Alternative approaches to ovarian stimulation for in vitro fertilization

Diederick de Jong

Proefschrift

Alternative Approaches to Ovarian Stimulation for In Vitro Fertilization

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Alternatieve methoden van ovariële hyperstimulatie voor in vitro fertilisatie

PROEFSCHRIFT

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'Life is what happens to you
while you`re busy making
other plans`

-Beautiful Boy-
1980 John Lennon

Voor Yannick

List of abbreviations

ACTH	adrenocorticotropic hormone
AD	androstenedione
ANOVA	analysis of variance
AST	all-subjects-treated
AUC	area under the curve
BMI	body mass index (weight/height^2)
CD	cycle day
CRF	corticotropin releasing factor
E_2	17b-estradiol
ET	embryo transfer
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HMG	human menopausal gonadotropin
HPG	human pituitary gonadotropin
ICSI	intracytoplasmic sperm injection
IGF	insulin-like growth factor
im	intramuscular
ITT	intent-to-treat
IU	international units
iv	intravenous
IVF	in vitro fertilization
IVRS	interactive voice response system
L	liter
LH	luteinizing hormone
mg	milligram
mm	millimeter
mL	milliliter
ng	nanogram
OPU	ovum pick-up
OHSS	ovarian hyperstimulation syndrome
P	progesterone
PMSG	pregnant mare serum gonadotropin
rFSH	recombinant follicle stimulating hormone
recFSH	recombinant follicle stimulating hormone
sc	subcutanous
TRF	thyroid stimulating hormone releasing factor
TVS	transvaginal sonography
VEGF	vascular endothelial growth factor
2PN	two-pronuclear

Contents

Chapter 1

General Introduction

1.1 In Vitro Fertilization

1.1.1 Historical aspects of in vitro fertilization

In vitro fertilization (IVF), literally meaning 'fertilization in glass', is characterized by co-culture of aspired oocytes with spermatozoa and subsequent transfer of the embryo(s) into the uterine cavity. The original indication for this treatment was subfertility resulting from impaired function of the fallopian tubes (*Steptoe* 1975). Since then, indications for the usage of IVF have increased considerably (*Diedrich et al.* 1992; *Macklon et al. 1991*).

The first efforts in the field of IVF in human were made in the 1940's. By incubating surgically harvested oocytes with spermatozoa *in vitro*, two-cell pre-embryos were observed (*Rock & Menkin* 1944). These embryos were not transferred into the uterus. A major breakthrough was achieved in 1959 by the fertilization of rabbit eggs *in vitro* and subsequently transferring the four-cell embryos into the fallopian tubes of recipient rabbits, followed by the delivery of healthy rabbits (*Chang* 1959). This was the first proof that the concept of fertilizing mammalian eggs *in vitro* could produce normal offspring.

The first attemps in IVF were characterized by the use of polyovulating agents and aspiration of human oocytes by laparoscopy resulting in an ectopic pregnancy (*Steptoe & Edwards* 1976). This technique failed to result in a life-born baby and was temporary abandoned. Edwards and Steptoe resorted to retrieving the single egg characteristics of the normal menstrual cycle. As a consequence, the best result was the recovery of solely one oocyte. On the 25th of July 1978 the first baby arising from IVF, Louise Brown, was born in the U.K. (*Steptoe & Edwards* 1978). Unfortunately, by aspirating the oocyte in the natural cycle only a few pregnancies were reported. Subsequently, the use of human menopausal gonadotropin (HMG) to stimulate multiple follicular development was re-introduced with more success (*Garcia et al.* 1983a; *Garcia et al.* 1983b).

1.1.2 Ovarian stimulation for IVF

Gonadotropins have been used in the treatment of anovulatory patients for many years. The first preparations were extracted from the serum of pregnant mares, the so-called pregnant mare serum gonadotropin (PMSG) (*Cole & Hart* 1930). The addition of human chorionic gonadotropin (hCG) increased the chance of success in terms of the occurrence of ovulation (*Hamblen & Davies* 1945). Due to the formation of antibodies against PMSG (*Leathum & Rakoff* 1948) their

clinical use in humans was abandoned. An alternative was found in extracted human pituitary gonadotropin (HPG). By using this preparation, treated patients were able to conceive while the formation of antibodies was avoided (*Gemzell* 1966). The application of HPG was abandoned after concerns were expressed that HPG may lead to transmission of prion diseases such as Jacob-Kreutzfeld disease (*Frasier & Foley* 1994). From the urine of menopausal women it became possible to extract HMG (*Albrecht* 1956). The estimated risk of Kreuzfeld Jacob disease after clinical application of HMG was considered to be minimal. The application of purified follicle stimulating hormone (FSH), lacking the luteinizing hormone (LH) activity of HMG, in ovarian hyperstimulation for IVF was introduced (*Acosta et al.* 1985). FSH alone, in the absence of LH, was capable in maintaining estradiol (E_2) synthesis and allowed the oocytes to mature up to the final stages (*Jones et al.* 1985).

When HMG became commercially available, gonadotropins were also applied for initiation of multiple follicular development for IVF (*Mettler et al.* 1982; *Garcia et al.* 1983a; *Garcia et al.* 1983b). Timing of induction final stages of oocyte maturation by human chorionic gonadotropin (hCG) was crucial for obtaining good quality oocytes (*Laufer et al.* 1984), requiring intensive ultrasound and hormonal monitoring (*DeCherney & Laufer* 1984; *Trounson & Calabrese* 1984). Despite intensive and frequent monitoring by both ultrasound and hormonal measurements, many IVF cycles had to be cancelled due to premature luteinization or ovulation (*Hamori et al.* 1987). In order to improve the efficacy of IVF treatment, the avoidance of a premature LH rise to occur was needed.

Indications that ovarian stimulation by clomiphene citrate lead less frequently to a premature ovulation compared to gonadotropins (*Wu* 1977; *Wu* 1978; *Testart & Frydman* 1982) encouraged the use of clomiphene citrate in IVF to aim for the recovery of more oocytes, resulting in a higher success rate (*Trounson et al.* 1981). Clomiphene citrate (*Greenblatt et al.* 1961) is an anti-estrogen interfering with the negative feedback mechanism at hypothalamic and pituitary level (*Odell & Swerdloff* 1975). After interruption of the negative feedback loop, an endogenous rise in FSH occurs (*Adashi* 1984). This endogenous rise in FSH may initiate the development of multiple dominant follicles and subsequent multiple pregnancies (*Goldfarb & Crawford* 1969). For induction of final oocyte maturation and timing of oocyte aspiration (*Hoult et al.* 1981) hCG was administered. In an effort to enhance the number of oocytes obtained after oocyte aspiration, some centres introduced clomiphene citrate in combination with human menopausal gonadotropin (HMG) (*Quigley et al.* 1984). However, no considerable improvement in number and quality of recovered oocytes

was obtained by means of this strategy (*Testart et al.* 1985; *Plachot et al.* 1985) and the risk of a premature ovulation remained.

It became clear early in the development of IVF that ovarian stimulation to initiate multiple dominant follicular development has improved the clinical outcome of assisted reproductive techniques, especially the results of IVF (*Quigley et al.* 1982; *MacDougall et al.* 1994). Although in the early days of IVF both gonadotropins and anti-estrogens were used, gonadotropins gained popularity in the following years (*Dor et al.* 1992). The rapid increase in serum estradiol levels (as a result of multiple dominant follicular development) frequently induces a premature rise in endogenous LH (*Yen* 1977; *Liu & Yen* 1983; *Vargyas & Marrs* 1987). Despite monitoring the cycle by the use of ultrasound and hormonal assessments, the risk of premature luteinization remained (*Buttery et al.* 1983). When a premature LH rise occurs, between 7% - 24% of the IVF cycles are cancelled prior to oocyte retrieval (*Jansen & Tucker 1999*; *Cummins et al. 1989*; *Smitz et al. 1988a*; *Hamori et al. 1987*). However, the development of gonadotropin releasing-hormone (GnRH) analogs contributed to the prevention of a premature LH surge (*Fraser & Baird* 1987; *Frydman et al.* 1988a; *Frydman et al.* 1988b).

