column effluent.

An ion chromatograph was constructed by computer which enabled the mass spectrum for each peak to be plotted. Derivated steroids were identified by comparison with mass spectra of reference compounds. Quantification of steroid peaks was made by an on board integrator in the data system. Peak areas were measured relative to the androstenediol, stigmasterol and cholesteryl butyrate internal standards for those steroids eluting between them. The main purpose of the TIC in this study was, however, to approximate the concentrations of the major steroids characteristic of follicular fluid by response relative to internal standards.

Selected ion monitoring (SIM) analysis by GC/MS:

The acquisition parameters on the MS data system were set up for the specific monitoring of the characteristic molecular ion of derivatised 11ß-OH-A_4 using a short GC temperature ramp programme of maintaining the column at 160°C for 2 minutes after sample injection then heating at 30°C per minute to 260°C after which the column was held at 260°C for 10 minutes. The GC run had been previously optimised using a 11ß-OH-A_4 standard derivative.

Study 2: Determination of steroids in serum and FFL.

Serum concentrations of steroids were measured using the commercially available RIA kits for testosterone, A_4, and cortisol. The 11ß-OH-A_4 was measured using the in-house method. Follicular fluid steroids were similarly assayed following extraction with dichloromethane and an HPLC separation step.

Study 3: Granulosa cell studies.

Follicular fluid containing granulosa cells was collected from 7 women undergoing follicular aspiration for IVF- ET. After repeated washings and centrifugation (Tsonis *et al* 1987), cells were gently suspended in Earl's culture medium which included NADPH, magnesium ions, 0.1% bovine serum albumin and 0.1% hyaluronidase. The volume of final suspension was adjusted to give approximately 50,000 cells / 0.5mls. Tritiated substrates (A_4 and cortisol) were used at 60,000 counts/20 minutes giving an approximate substrate concentration of 1nmol/L/incubation.

For the substrate incubations, cells were mixed with either of the tritiated steroids at 37°C for 24 hours, thus allowing sufficient time for completion of all reactions. This was followed by solvent extraction with 1ml of dichloromethane from which 700μl of the organic layer was removed, dried under OFN and resuspended in 300μl of initial HPLC mobile phase. An HPLC separation was then performed on 200μl of the resuspension according to conditions previously described. One minute eluent fractions from each run were collected directly into vials, followed by the addition of 5ml of scintillant, capping of vials and through mixing by vortex. Vials were counted in a liquid scintillation ß-counter for 20 minutes. Patterns of radioactivity in the HPLC radiograms were matched with the retention times of chromatograms obtained by the ultraviolet detection of known steroid standards which had been previously separated by an identical HPLC separation procedure.
Parallel incubations containing culture medium alone and culture medium with each of the subjects' whole blood, diluted 1:50, were performed as a check for background metabolism of the tritiated substrates.

Patients.

Serum and/or follicular fluid was obtained from women attending a variety of specialist clinics at the Middlesex Hospital, Charing Cross Hospital and the Hallam Medical Centre. Full ethical approval had been obtained by the Local Ethics Committees and consent obtained from the women to allow collection of samples.

Study 1.

Forty women attending the Hallam Medical Centre for IVF-ET had serum and follicular fluid saved prospectively on the day of oocyte collection. The samples were collected randomly over approximately 3 months and were stored at -20°C for later analysis.

For the total ion chromatography several samples of FFL were combined to give a total volume of 25 ml.

Study 2.

Serum and/or follicular fluid was obtained from five groups of women. There was no difference in age (range 28-43 years), BMI (range 18.2-27.8) or, where applicable, the duration of infertility (3-10 years) of the groups. All samples were collected between 8.30 and 12 am.

Group 1.

Fifty three women presenting to a gynaecology clinic with PCOS were consecutively recruited for the study. The women each had a pelvic ultrasound scan which showed the typical ultrasound appearance of PCO and were on no treatment. Serum 17α-hydroxyprogesterone concentrations taken in the morning were less than 5.0nmol/L unstimulated, or less than 10.0 nmol/L 60 minutes after synacthen (250ug) was given intravenously, thereby excluding patients with adrenal enzyme defects. In women with oligo- or amenorrhoea, serum samples were taken at the time of the appointment. If the women

were menstruating regularly, serum was collected during the early follicular phase of the cycle.

Group 2.

Thirteen healthy women volunteers were recruited from the staff as controls. The women had normal ovaries on scanning by ultrasound and were ovulating spontaneously.

Groups 3 and 4.

Serum and follicular fluid samples were collected at the time of oocyte collection from 51 women undergoing super-ovulation for IVF-ET. The women were recruited randomly over a 3 month period. Twenty seven women had incidentally diagnosed PCO (Group 3) and 24 had normal ovaries (Group 4). All of the women had regular ovulatory cycles.

Multi-follicular development was achieved using the standard combination of buserelin and human menopausal gonadotrophin. Clear follicular fluid was obtained by ultrasound directed percutaneous needle aspiration and only follicles with a mature oocyte were analysed.

Group 5.

Follicular fluid was obtained from the dominant follicles of 13 women with normal ovaries who had laparoscopies in the late follicular phase (Eden *et al* 1988). They were undergoing investigation of infertility found to be due to oligospermia alone in 3 women and pelvic adhesions in 10 women.

Study 3.

Granulosa cells were collected from the follicular fluid of 7 women undergoing oocyte collection prior to IVF-ET. The women all had ultrasound diagnosed PCO. The samples were collected on a random basis over a three day period.

Statistics.

Data were not normally distributed, therefore, all statistical comparisons were made using non parametric tests. The data are presented as median values and ranges. The data were analysed using the two tailed Mann-Whitney rank test of significance. To aid graphical representation in Fig *V*-7, 11ß-OH-A_4 results were plotted on a logarithmic scale.

Results.

Study 1: Establishment of reliable quantitative method for assessment of 11ß-OH-A_4 concentrations in biological fluids

Preparative chromatography of steroids.

Dichloromethane was found to be the most suitable solvent for the extraction of the steroids under study. Due to the wide range in polarities of the steroids, recoveries with this solvent ranged between 49% and 84% for 11ß-OH-A_4 (Table *V*-1).

Table *V*-1. Recoveries of steroids after extraction and HPLC (mean ± SD)

Steroid	n=	Recovery
11ß-OH-A_4	27	84 ± 20%
Testosterone	22	64 ± 23%
A_4	28	49 ± 23%
Cortisol	23	68 ± 20%

Different HPLC procedures were attempted. Although reverse phase systems with methanol/acetonitrile on ODS-C18 columns gave good separation of the steroids, subsequent RIAs were destroyed due to the presence of high background interference in the column effluent. This problem was not resolved despite using columns from different suppliers or from adopting a range of reaction protocols. A normal phase HPLC system, however, gave

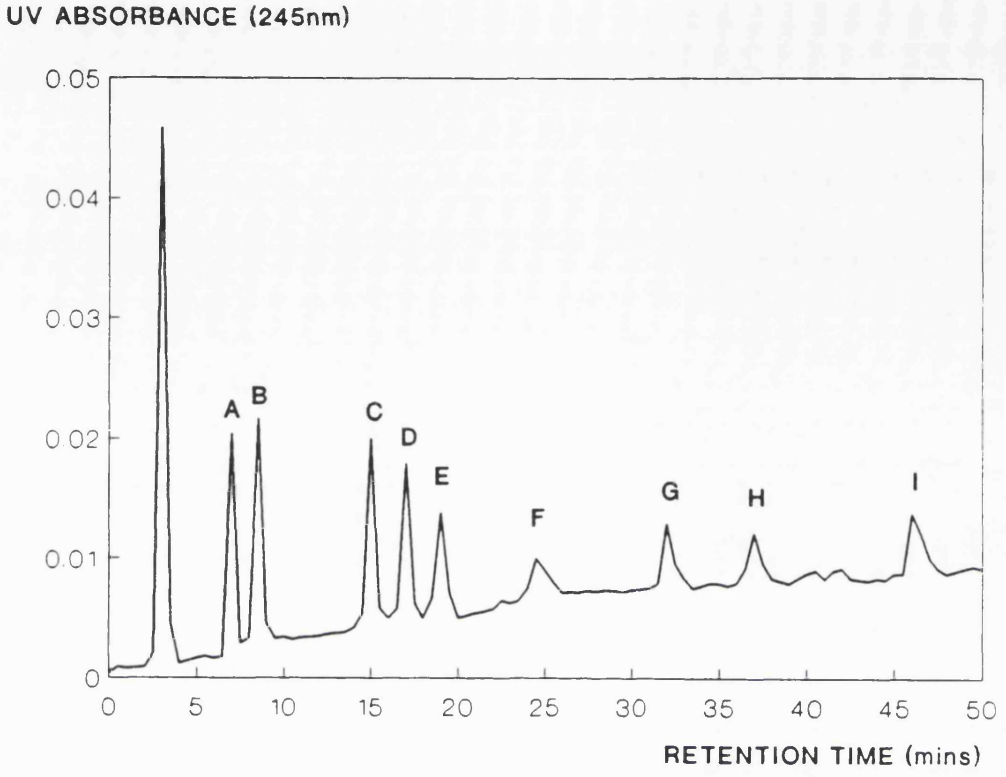


Figure *V*-1. HPLC chromatogram of steroids.

Key to steroid elution (retention times in mins)

A. Progesterone (7.22)
B. Androstenedione (9.35)
C. Testosterone (15.45)
D. 17 OH Progesterone
E. 11ß-OH-Androstenedione
F. 17ß-Oestradiol
G. Corticosterone (32.72)
H. Cortisone (37.45)
I. Cortisol (47.05)

insignificant background binding in subsequent RIAs and was thus adopted. Separation of the steroids of interest known to occur in follicular fluid was thus achieved, using the chromatographic conditions described above.

A 200μl aliquot of a mixture of these steroids (each at a concentration of 2ng/μl) gave a chromatogram as shown in Figure *V*-1. This demonstrated efficient separation of the steroids and thus enabled the collection, for subsequent RIA, of multiple eluent fractions from a single sample. The recoveries of steroids (Table *V*-1) were measured in parallel for every sample and were considered to be acceptable.

Synthesis of tritium labelled 11ß-OH-A_4 from cortisol

The synthesis of labelled 11ß-OH-A_4 was verified in two ways. First, the positions on the TLC plate corresponding to the cold 11ß-OH-A_4 standard gave peaks of radioactivity and immuno- reactivity. Furthermore when the reaction products were subjected to HPLC separation, the majority of radioactivity (53%) was found to reside in the fraction corresponding to 11ß-OH-A_4 as previously determined using an unlabelled standard. For all subsequent RIA analysis, the aforementioned HPLC fraction, collected between 18.5-20.5 min, constituted the purified preparation of label.

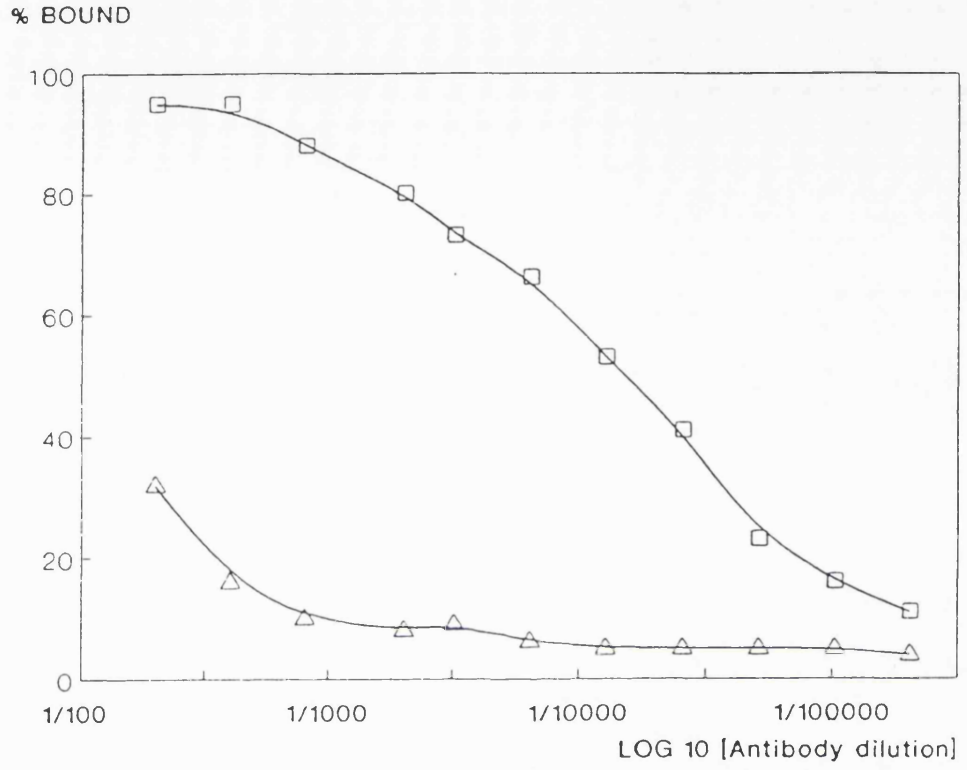
Radioimmunoassay

Background binding:

Using the normal phase diol column the background binding was found to be insignificant in the RIA (less than 0.3%).

Optimisation:

The optimum concentration of antibody in the RIA was determined from an antibody dilution curve (Fig *V*-2). A working dilution of 1:15,000 of antibody gave 50% binding when the total counts used per tube were approximately 20,000 counts/20 mins. Separation using a

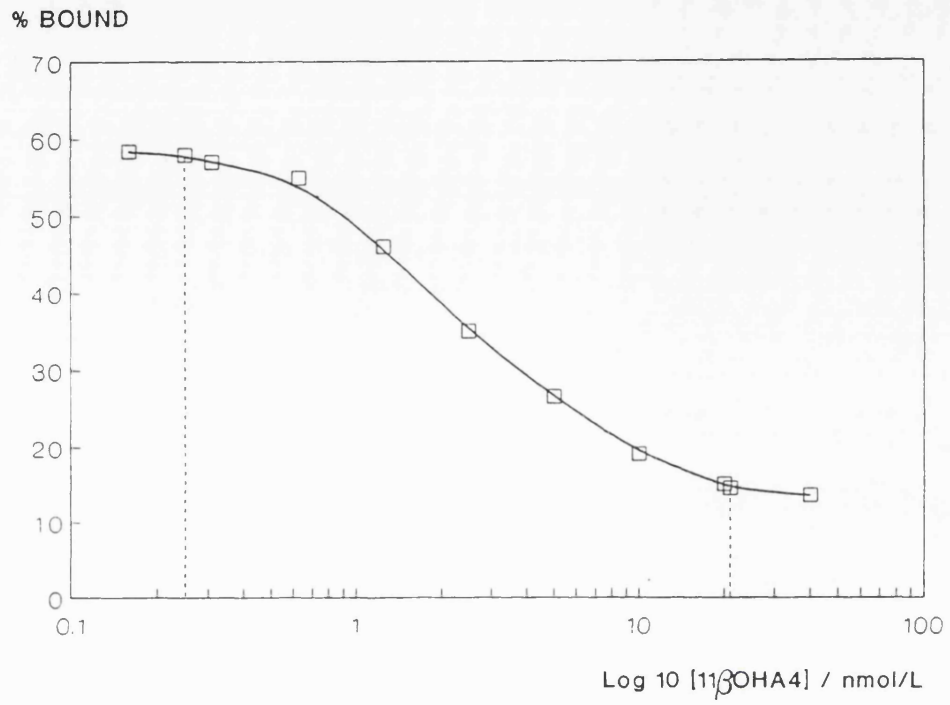


□ - Binding at an 11ß-OH-A_4 concentration of 40 nmol/L

△ - 0% binding

Figure *V*-2. 11ß-OH-A_4 antibody dilution curve for the in-house RIA

A)



B)

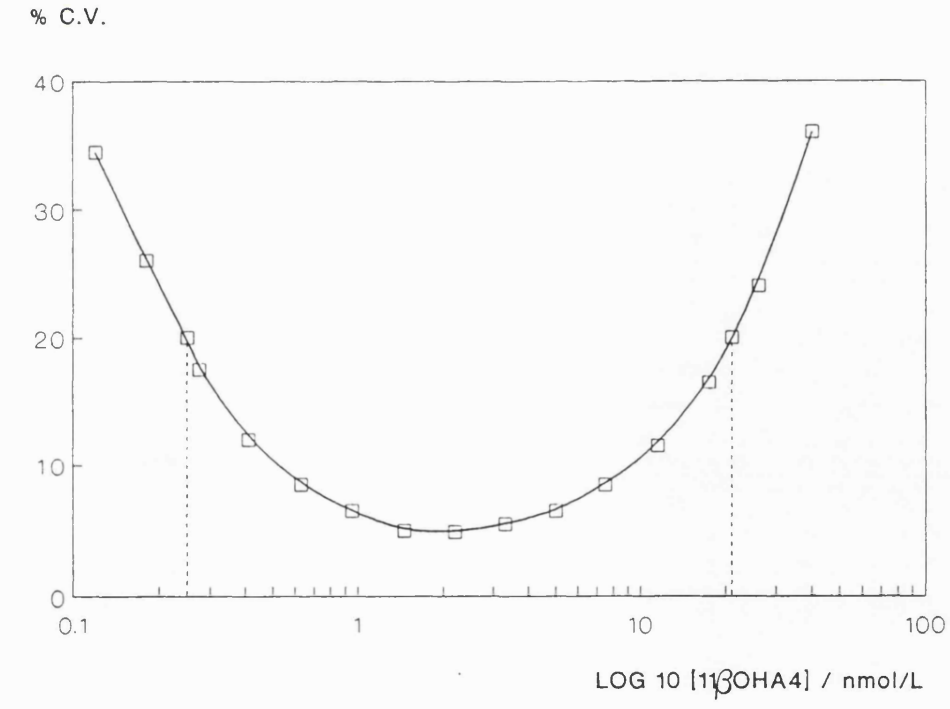


Figure *V*-3. 11ß-OH-A_4 radioimmunoassay.

A) Standard curve of in-house 11ß-OH-A_4 RIA.

B) Precision profile of in-house 11ß-OH-A_4 RIA

second antibody (DARS)-NRS-PEG assisted reagent was preferred as it was found to be the quickest, the most stable and the most accurate technique when compared with charcoal and saturated salt precipitation. A typical standard curve of the 11ß-OH-A_4 RIA is shown in Figure *V*-3.

Characterisation:

The precision profile (Fig *V*-3) was used to assess the detection limit of the assay (Chard 1985). At 20% coefficient of variation (CV) the sensitivity of the assay was found to be 0.25nM/L. The intra and inter assay precision was assessed by the CV of 3 serum quality controls (Q.C.). The results are presented in Table *V*-2.

Table *V*-2. Precision of in-house 11ß-OH-A_4 RIA (%c.v.)

Q.C.	Intra-assay variation (n=14 duplicates)	Inter-assay variation (n=7 RIAs)
1.13 nmol/L	10.6%	25.3%
4.72 nmol/L	8.3%	14.0%
10.55 nmol/L	7.9%	9.4%

Accuracy of the RIA was tested by a series of dilution and spiking experiments. Three serum samples, containing a known, low concentration of 11ß-OH-A_4, were spiked with increasing amounts of 11ß-OH-A_4 standard. A further three samples containing known high concentrations of 11ß-OH-A_4 were diluted. In all cases an RIA was performed to check for parallelism. There was no significant difference ($p > 0.3$) between observed and expected values (Table *V*-3).