1.2 Prevention of a premature LH surge

1.2.1 Native GnRH

Andrew Schally (figure 1.1) began working at the Allan Memorial Institute of Psychiatry's laboratory of experimental therapeutics with adrenocorticotropic hormone (ACTH) and adrenal cortical steroids in 1954. His choice of the hypothalamic endocrine field was most profoundly influenced by the work of one of his mentors, Geoffrey Harris, who first formulated the basic theories pertaining to hypothalamic control of secretion of the anterior pituitary gland. Schally's decades of research in this field were structured upon these theories. In 1955, Schally demonstrated the existence of corticotropin releasing factor (CRF) in hypothalamic and neurohypophysial tissue; this was the first experimental evidence of pituitary function being regulated by hypothalamic hormones (*Schally* 1978).

Figure 1.1
Andrew Schally

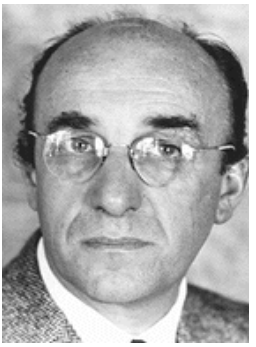

Figure 1.2
Roger Guillemin

Andrew Schally and Roger Guillemin (figure 1.2) began working with great enthusiasm at Baylor on the elucidation of the structure of CRF. However, their early findings were discouraging and frustrating because of problems with the isolation of CRF. Their failure to obtain enough CRF to determine its structure tended to cast doubt on the initial findings. Eventually, Schally and Guillemin ended their collaboration and started working separately on thyroid stimulating hormone releasing factor (TRF) and GnRH. They both succeeded simultaneously in isolation and identification of first TSH (*Guillemin et al.* 1965; *Schally et al.* 1966) followed by GnRH (*Amoss et al.* 1971; *Schally et al.* 1971) for which they shared a Nobel prize for physiology and medicine in 1977 with Rosalyn Yalow (for her work in radioimmunoassay).

GnRH is a small 10 amino acid large peptide (Table 1.1) which is intermittently secreted by the hypothalamus, inducing pulsatile secretion of FSH and LH from the anterior pituitary gland (Figure 1.3). The amino-acids at position number 6 are involved in enzymatic splicing, those at position number 2 and 3 are involved in gonadotropin release, while residues 1, 6, and 10 are important for the three-dimensional structure and receptor binding (*Clayton & Catt* 1980)

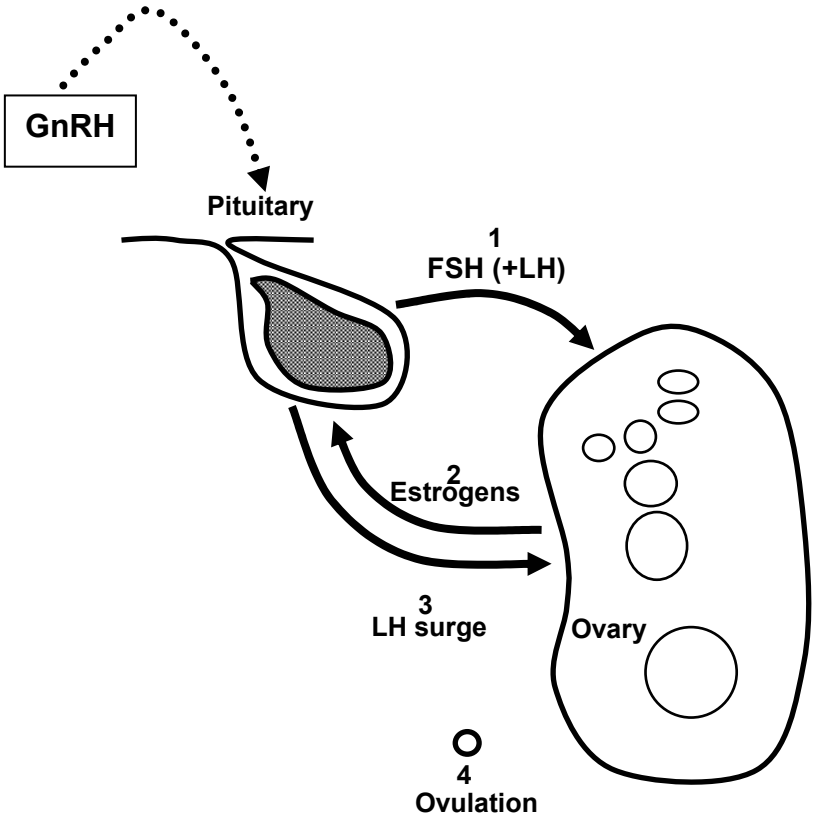

Figure 1.3
Scheme of pituitary-gonadal axis. In response to hypothalamic pulsatile gonadotropin releasing-hormone (GnRH) secretion, the pituitary releases follicle stimulating hormone (FSH) which stimulates growth of dominant follicles in the ovary (1). Growing follicles produce estrogens, which inhibit FSH release from the pituitary (2). By means of an as yet unclarified mechanism, when a Graafian follicle is present, the pituitary responds to further rising estrogen levels by releasing an luteinizing hormone (LH) surge (3) causing ovulation (4).

Position		1	2	3	4	5	6	7	8	9	10
Native GnRH		pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH2
GnRH agonists	buserelin	pGlu	His	Trp	Ser	Tyr	D-Ser	Leu	Arg	Pro	Ethylamid
	goserelin	pGlu	His	Trp	Ser	Tyr	D-Ser	Leu	Arg	Pro	AzGly
	leuprolin	pGlu	His	Trp	Ser	Tyr	D-Leu	Leu	Arg	Pro	Ethylamid
	triptorelin	pGlu	His	Trp	Ser	Tyr	D-Trp	Leu	Arg	Pro	Gly-NH2
	nafarelin	pGlu	His	Trp	Ser	Tyr	D-Nal	Leu	Arg	Pro	Gly-NH2
GnRH antagonists	Nal-Glu	D-Nal	D-Phe	D-Pal	Ser	Arg	D-Glu	Leu	Arg	Pro	D-Ala
	cetrorelix	D-Nal	D-Phe(4Cl)	D-Pal	Ser	Tyr	D-Cit	Leu	Arg	Pro	D-Ala
	ganirelix	D-Nal	DpClPhe	D-Pal	Ser	Tyr	D-hArg(Et2)	Leu	L-hArg(Et2)	Pro	D-Ala
	antide	D-Nal	D-Phe(4Cl)	D-Pal	Ser	Nic-Arg	D-Nic-Lys	Leu	Ip(Lys)	Pro	D-Ala
	antarelix	D-Nal	D-Cpa	D-Pal	Ser	Tyr	D-Hci	Leu	Ip(Lys)	Pro	D-Ala
	azaline-B	D-Nal	D-Cpa	D-Pal	Ser	Tyr	D-Aph(atz)	Leu	Ip(Lys)	Pro	D-Ala

Table 1.1
Amino-acid sequence of human gonadotropin-releasing hormone (GnRH) elucidated in 1971 by Schally and Guillemin. GnRH agonists and GnRH antagonists have replacements of the amino-acid sequence on various positions.

1.2.2 GnRH agonists

Soon after identification of the GnRH sequence, GnRH agonists were developed. An important feature leading to this development was the observation that a continuous administration of GnRH caused a decreased pituitary secretion of LH and FSH. By substituting amino acids on residue 6 and 10 of the GnRH sequence potent GnRH agonists were created (*Coy et al.* 1975) (Table 1.1). Administration of these compounds leads to a constant stimulation of the pituitary GnRH receptor system due to an extended half-life and an enhanced binding affinity for the GnRH receptor compared to native GnRH.

The mechanism of action of GnRH agonists is paradoxical. After administration of GnRH agonists an initial bolus release of gonadotropins is observed, the so-called 'flare-up' phase. Within 12 hours a 10, 5, and 4 times higher plasma concentration is observed for LH, FSH, and E_2, respectively (*Lemay et al.* 1984). Subsequently, pituitary secretion of LH and is suppressed by the formation of pituitary receptor/GnRH agonist complexes and internalization (*Hazum et al.* 1985; *Suarez-Quian et al.* 1986), the so called phase of 'down-regulation'. Approximately 90 days after cessation of GnRH agonist

treatment full pituitary recovery and physiological pituitary gonadotropin secretion is observed (*de Ziegler et al.* 1989).