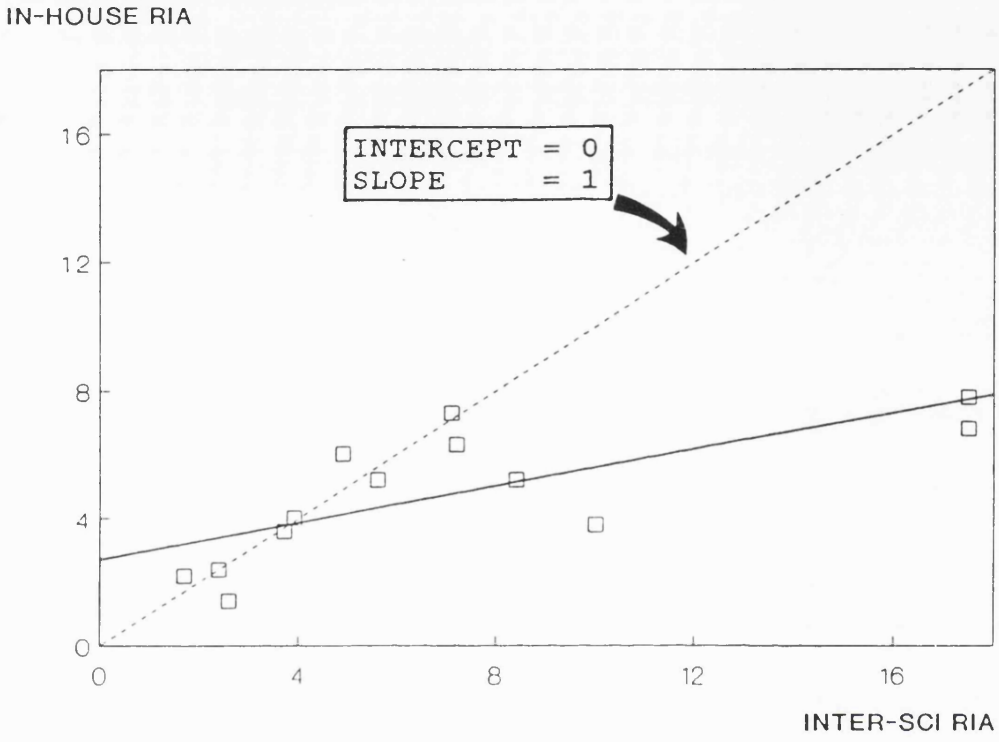


Fig *V*-4. Regression analysis between Intersci and in-house 11ß-OH-A_4 RIA (n=13)

(95% confidence limits: Intercept=2.73±1.55, slope=0.29±0.18)

Table *V*-3. Analytical recoveries of 11ß-OH-A_4

Sample No.	11ß-OH-A_4 added	11ß-OH-A_4 measured	Recovery
1	3.0	2.7	90%
	5.0	5.7	114%
	9.0	10.3	114%
2	3.4	3.6	106%
	5.4	4.8	89%
	9.4	9.0	96%
3	3.5	4.2	120%
	5.5	6.0	109%
	9.5	10.7	112%
4	1.4	1.2	88%
	2.7	1.7	63%
	5.5	5.1	93%
5	1.5	1.5	100%
	3.0	2.8	93%
	5.9	6.5	110%
6	1.8	1.2	68%
	3.6	3.0	83%
	7.1	7.9	111%

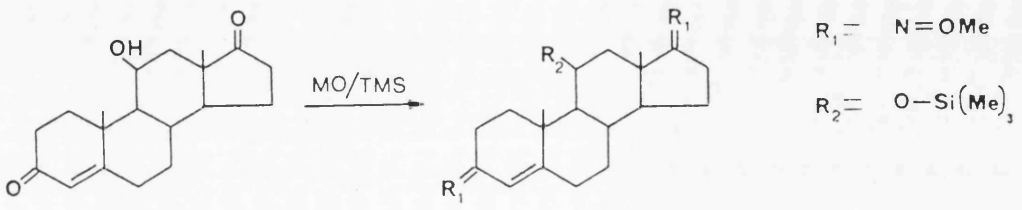
Specificity of the antisera has been reported elsewhere (Putz *et al* 1987). Most of the common steroids would not be present in the HPLC eluates found in the present study.

The results of our procedure were compared with the only commercially available method, the Intersci kit. The regression analysis demonstrated significant differences between the methods due to higher results with the Intersci kit: the intercept and slope lay outside the 95% confidence limits of 0 and 1.0 respectively (Fig *V*-4).

GC/MS of follicular fluid steroids

Authenticity of the 11ß-OH-A_4 in the relevant follicular fluid HPLC fraction was established using the technique of single ion monitoring-gas chromatography-mass spectrometry. The characteristic fragmentation pattern of derivatised 11ß-OH-A_4 is shown in Figure *V*-5 with

A)



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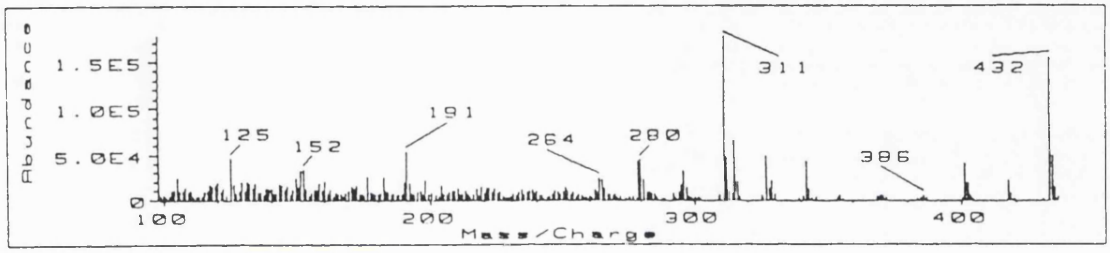
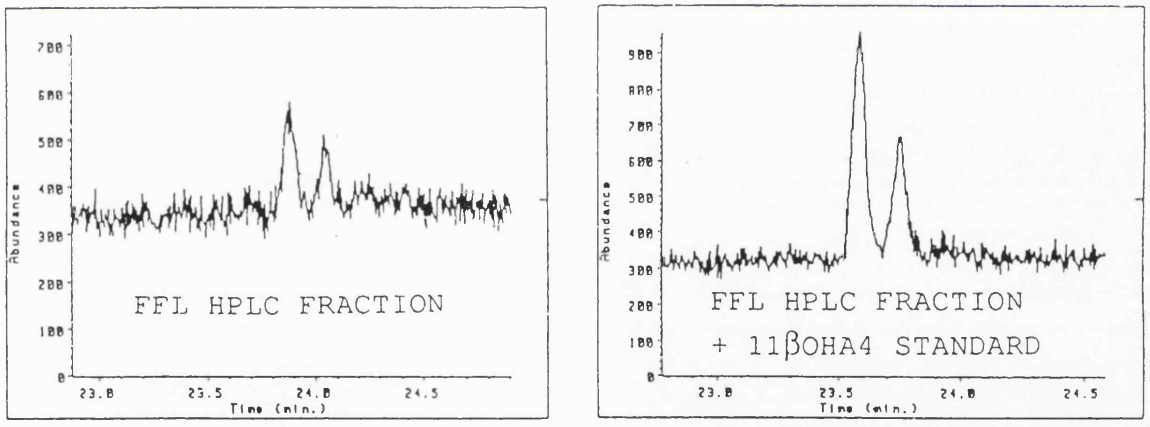


C)



(i) (ii)

Figure *V*-5. GC/MS analysis of 11ß-OH-A_4.

A) Derivatisation of 11ß-OH-A_4 for GC/MS analysis.

B) Mass spectrum of 11ß-OH-A_4-Mo-TMS derivative.

C) Single ion monitoring of 11ß-OH-A_4 in FFL

(i) before, (ii) after addition of 1ng 11ß-OH-A_4 standard

a molecular ion (*m/z*) of 432. Using single ion monitoring (SIM), 11ß-OH-A_4 was defined by the response for the molecular ion with a characteristic double peak (due to syn- and anti-isomers of the oxime derivatives) and the GC retention times. SIM analysis of follicular fluid samples demonstrated the presence of this 432 double peak at the identical time to that of the standard. On re-running the follicular fluid sample, spiked with approximately 1ng of derivatised 11ß-OH-A_4 standard, a reinforcement of this 432 double peak was obtained (Fig *V*-5).

In order to demonstrate which steroids are found in the highest concentrations in follicular fluid, a straightforward total ion chromatogram (TIC) was obtained by analysing 25 ml of derivatised FFL through the GC/MS. The resultant TIC trace is shown in Figure *V*-6 with a list of identified steroids together with an estimate of their concentrations. As can be seen 11ß-OH-A_4 was undetectable by this analysis and its presence could only be confirmed by the very sensitive SIM method.

Study 1 - Patient Samples.

11ß-OH-A_4, cortisol, A_4 and testosterone in follicular fluid were measured by the HPLC and RIA procedure (Table *V*-4). All results were corrected for background binding, recoveries and known procedural losses. The serum samples were measured by the specific in-house RIA after extraction alone.

Table *V*-4. Median (range) concentrations of steroids in serum and follicular fluid (nmol/L)

Steroid (n=)	Serum (n=25)	FFL (n=30)	FFL/serum
11ß-OH-A_4	3.0 (0.3-7.2)	11.7 (3.0-31)	3.9
Cortisol	838 (120-1580)	605 (41-4075)	1.4
A_4	6.2 (3.1-18.0)	308 (12-3525)	49.7
Testosterone	3.4 (0.3-11.8)	27.5 (5-74)	8.1

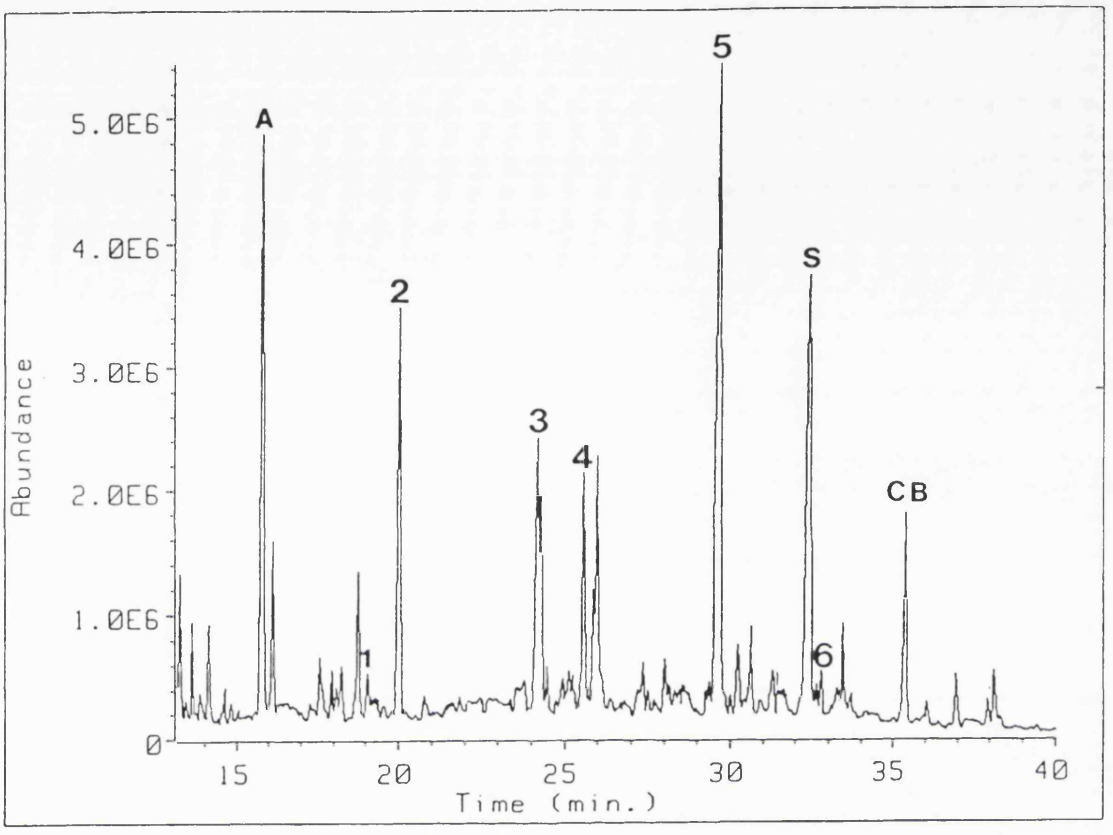


Figure *V*-6. Total ion chromatogram of FFL.

Key to steroids and concentrations (μmol/L).

A) Androstenediol (Internal standard)
1) Androstenedione (1.3)
2) 17ß-Oestradiol (13.9)
3) Progesterone (9.7)
4) 17OH-Progesterone (8.6)
5) Cholesterol (24.7)
S) Stigmasterol (Internal standard)
6) Cortisol (2.2)
CB) Cholesterol Butyrate (Internal standard)

Nineteen FFL samples were radioimmunoassayed for 11ß-OH-A_4 without HPLC (median = 42.7 nmol/L (range 8.9-59.8nmol/L). The results from samples without HPLC were significantly higher than the results from samples when preceded by HPLC (10.8 nmol/L (range 3.0-31nmol/L) p=0.03).

Study 2-Patient samples.

Comparison of serum results from women with PCOS and women with normal ovaries (Groups 1 and 2).

Serum concentrations of the androgens 11ß-OH-A_4, A_4 and testosterone, were significantly higher in untreated women with PCOS compared to control women with normal ovaries (Table *V*-5, Fig *V*-7). There was no difference in the concentrations of cortisol between the groups.

Table *V*-5. Median (range) concentrations of steroids in serum of 53 women with PCOS and in 13 controls (nmol/L)

Steroid	Women with PCOS	Women with normal ovaries	p=
11ß-OH-A_4	4.3 (1.0-14.4)	2.6 (1.0-9.0)	0.02
Cortisol	254 (113-642)	332 (165-434)	0.14
A_4	4.9 (2.6-16)	3.7 (1.4-8.2)	0.03
Testosterone	2.2 (1.1-4.3)	1.6 (0.9-2.4)	0.006

Gonadotrophin treatment; Comparison of serum results from women with PCO (Group 3) and women with normal ovaries (Group 4).

In the group of women receiving buserelin and gonadotrophins prior to IVF-ET, only

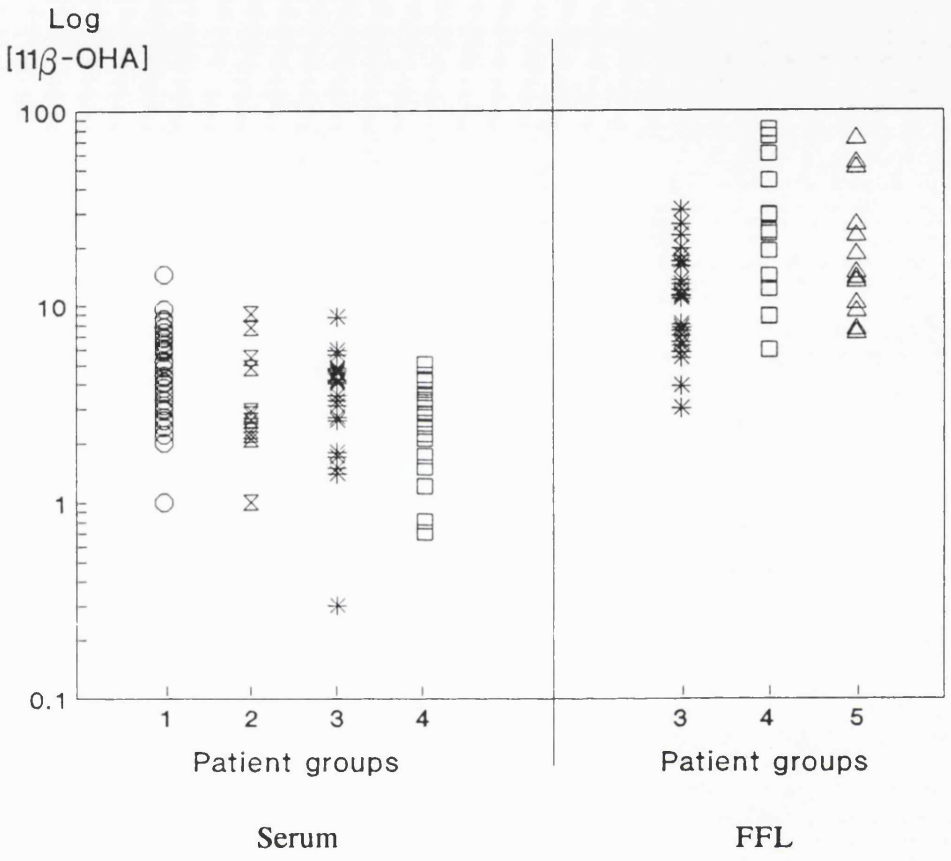


Fig *V*-7. Serum and FFL concentrations of 11ß-OH-A_4 (nmol/L) in different patient groups

circulatory 11ß-OH-A_4 and cortisol concentrations were significantly higher in women with PCO compared to those with normal ovaries (Table *V*-6).

Table *V*-6. Median (range) concentrations of steroids in serum of women with normal and polycystic ovaries undergoing ovarian stimulation for IVF-ET (nmol/L)

Steroid	Women with PCO (n=27)	Women with normal ovaries (n=24)	p=
11ß-OH-A_4	4.1 (0.3-8.7)	2.3 (0.7-5.0)	0.029
Cortisol	971 (470-1944)	503 (247-1367)	0.002
A_4	10.2 (3.1-15.8)	5.2 (3.1-16.6)	0.200
Testosterone	4.3 (1.8-14.8)	3.0 (0.3-9.0)	0.078

Measurements of 11ß-OH-A_4 concentrations in serum were similar in women receiving gonadotrophin treatment and those with no treatment, irrespective of ovarian morphology (Tables *V*-5 and *V*-6, Fig *V*-7). Serum androstenedione, testosterone and cortisol concentrations were, however, significantly higher ($p<0.001$) in women receiving gonadotrophin treatment compared with untreated women irrespective of ovarian morphology.

Follicular fluid measurements (Groups 3, 4 and 5).

High concentrations of all steroids under study were demonstrated in follicular fluid compared with the circulation. The high concentrations were observed both in women with natural cycles and women undergoing gonadotrophin stimulation prior to IVF-ET (Table *V*-7, Fig *V*-7). There were no significant differences in the follicular fluid concentrations of cortisol, androstenedione or testosterone between women with PCO and normal ovaries (p= 0.61, 0.11, 0.95 respectively). Significantly lower concentrations of 11ß-OH-A_4 were, however, found in follicular fluid in women with PCO compared to those with normal

ovaries (p=0.009). Furthermore, in women with normal ovaries, concentrations of testosterone in FFL were significantly higher (p<0.0001) in those women undergoing gonadotrophin stimulation compared with women in spontaneous cycles.

Table *V*-7. Median (range) concentrations of steroids in FFL of women with normal and polycystic ovaries undergoing ovarian stimulation for IVF-ET and of women with normal ovaries in spontaneous cycles (nmol/L)

Steroid	Women with PCO (n=27)	Women with normal ovaries (n=24)	Spontaneous cycles(n=13)
11ß-OH-A_4	11.3 (3-31)	23.3 (6-74)	14.7 (7-72)
Cortisol	701 (217-4141)	529 (141-4080)	-
A_4	435 (10-3527)	159 (12-1080)	325 (35-535)
Testosterone	21 (11-72)	25 (5-73)	7 (4-38)

Study 3 - Granulosa cell culture experiments.

Incubations of granulosa cells with A_4 demonstrated conversion to testosterone and 17ß-oestradiol in all 7 patients. None of the incubations, however, showed 11ß-OH-A_4 production from A_4 (Table *V*-8).