1.2.3 GnRH agonists in IVF

After reports of births following ovarian stimulation and pituitary 'down-regulation' for IVF (*Shaw et al.* 1985), various IVF protocols were developed (Figure 1.4). The 'short protocol' is characterized by administration of gonadotropins immediately after initiation of GnRH agonist treatment in the early follicular phase, utilizing the initial 'flare-up' of endogenous gonadotropins (*Howles et al.* 1987).

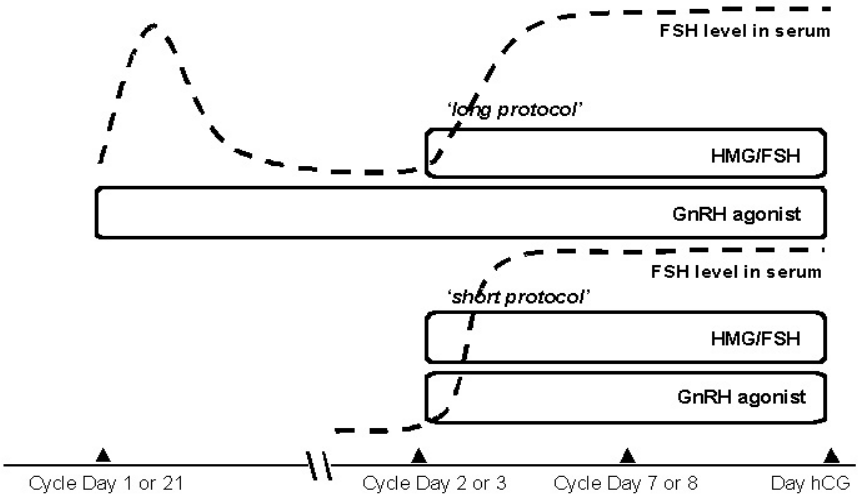

Figure 1.4
Scheme of ovarian stimulation for IVF and prevention of a premature LH rise with a gonadotropin-releasing hormone (GnRH) agonist, 'long protocol' (top panel) and 'short protocol' (bottom panel). In the 'long protocol', pituitary 'down-regulation' is initiated on cycle day 1 or on cycle day 21 of the previous cycle for at least 2 weeks (initial 'flare-up' phase is detectable), followed by co-treatment with human menopausal gonadotropin (HMG), follicle stimulating hormone (FSH), or recombinant FSH for ovarian hyperstimulation. In the 'short protocol' ovarian hyperstimulation is initiated on cycle day 2 or 3 with HMG, FSH, or recombinant FSH, together with daily co-treatment with a GnRH agonist to avoid a premature LH rise. Duration of the treatment period is at least two weeks longer in the 'long protocol' in comparison to the 'short protocol'.

The 'long protocol' is characterized by pituitary down regulation prior to ovarian stimulation by gonadotropins (*de Ziegler et al.* 1987; *Schmutzler et al.* 1988). The 'short protocol' may be favored in terms of patient convenience and costs (*Frydman et al.* 1988a). However, in terms of obtaining large quantities of both oocytes and embryos, pregnancy rate, and less cancellations the 'long protocol' may be superior compared to the 'short protocol' (*Hughes et al.* 1992).

1.2.4 Drawbacks arising from the use of GnRH agonists for VF

At present, the most commonly practised ovarian stimulation regimen in IVF includes pituitary down regulation with a GnRH agonist for at least two weeks followed by the co-administration of high dose exogenous gonadotropins. This so called 'long protocol' aims to stimulate ongoing growth of several follicles in normo-ovulatory women. The presence of many pre-ovulatory follicles allows for lower efficacy at oocyte pick-up, fertilization in-vitro, embryo culture and implantation. This approach to ovarian hyperstimulation involves prolonged, complex, expensive treatment protocols and intensive monitoring, while the risk of complications remains. Short-term risks include OHSS (*Friedman et al.* 1984; *Golan et al.* 1988) and higher order multiple pregnancies arising from the transfer of multiple embryos (*Feldberg et al.* 1986; *Simpson et al.* 1986; *Chetkowski et al.* 1989). In an effort to reduce the risks on complications the need for frequent and intensive monitoring increased further (*Mendelson et al.* 1985). With regards to long term complications, epidemiological studies have yet to clarify a possible association between ovarian hyperstimulation and ovarian cancer (*Shoham* 1994; *Bristow & Karlan* 1996).

In ovarian stimulation cycles without the application of GnRH agonists the possible need for luteal phase support was abandoned after the observation that although the luteal phase may be shorter following oocyte aspiration after ovarian stimulation by HMG (*Gronow et al.* 1985), it was shown not to be clinically important in terms of pregnancy rates (*Leeton et al.* 1985). In cycles characterized by ovarian stimulation by gonadotropins and pituitary down regulation by GnRH agonists, luteal phase support seems mandatory (*Smitz et al.* 1988b; *Balasch et al.* 1991; *Smitz et al.* 1992; *Beckers et al.* 2000).

In general, large quantities of oocytes are obtained after profound ovarian stimulation for IVF. Cryopreservation of supranumerical embryos and transfer in subsequent (unstimulated) cycles is often considered to justify the stimulation of a large number of follicles. Certainly, repeated ovarian stimulation and painful oocyte

pick-up procedures may be prevented using cryopreserved embryos. The added value of cryopreservation programs remains debatable however (*Jones et al.* 1995; *Jones et al.* 1997a) and the resulting increase in specific pregnancy rates (i.e.: increased chance of an ongoing pregnancy from a transfer of cryopreserved embryos after a failed fresh transfer) may be substantially less than generally perceived. Additionally, cryopreservation of excess embryos gives rise to complex ethical, religious and legal considerations.

Concerns over the complications arising from current strategies for ovarian hyperstimulation for IVF have been increasingly expressed (*Edwards et al.* 1996; *Fauser et al.* 1999) and novel approaches to ovarian stimulation for IVF are now needed. The application of GnRH antagonist may contribute to safer, less expensive, and milder protocols for ovarian stimulation for IVF (*Bouchard & Fauser* 2000).

1.2.5 GnRH antagonists

The clinical development of GnRH antagonists after identification in 1971 of the human GnRH sequence was more complex compared to the development of GnRH agonists. The main reasons for this were problems with solubility and histamine release. GnRH antagonists bind specifically and competitively to receptors on the gonadotrophic cells. When amino-acid on position number 2 of the sequence is replaced or removed, this compound is able to bind on the GnRH receptor without activating the receptor. Soon after administration of GnRH antagonists, gonadotropin release is suppressed and this lasts as long as the GnRH antagonist treatment is continued. GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotropin release and a rapid recovery of normal secretion of endogenous LH and FSH (*Ditkoff et al.* 1991).

The first generation of these compounds are characterized by modifications on position number 1, 2 and 6 of the sequence of human GnRH. The potency in suppressing gonadotropin release however, remained relatively poor. By enhancing its potency the histamine release was also increased due to the existence of GnRH receptors on mast-cells (*Kiesel & Runnebaum* 1992). The other problem, the hydrophobic character of the N-terminus which leads to gel-formation after injection, had to be resolved.

The third generation of GnRH anatagonists are characterized by modifications on position number 1, 2, 3, 6, and 10 of the sequence of human GnRH (Table 1.1). The histamine releasing properties and solubility of these compounds are within acceptable limits. Two of these compounds, cetrorelix and ganirelix, are recently introduced and could be valuable tools in reproductive medicine. Antide is currently are

in phase II trial research, while antarelix and azaline-B are in pre-clinical stage research.