Conversion of cortisol to cortisone occurred in all patients, however the presence of 11ß-OH-A_4 was clearly detected in only 2 out of 7 incubations where 29% of radiolabel co-eluted with 11ß-OH-A_4 (Table *V*-8). Contamination of granulosa cells by blood was small (0.3% on average).

Table V-8. Results of incubations of radiolabelled steroids with granulosa cells*

Elution times (mins)@	Mean radioactivities		
	Androstenedione incubation (n=7)	Cortisol incubation (n=5)	Cortisol incubation (n=2)
0-6.6	1	0	1
6.6-8.4 (a)	1	0	1
8.4-10.4 (b)	48 (27%)	1	0
10.4-14.3	3	0	0
14.3-16.3 (c)	21 (37%)	1	1
16.3-18.4 (d)	2	1	1
18.4-20.4 (e)	2	3	29 (2%)
20.4-23.4	1	1	1
23.4-25.4 (f)	16 (43%)	1	1
25.4-30.9	2	1	1
30.9-32.9 (g)	1	2	1
32.9-35.1	1	2	3
35.1-37.1 (h)	1	31 (61%)	23 (11%)
37.1-43.6	1	2	2
43.6-45.6 (i)	0	53 (36%)	35 (17%)
45.6-50.0	0	1	1

* = *Radioactivities of HPLC fractions following incubation with labelled substrates expressed as percentage of the total amount of radioactivity per incubation. Experimental variation (%CV) is given in brackets for each major peak.*

@ = *Elution of known steroids:*
(a) Progesterone; (b) A4; (c) Testosterone; (d) 17αOH progesterone; (e) 11β-OH-A4; (f) 17β-E2; (g) Corticosterone; (h) Cortisone; (i) Cortisol. (Small)

VI. Discussion

Summary

Studies on ovarian cell growth, differentiation and steroid production suggested that insulin-like growth factors play an important role in modulation of gonadal function. Clinical application of these in-vitro findings were investigated in the setting of a super-ovulation programme for IVF-ET. Women who had previously responded sub-optimally to various drug regimens were recruited into three separate studies. The ovarian response to gonadotrophin stimulation with additional GH treatment was assessed and the concentrations of IGFs in the circulation and the FFL were established.

Women with raised gonadotrophins did not have an improved response to ovarian stimulation when GH was added. Women with a normal appearance of the ovaries on ultrasound scanning of the pelvis did not show any clinical improvement with GH co-treatment. Women with a polycystic appearance of the ovaries did, however, demonstrate an improved response when treatment with hMG was augmented with GH. The improved response in women with PCO was seen both in the CC and hMG and the buserelin and hMG studies. GH administration was associated with a rise in the concentrations of IGF-I in the circulation. FFL concentrations were higher in GH treated women but significantly lower than serum concentrations.

Access to FFL through the IVF-ET procedures stimulated an interest in the investigation of an intraovarian marker for the subgroup of women with PCO. Detectable concentrations of 11ß-OH-A_4 had been demonstrated in the ovarian veins of an adrenalectomised woman with PCO and it was postulated that 11ß hydroxylase activity on C19 steroids may occur uniquely in women with PCO. Having developed a specific and sensitive technique for measuring 11ß-OH-A_4 in serum and FFL, the presence of this weakly androgenic steroid in the FFL of women with normal and polycystic ovaries was established. Although concentrations of 11ß-OH-A_4 were higher in the circulation of women with PCO, FFL concentrations were lower than in women with normal ovaries. 11ß-OH-A_4 concentrations are therefore not a useful marker for this enigmatic condition.

GH studies - methodology.

Suboptimal responders.

The participants in the studies were women who, despite requiring large doses of exogenous gonadotrophins, had suboptimal responses to ovarian stimulation regimens. Women with "poor" responses in IVF-ET programmes are difficult to treat and have an unfavourable outcome (Pellicer *et al* 1987). Treatment options currently include progressively increasing the dose of hMG or FSH, desensitising the pituitary with GnRH analogues prior to hMG administration or, occasionally, offering oocyte collection in natural or clomiphene stimulated cycles (Serafini & Marrs 1988, Benadiva *et al* 1988).

Increasing the dose of hMG does not necessarily result in a proportional improvement in the ovarian response and a plateau of ovarian response is often reached. Clinically this presents a dilemma of management and alternative treatments are needed for this small group of women.

IGF-I augmentation of the FSH-primed granulosa cell response has been well documented (Adashi *et al* 1984, 1985b, 1985c) and Jacobs *et al* (1987, 1988) used these cell studies as a basis for the clinical application of biosynthetic GH in ovulation induction. The work presented in this thesis complements the in-vivo fertilisation studies (Homburg *et al* 1988, 1990a,b) and describes the first use of GH administration performed in the IVF-ET setting.

Follicular development.

The aim of ovulation induction in amenorrhoeic women is unifollicular development. In those women resistant to CC, follicular stimulation is best achieved with daily injections of exogenous gonadotrophins (or pulsatile GnRH infusions) which may take several weeks (Hamilton-Fairley *et al* 1991). In IVF-ET programmes the aims are different. Multifollicular development is obligatory and the duration of treatment tends to be shorter. The ultimate aim of ovarian stimulation, prior to IVF-ET, is the collection of the maximum number of mature

oocytes with the transfer of healthy embryos.

With this is mind a suboptimal response was arbitrarily defined, for the CC and hMG studies, as one in which 3 or fewer oocytes were collected or two or fewer embryos transferred. This suboptimal response occurred despite the patient receiving a starting dose of at least 4 ampoules of hMG per day. These entry criteria defined a particularly resistant sub-group of "poor responders" but this was expedient as GH treatment in IVF-ET was untried. Apart from ever increasing doses of hMG, however, at that time, there was little other treatment to offer.

The randomised study of GH co-treatment with CC and hMG did not demonstrate an impressive improvement in ovarian response and the entry criteria were subsequently relaxed for the buserelin and hMG study. The aim of the latter study was to assess the use of GH in women who had some follicular development, but where improvement in the ovarian response was required. The entry criteria were therefore expanded to include women who had had up to 6 oocytes collected but where fewer than 4 embryos had been transferred. I consider that, in the buserelin and hMG study, the women did not necessarily constitute a true group of "poor responders" as the entry criteria were quite generous. The inclusion criteria were, however, appropriate as, without GH, larger doses of hMG would have been required in the next treatment cycle.

Polycystic ovaries.

The specific characteristics of women who respond suboptimally are difficult to define and the women form a heterogeneous group. Some women are found to have raised gonadotrophin concentrations and are thus either perimenopausal or have the resistant ovary syndrome. Other diagnostic tests tend not to be helpful. The finding of a normal appearance on scanning the ovaries does not predict a normal response to ovarian stimulation.

A pretreatment ultrasound scan of the ovaries was performed on all of the women in these studies and 34 of the 50 women recruited had co-incidentally diagnosed PCO. The finding of such a high percentage of women with ultrasound diagnosed PCO amongst the poor

response group has not been reported in the literature. This figure does, however, correspond with two other studies demonstrating a high percentage of women with ultrasound diagnosed PCO in populations attending IVF-ET centres (Owen *et al* 1989, Rutherford *et al* unpublished).

The women in the GH studies who were shown to have ultrasound diagnosed PCO did not have the classical signs and symptoms of PCO *syndrome*; most obviously they all had regular cycles. Hirsutism was not formally assessed but the group of women with PCO did not have higher BMIs or LH concentrations than the women with normal ovaries.

Women with PCO are typically regarded as potential hyper-responders and anxieties about ovarian hyperstimulation syndrome are axiomatic. That a high proportion of women with a previously suboptimal response have PCO reflects the heterogeneity of the latter condition. The wide range of ovarian response to gonadotrophins may reflect a diversity of action of some intraovarian modulator, for example the IGFs.

Study design.

Placebo controlled trial.

It was considered imperative that, having shown an improved ovarian response to GH in the open study, a placebo controlled study be initiated. Particularly in the emotive area of infertility, treatments can be introduced without suitable investigation or confirmation of efficacy. Within the setting of a private IVF-ET centre, financial motivation can easily lead to the introduction of unproven therapies.

After the initial open study was completed, it was made clear to the patients that GH was only available through the placebo controlled study. For ethical reasons, the design of the study included an open arm of GH treatment for women initially randomised to receive placebo. At least two cycles with no treatment were allowed to lapse before the GH cycle was undertaken.

Burger *et al* (1991) reported a reduced requirement for hMG in cycles following GH administration. The setting was an ovulation induction programme, however, and the women were amenorrhoeic and profoundly hypoestrogenic. The studies presented in this thesis do not address the phenomenon of a carry-over effect but I consider that women with normal hormone concentrations, undergoing ovarian stimulation prior to IVF-ET, form a fundamentally different group and a marked carry-over effect after two or more months is unlikely.

Age Criteria.

In both of the placebo controlled trials, there was an age restriction for entry. The women were less than 40 years in the CC and hMG studies and less than 38 years in the buserelin and hMG studies. The age exclusion criterion was changed for the second study as I aimed to recruit women with potentially more responsive ovaries. Perimenopausal women, defined herein as women with an early follicular phase serum concentration of FSH of greater than 15iu/L, were excluded from the placebo controlled studies, as 2 perimenopausal women had shown no response to GH in the open CC and hMG study.

Diagnostic Categories.

In the initial CC and hMG studies women whose partners had oligospermia were excluded. Since, however, the ovarian response to GH was considered the end point of the studies, not pregnancy rates, in the buserelin and hMG study women with any indication for IVF-ET were included. It had also been noted that the implantation rate, in the women with oligospermic partners achieving embryo replacement, was not different from the patients in the other diagnostic categories (Sharma *et al* 1988).

In the buserelin and hMG study there was a chance difference in the length of infertility between those women going on to receive GH and those receiving placebo. Since the end point of study was not fertility, but the effect of GH on the induction of multifollicular development with the collection of many oocytes, the difference was not, however, regarded as important in the design of the studies.

Administration of GH.

Pharmacological doses of GH (24iu on alternate days) were used in the studies with the aim of inducing high circulating concentrations of IGF-I. A dose of 20U GH had been shown, in a normal adult male, to result in serum IGF-I concentrations of a maximum of 2.2 U/ml at 36 hours post injection (Fig *I*-3). I wanted to induce a definite response in IGF-I concentrations in the study patients and therefore chose the 24u dose, rather than a smaller, more physiological dose.

End points of study.

The aims of the studies were to assess the impact of GH co-treatment on ovarian function. The most objective measurements for evaluation were the total dose of hMG used per cycle, the number of follicles observed and the urinary oestrogens or serum E_2 on the day of hCG administration, and the number of oocytes collected. Homburg *et al* (1988) had compared the number of days of treatment using GH or placebo treatment. I did not feel that assessing the duration of treatment gave any more information than the total dosage of hMG used, as in IVF-ET the length of therapy was so much shorter.

Because of the constraints of the clinic timetable, I could not be directly involved in the day to day management of every single one of the patients during ovarian stimulation, but the decision about hCG administration was governed by strict guidelines. The guidelines were that hCG should be given as soon as 3 or more follicles were greater than 14mm in diameter, with at least one follicle diameter greater than 17mm. This required careful ultrasound assessment of the number of follicles on the day of proposed hCG administration. The oestrogen results were used as corroboration of the ultrasound findings. I was satisfied that there was very little bias in the assessments of total hMG dosage, number of follicles and concentrations of oestrogens on the day of hCG administration as no extraordinary consideration was given to the women in the studies.

The objectivity of the number of oocytes collected may, however, be subject to criticism. During the period of the first studies I was not sufficiently technically experienced to provide

a constant level of skill at oocyte recovery. The most experienced operator present on the day performed the oocyte collection. I was, however, present for each procedure and was thus able to document the details of the follicles and oocytes and to supervise the collection of the follicular fluid.

By the time of the buserelin and hMG study, I had sufficient experience to feel confident that I could offer the women a reliable and repeatable level of expertise. Admittedly, I was enthusiastic for a favourable outcome of the oocyte collections, but as the study was double-blind, I must have been equally eager in the placebo as in the GH cycles. Indeed this point became evident in the evaluation of the results (Table *IV*-5 and -6). In the open arm of the study (GH cycles following placebo cycles), again lack of objectivity could be claimed but is unlikely, as a maximum effort had been expended in the placebo cycle and was expended again in the GH cycle. Although presented in the results sections, fertilisation, cleavage and pregnancy rates were not included in the formal end points of the studies as many variables exist to compound the effect of GH.

Embryo transfer.

At the time that the GH studies were being undertaken many centres were transferring a maximum of 4 embryos. This changed with the introduction of the Licensing Authorities in 1989 and 1990 and currently 3 is the maximum number of embryos transferable (RCOG 1990). The women recruited, particularly for the CC and hMG studies, were considered to be in a potentially poor outcome group and the guidelines from Hallam Medical Centre (1987-89) allowed transfer of 4 embryos under these circumstances. All the women were carefully counselled by the doctor performing the embryo transfer and the risk of multiple pregnancies stressed.

No pregnancies occurred in the first studies but of the 5 ongoing pregnancies in the buserelin and hMG study, 2 were twins. Both of these women had had 4 embryos transferred, one having had nine embryos cleaved and the other woman seven. The outcome was good in both of these cases but it is accepted that, under present circumstances, the management would be different.

GH studies - results

Clinical results.

Open study.

The results of the initial anecdotal case study reported in Chapter *III* demonstrated an ovarian effect of GH that mirrored the results reported by Homburg *et al* in ovulation induction cycles (1988,90a,b). Six women had 12 GH cycles and significantly more follicles developed and more oestrogen was excreted in the GH cycles compared with the prestudy cycles (Table *III*-1). This study was, however, uncontrolled and not blind. There was a lot of enthusiasm amongst all of the staff and great efforts were made to ensure the best possible outcome. Although women with a poor ovarian response tend to have repeatedly suboptimal cycles (Pellicer *et al* 1987, Jones 1984), the psychology of being involved in a new treatment regimen may itself have beneficial effects.

The two women who had raised gonadotrophins failed to show any improvement with GH co-treatment. Interestingly they were still having mainly regular cycles despite the predicted imminent failure of ovarian function. We were not able to establish whether the women had resistant ovary syndrome or premature menopause but in either condition increasing the doses of exogenous gonadotrophins does not necessarily result in an ovarian response. Whether healthy viable follicles no longer exist (premature menopause) or whether they can no longer respond to FSH stimulation (resistant ovary syndrome), GH did not improve the ovarian response.

Volpe *et al* (1989) reported, in women receiving hMG and pure FSH, that older women (age range 39 to 41 years) failed to show an improvement with GH co-treatment. Their FSH concentrations ranged from 6.6 to 18.4 mUI/ml but they did not clarify whether these results were in the normal or high range for their laboratories.

CC and hMG study.

The prospective, double blind, randomised, placebo controlled trial of GH cotreatment in women who previously had a poor response to treatment with CC and hMG for IVF-ET did not reproduce the results of GH use in ovulation induction (Homburg *et al* 1988, 1990a,b). No significant improvement in ovarian response to hMG with GH cotreatment was demonstrated (Table *III*-2). Patients with ultrasound diagnosed PCO did, however, show some response (Table *III*-7).

The women recruited for this study were extremely poor responders and had had up to four previous attempts at ovarian stimulation. Indeed only 10 of the 19 women had had any oocytes collected at all in their pretreatment cycles. They had required between 4 and 6 ampoules of hMG a day until hCG administration or until the treatment was abandoned which, in combination with CC, is unusual.

Group comparisons of the women receiving placebo compared with those receiving GH did not show any difference in outcome (Table *III*-2). Significantly less hMG was, however, used in the group receiving placebo. Because of the large interpatient variation in the patients' responses, an analysis of paired comparisons of the results of placebo or GH treatment with those from the prestudy cycles was performed (Tables *III*-3 & 4, 8 & 9). The pretreatment cycles were used as retrospective controls although the statistical weakness of this type of analysis is immediately recognised. Comparing the prestudy cycles with the placebo cycles, less hMG was used when placebo was administered (Table *III*-3). There was no difference in the amount of hMG used when the prestudy cycles were compared with the GH cycles (Table *III*-4). The use of less hMG in the placebo cycles compared either with the GH group or the prestudy cycles is difficult to explain and may merely reflect the heterogeneity of the groups.

Paired analyses were also performed on results from the placebo and subsequent GH cycles in those women who were initially randomised to receive placebo (Table *III*-5, 10). The GH cycles were open and therefore potentially subject to bias (see above). Despite the various statistical procedures, no improvement in outcome with GH in the overall group or in the

subgroups could detected.

The results from the first cycles of GH co-treatment in women with PCO and normal ovaries were compared (Table *III*-11). Higher urinary oestrogen excretion was noted during GH co-treatment in women with PCO compared with women with normal ovaries. The serum concentrations of E_2 were higher in women with PCO receiving GH compared the women receiving PL (Table *III*-7). The higher oestrogen production may reflect the augmenting effect of GH on steroidogenesis in the PCO group. When the pretreatment cycles had been compared there was no difference in oestrogen excretion between the women with PCO and normal ovaries.

When the results from all the GH cycles were compared, I found that more follicles developed and more oestrogen was excreted in the women with PCO than women with normal ovaries (Table *III*-12). The analysis was, however, flawed as some of the GH cycles were randomised and some were open. Although not very convincing statistically, the results did suggest some variation in response to GH depending on the ultrasound appearance of the ovaries.

The results of the CC and hMG studies did not show an effect of GH on the ovarian response.The group of women were, however, extremely resistant to hMG stimulation of the ovaries and a large effect was considered unlikely. Ibrahim *et al* also failed to show an improved response with GH in five CC and hMG cycles (1990), although Volpe *et al* showed some improvement in follicular response in six younger women receiving GH treatment in addition to hMG and pure FSH (1989).

Buserelin and hMG study.

In Homburg's study of in-vivo fertilisation (1990), most of the patients undergoing GH augmentation of hMG treatment were hypogonadotrophic. These women responded to GH treatment by requiring significantly less hMG over a shorter duration to achieve ovulation. The use of GnRH analogues in IVF-ET cycles had become increasingly common by 1988 and was recognised to induce a reversible form of hypogonadotrophic hypogonadism. The use

of GnRH analogues was also associated with the need for higher doses of hMG because of the suppression of endogenous FSH.

I therefore went on to perform a further prospective, randomised, double-blind, placebo controlled study of GH co-treatment to assess the ovarian response to GH in the presence of buserelin and hMG. The entry criteria were relaxed with the aim of recruiting patients less resistant to treatment.

Group comparison of the women randomised to receive GH or placebo demonstrated a significant improvement in ovarian response with GH (Table *IV*-3). Less hMG was required to achieve the criteria for hCG administration in the GH augmented cycles and despite this there was a tendency for more follicles to develop. More oocytes were collected in the GH group and more of these oocytes fertilised and cleaved. The improved response was, however, restricted to the women with PCO (Table *IV*-8 and -12). Comparing the results in women with PCO who received GH with those who received placebo, less hMG was used per cycle but more follicles developed and more oocytes were collected. The number of oocytes which fertilised and cleaved was also greater in the GH cycles (Table *IV*-12).

The women with normal ovaries did not show any improvement in ovarian response with GH when comparing placebo with GH treatment cycles (Table *IV*-8).

When the results from the prestudy cycles were compared with the treatment cycles, an improved response was demonstrated with both placebo and GH administration (Tables *IV*-5 and -6). The hMG usage was no different between prestudy cycles and placebo cycles and prestudy cycles and GH cycles. When the degree of improvement in the ovarian response was assessed, there was no significant augmentation of the ovarian response to GH. The number of oocytes which fertilised and cleaved were, however, greater in the GH cycles compared with the prestudy cycles, than in the placebo compared with the prestudy cycles.

In the women with PCO, although a significant placebo as well as a significant GH effect was demonstrated (Table *IV*-13 and -14), a far greater effect was noted with GH treatment (Table *IV*-15). The degree of improvement in ovarian response was much more striking with

GH treatment (Fig *IV*-2 and -3). In the women with normal ovaries, no effect of PL or GH was seen compared with prestudy cycles (Tables *IV*-9 and -10). Comparing the differences in ovarian response, however, the outcome of cycles with GH augmentation was actually worse compared with prestudy cycles, than the cycles of PL treatment compared with prestudy cycles (Table *IV*-15).

Ten women received placebo followed by treatment with GH. Because of the heterogeneity of the patients this analysis may be statistically appropriate, although assignment to the GH cycles was not blinded. Significantly more follicles developed in the GH cycles and higher serum E_2 concentrations were measured on the day of hCG administration (Table *IV*-16). Interestingly there was no significant improvement in the number of oocytes collected in the GH cycles which somewhat negates any criticism of bias at oocyte recovery.

Five pregnancies followed 19 ETs in 23 GH cycles. This is a low to average pregnancy rate in GnRHa and hMG cycles (Rutherford *et al* 1988) and is far less than the pregnancy rate reported recently from another centre using GH (Ibrahim *et al* 1991). In that study, Ibrahim *et al* (1991) treated 10 women with GH in addition to the long regimen of buserelin and hMG. The recruitment criteria were not described and the study was not controlled. They confirmed an improvement in the oocyte collection rate with GH cotreatment despite using less hMG per cycle. Six of the ten women became pregnant.

GH therapy has also been used with the short regimen of buserelin and hMG. (Rönnberg *et al* 1989, Ibrahim *et al* 1990). Rönnberg *et al* (1989) presented data on 38 "endocrinologically healthy" women treated with buserelin for 4 days prior to hMG who were randomised to receive placebo or GH co-treatment. There was no difference in outcome between women receiving placebo and women receiving GH. Ibrahim *et al* (1990) reported on five women who were previously poor responders who received GH in addition to the "ultrashort" regimen of buserelin (Day 2 to 4) with hMG. Four of the five women had oocyte collections but in only two women were oocytes recovered. Premature LH surges were noted in four of the GH cycles.

It therefore appears that GH augmentation of the ovarian response is only effective once pituitary desensitisation with the long regimen of GnRH analogues has been produced.

Ovarian morphology.

In the studies described in this thesis the subgroup of women with normal ovaries was small which makes interpretation of the data difficult. Whether women who respond poorly to ovarian stimulation regimens, and have a "non PCO" appearance on scanning, can strictly be defined as normal deserves further investigation. These women possibly have a tightly regulated control of unifollicular development which can not be overcome by high concentrations of gonadotrophins or growth factors. The suboptimal response in IGF-I production in these women is discussed below.

In patients with ultrasound diagnosed PCO there are many potential follicles present and an effect on their ovarian response might create problems of bias, if this diagnosis were not considered in the interpretation of results. Ideally, with large numbers, group analyses would be possible but there remain many problems of patient-to-patient variation.

At present there are no data to support the use of GH treatment in women with normal ovaries on ultrasound scan. In the group of women who respond poorly to standard ovarian stimulation regimens, ovarian ultrasound should be used to identify the women with PCO and only these women should be assessed for treatment with GH. We attempted to establish if a biochemical marker, 11ß-OH-A_4, could differentiate between women with normal and polycystic ovaries but were unsuccessful (Chapter *V*).

GnRH analogues.

Specific receptors to GnRH have been identified on granulosa cells and luteal cells of rat ovaries (Clayton *et al* 1979, Pieper *et al* 1981, Clayton 1982) but it is not clear if receptors for GnRH are present in other animal, or human, ovaries (Asch *et al* 1981, Clayton 1982, Brown and Reeves 1983, Popkin *et al* 1983, Bramley *et al* 1985). Significant concentrations of GnRHa have been found in FFL following administration in superovulation programmes (Loumaye *et al* 1989) but the exact effect on ovarian function is uncertain.

In work on hypophysectomised rats, GnRH and its analogues have been shown to have direct

inhibitory effects, for example the peptides antagonised the effects of exogenous gonadotrophins on ovarian weight gain and follicular development as well as ovulation (Rippel and Johnson 1976, Hsueh and Erickson 1979, Ying and Guillern 1979, Hammond 1981, Ranta *et al* 1983).

In cultured granulosa cells of the rat, administration of GnRH agonists resulted in inhibition of FSH induced aromatase activity and LH/hCG receptor induction (Daughaday *et al* 1972, Hsueh and Erickson 1979, Hsueh *et al* 1980, Dorrington 1983). In rat luteal cells, the agonists prevented much of the FSH stimulated increase in cholesterol side chain cleavage activity, thereby affecting progesterone synthesis (Clayton *et al* 1979, Jones and Hsueh 1980). Inhibitory effects have also been demonstrated on cAMP accumulation and steroidogenesis in porcine granulosa cells (Massicotte et al 1980).

Adashi *et al* demonstrated in rat granulosa cells that the ability of FSH to enhance IGF-I binding was significantly inhibited by GnRH agonists (Adashi *et al* 1988b, Adashi *et al* 1991a). GnRH agonist analogues were also shown to have an inhibitory effect on IGF-I supported proteoglycan synthesis and hCG stimulated progesterone accumulation (Adashi *et al* 1991a). GnRH is known to exert some of its inhibitory effect in vitro by attenuating FSH-stimulated granulosa cell cAMP generation (Hsueh *et al* 1980).

The situation in the human is, however, less clear. In human granulosa cells no inhibitory effect of GnRH or its agonist on steroidogenesis was demonstrated (Casper *et al* 1982, Casper *et al* 1984). One report (Tureck *et al* 1981) has, however, demonstrated inhibition of progesterone secretion by GnRH agonist after 96 hours in culture.

It is interesting to postulate, if GnRH analogues had an inhibitory action on the steroidogenic function of human ovarian cells, whether increasing local concentrations of growth factors may overcome these effects.

Safety factors.

Blood pressure, glucose concentrations and U&Es and LFTs (except alanine transferase) did

not alter throughout the treatment cycles. The GH therapy was given for a maximum of 12 days and was thus unlikely to have any profound biochemical effects. Six healthy men given GH daily for 5 days had increases in their fasting glucose concentrations but other relevant parameters remained normal (Ho *et al* 1989). I did not find any changes in random glucose concentrations over the period of the study.

The only significant biochemical change noted was a decrease in serum alanine transferase concentrations. This is in contrast to the results of Homburg *et al* (1988) where a transient rise in this enzyme was demonstrated during GH co-treatment. One explanation is that women who have been oestrogen deficient for a prolonged period before treatment, as in Homburg's study, may have an increased hepatic response both to the GH and the rapid rise in circulating steroid concentrations.

The studies presented in this thesis support the premise that short term GH therapy is safe, even in the high doses used here. Although not tested for in my studies, GH antibodies are unlikely to occur with the biosynthetic hGH.

IGF-I.

In experiments on granulosa cells, co-culture with IGF-I increased steroid output in response to gonadotrophin stimulation (Adashi *et al* 1984,1985b,1985c, Davoren *et al* 1986). The main source of circulating IGF-I in the human is the liver and production of this peptide is known to be under GH control (Daughaday *et al* 1976). As well as investigating the effect of systemic GH on the ovarian response to gonadotrophins I wished to establish what, if any, changes occurred in the insulin like growth factors in both the circulation and the ovary during GH treatment.

Baseline concentrations.

The results from the three GH studies (Chapters *III* and *IV*) demonstrated that serum IGF-I concentrations were not altered by gonadotrophin treatment alone, but were increased by intramuscular administration of GH (Figs *III*-1, Fig *IV*-4). There was no difference in

baseline (early follicular phase) concentrations of IGF-I between women with normal and polycystic ovaries. Neither of my studies was designed to investigate the differences in concentrations of IGF-I in women with PCO and normal ovaries but it is interesting that the lack of difference in concentrations is supported by the findings of other workers.

Lanzone *et al* (1990), for example, investigated 19 women with PCO and 15 controls. The different assays for IGF-I do not allow comparison of absolute concentrations but no differences were noted in IGF-I concentrations between the two groups. Several other workers have investigated women with PCOS, the majority of whom had high testosterone concentrations, and found no difference in serum concentrations of IGF-I compared with controls (Urdl 1987, Sharp *et al* 1991, Kiddy *et al* 1989, Conway *et al* 1990, Kazer *et al* 1990, Eden *et al* 1988). Two other groups have, however, demonstrated higher serum concentrations of IGF-I in women with PCOS compared with controls (Iwashita *et al* 1990, Laatikainen *et al* 1990). It is not clear whether this difference in results is due to differences in assay methodology or patients.

Treatment with buserelin for a minimum of two weeks in the buserelin and hMG study was not associated with any changes in IGF-I concentrations. Lanzone *et al* reported that IGF-I concentrations were not altered by 8 weeks of buserelin administration (Lanzone *et al* 1990). This suggests that gonadotrophins may not be important clinically as regulators of IGF-I concentrations. Lanzone *et al* and other workers have also shown that insulin concentrations are not altered by pituitary suppression with GnRH analogues (Lanzone *et al* 1990, Geffner *et al* 1986).

GH treatment.

In the CC and hMG studies only women with PCO showed a rise in IGF-I concentrations in response to GH (Fig *III*-2). In the buserelin and hMG study both the group of women with normal ovaries and the group with PCO demonstrated increases in IGF-I concentrations during GH cotreatment (Fig *IV*-5). The concentrations in the women with PCO were, however, significantly higher than the women with normal ovaries. That this augmentation of circulating IGF-I concentrations by GH occurred to a far greater degree in patients with

ultrasound diagnosed PCO is a novel observation.

IGF-I concentrations in serum are recognised to be controlled by many factors including age, nutrition and gonadal steroids but predominantly GH. GH administration in IVF and in-vivo fertilisation has now been reported by several other workers (Jacobs 1987, Homburg *et al* 1988, Volpe *et al* 1989, Genazzani *et al* 1989, Rönnberg *et al* 1989, Blumenfeld & Lunenfeld 1989, Volpe *et al* 1990, Homburg *et al* 1990a,b, Ibrahim *et al* 1990, 1991). In the in-vivo fertilisation work, all of the women were amenorrhoeic due to a variety of causes, including PCOS, but none of the authors differentiated between the diagnoses of the women when describing the GH or IGF-I concentrations (Homburg *et al* 1990a,b, Burger *et al* 1991).

IGF-I concentrations have not been reported in any of the IVF-ET studies nor did any other study give descriptions of ovarian morphology. Kiddy *et al* (1989) demonstrated a decrease in serum concentrations of IGF-I in women with PCO after a calorie restricted diet but there are no other reports of changes in IGF-I concentrations, specifically in women with PCO, following different therapies. Kazer *et al* (1990) demonstrated lower circulating concentrations of GH in untreated women with PCO compared with controls and suggested there was lower GH secretion in PCOS. They suggested that the low concentrations may be due to negative feed back on GH secretion by insulin or by IGF-I but suggested that further work was needed to elucidate the role of GH and IGF-I in PCOS. The work presented in the thesis also suggests differences in the response of women with PCO to GH and/or IGF-I compared with controls, although GH concentrations in serum were not assessed.

Of note is the observation that the IGF-I cconcentrations in serum declined after the initial peak despite continuing GH administration. This was also noted by Homburg *et al* (1990) and may reflect the limitation of hepatic production of IGF-I despite continued GH stimulation.
Ovarian morphology.

In the CC and hMG studies the women who had normal ovaries showed no effect of GH co-treatment on IGF-I concentrations in serum. This group of women were particularly resistant to gonadotrophin treatment and it is possible that their lack of growth factor response to

treatment with GH may reflect an underlying abnormality in either their hepatic or ovarian function, exemplified clinically by the repeated difficulty in obtaining multifollicular development. The women with normal ovaries in the buserelin and hMG studies showed a response to GH by increasing their IGF-I concentrations in serum. These women were not as resistant a group as in the CC and hMG studies, but even so did not respond clinically to GH treatment despite having an increase in IGF-I concentrations.

One concept of follicular growth incorporates a fixed number of follicles which can be stimulated each month, with women who have "normal" ovaries having fewer than those with polycystic ovaries. Thus an increase in growth factors produced by systemic GH administration might have limited potential for increasing the follicular response in women with a predetermined smaller number of oocytes. The improved response to exogenous gonadotrophins by GH co-treatment was seen only in those women where greater numbers of potential follicles were present ie. those women with PCO.

In the women with PCO some abnormality of intraovarian control had possibly led to a poor response to gonadotrophin stimulation in the past. The women with PCO showed the expected physiological response to GH administration by increasing their circulating IGF-I concentrations. They subsequently showed an improved ovarian response to gonadotrophin stimulation. This association suggests that even though the women with PCO had previously responded suboptimally to gonadotropin stimulation, when co-treated with GH, they responded normally to gonadotropin therapy. The GH treatment may act by ameliorating an underlying abnormality of intraovarian growth factor regulation in these patients or may have a direct effect on ovarian function.

FFL concentrations.

In both the CC and hMG and the buserelin and hMG studies, there was no significant difference in the serum concentrations of IGF-I on the day of oocyte collection between those women receiving GH and those receiving placebo (Fig *III*-1, Fig *IV*-4, Table *IV*-18). Significantly higher concentrations of IGF-I were, however, found in the FFL of patients treated with GH compared with placebo (Table *III*-13). These results are in contrast to the data published by Volpe *et al* (1989) and Barreca *et al* (1989) who showed no significant

difference in FFL concentrations of IGF-I between GH-treated patients and controls. These workers appear, however, to have analysed an unspecified number of samples of follicular fluid (up to 16) from each patient which may have biased their results.

When the serum and follicular fluid concentrations of IGF-I were compared in my studies there was a significant difference in the results (Figs *III*-3 and -4, Table *IV*-18). Both overall, and in the different subgroups, serum concentrations were higher than follicular fluid concentrations. Together with the data demonstrating that IGF-I concentrations in FFL are higher in women receiving GH than in women receiving placebo, these results are consistent with diffusion of the peptide into the follicle rather than intraovarian production of IGF-I. It is known that IGF-I is synthesized mainly in the liver and an hepatic source is most probably the main contributor to the FFL concentrations. The differences in the follicular fluid concentrations of IGF-I between the two treatment groups, and the different ovarian morphology types, thus probably reflects the differences in serum concentrations over the preceding few days. The women with PCO had higher serum concentrations of IGF-I and it is they who had the highest follicular fluid concentrations and also the better ovarian response.

Alternatively, the significantly higher concentrations of IGF-I in the FFL after GH treatment may reflect local production of IGF-I by granulosa cells, as has been demonstrated in in vitro experiments (Steinkampf *et al* 1988). IGF-I may be produced in the human ovary but in insignificant concentrations compared with circulating concentrations. Moreover only minimal amounts of IGF-I mRNA were detected in cultured human granulosa cells, with no change after gonadotropin or ovarian steroid administration (Voutilainen & Miller 1987).

In women studied in unstimulated cycles, Eden *et al* (1988) also demonstrated higher IGF-I concentrations in serum compared with follicular fluid. Significantly higher concentrations of IGF-I were found in dominant follicles compared with cohort follicles and the concentrations were related to the follicle volume. No significant differences were found between women with normal and polycystic ovaries. I also found no difference in IGF-I concentrations between women with PCO and those with normal ovaries when the subjects were receiving placebo.

In a later paper, however, Eden *et al* (1990) did report higher concentrations of IGF-I in women with PCO than in women with normal ovaries when the size of the follicles was controlled for. The women were in unstimulated cycles and the follicles were small. IGF-I concentrations in FFL did not correlate with serum concentrations in this study but serum concentrations of IGF-I were again higher than FFL concentrations.

Jesionowska *et al* (1990) studied IGF-I concentrations in the serum of nine women undergoing abdominal hysterectomy and showed no significant difference between peripheral and ovarian vein concentrations nor between follicular and luteal phase concentrations. They concluded that the majority of circulating IGF-I was derived from extraovarian sources.

In women undergoing IVF-ET, other workers (Rabinovici *et al* 1990, Geishovel *et al* 1989, Hamori *et al* 1991) have found that IGF-I concentrations in FFL were significantly lower than serum concentrations. These three groups also found a correlation between serum and follicular fluid concentrations of IGF-I. I was unable to demonstrate a correlation in my studies but it may be that the GH induced elevations of IGF-I concentrations in the serum confounded the analysis.

Roussie *et al* (1989) reported IGF-I concentrations in the follicular fluid of women undergoing different ovulation induction regimens prior to IVF-ET. The concentrations were much higher than those reported here, presumably because of differences in assay technique. Because IGF-I is closely bound to at least two binding proteins, acid extraction is required to assess total IGF-I content. It is difficult to compare the result from Roussie's study with mine, because they did not state whether acid extraction was performed. The group reported higher concentrations of IGF-I in the follicular fluid of women receiving GnRH analogues compared with those receiving CC. My data do not support this as, although not formally tested, the concentrations of IGF-I in the FFL of women in the first study were higher than the concentrations from women receiving buserelin.

Roussie *et al* (1989) demonstrated a correlation between IGF-I and E_2. These data support the work by Eden *et al* (1988) who suggest that IGF-I may be important in ovarian steroid production. Although I did not find a difference in the oestradiol concentrations in the

follicular fluid of patients with higher IGF-I concentrations, I did find an increase in urinary oestrogen excretion in patients with PCO who received GH.

The production and reception of IGF-I in the human ovary.

IGF-I gene expression in the liver is known to be GH dependent and it has been suggested that extrahepatic expression of the IGF-I gene may also be GH dependent (Davoren *et al* 1986, Hynes *et al* 1987, Mondschein and Hammond 1988, Roberts and Leroith 1988, Murphy and Friesen 1988). Pivotal to the work in this thesis is the demonstration of modulation, by IGF-I, of gonadotrophin-induced ovarian cell function (Adashi *et al* 1985a). Whether, however, significant intraovarian production of IGF-I occurs in the human ovary is still uncertain.

IGF-I gene expression has been demonstrated in murine (Murphy *et al* 1987, Oliver *et al* 1989, Hernandez *et al* 1989,) and porcine (Hsu and Hammond 1987a, Hsu and Hammond 1987b, Mondschein and Hammond 1988) granulosa cells and appears to be enhanced by gonadotrophins, E_2 and GH (Hsu and Hammond 1987a, Hsu and Hammond 1987b). Davoren and Hseuh (1986) showed that a single injection of ovine GH in immature, hypophysectomised, oestrogen treated female rats resulted in an increase in immunoreactive IGF-I in the ovaries. They concluded that GH may affect ovarian differentiation by inducing the local production or accumulation of IGF-I. The studies mentioned earlier do not, however, support the hypothesis that GH stimulates significant intraovarian production of IGF-I in the human ovary.

Very recently Hernandez *et al* (1992) demonstrated IGF-I gene expression in the whole premenopausal ovary but not in luteinised granulosa cells. Immunohistochemical determination demonstrated that the IGF-I protein was almost entirely localised to the thecal-interstitial compartment. This confirms other work showing that human granulosa cells do not express the gene for IGF-I (Ramasharma *et al* 1986, Ramasharma d Li 1987, Voutilainen and Miller 1987) and so the site, and control, of ovarian IGF-I production is still unclear.

It has not been established in humans whether treatment with GH increases intraovarian

production of IGF-I or merely increases hepatic output. If production of IGF-I in the human ovary occurs, but is restricted to the theca-lutein cells, this suggests a predominantly paracrine role for IGF-I in the ovary. Despite these controversies, if IGF-I were found to play an important role in modulating the ovarian response to gonadotrophins in the human, abnormalities in production or reception of this growth factor might contribute to the dysfunction in women who respond suboptimally to ovulation induction regimens.