1.2.6 GnRH antagonists for IVF

After 2 decades of preclinical development of GnRH antagonists (*Loy* 1994; *Reissmann et al.* 1994; *Reissmann et al.* 1995) these compounds are now in clinical use. Although the purpose for which this drug was developed was originally a non-steroid contraceptive drug (*Kenigsberg & Hodgen* 1986) GnRH antagonists have potential benefit in assisted reproduction. The main objective of using GnRH antagonists in IVF is the avoidance of a premature LH rise. GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotropin release and a rapid recovery of normal secretion of endogenous LH and FSH (*Ditkoff et al.* 1991). Preliminary clinical trials in the field of assisted reproduction have already been initiated with the second generation GnRH antagonist Nal-Glu (*Frydman et al.* 1991). A small comparative study of Nal-Glu with leuprolide acetate for IVF in seven patients resulted in equal oocyte and embryo assessments for both treatment groups and pregnancies were reported in the Nal-Glu group (*Cassidenti et al.* 1991). The effectiveness of Nal-Glu for avoiding the premature LH surge was compared with leuprolide acetate for IVF in thirty patients. In this study the serum luteinizing hormone and estradiol levels in the late follicular phase were lower and more mature oocytes and embryos of better quality were obtained in the Nal-Glu group compared with the leuprolide acetate group (*Minaretzis et al.* 1995a). Due to the acute suppression of LH release by Nal-Glu timing of oocyte aspiration in natural cycle IVF seems to be possible and pregnancies were reported (*Paulson et al.* 1994).

While the main objective of using GnRH antagonists in IVF is the avoidance of a premature LH rise, they could have potential additional advantages over GnRH agonists since it lacks the undesirable ‘flare-up’ effect and the relatively long period necessary for the pituitary to obtain a status of ‘down-regulation' of GnRH agonists. The use of a smaller total amount of gonadotropins for IVF appears to be possible with GnRH antagonists in comparison to GnRH agonists (*de Jong et al.* 1999a). The mechanism of action of GnRH antagonists is characterized by rapid suppression of pituitary LH release. Therefore, administration could be restricted to the time when to expect a premature LH rise, i.e. the late follicular phase. Up to the mid-follicular phase the endogenous gonadotropin secretion is could be left undisturbed by using GnRH antagonists.

Cetrorelix [N-Ac-D-Nal(2)^{1}, D-Phe(4Cl)^{2}, D-Pal(3)^{3}, D-Cit^{6}, D-Ala^{10}]-GnRH is a stable solvable third generation GnRH antagonist with minimal histamine-releasing properties. Within several hours after administration of this drug a rapid reversible suppression of the pituitary-gonadal axis is obtained. Recent studies have shown that the GnRH antagonist cetrorelix, either at single or multiple doses, is effective in preventing a premature LH rise during ovarian stimulation for IVF (*Diedrich et al.* 1994; *Olivennes et al.* 1994; *Olivennes et al.* 1995). Pregnancies were reported in all trials. The minimal effective dose of cetrorelix was 0.25 mg (*Albano et al.* 1997) and 3 mg (*Olivennes et al.* 1998a) for the multiple and single dose respectively. Cetrorelix has no negative influence on the luteal phase (*Albano et al.* 1996; *Lin et al.* 1999). Without the provision of subsequent luteal support the luteal phase is comparable with the normal menstrual cycle (*de Jong et al.* 1999a), although a shortening of the luteal phase and an impairment of corpus luteum function have been reported (*Albano et al.* 1998).

Ganirelix [N-Ac-D-Nal(2)^{1}, DpClPhe^{2}, D-Pal(3)^{3}, D-hArg(Et$_2$)^{6}, L-hArg(Et$_2$)^{8}, D-Ala^{10}]-GnRH is a stable solvable potent third generation GnRH antagonist with minimal histamine-releasing properties (*Nestor et al.* 1992; *Nelson et al.* 1995). This compound is characterized by an immediate reversible suppression of pituitary gonadotropin release within several hours after administration (*Nelson et al.* 1995). A large randomized controlled trial has shown that the GnRH antagonist ganirelix is effective in preventing a premature LH rise during ovarian stimulation with recombinant FSH for IVF (*The ganirelix dose-finding study group* 1998). Pregnancies were reported (*Itskovitz-Eldor et al.* 1998; *The ganirelix dose-finding study group* 1998). A possible beneficial effect of a high dose ganirelix in the treatment of imminent ovarian hyperstimulation syndrome has been suggested (*de Jong et al.* 1998).

Antide [Ac-D-Nal^{1}, D-Phe(4Cl)^{2}, D-Pal^{3}, Nic-Lys^{5}, D-Nic-Lys^{6}, Ip-Lys^{8}, D-Ala^{10}]-GnRH is a very potent long-acting stable third generation GnRH antagonist. Treatment with antide produced profound long-term inhibition of tonic gonadotropin levels, yet hyper-responsiveness to exogenous GnRH administration was maintained (*Leal et al.* 1989). Anti-reproductive effects and induction of luteolysis have been reported in animal studies (*Habenicht et al.* 1990; *Gordon et al.* 1991; *Srivastava & Sridaran* 1991; *Srivastava et al.* 1994). The development of this drug is in phase II trial research.

Antarelix [Ac-D-Nal^{1}, D-Cpa^{2}, D-Pal^{3}, D-Hci^{6}, Ip-Lys^{8}, D-Ala^{10}]-GnRH is a potent soluble third generation GnRH antagonist (*Deghenghi et al.* 1993; *Sorensen et al.* 1996). This drug is in pre-clinical phase.

Azaline-B [Ac-D-Nal^{1}, D-Cpa^{2}, D-Pal^{3}, Aph^{5}(atz), D- Aph^{6}(atz), Ip-Lys^{8}, D-Ala^{10}]-GnRH is a potent soluble third generation GnRH

antagonist (*Campen et al.* 1995; *Rivier et al.* 1995). This drug is in pre-clinical stage.

1.2.7 A comparison of GnRH agonists and GnRH antagonists

Both GnRH agonists and GnRH antagonists are used to avoid a premature LH rise. Their mechanism of action and timing of application is totally different. The main differences are summarized in Table 1.2.

GnRH antagonists have a direct dose dependent action whereas GnRH agonists have an initial 'flare-up' phase after which the pituitary is 'down-regulated'. This affects the timing of ovarian hyperstimulation for IVF (Figure 1.5). An IVF regimen with a GnRH antagonist reduces the duration of treatment by two to three weeks, leading to an increase in patients convenience. Besides that, GnRH antagonist could have potential advantages over GnRH agonists since it lacks the undesirable 'flare-up' effect and the relatively long period necessary for the pituitary to obtain a status of 'down-regulation' of GnRH agonists.

GnRH agonists	GnRH antagonists
Avoidance of premature LH rise	Avoidance of premature LH rise
Increasing doctor's convenience	Increasing patient's convenience
IVF regimen is time consuming, complicated, and expensive	IVF regimen is less time consuming, less complicated, and likely less expensive
Large amounts of exogenous gonadotropins needed	Less exogenous gonadotropins needed
Slow recovery of pituitary function after cessation	Rapid recovery of pituitary function after cessation
Induction of an endogenous LH surge impossible	Induction of an endogenous LH surge possible

Table 1.2
Main differences between the mechanisms of action of GnRH agonists and GnRH antagonists when used to prevent a premature LH rise during ovarian hyperstimulation for IVF.

Since timing of treatment largely depends on the normal menstrual cycle of patients, there are implications for the doctor's convenience. An IVF treatment cycle with the GnRH antagonist can commence with an undisturbed menstrual cycle and recruitment of a normal cohort of follicles, allowing normal pituitary function in the early to mid-follicular

phase. On top of endogenous FSH release, exogenous FSH is administered. This could lead to a reduction in gonadotropin consumption of patients in comparison to the 'long protocol'. Following GnRH agonist co-treatment during the follicular phase, luteolysis is initiated earlier due to slow recovery of endogenous pituitary secretion of gonadotropins (*Smitz et al.* 1988b). Therefore, provision of luteal support is common practice following ovarian stimulation for IVF (*Soliman et al.* 1994).The pituitary has been shown to recover rapidly after cessation of GnRH antagonist treatment (*Ditkoff et al.* 1991), which may permit abandoning additional luteal support.