Over the past few years, IGF-I binding and the presence of type I receptors have been clearly demonstrated in the human ovary (Gates *et al* 1987, Hernandez *et al* 1989, Poretsky *et al* 1985, Poretsky *et al* 1990, Balboni *et al* 1987). The action of IGF-I in the human ovary appears similar to that in animal models. IGF-I regulation of aromatase activity has, for example, recently been shown in human granulosa cells in vitro (Spicer *et al* 1988, Erickson *et al* 1989, Erickson *et al* 1990, Bergh *et al* 1991). IGF-I and FSH were shown to act synergistically on E_2 production and similar effects were demonstrated on human luteal cells (Erickson *et al* 1989, Bergh *et al* 1991, Christman *et al* 1991). Type I receptors have also recently been demonstrated in human ovarian stroma which suggests that IGF-I (or other factors stimulating the type I receptor) may play a part in modulation of androgen biosynthesis (Nagamani & Stuart 1990, Poretsky *et al* 1990). This possible effect has implications for the role of growth factors in clinical conditions such as PCOS.

Erickson *et al* (1990) suggested that in women with PCOS there is a deficiency in P450arom activity, resulting in cessation of full follicular development. They demonstrated that both FSH and IGF-I stimulated E_2 synthesis by cultured granulosa cells of women with PCOS but proposed that the FFL from women with PCOS contained inhibitors to IGF-I and FSH action. Franks and Mason (1991) also suggest that an inhibitor of FSH action exists in the polycystic ovary, namely epidermal growth factor. If intraovarian inhibitors were to exist specifically in women with PCOS, the improved response to GH treatment in this subgroup may reflect suppression of the inhibitor or increased activity of IGF-I.

In the human, IGF-I may also modulate E_2 action. In rats, although not yet substantiated in humans, Murphy and Friesen (1988) suggest that both E_2 and GH are potent stimulators of IGF-I gene expression in the appropriate target tissues where these hormones induce growth

(for example the uterus). E_2 increases ovarian IGF-I gene expression in murine (Hernandez *et al* 1989) and porcine granulosa cells (Hsu & Hammond 1987b) and thus, in animals at least, it appears that E_2, gonadotrophins and IGF-I work together to modulate folliculogenesis.

Further recent work has demonstrated that IGF-I significantly stimulated ^{3}H-thymidine incorporation into human granulosa cells thus indicating that IGF-I may be important in proliferation of cells during the development of the follicle (Olsson *et al* 1990). FSH and E_2 stimulate granulosa cell proliferation in vivo and thus again FSH, E_2 and IGF-I may be collaborating during follicular development. Whether, however, the action of IGF-I is paracrine or endocrine has yet to be established.

IGF-II

Serum concentrations

Baseline concentrations of IGF-II in serum were no different between the treatment groups nor between the women with PCO and those with normal ovaries (Fig *III*-5). Treatment with hMG, with or without GH, did not result in any changes in IGF-II concentrations. The hormonal regulation of IGF-II is different to IGF-I. The production of IGF-II from human granulosa cells is under multihormonal regulation and has been shown to be increased by hCG, FSH and LH, although data on control by PRL and GH is equivocal (Ramasharma and Li 1987, Voutilainen and Miller 1987). IGF-II concentrations are not raised in acromegaly, although they are somewhat decreased in GH deficiency (Zapf and Froesch 1981). In general the production of IGF-II is not thought to be predominately under GH control (Zapf and Froesch 1981, Clemmons and Van Wyk 1984, Grant *et al* 1986) and we have demonstrated that concentrations of this growth factor did not alter during cotreatment with GH.

The results from this study also suggest that neither gonadotrophins nor E_2 have an effect on in-vivo production of IGF-II. Similar concentrations of IGF-II in the follicular and luteal phase of natural cycles have been demonstrated (Jesionowska *et al* 1990) and it is interesting

to note that there are no significant changes in IGF-II concentrations during puberty (Daughaday and Rotwein 1989). These data support the notion that steroids are not important modulators of IGF-II production.

FFL concentrations.

There were no differences in follicular fluid concentrations of IGF-II between the different subgroups (Table *III*-15). Serum concentrations were significantly higher than follicular fluid concentrations suggesting that IGF-II is not produced in significant amounts in the ovary (Fig *III*-6). The finding of higher serum than FFL concentrations of IGF-II is in contrast to the data from Ramasharma *et al* who demonstrated that concentrations of IGF-II were higher in FFL than serum (1986). Other workers, however, found that serum concentrations were not significantly different from FFL concentrations but proposed that IGF-II may be being produced in the ovary (Hamori *et al* 1991).

The main production site of IGF-II is the liver but several other organs including the ovary have been shown to be capable of producing IGF-II. IGF-II was first demonstrated in human FFL by Ramasharma *et al* in 1986 and human ovarian follicles have been shown to express mRNA for IGF-II and to secrete the growth factor (Ramasharma and Li 1987, Voutilainen and Miller 1987, Geisthovel *et al* 1989). More detailed studies have recently demonstrated mRNA for IGF-II in whole premenopausal ovaries and luteinised granulosa cells, whereas IGF-I mRNA was localised to the thecal-interstitial department (Hernandez 1992). The ovarian contribution to circulating IGF-II concentrations has yet to be established.

IGF-II is bound to the large IGFBP complex in FFL but the role of this BP in relation to the action of IGF-II in the ovary is unknown (Holly *et al* 1990).

The role of IGF-II in the ovary.

In the rat, although IGF-II preferentially binds to type II /mannose-6-phosphate receptors, the hormonal action of IGF-II is thought to be through the type I receptors (Adashi *et al* 1989). Type I receptors are regulated by gonadotrophins, steroids and GH and also mediate

IGF-I action (Adashi *et al* 1989, Adashi *et al* 1988b, Adashi *et al* 1986a, Veldhuis *et al* 1986). Both type I and type II receptors, as well as insulin receptors are present in the human ovary (Hernandez *et al* 1992).

Although the precise role of IGF-II is unknown it is considered to be primarily a fetal hormone with high serum concentrations present in fetal and neonatal life (Moses *et al* 1980). As high concentrations of IGF-II have been demonstrated in human ovarian tissues, a paracrine or autocrine role in steroidogenesis is possible (Voutilainen and Miller 1987). Although stimulation by IGF-II of androgen production by theca-interstitial cells has been demonstrated, no effect on oestradiol production from granulosa cells was seen (Barbieri *et al* 1986, Erickson *et al* 1990).

Data from rats transplanted with IGF-II secreting tumours, suggest that IGF-II may be involved with the control of IGF-I secretion but the exact role is unclear (Wilson *et al* 1989). IGF-II may have a direct inhibitory effect upon secretion of IGF-I in the peripheral tissues. High concentrations of IGF-II did not alter GH concentrations although feedback of growth factors on GH secretion from the pituitary is recognised (Berelowitz *et al* 1981, Ceda *et al* 1987, Wilson *et al* 1989).

IGFBPs.

Ovarian cells secrete a heterogeneous and complex family of IGFBPs which modulate the function of the IGFs. The secretion of these proteins by ovarian cells is regulated by gonadotrophins and locally produced ovarian factors. The IGFBPs are thought to affect IGF binding and action (Elgin *et al* 1987, Rutanen 1988, Blum *et al* 1989, Taylor *et al* 1990).

The group of IGFBPs is made up of many different binding proteins, several of which have not yet been fully characterised. The two main IGFBPs are IGFBP-3, in the large 150kDa complex, and the low molecular weight, IGFBP-1. Both are produced in the liver and other rapidly growing tissues. IGFBP-3 is the major carrier of IGFs in the serum and its production is controlled by GH. The majority of IGF-I in follicular fluid is bound to IGFBP-1 (Seppälä *et al* 1984).

Human granulosa cells and follicular fluid are known to contain IGFBPs (Suikkari *et al* 1989a, Seppälä *et al* 1984, Holly *et al* 1990, Giudice *et al* 1991) and Koistinen *et al* (1990) demonstrated that human granulosa cells contain mRNA for IGFBP-1. These findings have recently been confirmed with the detection of mRNA transcripts for IGFBP-1,-2 and -3 in human luteinised granulosa cells (Giudice *et al* 1991). Four different IGFBPs were found to be secreted into the culture medium.

FSH has recently been shown to be one of the factors regulating IGF binding activity in the mouse ovary (Adashi *et al* 1990, Adashi *et al* 1991b). Several other pituitary derived factors are thought to affect IGFBP release from granulosa cells. By decreasing the binding of IGF-I, FSH and other factors may be important in allowing the presentation of increased amounts of IGF-I to the granulosa cell. Modulation of the availability of IGF-I to the developing follicle may be vital in the process of selection and dominance.

Serum concentrations of the low molecular weight IGFBP have been shown to be inversely related to insulin concentrations and patients with PCOS have lower concentrations of this IGFBP (Perkonen *et al* 1989, Suikkari *et al* 1988, 1989b, Conway *et al* 1990). Low IGFBP concentrations occurred mainly in obese women who have higher fasting insulin concentrations but even slightly elevated fasting insulin concentrations were associated with a significant reduction in BPs (Perkonen *et al* 1989, Suikkari *et al* 1989). Exciting areas of research are opening up to investigate whether the increased concentrations of free IGF-I, consequent on the lower IGFBP concentrations, may play a part in the pathogenesis of PCO by stimulating the proliferating and differentiating granulosa cells. LH-induced production of androgens in theca cells is also modulated by IGF-I and alterations in IGFBP concentrations, secondary to high insulin concentrations, could provide a mechanism for hyperandrogenism in PCOS (Perkonen *et al* 1989).

Although not directly related to the clinical studies presented in this thesis, IGFBPs appear to have an important role in modulating ovarian physiology and pathology. Uncertainty still remains as to the mechanism of action of GH in the clinical setting and an effect upon the production and/or action of the binding proteins is possible.

Serum and follicular fluid oestradiol concentrations.

Serum concentrations

Oestradiol concentrations rose during hMG therapy in both the CC and hMG and the buserelin and hMG studies (Figs *III*-7, *IV*-6). There were no differences in E_2 concentrations between the placebo and GH cycles in the CC and hMG study. In the buserelin and hMG study, however, women with PCO who received GH had higher concentrations of E_2 on the day of hCG administration than women with normal ovaries.

On the day of oocyte collection, E_2 concentrations were lower than on the day of hCG administration in both studies. The decline in E_2 concentrations associated with the LH surge is well recognised in IVF-ET and is thought to be due to the suppression of 17 hydroxylase activity resulting in less available androstendione (Baird 1991).

In the CC and hMG study there were no differences in the concentrations of E_2 between treatment groups on the day of oocyte collection. In the buserelin and hMG study, however, E_2 concentrations were higher in women receiving GH than those receiving placebo. This was true, independent of ovarian morphology.

Overall, the E_2 results suggest that there may be some effect of GH on oestradiol production, particularly in women with PCO. In super-ovulation programmes, however, steroidogenesis is occurring at a considerable rate and there is probably little capacity for increasing production of E_2. The increased output was not obviously related to the number of follicles nor oocytes collected, nor did it correlate with IGF-I concentrations. Because of the many factors affecting E_2 production it was unlikely that a clear relationship between IGF-I and E_2 would emerge. The E_2 results reflect the problems in applying in-vitro cell work to the clinical situation.

In the buserelin and hMG study, desensitisation of the pituitary was assessed in the clinic by measurement of E_2 using a rapid kit designed for IVF. When the stored samples were later assayed at the Middlesex, three women were found to have mid follicular range

concentrations of E_2, although they had low FSH concentrations. It is not clear whether these were erroneous results from the Middlesex Hospital, although the results fall in the middle of the assay range. Alternatively, ovarian suppression by GnRHa may have been inadequate and the IVF kit assay incorrect. Lack of full ovarian suppression is unlikely to have affected the final follicular response.

Follicular fluid concentrations.

In the CC and hMG study there were no differences in the concentrations of E_2 in FFL between the different treatment groups. The higher concentration of E_2 found in the FFL of women with normal ovaries compared with those with PCO was probably due to the small sample size and represents a type 1 error (Table *III*-16).

Oestradiol concentrations in FFL were not measured in the buserelin and hMG study as it was felt unlikely that further insight into the response to GH could be gained.

Other peptides and steroids

FSH

Baseline concentrations of FSH were no different between the women who went on to receive GH and those who went on to receive placebo in either of the randomised studies. In the buserelin and hMG study, however, the group of women with normal ovaries had higher concentrations of FSH than the group of women with PCO, although the results were all below 15IU/L. These results may suggest that women with normal ovaries, who respond poorly to hMG stimulation, may be more resistant to their endogenous gonadotrophins. Certainly this group did not show an improved ovarian response with GH, although I do not think that variations within the normal range of FSH concentrations can be used to differentiate between women who may or may not respond to GH. Early follicular phase concentrations of FSH greater than 15IU/L, however, probably reflect incipient ovarian failure. GH is not an appropriate treatment for these women.

FSH concentrations were high throughout the treatment period with hMG (see Appendices). The absorption from an intramuscular injection is variable and the concentration of exogenous FSH far exceeds the endogenous concentrations. Any possible impact of GH on FSH concentrations was therefore not investigated.

LH

The LH concentrations were no different between the treatment groups nor between women with normal and polycystic ovaries. The samples were taken in the early follicular phase when LH concentrations are normally low. The observation that women with PCO did not have higher LH concentrations than women with normal ovaries supports the concept of the heterogeneity of the condition. The women with PCO in the GH studies had regular ovulatory cycles and were not obviously hirsute; that is to say, they did not have PCO *syndrome*. Although a high LH concentration, or raised androgens, is regarded by many as obligatory for the diagnosis of PCOS, essential to an understanding of the work presented in this Thesis is the difference between ultrasound diagnosed PCO and PCOS. The morphological appearance on ultrasound was the only prerequisite to the diagnosis of PCO.

In the placebo controlled CC and hMG study none of the 12 women with PCO had LH surges in GH cycles. Three of the seven women with normal ovaries who received GH had spontaneous LH surges prior to hCG administration. The premature LH surges may explain the particularly poor performance of the women with normal ovaries. Ibrahim *et al* (1990) reported an uncontrolled trial of GH augmentation of CC and hMG treatment in IVF-ET. In four of the five cycles a spontaneous LH surge occurred. The ovarian morphology of the recruits was not described.

The LH surge is considered to be due to feedback of E_2 on the hypothalamus and pituitary (Baird 1991, Schaison and Couzinet 1991). It is unlikely that GH co-treatment had the effect of inducing a premature LH surge through E_2 as no differences in oestrogen concentrations were noted in my studies between women on placebo and those on GH. There is a theoretical possibility, however, that GH may have a direct effect of enhancing the hypothalamic-pituitary response to E_2. In my study, premature LH surges only occurred in women with

normal ovaries who received GH.

LH concentrations remained suppressed during buserelin administration and there was no evidence of any spontaneous LH surges.

Progesterone concentrations

Progesterone concentrations in serum were low on the day of hCG administration in the buserelin and hMG cycles whether GH or placebo were added. This reflects the desensitisation of the pituitary and demonstrates that luteinisation does not occur without the LH (or hCG) surge.

FFL concentrations of progesterone were only assessed in the CC and hMG studies (Table *III*-16). There was no difference in progesterone concentrations between treatment groups but women with PCO were found to have lower concentrations than women with normal ovaries. This was in direct contrast to the serum results from the buserelin and hMG study (Table *IV*-19). In the latter study significantly higher concentrations of progesterone were found on the day of oocyte collection in women with PCO who received GH compared with women with normal ovaries. Serum concentrations of progesterone on the day of oocyte collection reflect the response of the ovary to the hCG. These results are not easily explained but probably do not contribute to the assessment of the clinical application of GH.

Testosterone, insulin and GH concentrations in FFL.

The content of the FFL in the CC and hMG studies was assayed for several steroids and peptides including testosterone, insulin and GH (Tables *III*-17). There were no differences in insulin or GH concentrations between any of the groups (Table 16-Chapter III). This was reassuring as the last GH injection was given several days before the oocyte collection and the results demonstrate that neither peptide is concentrated in the intrafollicular environment.

Follicular fluid concentrations of testosterone were lower in women with PCO than women with normal ovaries (Table *III*-16). Serum testosterone concentrations were not measured but,

as mentioned earlier, the women with PCO were considered to have no signs of hyperandrogenicity. If anything however, higher concentrations of testosterone would have been expected in the women with PCO. The higher concentration of testosterone that I found in the women with normal ovaries is indeed a surprising result.

The role of GH in the ovary

Rat granulosa cells have been shown to be the site of GH reception and action (Jia *et al* 1986). GH amplified FSH hormonal action by augmenting granulosa cell cAMP elaboration and also augmented FSH-supported LH receptor acquisition and progesterone biosynthesis (Jia *et al* 1986). It has been suggested that GH may also enhance FSH supported IGF-I binding, either directly at the level of the ovary or indirectly via generation of hepatic IGF-I (Adashi *et al* 1988a). Hutchinson *et al* (1988) also showed an effect in rats of GH on FSH stimulated granulosa cell action, demonstrating an augmenting effect of GH on aromatase activity. Jorgensen and Nowak (1989) demonstrated a significant effect on ovarian weight and number and size of follicles with GH cotreatment in hypophysectomised rats given exogenous gonadotrophins (Pergonal or Metrodin). The augmented response was not seen in intact animals and it was suggested that this was because maximal IGF-I production was already occurring. GH alone caused a significant increase in ovarian weight but the effect was less than with gonadotrophins alone. The authors conclude that GH plays a role in the regulation of ovarian function but that the effect is mainly through stimulation of IGF-I concentrations.

The background for the research presented in this thesis was the work demonstrating augmentation of FSH stimulated granulosa cell function by IGF-I. To induce high circulating concentrations of IGF-I, biosynthetic GH was given. The direct action of GH on ovarian function had not, at that time, been demonstrated. Indeed Ramasharma *et al* (1987) showed that GH was not capable of stimulating progesterone production in human granulosa cells.

In in-vitro studies of human granulosa cells, Mason *et al* (1990) have, however, shown that GH directly stimulated E_2 secretion by human granulosa cells. GH was demonstrated to stimulate E_2 accumulation even in the absence of FSH. The augmented E_2 response to FSH by GH was additive not synergistic, suggesting a mechanism independent of FSH. IGF-I

could not be detected in the medium either before or after GH stimulation. These data are consistent with the observations of Voutilanen and Miller (1987) and Ramasharma and Li (1987) that mRNA for IGF-I was not detectable in human granulosa cells after GH treatment.

Studies on animal and human ovarian tissue suggest that GH plays an important role in the physiology of ovarian function. Whether the gonadal action of GH is primarily direct or via IGF-I has yet to be established. The significant role of these two hormones in the onset and maintenance of puberty is becoming more apparent. Ojeda and Jamieson (1977) have shown that a rise in circulating concentrations of GH occurs prior to puberty and it is recognised that suppression of endogenous release of GH in pre-pubertal female rats leads to delayed puberty. In humans, GH deficiency results in delayed puberty and GH has been shown to be necessary for normal pubertal development (Burns *et al* 1981, Darendeliler *et al* 1990). Darendeliler *et al* (1990) studied 134 children with isolated GH deficiency on GH treatment and showed that the duration of puberty was significantly shortened with GH therapy. They suggest that GH synergises with gonadotrophins during puberty and that treatment with GH accelerates the pubertal process.