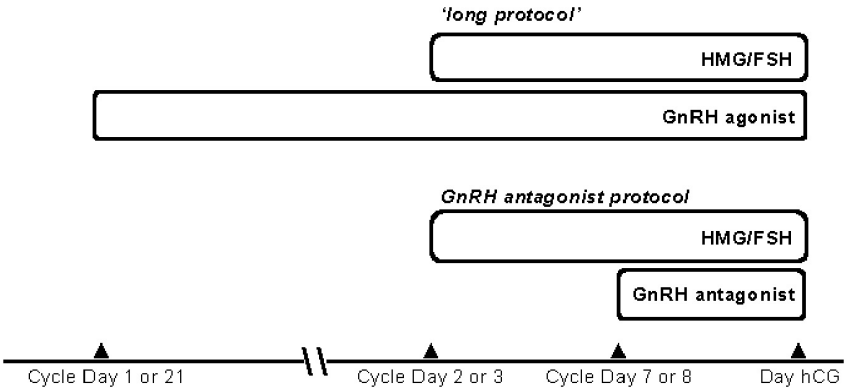

Figure 1.5
Scheme of ovarian stimulation for IVF and prevention of a premature LH rise with a gonadotropin-releasing hormone (GnRH) agonist ('long protocol') or a GnRH antagonist ('Lübeck protocol'). In the 'long protocol', pituitary 'down-regulation' is initiated on cycle day 1 or on cycle day 21 of the previous cycle for at least 2 weeks, followed by co-treatment with human menopausal gonadotropin (HMG), follicle stimulating hormone (FSH), or recombinant FSH for ovarian hyperstimulation. In the GnRH antagonist protocol, ovarian hyperstimulation is initiated on cycle day 2 or 3 with HMG, FSH, or recombinant FSH, followed by daily co-treatment with a GnRH antagonist to avoid a premature LH rise. Duration of the treatment period is at least two weeks longer in the 'long protocol' in comparison to the GnRH antagonist protocol.

1.2.8 New drugs and new paradigms for IVF treatment

Over the last decades the clinical outcome of IVF, in terms of achieving an ongoing pregnancy, has improved considerably by applying the profound ovarian stimulation by polyovulating agents and pituitary down regulation by a GnRH agonist. However, the dramatically increasing number of achieved (multiple) pregnancies as a result of applying this regimen should be considered as a drawback. A recent

study by the National Center for Health Statistics in the United States shows that in the year 1997, the number of triplets and higher order of multiple births has risen by more than 400% since 1980, mainly attributed to the use of those polyovulating agents (*National center for heath statistics* 2000). In recent years outcomes of IVF using the natural menstrual cycle have improved. Data from several series indicate cancellation rates of 10-30%, egg recovery rates of 75-90%, fertilization rates of 60-80%, implantation rates of between 20-30% and ongoing pregnancy rates per started cycle of 5-15% (*Garcia* 1989; *Lenton et al.* 1992; *Lenton & Woodward* 1993; *Seibel* 1994; *Society for assited reproductive technology (SART)* 1996). Analysis of the cost-effectiveness of natural cycle IVF shows that natural cycle IVF offers a low-cost alternative that may be more accessible to patients (*Daya et al.* 1995). However, intense monitoring still is required and this regimen could only be applied under specific conditions.

Minimal ovarian stimulation for IVF by extending the 'FSH window' (*Baird* 1987; *Fauser & Van Heusden* 1997; *Schipper et al.* 1998) may offer an alternative approach which deals with further simplification of IVF treatment and allows a reduction in complication rates and costs. Exogenous FSH may therefore be used during the mid- to late-follicular phase to ensure growth of follicles up to the preovulatory stage and a GnRH antagonist may be used to avoid a premature LH rise. By making optimal use of endogenous FSH, the amount of exogenous FSH required should be substantially reduced.

In addition, with the use of GnRH antagonists during the late follicular phase, final stages of oocyte meiotic maturation can be induced by cessation of the GnRH antagonist, recombinant LH (*The european recombinant human LH study group* 1998), native GnRH (*Gordon et al.* 1990), or GnRH agonist instead of hCG (*Olivennes et al.* 1996). Since these approaches may reduce the risk on OHSS, and therefore deserve attention.

In conclusion, the expectation of the introduction of GnRH antagonists for avoiding the premature LH rise in IVF may offer the already mentioned less expensive, less time-consuming, and more user-friendly treatments with possibly lower complication rates. But also, perhaps most importantly, the possibility of ‘softer’ IVF treatment strategies, high singleton pregnancy rates while avoiding multiple gestations and their concomitant problems. Studies are needed to establish the optimal starting day and dose of FSH administered, the options for induction of final stages of oocyte meiotic maturation, and the role of luteal support following GnRH antagonist administration.

1.3 Aim and outline of the thesis

This thesis compromises eight studies dealing with the application of GnRH antagonists in IVF. It is clear that GnRH antagonists in IVF may offer new opportunities for improving various regimens for ovarian stimulation for IVF. Questions with regard to complications of profound ovarian stimulation for IVF received special attention in this thesis.

The first objective was to establish the minimal effective dose of the GnRH antagonist ganirelix for the prevention of a premature LH surge during ovarian stimulation with recombinant FSH for IVF. A double blind randomized study (phase II trial) was carried out to address this issue. Secondly, the possible influence of different doses of the GnRH antagonist ganirelix on follicular development and endocrine parameters was determined.

After establishing the minimal effective dose of the GnRH antagonist ganirelix (0.25 mg/d) for the prevention of a premature LH surge during ovarian stimulation for IVF, a non-inferiority trial was conducted (phase III trial). The suggested GnRH antagonist IVF protocol, derived from the already mentioned phase II trial, was compared with the 'long protocol' as gold standard.

By means of a observational longitudinal study the additional benefit of the cryopreservation program in addition to the 'long protocol' is investigated. The results of this study added legitimacy to the design of a strategy of ovarian stimulation for IVF aiming at less follicle, oocytes, and embryos. To test this concept, a pilot study was conducted in which the 'FSH window' is extended by minimal intervention in the mid-follicular phase of the normal menstrual cycle. In addition, a GnRH antagonist is used for the prevention of a premature LH surge in the late follicular phase.

Minimal ovarian stimulation for IVF may reduce the chance for complications such as OHSS. Inducing an endogenous LH surge for final oocyte maturation by using a GnRH agonist is possible in case a GnRH antagonist for the prevention of a premature LH surge during ovarian stimulation for IVF is applied, in contrast to the the case with 'long protocol'. In addition, this approach may further reduce the chance on OHSS. The possibilty of conceiving after induction of final oocyte maturation is illustrated by a case report. Finally, by a randomized multi-center study, the endocrine physiology of the luteal phase after ovarian hyperstimulation for IVF and prevention of a premature LH surge by a GnRH antagonist, is explored comparing induction of the final stages of oocyte maturation with a GnRH agonist to hCG as gold standard.

Chapter 2

In vitro fertilization: GnRH antagonist for the prevention of a premature LH surge

2.1 Dose-finding study of the GnRH antagonist ganirelix

2.1.1 Introduction

Gonadotropin-releasing hormone (GnRH) analogues are used for a variety of disorders in which reversible suppression of the pituitary-gonadal axis is desired (*Conn & Crowley, Jr.* 1994). This can be achieved with GnRH agonists as well as with GnRH antagonists. GnRH agonists are currently applied for the treatment of endometriosis, uterine fibroids, precocious puberty, prostatic hyperplasia and prevention of endogenous luteinizing hormone (LH) surges during ovarian stimulation. With respect to the latter indication, various dosages and regimens are applied, but the minimal effective dose of GnRH agonists remains to be established.

Disadvantages of GnRH agonists during ovarian stimulation are the initial release of gonadotropins ('flare-up'), the rather long period until pituitary suppression becomes effective, and possibly the higher therapeutic dose of follicle stimulating hormone (FSH) required due to suppression of endogenous FSH release. In contrast to GnRH agonists, GnRH antagonists immediately suppress pituitary gonadotropins by GnRH receptor competition and permit flexibility in the degree of pituitary-gonadal suppression. Moreover, discontinuation of GnRH antagonist treatment leads to a rapid and predictable recovery of the pituitary-gonadal axis (*Gordon et al.* 1990; *Felberbaum et al.* 1995). In women undergoing ovarian stimulation, GnRH antagonist treatment is required for only a few days when a premature LH surge is imminent. For this purpose the applied dose of GnRH antagonist should prevent the occurrence of LH surges but also retain sufficient endogenous LH to support FSH-induced steroidogenesis. Since the inhibitory effect of GnRH antagonists on LH is more pronounced than on FSH, a low therapeutic dose also minimizes the suppression of endogenous FSH, if any.

The successful application of GnRH antagonists to prevent premature LH surges during ovarian stimulation for in-vitro fertilization (IVF) was first published by Cassidenti et al. (1991) and Frydman et al. (1991) using the GnRH antagonist Nal-Glu. Thereafter, studies on single, dual or daily administration of different doses of the GnRH antagonist cetrorelix in women undergoing ovarian stimulation with human menopausal gonadotropins (HMG) were reported (*Diedrich et al.* 1994; *Olivennes et al.* 1994; *Felberbaum et al.* 1996; *Albano et al.* 1997).

The current study was designed to select the minimal effective dose of the third-generation GnRH antagonist Org 37462 (ganirelix) to prevent premature LH surges during ovarian stimulation

when administered once daily by s.c. injection. Org 37462 was selected for this purpose since it induces rapid, profound and reversible suppression of the pituitary-gonadal axis, has a high aqueous solubility, and minimal histamine-releasing properties (*Rabinovici et al.* 1992; *Nelson et al.* 1995). In particular, the latter is an advantage over previous generations of GnRH antagonists which caused local cutaneous anaphylactoid-like reactions. The final dose selection was based on the incidence of LH surges $\geq$10 IU/L during Org 37462 treatment, the clinical outcome and the overall tolerance.

2.1.2 Materials and Methods

Patients

A total of 333 patients, for whom ovarian stimulation and IVF with or without intracytoplasmic sperm injection (ICSI) were indicated, were screened, randomized to one of the six treatment groups and started ovarian stimulation with recombinant FSH (recFSH). One patient who started recFSH discontinued after one injection; thus, 332 patients underwent Org 37462 treatment. In total, 13 IVF centres in nine different countries participated, the number of patients per centre ranging from 10 to 60. Main inclusion criteria were an age of at least 18 but not more than 39 years, a bodyweight of 50-75 kg and body mass index (BMI) of 18-29 kg/m^2, and regular menstrual cycle ranging from 24 to 35 days. Patients with either a history of or current type I hypersensitivity (urticaria, eczema, hay fever, asthma) or endocrine abnormality were excluded.

Study design

This study was a Phase II, multicentre, double-blind, randomized dose-finding study to assess the efficacy of the GnRH antagonist Org 37462 to prevent premature LH surges in women undergoing ovarian stimulation with recFSH. Org 37462 (NV Organon, Oss, The Netherlands) was tested as a solution for injection (0.5 ml) in six doses, i.e. 0.0625, 0.125, 0.25, 0.5, 1.0 and 2 mg, and was administered subcutaneously once daily. Ovarian stimulation was carried out with recFSH (Puregon®, NV Organon).

A schematic description of the applied treatment regimen is given in Figure 2.1. RecFSH treatment was started in patients on day 2 of the menstrual cycle by a once-daily s.c. injection. Just before the first injection of recFSH, spontaneous pregnancy was excluded. During the first five treatment days, the daily dose of recFSH was fixed at 150 IU. After 5 days of recFSH treatment, Org 7462 treatment was begun with daily s.c. administration, the recFSH dose being adjusted depending on the individual ovarian response as assessed

by daily ultrasound. Org 37462 treatment was continued up to and including the day of human chorionic gonadotropin (hCG) administration. hCG (10 000 IU; Pregnyl®, NV Organon) was administered when at least three follicles ≥17 mm diameter were observed, and 30-36 h thereafter oocyte retrieval was performed and follicle fluid of one of the three largest follicles punctured was stored. Oocyte retrieval was followed by IVF with or without ICSI, and no more than three embryos were to be replaced 2-4 days thereafter. Luteal phase support was given as per the clinics' routine practice and standard care and was started at latest on the day of embryo transfer.

Org 37462						✱	✱	✱	✱	✱
Rec FSH	•	•	•	•	•	o	o	o	o	o
Cycle day	2	3	4	5	6	7	8	9	10	**3 follicles ≥ 17 mm diameter 10000 IU hCG**

Figure 2.1
Schematic description of the treatment regimen using recombinant follicle stimulating hormone (recFSH) for ovarian stimulation and Org 37462 for the prevention of premature luteinizing hormone (LH) surges. hCG = human chorionic gonadotropin

Assessments
During the first 5 days of only recFSH treatment, blood samples for hormone analysis were collected once daily in the morning just before recFSH administration. During Org 37462 treatment, blood samples were withdrawn twice daily, viz. just before Org 37462 administration and about 8 h thereafter. Serum FSH, LH, estradiol and progesterone concentrations were measured by a central laboratory, using a fluoroimmunoassay (Delfia®, Wallac OY, Finland) and androstenedione by a coat-a-count direct radioimmunoassay (Diagnostics Products Corporation, Los Angeles, CA, USA).

Org 37462 was measured in all blood samples collected from the first day of Org 37462 treatment onwards, and in the follicle fluid of one large punctured follicle. The method of analysis was previously described (*Nerenberg et al.* 1993); the detection limit was 0.02 ng/mL.

During Org 37462 treatment, transvaginal ultrasonography was performed every day to measure the growth of individual follicles (≥11 mm).

Local tolerance after s.c. administration of Org 37462 was assessed at 1 and 24 h after administration. Local reactions were scored as either none, mild, moderate or severe for redness, swelling, bruising, pain and itching, respectively.

External independent advisory committee
An external independent advisory committee, consisting of one gynaecologist (IVF expert) and one statistician, was appointed at the start of the study in order to advise on stopping a treatment arm if LH surges ≥10 IU/L occurred during Org 37462 treatment.

During the study, several subjects had an LH surge, while a few with an initially normal ovarian response to recFSH were reported to have follicular growth arrest and falling serum estradiol concentrations after starting Org 37462 treatment. Based on these observations and a review of all other data available, the advisory committee indicated that the lowest and highest dose groups were to be stopped during the study.

Analysis and dose selection
Three patients were excluded from the efficacy analysis because of protocol violations related to drug compliance or inclusion/exclusion criteria. In addition, the efficacy data of seven patients were excluded from the moment that a protocol deviation occurred: five patients began recFSH treatment but were switched during Org 37462 treatment to HMG, one patient stopped Org 37462 treatment 1 day before stopping recFSH treatment, and one patient donated her oocytes.

Dose groups (0.0625 and 2 mg) which were stopped prematurely were considered non-eligible. Possibly eligible dose groups were evaluated for the incidence of LH surges, and a statistical subset selection procedure for the ranking and selection of these dose groups was applied to the number of follicles ≥11 and ≥15 mm on the day of the hCG injection, the number of cumulus-oocyte complexes retrieved, the number of good-quality embryos obtained, and the intrauterine vital pregnancy rate 5-6 weeks after embryo transfer.

For the response variables mentioned above, the dose groups were ordered in terms of the calculated treatment effect estimates. Subsequently, a statistical subset selection procedure was performed which selected the dose group with the best effect estimate as well as the doses which were within a certain 'distance' from this best dose group in terms of effect estimates based on an analysis of variance (ANOVA) model for continuous variables and a logistic regression model for binary variables (*Driessen* 1991; *Chang & Hsu* 1992). The procedure was defined such that with 95% confidence the selected subset of dose groups contained the true best dose group. This meant that a dose that was not selected was unlikely to be the 'truly best' dose for that specific variable.
Efficacy parameters were summarized using descriptive summary statistics (mean, SD, median minimum and maximum), unadjusted for centre. For the statistical subset selection procedure, efficacy

parameters were adjusted for centre, but only unadjusted means are presented. Summary statistics of the outcome of the efficacy parameters are presented for all patients who started Org 37462 treatment ('per attempt' basis), unless otherwise indicated.

2.1.3 Results

Patient characteristics
The six treatment groups were comparable in demographic and subfertility characteristics (data not shown). The overall (*n* = 329) mean age was 31.6 years and body mass index 22.7 kg/m^2. Causes of subfertility were 45.9% male factor, 26.4% tubal factor, 7.9% both male and tubal factor, and 19.8% other factors. Overall, the percentages of women with primary and secondary infertility were 58.4% and 41.6%, respectively.

Serum hormone concentrations before Org 37462 treatment
Median serum hormone concentrations measured just before the first recFSH administration (cycle day 2) and at treatment day 6 (cycle day 7) just before the start of Org 37462 are presented in Table 2.2.

A fixed daily dose of 150 IU recFSH for 5 days increased median serum FSH concentrations by 1.2 IU/L. Serum estradiol concentrations increased on average 10-fold, while median serum LH concentrations fell from 4.6 IU/L on the first day of stimulation to 1.8 IU/L on day 6 of stimulation. Circulating concentrations of androstenedione and progesterone remained unchanged during the first 5 days of ovarian stimulation.