Circulating concentrations of IGF-I in pubertal children are higher than in normal adults and the rise in serum IGF-I correlates with sexual developmental stage, serum concentrations of E_2 and androgens and growth velocity (Rosenfield *et al* 1983, Rosenfield and Furlanetto 1985, Cara *et al* 1987). Although the exact mechanisms are not clear, increases in endogenous GH are thought to play an important role in inducing the high concentrations of IGF-I. Sex steroids are also pivotal to pubertal development and may have a direct effect in increasing IGF-I concentrations (Moll *et al* 1986). Alternatively the sex steroids may increase GH production with a secondary increase in IGF-I (Cara *et al* 1989). Devesa *et al* (1991) suggest that E_2 inhibits the hypothalamic release of somatostatin and thereby modulates GH release.

Whatever the exact mechanisms, it is well recognised that sexual maturation and the active growth phase are associated and there is increasing evidence for a link between GH, ovarian function and the onset of puberty.

11ß-hydroxyandrostenedione as a marker for PCO

Study 1.

Rationale

Having established that women with PCO formed a subgroup in whom GH therapy augmented their ovarian response to gonadotrophins, we investigated whether 11ß-OH-A_4 could be used as a marker for the condition.

The inactive androgen, 11ß-OH-A_4, has been considered to be exclusively adrenal in origin. Predominantly arising from the 11ß hydroxylation of androstenedione, production of 11ß-OH-A_4 was thought to divert synthesis away from active androgens (such as testosterone and dihydrotestosterone) (Jeanloz *et al* 1952, Cohn and Mulrow 1963, Goldzieher and Beering 1969, Axelrod *et al* 1973). 11ß-OH-A_4 then plays no further part in androgen production as it is subsequently metabolised by the liver and adrenal to the 11ß hydroxy-androsterone and 11ß hydroxy-aetiocholanone which are excreted in the urine. 11ß-OH-A_4 synthesis by side chain cleavage of cortisol has also been demonstrated (Deshpande *et al* 1970, Axelrod *et al* 1973, Hudson *et al* 1974). Overall it was, however, considered that this pathway constituted a relatively minor contribution to overall 11ß-OH-A_4 synthesis.

11ß hydroxylation of C21, but not C19, steroids was suggested to occur in the ovary but recently there was a report of measurable amounts of 11ß-OH-A_4 in the ovarian vein of an adrenalectomised patient (Polson *et al* 1987). The presence of 11ß-OH-A_4 in an adrenalectomised woman was thought to reflect 11ß hydroxylase activity on C19 steroids in the ovary. The adrenalectomised woman studied by Polson *et al* (1987) had PCO. We therefore investigated the use of measurements of 11ß-OH-A_4 concentrations as a marker for this condition.

There is increasing evidence that PCO has a genetic component and an enzyme defect in androgen metabolism is a possibility (Cooper *et al* 1968, Givens *et al* 1971, Hutton and

Clark 1984, Givens 1988, Hague *et al* 1988). A plausible candidate is the 11ß hydroxylase (P450c11 enzyme complex) gene which has a central role in the control of steroid and possibly androgen metabolism. The P450c11 enzyme complex itself exhibits different degrees of substrate specificity, for example in the adrenal 11-deoxy-corticosteroid (C21) is 11ß hydroxylated at three times the rate of a C19 steroid such as androstenedione (Finkelstein 1973).

High performance liquid chromatography and radioimmunoassay.

The first step in our search for a candidate gene for PCO was to establish a reliable assay for assessment of 11ß-OH-A_4 concentrations in serum and FFL. The only commercially available kit (Interscience Laboratories USA) had not been validated for use in analysing FFL and was expensive. We therefore developed an in-house RIA in which a preparative HPLC separation was a prerequisite. This initial step was necessary in order to eliminate potentially cross reacting steroids (Dehennin *et al* 1987), which were present in high concentrations in follicular fluid (Lobo *et al* 1985, McNatty *et al* 1973, Carson *et al* 1982); for example A_4 was present in micromolar amounts, as confirmed in our own gas chromatography and mass spectrometry analysis of a pooled sample of FFL (Fig *V*-6).

Follicular fluid samples, the steroids from which were separated by HPLC prior to RIA, yielded lower results when compared with samples assayed after extraction alone. When comparing serum sample results, using the commercially available method and our in-house method, the latter resulted in significantly lower 11ß-OH-A_4 concentrations (Fig *V*-5). This is consistent with a greater specificity of the in-house method. There was also an improvement in sensitivity using the in-house method (0.25nmol/L) over the value of 0.4nmol/L using the Intersci kit.

The HPLC, followed by RIA, system was demonstrated to be a reliable technique as shown by the RIA validation, high recoveries and consistent retention times (Table *V*-3). The non-specific binding, post extraction and post HPLC, was negligible when sample blanks were immunoassayed. Much work was done to establish the most reliable HPLC system. The solvent eluent from many HPLC systems contained material that destroyed the subsequent

RIA, which is a common problem plaguing RIA measurement after HPLC. The normal phase system, using a diol column as described in this study, was shown to overcome this difficulty (Schoneshofer *et al* 1981). The HPLC method, by virtue of the chromatographic separation of steroids, enabled the simultaneous measurement of other steroids pertinent to this study (for example A_4, testosterone and cortisol) from a single sample.

Applying the technique of HPLC separation followed by RIA to the serum and FFL samples, we demonstrated the presence of 11ß-OH-A_4 in all samples. The presence of 11ß-OH-A_4 was confirmed by selected ion monitoring by gas chromatography and mass spectrometry. 11ß-OH-A_4 concentrations were approximately six times higher in FFL compared with serum. This was the first demonstration of 11ß-OH-A_4 in FFL.

Gas chromatography and mass spectrometery analysis of steroids.

The concentrations of steroids in follicular fluid were much higher than circulating concentrations (Table *V*-4) and this included steroids involved in the pathways of 11ß-OH-A_4 synthesis. The ratio of FFL to serum was particularly high for A_4, consistent with the synthesis of this androgen in the ovary. The ratio was, however, approximately 1 for cortisol because, in this study, serum levels were high (median 816nmol/L).

GC/MS analysis of steroids from the pooled sample of FFL (Fig *V*-6) demonstrated concentrations of steroids within limits reported in the literature (Dehennin *et al* 1987a, Dehennin *et al* 1987, Vanluchene *et al* 1990, Lobo *et al* 1985, McNatty *et al* 1973, Carson *et al* 1982). The presence of 11ß-OH-A_4 in follicular fluid was confirmed by the alternative analytical method of selected ion monitoring GC/MS. The application of this sensitive technique was necessary as 11ß-OH-A_4 concentrations in FFL were too low to be demonstrated by standard GC/MS.

Summary.

Having established that women with PCO formed the subgroup who responded to GH in the IVF-ET studies, we investigated the use of 11ß-OH-A_4 concentrations as a marker of the

condition. We developed a reproducible and validated method for measuring 11ß-OH-A_4 concentrations in serum and follicular fluid with an initial HPLC separation prerequisite. FFL concentrations of 11ß-OH-A_4 were many times higher than serum and this was the first demonstration of 11ß-OH-A_4 in human, or animal, FFL. The presence of 11ß-OH-A_4 in follicular fluid was validated by selected ion monitoring GC/MS. The concentrations of the other steroids measured was in keeping with the recognised milieu of the ovary.

Studies 2 and 3.

Rationale

The origin and implication of the high concentrations of 11ß-OH-A_4 in FFL was of great interest in relation to ovarian androgen metabolism and particularly to the potential use in differentiating women with PCO. We therefore measured the various steroids concentrations, including 11ß-OH-A_4, in several groups of women, with normal or polycystic ovaries, receiving no treatment or undergoing superovulation regimens prior to IVF-ET.

Serum results in the untreated groups.

The serum concentrations of androgens were significantly higher in women with the PCOS compared with women with normal ovaries (Table *V*-5). There was no difference in the cortisol concentrations between the two groups.

The overwhelming evidence supports an ovarian source of androgen excess in PCOS (Wajchenberg *et al* 1986, Jacobs 1987a). The high serum concentrations of 11ß-OH-A_4 in the hyperandrogenic women may also be derived from synthesis within the ovary. Alternatively, the higher concentrations of 11ß-OH-A_4 in women with PCO may suggest an adrenal source of this androgen, reflecting metabolism of the high concentrations of its main precursor, androstenedione.

Serum concentrations during gonadotrophin stimulation.

The serum concentrations of 11ß-OH-A_4 and cortisol following gonadotropin stimulation were higher in women with ultrasound diagnosed PCO compared with women with normal ovaries (Table *V*-6). The association of raised concentrations of 11ß-OH-A_4 and cortisol in the group of women with PCO suggested that the excess 11ß-OH-A_4 might be a metabolite of cortisol. The high serum concentrations of cortisol are probably a reflection of the adrenal response to the stress of the oocyte collection procedure for IVF-ET.

Comparisons were made between the results from the untreated women and the women who received exogenous gonadotrophins prior to IVF-ET (Tables *V*-5 and -6). 11ß-OH-A_4 concentrations were similar between the groups but the other steroids measured were higher in women undergoing treatment for IVF-ET. This was not a particularly valid comparison as the women formed such different groups. In the untreated group were women with normal ovaries who were asymptomatic and also women who were attending a specialist clinic with symptoms of PCOS. In the group receiving gonadotrophin treatment were women with a variety of indications for IVF-ET. The diagnosis of PCO had been made on the ultrasound appearance only and the women had regular ovulatory cycles.

It was interesting that there were significant differences in the 11ß-OH-A_4 concentrations in both the untreated and treated groups between the women with normal and polycystic ovaries (Tables *V*-5 and -6). There was, however, considerable overlap in the range of results and thus serum concentrations of 11ß-OH-A_4 cannot be used to differentiate between normal and polycystic ovaries.

Follicular fluid concentrations.

The intra-follicular environment appeared to be different to serum, particularly for women in hyperstimulated cycles. Within the ovary, 11ß-OH-A_4 concentrations were significantly lower in women with polycystic ovaries compared to those with normal ovaries, whereas androstenedione concentrations were not significantly different (Table *V*-7). There was no influence of ovarian morphology on intrafollicular fluid concentrations of testosterone or

cortisol. The difference in the relative amounts of androstenedione and 11ß-OH-A_4 in follicular fluid might be explained by a local diversion of the ovarian metabolism of androstenedione towards testosterone, at the expense of 11ß-OH-A_4.

Testosterone concentrations in follicular fluid were similar in women receiving gonadotropin treatment whether they had normal or polycystic ovaries (Table *V*-7). Testosterone concentrations were, however, much higher in women with normal ovaries who were given gonadotrophins compared with those in the untreated group. This suggested that the hyperstimulated ovary was producing large quantities of active androgen, reflected in the follicular fluid concentrations.

All of the follicular fluid steroids measured were present in considerably higher concentrations than in the circulation. In addition to denovo synthesis, the ovary has the ability to concentrate all commonly occurring steroids from the circulation because of the large quantities of steroid binding proteins therein, such as albumin, sex hormone binding globulin (Ben-Rafael *et al* 1986) and cortisol binding globulin. The presence of 11ß-OH-A_4 in follicular fluid may represent concentration of the steroid from the circulation.

Granulosa cell studies

Whether the novel finding of high concentrations of 11ß-OH-A_4 in the FFL reflected ovarian synthesis or merely concentration from serum required further investigation. We therefore undertook incubation studies with granulosa cells and the potential precursors.

Both androstenedione and cortisol are potential substrates for the synthesis of 11ß-OH-A_4. The in-vitro studies of granulosa cells incubated with labelled substrates gave no evidence of 11ß hydroxylase activity, however, synthesis of 11ß-OH-A_4 by side chain cleavage of cortisol was unexpectedly demonstrated in two of the seven patients (Table *V*-8). This has never before been demonstrated in the ovary. Peripheral synthesis of 11ß-OH-A_4 has been reported to be relatively small (Axelrod *et al* 1973), which should limit any possible effects of blood contamination present in the incubations. Indeed, control incubations with blood alone did not demonstrate any synthesis of 11ß-OH-A_4.

Steroid synthesis in the ovary is compartmentalised (Lobo *et al* 1985) and it may therefore be appropriate for future studies to perform similar experiments on isolated thecal cells. The high concentrations of 11ß-OH-A_4 in follicular fluid could be explained either by the steroid enriched milieu, characteristic of follicular fluid, or some synthesis from cortisol.

PCO.

The extensive range of steroid values within each of the study groups reported herein is consistent with the currently held view that the PCO syndrome is characterised by a heterogeneity of endocrine as well as other clinical features (Conway *et al* 1989). The aetiology of this condition is unlikely to be ascribed to any one particular pathological condition (such as hyperandrogenism) nor to an abnormality of expression of some single and all encompassing mechanism (Hague *et al* 1988). Rather, a complex combination of factors, acting in different degrees and at various levels such as the hypothalamic, pituitary, adrenal or gonadal must be responsible.

The high serum concentrations of 11ß-OH-A_4 in women with PCOS reflect the overall pattern of raised androgens in this condition. Lower concentrations of 11ß-OH-A_4 in the follicular fluid of women with PCOS are consistent with a diversion of synthesis away from the inactive androgen, 11ß-OH-A_4, and towards testosterone. Evidence for this hypothesis was not, however, provided by our in-vitro granulosa cell studies.

A combination of the following phenomena most likely explain our results: in some cases, intraovarian side-chain cleavage of cortisol; the ovary's ability to concentrate steroids from the circulation; an overall increase in androgens within the wide ranges of sampling error observed; an abnormality in androgen metabolism in women with PCO and PCOS.

Conclusions.

In conclusion, neither serum nor follicular fluid concentrations of 11ß-OH-A_4, can be regarded as a reliable marker of hyperandrogenism in women with polycystic ovaries. We did not demonstrate synthesis of 11ß-OH-A_4 from A_4 by P450c11 activity in granulosa cells.

We suggest that 11ß-OH-A_4 may be being synthesised within the ovaries from side-chain cleavage of cortisol, but most likely arises from concentration from the circulation.

11ß-OH-A_4 concentrations can not, therefore, be used as a marker of adrenal androgenisation as suggested by Hudson *et al* (1990). The authors argue that 11ß hydroxylase activity on A_4 is strictly adrenal and therefore 11ß-OH-A_4 concentrations in serum reflect adrenal 17-ketosteroid production. Although we were unable to demonstrate P450c11 activity in the ovary, 11ß-OH-A_4 production form cortisol was suggested. The studies presented herein demonstrate clearly the presence of 11ß-OH-A_4 in FFL and raise doubt about the use of this weak androgen as an adrenal marker. The results lend further support to the overlap in adrenal and ovarian function and thus the difficulty in defining a single marker for the heterogenous condition of PCO.

Future areas of research.

The clinical application of result from studies of growth factor modulation of granulosa cell function formed the basis for the work presented in this thesis. Reports of the clinical effects of GH treatment, in both in-vivo and in-vitro fertilisation, have stimulated much further work both in the laboratory and with patients. The role of growth factors, and of GH itself, within the ovary is still far from clear and much work will be needed over the next few years to clarify the complex phenomenon of folliculogenesis.

Hopefully, consequent upon this elucidation, will come further treatment options for those women undergoing ovulation induction, whether prior to IVF-ET or natural conception. Women responding suboptimally to ovarian stimulation form a small but frustrating group for those wishing to help them conceive. GH augmentation of exogenous gonadotrophin treatment may have a place in the management of these women but care must be taken to establish the correct individuals suitable for treatment. Unfortunately none of the other studies on the use of GH in IVF-ET published to date have been randomised or placebo controlled. The consequence of this may be the indiscriminate administration of GH to women with inappropriate indications and a responsive subgroup may never be properly defined.

Further studies to determine the minimum dose of GH that sensitizes the human ovary, to select the most appropriate group of patients to be treated, to explore the mechanism of action of GH and to define the role of IGF-I in the process of the human follicular development are, therefore, just some of the areas needed to be investigated.

The polycystic ovary syndrome is a fascinating condition and the high prevalence of the morphological appearance of the ovaries requires further investigation. Although the P450c11 enzyme complex does not appear to hold the key to the genetic component of this condition, alterations in androgen and other steroid biosynthesis will need to continue to be studied. The possibility that abnormalities in growth factor production or action may be important in the aetiology of the syndrome will no doubt be the basis of much further research.

Conclusions.

Work over the last decade has demonstrated that growth factors play an important role in the complex formation of the developing follicle and consequent ovulation. Modulation of gonadotrophin induced steroidogenesis may be one of the principal actions of these growth factors. There is increasing evidence for a link between growth, growth hormone, puberty and ovarian function.

Based on the work presented in this thesis and the notion that IGF-I is capable of augmenting FSH hormonal action in the human ovary, we hypothesize that GH-FSH synergy may, in part, be due to the ability of GH to increase IGF-I production and augment the action of FSH.

We have demonstrated that, in women previously responding suboptimally to exogenous gonadotrophin stimulation prior to IVF-ET, GH cotreatment is associated with an increase in ovarian response and in circulating IGF-I concentrations, most markedly in women with ultrasound diagnosed PCO. Women who received treatment with GH had higher concentrations of IGF-I in their follicular fluid than those who received placebo, but these concentrations were significantly lower than in serum. This is consistent with GH stimulated *hepatic* production of IGF-I, but does not support the hypothesis of significant *intraovarian* IGF-I production.

The application of results from in-vitro experiments to in-vivo studies is exemplified by the work presented in this thesis. The difficulties of applying the conclusions of one set of work to another is also represented herein. Treatment with GH appears to have some augmenting effect upon gonadal response to gonadotrophins, but only in a subgroup of women.

Establishing the characteristics of women who respond to GH will be an important contribution to the use of this drug in ovarian stimulation regimens. Women with PCO appear to form the group of potentially responsive patients. At present there is no reliable biochemical marker for this heterogeneous condition. Measurement of 11ß-OH-A_4

concentrations in the serum or FFL of women can not be used to differentiate between those with PCO or normal ovaries.

In conclusion, the work presented in this thesis suggests that there is a role for GH augmentation of gonadotrophin treatment in a small group of women who have previously responded suboptimally and who have PCO.

Acknowledgements and Publications

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Without Professor Howard Jacobs' inspiration none of this work would have been undertaken and I am for ever indebted to his patience, encouragement and conviction that "statistics can be fun". Peter Holownia and John Honour demonstrated limitless tolerance in teaching me the laboratory techniques and I am particularly grateful to Peter for his support in completing the work. Without Gerry Conway's assistance I would still be computer-terrified and I will forever appreciate his help in the development of my computer skills.

Finally I would like to thank my family, friends and work colleagues who have provided constant support and encouragement and without whom I would have given up before I started. I am particularly grateful to Liz and Frances Mary for always being there over the past few months.

Publications

The following publications contain data included in this dissertation.

Cotreatment with growth hormone of sub-optimal responders in IVF-ET.
Owen EJ, West C, Jacobs HS, Mason BA.
Human Reproduction (1991) **6**, 524-528.

Serum and Follicular Fluid Insulin-like Growth Factors-1 and 11 during Growth Hormone Cotreatment for In-Vitro Fertilisation and Embryo Transfer.
Owen EJ, Torresani T, West C, Mason BA, Jacobs HS
Clinical Endocrinology (1991) **35**, 327-334

Cotreatment with Growth Hormone, after pituitary suppression, for ovarian stimulation in in vitro fertilization: a randomized, double blind, placebo-control trial.
Owen EJ, Shoham Z, Mason B, Ostergaard H, Jacobs HS
Fertility and Sterility (1991) **56**, 1104-1110

The Determination of 11-Beta-Hydroxyandrostenedione in Human Follicular Fluid and Plasma
Holownia P, Owen EJ, Hampl R, Jacobs HS, Honour JW
Journal of Steroid Biochemistry and Molecular Biology (1991) **38**, 389-398

Studies to confirm the source of 11ß-hydroxyandrostenedione.
Holownia P, Owen EJ, Conway GS, Round J, Honour JW.
Journal of Steroid Biochemistry and Molecular Biology (1992) **41**, 875-880

11-Beta-Hydroxyandrostenedione in the plasma, follicular fluid and granulosa cells of women with normal and polycystic ovaries.
Owen EJ, Holownia P,Conway GS, Jacobs HS, Honour JW
Fertility and Sterility (1992) In Press

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Review Articles

The following review articles contain some of the hypotheses that are developed in this thesis.