Disposition and cancellations
The final number of patients per treatment group who were treated with Org 37462 and included in the efficacy analysis is presented in Table 2.1. Thirty-nine of the 329 patients who began Org 37462 treatment did not undergo embryo transfer because of either fertilization failure (*n* = 16), insufficient ovarian response (*n* = 11) or other reasons. In total, 12 women experienced an LH surge during Org 37462 treatment, but only one of these led to cancellation. In addition, four patients in the 2 mg group and one patient in the 1 mg group were switched from recFSH to HMG because of insufficient ovarian response. All five women finally had embryo transfer, and one became pregnant. The lowest and highest Org 37462 dose groups, were stopped during the study as advised by an External Independent Advisory Committee and therefore considered non-eligible.

	Daily dose of Org 37462 (mg)						Total
	0.0625	0.125	0.25	0.5	1.0	2.0	
recFSH + Org 37462	31	65	69	69	65	30	329
Embryo transfer	27	60	62	54	60	27	290
LH rise $\geq$ 10 IU/L	5	6	1	-	-	-	12
Switched to HMG	-	-	-	-	1	4	5

Table 2.1
Numbers of subjects per Org 37462 treatment group included in the efficacy analysis. Human menopausal gonadotropin (HMG), luteinizing hormone (LH), and recombinant follicle stimulating hormone (recFSH).

Serum hormone concentrations before Org 37462 treatment
Median serum hormone concentrations measured just before the first recFSH administration (cycle day 2) and at treatment day 6 (cycle day 7) just before the start of Org 37462 are presented in Table 2.2.

	FSH (IU/L)	LH (IU/L)	Androstenedione (ng/L)	Estradiol (pg/L)	Progesterone (ng/L)
Day 1 of recFSH	6.5 (4.0-10.0)	4.6 (2.2-7.7)	1.8 (1.0-3.2)	30.7 (15.1-57.6)	0.5 (0.29-1.3)
Day 6 of recFSH	7.7 (5.8-11.1)	1.8 (0.66-6.4)	2.0 (1.1-3.5)	301 (84.2-777)	0.4 (<0.25-0.85)

Table 2.2
Median and 5th and 95th percentiles (in parenthesis) of serum hormone concentrations at the start of recombinant follicle stimulating hormone (recFSH) treatment (stimulation day 1) and before the first Org 37462 injection (stimulation day 6). FSH = follicle stimulating hormone, LH = luteinizing hormone.

A fixed daily dose of 150 IU recFSH for 5 days increased median serum FSH concentrations by 1.2 IU/L. Serum estradiol concentrations increased on average 10-fold, while median serum LH concentrations fell from 4.6 IU/L on the first day of stimulation to 1.8 IU/L on day 6 of stimulation. Circulating concentrations of androstenedione and progesterone remained unchanged during the first 5 days of ovarian stimulation.

Serum Org 37462 concentrations

Figure 2.2 (upper panel) shows serum concentrations of Org 37462 measured in each dose group twice daily for all patients who received at least 5 days of Org 37462 treatment. Circulating Org 37462 increased in a linear, dose-proportional manner and steady state was reached within 2 days in all dose groups. At steady state (day 3), mean concentrations were 0.2, 0.3, 0.6, 1.1, 2.7 and 5.5 ng/mL in the morning (a.m.) prior to Org 37462 administration, and 0.7, 1.5, 3.0, 6.1, 11.4 and 23.0 ng/mL about 8 It after injection (p.m.) in the 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 mg dose groups, respectively. In follicular fluid collected at oocyte retrieval, Org 37462 concentrations were similar to those in the circulation (data not shown). On the day of embryo transfer, mean serum Org 37462 concentrations were undetectable (<0.02 ng/mL) in the three lowest dose groups, and very low in the 0.5, 1.0 and 2.0 mg dose groups (0.04, 0.07 and 0.17 ng/mL respectively).

LH rises before and during Org 37462 treatment

In total, six (1.8%) patients showed an LH rise (≥10 IU/L) before the first Org 37462 administration. Two of these six women were non-responders, as their serum estradiol concentration after 5 days of once-daily 150 IU recFSH remained unchanged from baseline. The other four were high-responders in whom an LH rise was seen on stimulation day 6, just before the first Org 37462 administration. In these four patients, the increases in serum estradiol during the first five stimulation days were relatively high, ranging from 700 to 1860 pg/mL on the first day of Org 37462 treatment.

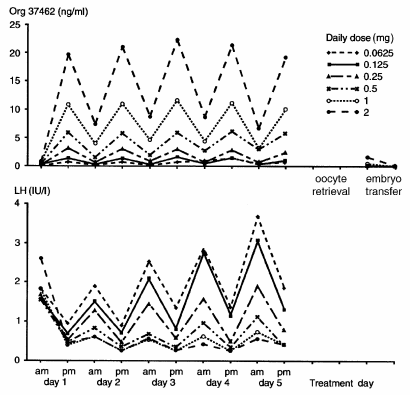

Figure 2.2
Serum Org 37462 concentrations (upper panel) and immunoreactive luteinizing hormone (LH) concentrations (lower panel) measured just before each Org 37462 injection in the morning (a.m.) and measured approximately 8 h later in the afternoon (p.m.) for patients with at least 5 days of Org 37462 treatment.

All subjects with an LH rise during Org 37462 treatment were evaluated for daily compliance of Org 37462 by monitoring their serum concentrations of the drug. Among these subjects (*n* = 15), three had a rise while serum Org 37462 concentrations had fallen

temporarily to baseline, showing that the Org 37462 had either been wrongly injected or not administered. A typical example of an LH surge occurring in the 0.25 mg group is shown in Figure 2.3. The remaining 12 patients with an LH rise showed daily drug compliance during Org 37462 treatment (Figure 2.4), the incidence of rises being 16. 1 % (*n* = 5), 9.2% (*n* = 6) and 1.4% (*n* = 1) in the 0.0625, 0. 125 and 0.25 mg groups respectively. In the 0.25 mg group, only one patient had an LH value ≥10 IU/L (10.6 IU/L) during Org 37462 treatment, indicating that a circulating concentration of 1-4 ng/mL Org 37462 is sufficient to prevent LH surges from occurring (see Figure 2.2).

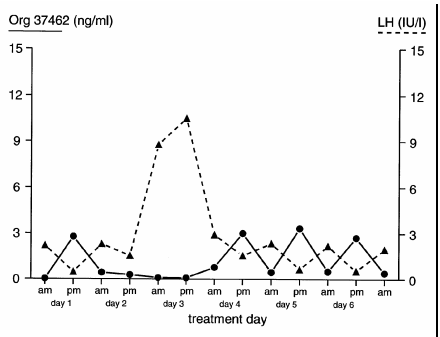

Figure 2.3
Typical example of a patient with an immediate luteinizing hormone (LH) rise due to non-compliance toward daily treatment with Org 37462.

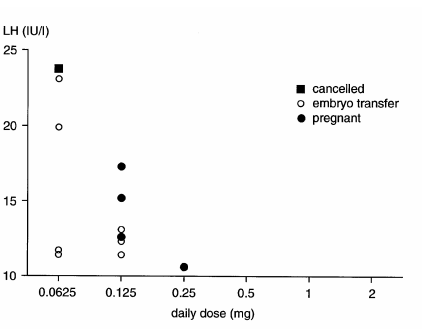

Figure 2.4
Scatter plot of individual luteinizing hormone (LH) values ≥ 10 IU/L measured during Org 37462 treatment.

Of the 12 patients with LH values ≥10 IU/L, seven also had rises of serum progesterone (≥1 ng/mL). One patient (0.0625 mg group) out of 12 patients with an LH rise was cancelled: this patient had serum LH values ≥10 IU/L prior and during treatment with 0.0625 mg Org 37462. The other 11 patients underwent embryo transfer, with four becoming pregnant, though three of these women showed a rise in serum progesterone (at least one value ≥1.0 ng/mL) before hCG administration.