Use of growth hormone to augment the ovarian response to treatment with gonadotrophins.
Jacobs HS, Homburg R, Owen EJ, Eshel A, Abdalla HI, West C.
(1988) In: Advances in Gynaecological Endocrinology. Ed: Genazzani AR, Petraglia F, Volpe A, Farchinetti F. Parthenon Publishing.

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Shoham Z, Homburg R, Owen EJ, Conway GS, Ostergaard H, Jacobs HS
(1991) In: Clinical Obstetrics and Gynaecology - Assisted Reproduction. Ed: Hamberger and Wikland. Bailliere Tindall London

Role of Growth Hormone in Infertility.
Jacobs HS, Bouchard P, Conway GS, Homburg R, Lahlou N, Mason B, Ostergaard H, Owen EJ, Shoham Z
Hormone Research (1991) **36**, 61-65

Prizes

Society of Reproductive Endocrinologists Prize Paper (1988)

The paper presented to the American Fertility Society entitled Combined Growth Hormone and Gonadotrophin Therapy for Ovulation Induction was awarded the prize for an "outstanding contribution to the subject of reproductive endocrinology".

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Appendices

Abbreviations used in the appendices.

PtN = Patient number
A = Open study
PL = Placebo cycle
GH = Growth Hormone cycle
hMG = Total dose of hMG used per cycle
Foll = Number of follicles $\geq$ 14mm in diameter on the day of hCG administration
UE = Total urinary oestrogens on the day of hCG administration (nmol/24hrs)
S E_2 = Serum concentration of E_2 on the day of hCG administration (pmol/L)
Ooc = Number of oocytes collected
Fer = Number of oocytes fertilised (excluding polyspermia)
Clv = Number of embryos cleaved
Ab = Abandoned cycles
- = Insufficient sample for analysis or no sample obtained

CC and hMG studies:

D2 = Day 2 of cycle; first day of CC administration
D8 = Day 8 of cycle
D9 = Day 9 of cycle
D10-14 = Mean results from days 10,11,12,13 and/or 14 of cycle
Day OC = Day of oocyte collection
MFFL = Mean concentration in follicular fluid

Buserelin and hMG study:

D-14 = First day of buserelin administration
D 1 = First day of hMG administration
D 6-7 = Days 6 or 7, or mean of two values
D 8-9 = Days 8 or 9, or mean of two values
D 10-11 = Days 10 or 11, or mean of two values
D 12-14 = Mean results from days 12, 13 and/or 14 of cycle
Day OC = Day of oocyte collection
MFFL = Mean concentrations in follicular fluid

Clinical results from open CC and hMG study

PtN	Prestudy cycle				First GH cycle				Second GH cycle			
	hMG	Foll	UE	Ooc	hMG	Foll	UE	Ooc	hMG	Foll	UE	Ooc
Normal ovaries												
2	48	1	188	Ab	60	3	3188	0	64	2	491	2
5	54	1	245	Ab	48	2	449	2	70	5	891	5
Polycystic ovaries												
4	80	1	613	Ab	64	3	1039	4	78	5	1183	1
7	36	6	663	3	36	5	618	9	36	5	346	5
8	72	4	174	4	48	4	551	4	80	6	521	6
9	42	0	124	Ab	36	2	227	2				

Clinical data from placebo controlled CC and hMG study

	Prestudy cycles				Placebo cycles					GH cycles				
PtN	hMG	Foll	UE	Ooc	hMG	Foll	S E_2	UE	Ooc	hMG	Foll	S E_2	UE	Ooc
Normal ovaries														
1	36	2	279	0	24	3	3520	708	2	32	3	2560	328	2
2	30	2	256	5						48	3	1805	603	3
3	36	1	566	0	32	1	1084	139	0	32	1	1301	391	0
4	24	3	304	1						20	2	2339	316	0
5	28	2	242	0						32	3	2431	317	2
6	28	2	221	0	24	2	2616	484	6	32	3	1852	356	2
7	42	4	314	2						52	0	200	125	0
PCO														
21	24	2	110	0	28	2	1087	169	0	24	3	2630	810	1
22	24	2	211	0	24	4	2030	574	6	0	0	0	0	0
23	36	3	549	2						36	2	2144	1013	2
25	20	4	540	3	20	3	2849	328	3	20	3	2144	391	2
26	36	4	409	3						30	3	1834	406	3
27	44	2	437	0						28	6	4222	354	3
28	36	2	390	2	24	3	903	266	1	36	4	2350	456	5
29	28	3	526	5						28	4	3200	500	5
30	35	4	855	2	30	7	4185	796	4	30	7	1723	544	3
31	30	3	610	3						36	5	6360	637	6
32	24	2	238	0	24	2	1598	168	3	24	2	2210	354	3
33	36	2	312	0						30	2	2162	475	3

Serum concentrations of FSH (IU/L) in women with normal ovaries - CC and hMG studies

Pt N	Cycles	PL/GH	D 2	D 8	D 9	D 10-14	Day OC
1	1	PL	8.0	24.5			24.5
1	2	GH	15.0	23.0	>20.0	24.4	>20.0
2	1	GH	5.5	15.6	20.1	>20.0	17.9
2	2	GH	>22.0	22.2			14.3
3	1	PL	12.4			28.1	
3	2	GH	7.1	>30.0			
4	1	GH	4.1	16.6	18.5		
5	1	GH	11.4	21.5	21.1	26.8	24.0
5	2	GH	10.0	>40.0	38.7	>40.0	>40.0
6	1	PL	5.6	12.3			9.1
6	2	GH	11.9	21.2	18.7	21.7	16.9
7	1	GH	3.3	31.7	>35.0		
A2	1	GH	7.7		14.4	>35.0	27.9
A2	2	GH	8.9	33.3	>40.0	>35.0	>40.0
A5	1	GH	11.7		>18.0	>18.0	17.0
A5	2	GH	0.5	>20.0	>22.0		15.1

Serum concentrations of FSH (IU/L) in women with PCO - CC and hMG studies

Pt N	Cycles	PL/GH	D 2	D 8	D 9	D 10-14	Day OC
21	1	PL	4.4	17.3	21.0		
21	2	GH	8.2	16.7	15.0	10.2	10.2
22	1	PL	7.4	13.0	7.9	10.2	8.4
23	1	GH	5.6	22.0			16.9
25	1	PL	7.7	20.7	17.3		11.9
25	2	GH	10.2	16.6	20.2		14.1
26	1	GH	9.5	14.1	13.5		18.9
27	1	GH	5.1	16.4	15.5		16.0
28	1	PL	9.5	>25.0			
28	2	GH	9.2	>34.0	>40.0	39.0	33.0
29	1	GH	9.5	12.7	16.0		12.3
30	1	PL	6.9	8.4	7.4	15.9	6.1
30	2	GH	5.9	3.4	8.0	8.7	8.4
31	1	GH	9.9	15.4		18.5	11.9
32	1	PL	7.0	19.9	23.3		15.7
32	2	GH	6.3	11.6	10.6	10.0	15.4
33	1	GH	7.4	31.0			34.6
A4	1	GH	9.8	>35.0	38.7	13.2	5.0
A4	2	GH	11.4	>35.0	23.7	>35.0	20.0
A7	1	GH	1.1	13.1	14.7	13.5	9.8
A7	2	GH	2.1	14.1	13.7	12.4	6.7
A7	3	GH	0.6	8.5	6.8	8.9	7.9
A8	1	GH	6.9	>20.0			22.2
A8	2	GH	9.5	24.0	16.6	24.3	21.7
A9	1	GH	5.0	17.0	15.1	11.5	-

Serum concentrations of LH (IU/L) in women with normal ovaries - CC and hMG studies

Pt N	Cycles	PL/GH	D 2	D 8	D 9	D 10-14	Day OC
1	1	PL	4.9	8.1			25.6
1	2	GH	8.0	18.4	19.9	24.3	>36.0
2	1	GH	2.3	5.7	5.1	10.9	11.4
2	2	GH	8.9	5.4			16.5
3	1	PL	4.1			-	
3	2	GH	3.7	25.4			
4	1	GH	5.2	9.0	8.8		
5	1	GH	10.4	14.5	12.1	13.9	48.5
5	2	GH	8.8	15.8	8.0	12.2	>40
6	1	PL	3.0	8.1			16.3
6	2	GH	7.4	8.6	7.4	5.2	33.1
7	1	GH	1.2	11.7		26.6	
A2	1	GH	4.2		13.9	25.9	18.4
A2	2	GH	3.6	9.9	10.2	10.7	>50
A5	1	GH	6.9		8.7	42.3	>38
A5	2	GH	0.9	7.9	17.2		23.4

Serum concentrations of LH (IU/L) in women with PCO - CC and hMG studies

Pt N	Cycles	PL/GH	D 2	D 8	D 9	D 10-14	Day OC
21	1	PL	5.2	14.2	14.8		
21	2	GH	4.2	10.1	8.2	11.7	24.2
22	1	PL	4.7	4.8	3.4	4.1	17.8
23	1	GH	3.5	5.4			33.1
25	1	PL	4.6	7.5	6.3		23.1
25	2	GH	5.0	4.8	4.8		20.1
26	1	GH	4.4	5.2	5.0		30.1
27	1	GH	4.7	4.1	3.8		>50
28	1	PL	4.5	12.5			
28	2	GH	8.0	6.8	6.9	6.0	30
29	1	GH	7.0	6.5	6.5		27.7
30	1	PL	9.9	3.7	4.4	31.5	13.1
30	2	GH	4.2	8.2	3.0	3.7	15.1
31	1	GH	4.3	7.2		5.4	12.3
32	1	PL	5.3	7.4	7.8		26.0
32	2	GH	7.4	6.9	5.2	4.7	21.2
33	1	GH	4.5	8.6			>35
A4	1	GH	7.3	7.4	8.0	9.4	3.1
A4	2	GH	15.8	10.1	8.7	28.5	21.0
A7	1	GH	1.5	3.6	3.3	3.4	14.2
A7	2	GH	1.7	3.7	3.4	3.2	11.4
A7	3	GH	2.2	2.4	2.1	3.2	9.3
A8	1	GH	3.1	4.9			18.1
A8	2	GH	4.4	6.5	4.9	7.2	18.3
A9	1	GH	5.1	5.4	3.8	6.8	-

Serum (pmol/L) and mean follicular fluid (nmol/L) concentrations of E_2 in women with normal ovaries - CC and hMG studies

Pt N	Cycles	PL/GH	D 2	D 8	D 9	D 10-14	Day OC	MFFL
1	1	PL	223	3520			1676	954.2
1	2	GH	93	1088	1844	2560	1205	789.1
2	1	GH	329	2314	2579	2486	1451	697.3
2	2	GH	74	4560			2439	761.6
3	1	PL	182	825		1084		
3	2	GH	246	1301				
4	1	GH	440	1735	2339			
5	1	GH	105	1368	2066	2431	1191	804.4
5	2	GH	119	1630	1825	1800	1521	
6	1	PL	289	1703		2616	2500	887.7
6	2	GH	68	634	847	1463	1070	587.2
7	1	GH	74	689	200			
A2	1	GH	179		1837	3780	1170	
A2	2	GH	74	1082	1554	1603	1078	789.1
A5	1	GH	115		1276	1759	395	767.1
A5	2	GH	53	3500	4500		1024	789.1

Serum (pmol/L) and mean follicular fluid (nmol/L) concentrations of E_2 in women with PCO - CC and hMG studies

Pt N	Cycles	PL/GH	D 2	D 8	D 9	D 10-14	Day OC	MFFL
21	1	PL	109	600	1087			
21	2	GH	240	1804	2077	2630	1506	685.1
22	1	PL	150	1754	2111	2030	1811	844.1
23	1	GH	126	2144			2313	813.5
25	1	PL	160	2164	2849		1639	1018.4
25	2	GH	176	2404	3475		1079	1101.0
26	1	GH	119	1467	1834		1529	954.2
27	1	GH	85	2260	4222		1665	1275.4
28	1	PL	102	903				504.7
28	2	GH	133	1215	2408	2350	1118	706.5
29	1	GH	153	2058	3200		2208	890.0
30	1	PL	204	2100	2875	4073	1225	821.2
30	2	GH	128	734	1026	1723	1450	513.8
31	1	GH	206	5606		6360	1118	1137.7
32	1	PL	139	1506	1598		1803	1651.5
32	2	GH	69	1270	1780	2210	1375	981.5
33	1	GH	143	2162			-	917.0
A4	1	GH	121	1806	2640	5940	2140	1064.3
A4	2	GH	162	2510	2937	3015	1790	711.0
A7	1	GH	48	877	1666	3050	2760	758.4
A7	2	GH	43	988	1568	2560	1768	660.6
A7	3	GH	20	452	654	1612	1818	697.6
A8	1	GH	122	2600			1984	513.8
A8	2	GH	91	1699	3009	3310	1637	679.0
A9	1	GH	93	504	727	1488	752	743.2

Serum and mean follicular fluid concentrations of IGF-I in women with normal ovaries - CC and hMG studies (nmol/L)

PtN	Cycle	PL/GH	D 2	D 8	D 9	D10-14	Day OC	MFFL
1	1	PL	40.8	6.8			49.8	22.0
1	2	GH	53.4		13.3	-	56.7	54.6
2	1	GH	28.8		23.6	-	22.6	19.0
2	2	GH	12.1	9.3			44.8	28.6
3	1	PL	32.7			32.2		
3	2	GH	33.0	51.6				
4	1	GH	31.4	29.9	20.1			
5	1	GH	22.0		50.2	-	26.7	28.0
5	2	GH			10.2	-	23.2	30.6
6	1	PL	17.7	52.5			21.2	61.5
6	2	GH	43.9		97.3	-	92.1	17.1
7	1	GH	29.6	50.7		36.8		
A2	1	GH	26.9		51.2	-	35.6	40.6
A2	2	GH	45.0		47.2	-	34.0	-
A5	1	GH	32.9		40.8	-	30.7	22.5
A5	2	GH	37.0		40.8		27.8	34.8

Serum and mean follicular fluid concentrations of IGF-I in women with PCO - CC and hMG studies (nmol/L)

PtN	Cycle	PL/GH	D 2	D 8	D 9	D10-14	Day OC	MFFL
21	1	PL	15.3		18.3			
21	2	GH	35.9		8.9	-	35.3	28.7
22	1	PL	29.0		11.9	-	24.3	27.9
23	1	GH	32.2	64.7			21.6	29.7
25	1	PL	28.3		27.2		38.2	14.3
25	2	GH	-	38.2	56.0		-	24.7
26	1	GH	24.5		19.1		37.6	37.8
27	1	GH	34.8		55.6		37.4	26.7
28	1	PL	44.8	49.0				33.9
28	2	GH	54.6		58.2	-	72.4	51.0
29	1	GH	16.8		64.1		52.8	26.0
30	1	PL	37.8		32.0	34.8	36.6	28.2
30	2	GH	35.9		14.8	-	51.3	33.7
31	1	GH	38.8	45.6		37.4	-	18.1
32	1	PL	40.4		34.4		27.4	30.6
32	2	GH	29.8		-	48.6	35.3	36.4
33	1	GH	27.6	50.6			50.4	28.1
A4	1	GH	47.2		82.0	-	42.2	42.9
A4	2	GH	33.0		52.0	-	41.8	43.3
A7	1	GH	18.2		61.6	-	31.3	37.4
A7	2	GH	28.5		76.6	-	14.0	46.9
A7	3	GH	43.4		103.0	-	54.0	42.8
A8	1	GH	35.3	48.2			31.4	23.2
A8	2	GH	43.7		80.1	-	62.0	40.5
A9	1	GH	36.2		81.0	-	43.6	38.1

Serum and mean follicular fluid concentrations of IGF-II in women with normal ovaries - CC and hMG studies (nmol/L)

Pt N	Cycle	GH/PL	D 2	D 8	D 9	D 10-14	Day OC	MFFL
1	1	PL	65.2	120.0			82.4	61.5
1	2	GH	91.4		40.2	-	75.6	66.5
2	1	GH	70.6		73.2	-	61.8	40.4
2	2	GH	39.8	40.3			71.6	54.3
3	1	PL	99.9			94.0		
3	2	GH	67.6	132.0				
4	1	GH	62.4	66.8	67.6			
5	1	GH	73.2		80.0	-	107.0	70.1
5	2	GH			56.6	-	55.8	67.1
6	1	PL	82.3	147.9			50.2	128.5
6	2	GH	82.7		100.2	-	142.1	32.4
7	1	GH	86.0	112.0		89.2		
A2	1	GH	73.6		95.3	-	79.5	84.3
A2	2	GH	148.0		158.0	-	119.0	41.5
A5	1	GH	101.2		126.0	-	81.6	57.1
A5	2	GH	219.0		113.0		105.0	77.2

Serum and mean follicular fluid concentrations of IGF-II in women with PCO - CC and hMG studies (nmol/L)

Pt N	Cycle	GH/PL	D 2	D 8	D 9	D 10-14	Day OC	MFFL
21	1	PL	57.8		52.8			
21	2	GH	78.4		71.9	-	72.2	68.5
22	1	PL	58.6		51.4	-	59.8	55.9
23	1	GH	66.6	95.3			45.8	61.7
25	1	PL	118.0		83.2		98.7	46.2
25	2	GH		70.8	109.0		-	60.0
26	1	GH	50.2		135.2		111.0	92.0
27	1	GH	97.2		124.8		108.0	60.6
28	1	PL	81.4	129.0				65.5
28	2	GH	119.0		98.7	-	139.0	90.8
29	1	GH	35.2		117.7		44.4	48.9
30	1	PL	110.4		107.2	132.0	120.0	75.0
30	2	GH	95.4		76.2	-	129.0	56.3
31	1	GH	105.6	109.2		75.6	-	40.4
32	1	PL	88.8		85.6		100.4	78.6
32	2	GH	102.2		-	139.3	144.8	127.5
33	1	GH	79.3	135.2			131.4	49.4
A4	1	GH	153.0		183.0	-	77.4	59.3
A4	2	GH	79.6		107.0	-	145.0	117.0
A7	1	GH	36.4		122.0	-	57.4	61.0
A7	2	GH	62.4		147.0	-		69.3
A7	3	GH	115.0		115.0	-	112.0	66.4
A8	1	GH	76.0	115.0			63.4	51.4
A8	2	GH	80.6		115.5	-	113.8	69.9
A9	1	GH	147.6		126.0	-	110.0	76.8

Mean follicular fluid concentrations of insulin (pmol/L), progesterone (μmol/L), testosterone (nmol/L) and GH (mU/L) in women with normal ovaries - CC and hMG study

Pt N	Cycle	PL/GH	Insulin	Prog	Testo	GH
1	1	PL	104.0	54.9	12.4	6.3
1	2	GH	47.0	40.6	13.4	8.0
2	1	GH	49.5	36.6	6.7	1.6
2	2	GH	135.0	67.2	10.3	11.2
5	1	GH	49.0	39.2	12.8	2.1
5	2	GH	199.5	85.9	11.5	2.9
6	1	PL	-	39.8	10.2	-
6	2	GH	30.0	33.4	10.4	3.4
A2	1	GH	86.0	65.2	12.9	6.7
A2	2	GH	40.0	34.2	8.9	3.5
A5	1	GH	71.0	67.6	21.8	1.9
A5	2	GH	30.0	31.8	12.0	1.3

Mean follicular fluid concentrations of insulin (pmol/L), progesterone (μmol/L), testosterone (nmol/L) and GH (mU/L) in women with PCO - CC and hMG study

Pt N	Cycle	PL/GH	Insulin	Prog	Testo	GH
21	2	GH	51.5	31.7	8.5	3.6
22	1	PL	49.0	19.9	11.6	3.3
23	1	GH	74.0	19.0	11.7	0.8
25	1	PL	-	31.5	12.9	4.8
25	2	GH	-	32.2	12.3	3.1
26	1	GH	92.0	21.5	11.3	0.4
27	1	GH	54.0	31.0	4.5	8.7
28	1	PL	118.5	12.3	9.5	1.4
28	2	GH	70.0	19.5	8.1	2.3
29	1	GH	51.5	20.3	11.9	4.5
30	1	PL	192.5	81.9	11.7	0.7
30	2	GH	251.0	47.7	5.9	0.7
31	1	GH	30.0	21.9	-	2.1
32	1	PL	36.5	47.7	5.7	1.8
32	2	GH	32.5	54.1	8.1	2.4
33	1	GH	133.0	36.6	10.7	2.9
A4	1	GH	47.0	26.6	10.1	4.2
A4	2	GH	79.5	90.6	12.6	5.2
A7	1	GH	30.0	12.5	8.6	1.6
A7	2	GH	30.0	12.2	4.9	1.3
A7	3	GH	30.0	12.2	8.8	2.7
A8	1	GH	30.0	41.3	9.3	1.0
A8	2	GH	30.0	24.7	6.4	1.0
A9	1	GH	122.5	38.2	11.6	0.4

Mean follicular fluid concentrations of IGF-I (nmol/L), IGF-II (nmol/L), insulin (pmol/L), progesterone (μmol/L), testosterone (nmol/L) and GH (mU/L) analysed from 5 women in prestudy cycles:

Pt N	IGF-I	IGF-II	Insulin	Prog	Testo	GH
2	15.9	61.7	94.5	73.6	9.5	2.8
3	19.7	60.5	61.0	44.9	7.4	1.4
23	37.8	55.1	52.0	17.5	15.2	1.9
29	15.0	30.0	37.0	6.8	5.0	0.4
A8	30.0	85.2	45.5	43.8	8.5	1.6

Clinical results from placebo controlled buserelin and hMG study

	Prestudy cycles					Placebo cycles						GH cycles					
PtN	hMG	Foll	Ooc	Fer	Clv	hMG	Foll	S E_2	Ooc	Fer	Clv	hMG	Foll	S E_2	Ooc	Fer	Clv
Normal ovaries																	
1	63	4	4	3	3	27	4	1495	4	2	1	27	8	2555	6	1	1
2	60	3	2	0	0							85	3	1397	3	1	0
3	24	7	6	4	3							24	4	2222	2	1	1
4	62	2	2	1	1	48	2	2992	3	3	2	48	2	3061	2	2	2
5	24	3	4	1	1	30	5	5060	7	0	0						
6	53	4	4	2	2							65	3	3660	3	0	0
8	32	4	3	0	0	48	4	2290	4	2	0						
Polycystic ovaries																	
20	28	6	6	4	1							28	8	3816	11	5	3
21	36	4	8	2	1							28	11	-	16	5	4
23	46	3	5	4	3							42	8	3791	12	11	7
24	18	5	3	0	0	30	9	5465	13	5	4	27	16	10000	8	7	6
25	28	3	2	1	1	24	7	2033	9	3	2	32	7	4490	9	3	1
26	36	2	0	0	0							27	4	2355	6	6	4
27	24	7	6	1	1							24	11	8960	16	11	10
28	32	5	3	3	1	32	5	2526	3	3	2	36	10	3500	8	2	2
29	46	4	4	3	2	32	6	3286	8	4	4	36	8	4260	7	3	1
31	27	3	6	2	2							30	7	8339	8	5	4
32	24	4	6	1	1							24	11	3087	12	10	9
33	35	6	6	0	0	40	7	8440	6	5	5	45	5	1749	2	0	0
34	64	3	4	2	1	100	4	4970	4	2	2	90	6	5620	6	4	4
35	14	5	6	0	0							20	9	5810	12	5	5
36	21	5	3	2	2							30	7	8840	10	8	6
37	28	3	3	2	1	46	4	4695	2	0	0	20	3	4919	4	0	0
38	60	4	5	3	3	48	9	1613	11	6	5	60	11	3402	13	7	7
39	28	7	3	0	0							24	19	8900	11	6	6

Serum concentrations of FSH (IU/L) - buserelin and hMG study

PtN	PL/GH	D-14	D1	D6-7	D8-9	D10-11	D12-14	Day OC
Normal ovaries								
1	PL	7.1	3.1	-	10.0	9.4		5.5
2	GH	9.1	2.0	30.0	30.0	26.0	30.0	28.7
3	GH	10.6	5.8	12.0	11.3			6.7
4	PL	5.0	7.0	25.0	14.6			14.0
5	PL	7.0	0.8	13.0	12.0	13.5		8.4
6	GH	11.0	6.1	3.1	23.5	14.0	14.3	8.7
8	PL	7.7	5.3	16.5	17.5	16.5	19.0	9.7
Polycystic ovaries								
20	GH	5.0	-	12.5	12.0			8.0
21	GH	1.7	5.9					19.4
23	GH	6.8	4.1	15.5	15.2			9.0
24	PL	7.2	1.5	13.5	12.4	8.9		6.4
25	PL	4.2	3.0	8.0				7.4
26	GH	8.8	4.9	20.0	17.3	18.1		6.9
27	GH	5.4	3.4	10.0	8.8			5.8
28	PL	4.6	4.1	16.5	16.9			11.8
29	PL	6.1	2.0	17.5	18.0			5.0
31	GH	7.6	2.8	15.0	19.0	17.5		9.7
32	GH	5.2	5.0	9.7	9.0			6.1
33	PL	2.0	3.4	21.5	17.0			11.0
34	PL	11.0	1.4	17.0	20.5	23.0		23.0
35	GH	6.4	2.4	7.5	10.1	10.3		-
36	GH	6.4	1.9	11.5	9.1	14.7		10.2
37	PL	6.6	1.0	11.0	8.3	11.5		9.0
38	PL	7.7	3.4	14.0	14.5			5.3
39	GH	3.1	9.1	10.5				5.1

Serum concentrations (IU/L) of LH - buserelin and hMG study

PtN	PL/GH	D-14	D1	D6-7	D8-9	D10-11	D12-14	Day OC	Prog OC
Normal ovaries									
1	PL	6.3	3.5	-	3.6	3.4		10.2	6.9
2	GH	5.2	1.9	4.1	3.9	4.5	5.2	13.6	9.9
3	GH	6.8	8.5	6.8	5.5			9.3	7.9
4	PL	5.0	9.0	5.8	6.5			11.0	13.0
5	PL	3.7	1.8	2.2	2.5	2.6		21.0	32.0
6	GH	5.5	4.3	5.3	5.4	4.2	4.5	10.0	9.0
8	PL	6.6	3.1	3.4	2.5	2.7	2.4	12.0	19.0
Polycystic ovaries									
20	GH	6.3	-	2.5	2.0			13.0	41.0
21	GH	4.2	10.5					12.7	23.0
23	GH	9.0	3.0	4.3	3.8			8.8	21.0
24	PL	11.0	2.1	2.9	2.9	2.7		11.7	45.0
25	PL	4.7	2.0	2.5				15.0	15.0
26	GH	7.0	6.0	6.5	6.7	7.8		15.9	11.0
27	GH	25.8	5.0	3.8	4.4			9.0	27.0
28	PL	3.7	3.1	3.2	4.2			12.0	14.0
29	PL	7.1	2.0	3.0	3.0			11.0	16.0
31	GH	6.6	2.8	2.5	3.2	2.7		11.0	41.0
32	GH	6.1	3.7	3.1	2.0			9.9	26.0
33	PL	4.0	6.0	10.8	3.5			15.0	52.0
34	PL	6.0	7.2	8.0	5.9	5.5		9.0	21.0
35	GH	7.5	3.4	3.0	3.1	2.6		-	-
36	GH	5.5	3.8	4.7	4.0	4.7		20.1	48.0
37	PL	5.0	2.0	3.0	3.0	3.5		10.0	35.0
38	PL	4.9	1.6	2.7	2.7			8.1	15.0
39	GH	5.0	18.0	10.2				8.2	27.0

Progesterone concentrations on the day of hCG administration were less than 8 nmol/L in all women.

Serum concentrations (pmol/L) of E_2 - buserelin and hMG study

PtN	PL/GH	D-14	D1	D6-7	D8-9	D10-11	D12-14	Day OC
Normal ovaries								
1	PL	109	46	-	588	1495		1195
2	GH	150	41	164	363	576	1244	700
3	GH	191	59	702	1814			1685
4	PL	92	41	1118	2429			1156
5	PL	196	90	1150	2574	4175		2078
6	GH	104	52	292	742	1322	2948	1930
8	PL	151	40	268	612	1567	2511	908
Polycystic ovaries								
20	GH	166	434	2793	3816			3398
21	GH	160	201					8110
23	GH	288	60	3042	3791			3904
24	PL	105	68	1090	1813	4255		2615
25	PL	103	69	2130				4125
26	GH	103	104	770	1092	2355		1582
27	GH	167	67	2766	7173			6560
28	PL	121	58	1020	2718			1396
29	PL	184	49	1166	2067			1100
31	GH	260	40	355	2466	7934		5103
32	GH	110	55	1682	2790			2995
33	PL	185	88	3473	8140			5375
34	PL	101	415	1406	3500	4838		2829
35	GH	91	115	1223	3158	4755		-
36	GH	262	437	3088	3950	7930		4870
37	PL	123	41	2139	2834	4330		1196
38	PL	119	64	2595	1613			3226
39	GH	154	118	6875				7220

IGF-I concentrations (nmol/L) in serum and follicular fluid
- buserelin and hMG study

PtN	PL/GH	D-14	D1	D6-7	D8-9	D10-11	D12-14	Day OC	MFFL
Normal ovaries									
1	PL	26.8	23.0		25.1	22.8		23.8	20.9
2	GH	26.8	25.3	64.5	53.0	-	48.2	42.3	24.6
3	GH	33.6	33.9	57.9	44.4			34.1	29.4
4	PL	24.1	18.4	26.7	27.3			27.7	9.8
5	PL	44.1	42.7	49.6	44.2	37.7		40.2	25.2
6	GH	32.4	35.0	73.3	-	52.6	55.0	39.3	30.9
8	PL	26.3	28.8	27.0	30.2	-	29.3	28.8	-
Polycystic ovaries									
20	GH	19.8	19.0	40.2	34.8			19.7	15.7
21	GH	28.5	27.5					34.8	25.8
23	GH	24.7	35.6	42.5	37.5			22.9	23.0
24	PL	36.2	30.0	29.5	-	23.0		22.9	20.2
25	PL	31.0	32.4	29.5				28.9	20.3
26	GH	16.0	31.5	57.9	-	59.2		36.6	32.7
27	GH	25.7	20.4	45.0	24.5			26.1	18.3
28	PL	15.1	13.7	16.0	17.6			16.0	14.3
29	PL		28.0	21.3	25.2			24.2	19.8
31	GH	30.3	27.9	44.6	59.1	38.0		32.4	30.4
32	GH	29.4	22.3	35.6	23.7			20.6	16.6
33	PL	28.2	24.6	21.0	22.1			16.6	16.6
34	PL	25.4	29.3	21.6	-	23.6		21.3	8.0
35	GH	27.0	22.9	35.5	-	26.4		18.0	17.9
36	GH	56.9	58.5	57.6	-	45.3		29.1	37.6
37	PL	24.6	26.3	28.7	-	26.2		30.3	-
38	PL	30.3	22.9	34.1	34.0			34.3	23.1
39	GH	21.7	22.7	43.9				20.7	20.6

11ß-OH-A_4 study 1. Steroid results in serum and follicular fluid (nmol/L).

Serum

11ß-OH-A_4	A_4	Testo	Cortisol
1.8	12.4	2.8	637
3.2	10.5	5.8	929
4.7	11.3	4.9	1294
1.5	4.3	4.5	410
0.3	5.2	3.7	973
4.6	5.7	3.5	971
2.6	14.9	1.8	1276
5.9	14.1	9.6	1186
4.1	3.1	7.1	557
4.2	15.8	8.2	1231
5.6	3.5	9.3	1230
2.7	13.6	3.0	1054
4.5	12.7	2.6	819
4.1	6.2	11.8	838
3.2	5.2	1.8	898
4.2	10.2	3.4	253
0.7	7.7	3.3	399
2.8	4.5	0.3	469
1.7	8.0	6.2	544
3.0	5.2	2.6	727
5.6	3.1	1.9	1580
2.2	13.5	2.0	120
2.2	3.1	3.2	400
1.5	10.3		
1.7	4.5		
7.2	6.1		
2.2	18.0		
	4.2		

Follicular fluid

11ß-OH-A_4	A_4	Testo	Cortisol
6.5	19	29	270
3.0	88	26	270
16.8	49	44	701
26.2	10	63	189
15.9	435	28	410
16.9	433	19	1202
12.0	321	19	1049
13.5	610	12	1222
18.0	882	72	210
11.2	790	74	4140
17.9	434	12	270
16.1	295	18	1400
3.9	227	73	467
12.8	3525	16	620
7.7	3021	57	347
23.0	1432	70	639
6.5	373	18	41
11.3	191	29	590
10.8	72	5	239
7.0	385	10	693
19.7	12	11	402
31.0	102	15	2653
5.4	19	33	4075
8.0	1080		
6.2	279		
5.8	127		
7.4	86		
	619		

11ß-OH-A_4 concentrations in serum (Groups 1-4) and follicular fluid (Groups 5-7)(nmol/L)

Serum - no R_x			Serum - IVF		Follicular fluid - IVF		FFL - no R_x
Normal ovaries	Polycystic ovaries	cont.	Normal ovaries	Polycystic ovaries	Normal ovaries	Polycystic ovaries	Normal ovaries
1.0	7.3	4.8	2.4	4.8	60.0	6.5	13.8
2.8	5.2	2.0	3.7	8.7	29.0	3.0	9.3
1.0	7.8	3.1	4.4	4.0	12.1	16.8	7.5
2.1	3.6	4.3	5.0	1.7	73.7	26.2	7.2
5.4	4.0	4.4	3.2	1.4	8.8	15.9	51.0
2.9	6.3	9.6	4.1	4.6	5.9	16.9	10.2
2.2	6.0	3.8	2.1	3.1	29.5	12.0	72.0
4.8	2.2	5.2	3.3	1.5	19.0	13.5	54.0
9.0	3.0	7.7	1.2	3.5	23.3	18.0	22.8
7.7	4.3	4.0	1.7	2.6	14.1	11.2	26.1
2.6	2.6	1.0	2.2	0.3	8.7	17.9	13.2
2.3	4.0	7.3	0.8	4.7	43.8	16.1	18.3
2.6	4.3	4.4	2.2	4.8	8.8	3.9	14.7
	5.1	4.1	3.0	1.8	24.0	12.8	
	3.1	8.5	0.7	3.3	80.0	7.7	
	3.8	4.1	4.2	4.5		23.0	
	6.8	6.5	2.8	2.7		6.5	
	3.1	4.4	2.2	5.6		11.3	
	3.6	6.1	1.5	4.1		10.8	
	2.9	4.1	1.7	4.2		7.0	
	2.7	2.4		5.9		19.7	
	4.8	3.3		4.7		31.0	
	8.3	3.6				5.4	
	5.9	5.6				8.0	
	6.9	6.3				6.2	
	14.4	5.8				5.8	
	5.1					7.4	

A_4 concentrations in serum (Groups 1-4) and follicular fluid (Groups 5-7)(nmol/L)

Serum - no R_x			Serum - IVF		Follicular fluid - IVF		FFL - no R_x
Normal ovaries	Polycystic ovaries	cont.	Normal ovaries	Polycystic ovaries	Normal ovaries	Polycystic ovaries	Normal ovaries
4.9	7.3	4.6	13.5	6.2	373	19	443
2.7	3.4	4.6	10.3	15.8	191	88	188
3.6	7.5	2.6	4.2	12.7	72	49	35
3.3	4.3	5.5	4.5	10.2	385	10	158
5.3	4.5	6.9	3.1	14.1	12	435	230
8.2	6.5	6.7	3.1	5.2	102	433	413
3.9	3.4	8.8	7.4	13.6	19	321	899
1.4	4.4	4.9	16.6	7.7	1080	610	535
5.2	4.1	7.5	18.0	3.5	279	882	320
3.7	3.3	16.0	6.4	3.1	127	790	505
1.6	6.5	11.0	6.1	12.4	86	434	283
5.8	5.3	4.7	5.7	10.5	619	995	325
	10.0	3.0	3.6	11.3	274	227	398
	4.7	6.2	8.0	4.3	754	3527	
	8.6	4.3	4.5	5.2		3021	
	11.0	4.4	5.2	8.5		1432	
	4.3	4.6		14.9			
	7.7	5.5					
	2.6	9.0					
	5.1						
	7.2						
	4.3						

Testosterone concentrations in serum (Groups 1-4) and follicular fluid (Groups 5-7)(nmol/L)

Serum - no R_x			Serum - IVF		Follicular fluid - IVF		FFL - no R_x
Normal ovaries	Polycystic ovaries	cont.	Normal ovaries	Polycystic ovaries	Normal ovaries	Polycystic ovaries	Normal ovaries
1.5	2.1	1.1	9.0	2.8	72	29	7
0.9	1.1	3.1	3.2	5.8	16	26	5
1.3	2.1	1.7	2.8	4.9	57	44	4
1.4	1.9	2.3	7.1	4.5	72	63	4
1.9	2.9	2.3	4.5	3.7	18	28	6
2.3	2.9	1.4	2.3	3.5	29	19	7
1.5	3.1	2.1	2.0	3.0	5	19	44
1.8	1.7	3.3	2.6	6.8	10	12	38
2.4	2.1	2.4	1.8	4.0	11	72	7
1.6	1.4	4.2	3.8	8.2	15	74	11
2.1	1.7	2.3	4.2	9.3	52	12	8
1.4	1.4	1.8	7.8	6.4	32	18	10
2.1	3.5	3.8	2.7	2.6	33	43	10
	2.0	3.1	2.9	11.8	53	39	
	2.1	1.5	3.0	1.8	11	11	
	3.8	1.5	3.0	3.4	13	15	
	2.2	2.9	3.2	3.3	63	14	
	2.6	2.3	0.3	14.8	52	17	
	4.3	1.7		6.2	17	30	
	2.2	2.1		4.5	21	30	
	2.8			1.9	6	15	
	3.8			2.0	72	19	
	2.1				73	20	
	2.0				9	28	

Cortisol concentrations in serum (Groups 1-4) and follicular fluid (Groups 5-7)(nmol/L)

Serum - no R_x			Serum - IVF		Follicular fluid - IVF	
Normal ovaries	Polycystic ovaries	cont.	Normal ovaries	Polycystic ovaries	Normal ovaries	Polycystic ovaries
362	328	334	727	679	467	270
241	391	481	1367	981	624	270
434	642	250	544	912	347	701
332	304	244	365	1944	639	914
372	281	164	819	536	141	189
310	238	238	339	1165	590	410
374	200	173	247	1190	239	1202
165	172	268	469	929	693	1049
300	206	296	537	1275	402	1222
	164		385	922	2653	217
	362		374	891	4080	4140
	402		597	929	246	270
	161		253	470	859	1405
	344		898	1230	3572	
	257		399	557	1090	
	321		973	929	752	
	401		410	1276	423	
	246		637	971		
	131			1294		
	177			1251		
	243			1186		