Total dose of recFSH and duration of Org 37462 treatment
Little difference was noted between the dose groups with respect to the total amount of recFSH administered, the mean daily dose ranging from 181 IU (0.25 mg group) to 204 IU (1.0 mg group) and the median daily dose from 150 IU (0.0625 mg group) to 183 IU (1 mg group). The duration of Org 37462 treatment ranged between 2 days (minimum, all groups) and 12 days (maximum, 1 mg group) and 4. or 5 days (median values) in the six dose groups. Overall, the duration of

Org 37462 treatment was 5 days, while that of ovarian stimulation was 10 days.

Hormone values during Org 37462 treatment and on the day of hCG

Serum LH concentrations decreased with increasing Org 37462 concentrations in a dose-related manner (Figure 2.2, lower panel). Serum LH concentrations tended to increase during Org 37462 treatment, especially in the two lowest dose groups. In the two highest dose groups, serum LH concentrations were mostly ≤1 IU/L (for the 1 and 2 mg groups, 75% and 95% of all subjects, respectively), indicating profound pituitary suppression.

During ovarian stimulation from day 6 of recFSH treatment onwards, increases in serum estradiol concentrations fell in relation to the increasing doses of Org 37462. In addition, in the 2 mg dose group, a slight decline in serum estradiol was seen after the first Org 37462 administration (Figure 2.5).

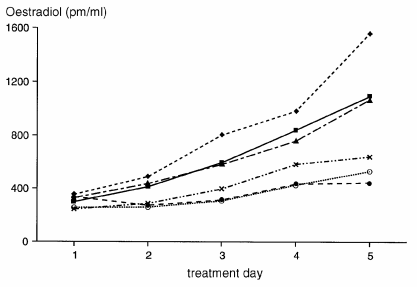

Figure 2.5
Serum estradiol concentrations measured once daily just before each Org 37462 injection for patients with at least 5 days of Org 37462 treatment. See Figure 2.2 for key.

Median values of hormone concentrations measured on the day of hCG administration are shown in Table 2.3. Serum FSH concentrations were similar among dose groups, and ranged from 8.8 to 10.2 IU/L Median serum LH concentrations fell with increasing doses of Org 37462, from 3.6 IU/L in the lowest dose group to 0.4 IU/L in the highest. In the 0.25 mg treatment group, the amounts of endogenous LH at the start of Org 37462 treatment (1.8 IU/L) and on the day of hCG administration (1.7 IU/L) were similar (Table 2.2). In parallel to endogenous LH, rises in serum estradiol also decreased largely with increasing Org 37462 doses. Serum estradiol concentrations were lower before the second Org 37462 administration than before the first in 23%, 47% and in 96% of women treated with 0.5, 1 and 2 mg Org 37462, respectively. Only in the three lowest dose groups did all patients have serum estradiol values >200 pg/mL on the day of hCG administration (data not shown). In addition, serum androstenedione concentrations also tended to decrease in a dose-related manner, whereas serum progesterone concentrations were similar among the different dose groups.

Number of follicles on the first day of Org 3 7462 treatment and on the day of hCG

On day 6 of recFSH stimulation, before the first Org 37462 administration, the overall number of follicles ≥11 and ≥15 mm was 4.1 and 0.5, respectively. On the day of hCG administration, the mean number of follicles ≥11 and ≥15 mm was similar between the treatment groups, and ranged from 9.4 to 11.4 and from 6.2 to 7.5, respectively. The statistical selection procedure selected the 0.125, 0.25 and 1 mg dose groups as eligible for both parameters. The mean number of follicles of ≥ 17 mm diameter before hCG administration was also similar between the dose groups, and ranged from 3.7 to 4.7. The 0.25 mg dose group had the largest number of follicles of ≥11 and ≥15 mm diameter.

	Daily dose of Org 37642 (mg)					
	0.0625	0.125	0.25	0.5	1.0	2.0
FSH (IU/L)	9.1 (6.9-25.8)	9.0 (6.2-20.8)	9.1 (6.4-19.2)	10.2 (6.0-20.6)	9.8 (6.2-25.4)	8.8 (6.8-26.3)
LH (IU/L)	3.6 (0.6-19.9)	2.5 (0.6-11.4)	1.7 (<0.25-6.4)	1.0 (0.4-4.7)	0.6 (<0.25-2.2)	0.4 (<0.25-0.8)
AD (ng/mL)	2.6 (1.3-5.4)	2.6 (1.1-4.4)	2.4 (1.3-4.4)	2.2 (1.3-3.5)	2.0 (1.1-3.6)	1.5 (1.1-2.5)
E_2 (pg/mL)	1475 (645-3720)	1130 (462-3780)	1160 (384-3910)	823 (279-2720)	703 (284-2340)	430 (166-884)
P (ng/mL)	0.8 (0.4-2.8)	0.6 (0.3-1.9)	0.7 (0.3-1.8)	0.6 (0.3-1.4)	0.6 (0.3-2.0)	0.5 (0.3-1.6)

Table 2.3
Median and 5th and 95th percentiles (in parentheses) of serum hormone concentrations on the day of human chorionic gonadotrpohin (hCG) just before the last Org 37462 administration. FSH = follicle stimulating hormone, LH = luteinizing hormone, AD = androstenedione, E_2 = estradiol, P = progesterone.

Treatment outcome

The clinical outcome for the main parameters is given in Table 2.4. The mean numbers of cumulus-oocyte complexes, embryos obtained and embryos transferred were similar between the different treatment groups, and the statistical subset procedure selected all four completed treatment groups as eligible.

The mean number of embryos transferred in the various dose groups ranged from 2.3 to 2.7. Although comparable numbers of embryos were replaced, the implantation rate (number of gestational sacs divided by the number of replaced embryos) was relatively low in the three highest dose groups, especially in the 2.0 mg group. In addition, the number of pregnancy losses during the first 6 weeks

after embryo transfer was relatively higher in the 1.0 and 2.0 mg treatment groups.

Overall, a total of 67 intrauterine pregnancies with heart activity (52 single and 15 multiple gestations) was established by ultrasound at 5-6 weeks after embryo transfer. The pregnancy rate was highest in the 0.25 mg group (36.8% per attempt and 40.3% per transfer, *n* = 25; see Table 2.4), but the dose selection procedure indicated both 0. 125 and 0.25 mg as eligible doses. In total, 13 miscarriages were reported, but in four of these only a positive hCG test was available to document the early pregnancy. Because of the lower implantation rates and higher miscarriage rates, the vital pregnancy rates were lowest in the three highest dose groups, especially the 2.0 mg treatment group. Serum estradiol concentrations on the day of hCG, or at the day of oocyte retrieval, of women who became pregnant were within the same range as those from women who did not become pregnant or who had an early miscarriage (Figure 2.6).

	Daily dose of Org 37462 (mg)					
	0.0625	0.125	0.25	0.5	1.0	2.0
Recovered cumulus-oocyte complexes	9.0 ± 5.7	9.5 ± 5.5	10.0 ± 5.4	8.8 ± 6.6	9.3 ± 6.0	8.6 ± 4.4
Embryos:						
Total	5.4 ± 3.6	5.9 ± 4.3	5.4 ± 4.4	4.6 ± 4.2	5.3 ± 3.9	4.9 ± 3.7
Good quality	3.8 ± 2.8	3.3 ± 2.6	3.3 ± 3.0	2.5 ± 2.7	3.3 ± 2.7	3.5 ± 3.7
Implantation rate (%)	14.2	16.6	21.9	9.0	8.8	1.5
Early miscarriage rate per embryo transfer (%)	0 (0/27)	3.3 (2/60)	1.6 (1/62)	3.7 (3/54)	8.5 (5/59)	13.0 (3/23)
Vital pregnancy rate:						
Per attempt (%)	23.3 (7/30)	26.2 (17/65)	36.8 (25/68)	11.6 (8/69)	14.1 (9/64)	3.8 (1/26)
Per embryo transfer (%)	25.9 (7/27)	28.3 (17/60)	40.3 (25/62)	14.8 (8/54)	15.3 (9/59)	4.3 (1/23)
Ongoing pregnancy rate:						
Per attempt (%)	23.3 (7/30)[a]	23.1 (15/65)	33.8 (23/68)	10.1 (7/69)	14.1 (9/64)	0 (0/26)
Per embryo transfer (%)	25.9 (7/27)[a]	25.0 (15/60)	37.1 (23/62)	13.0 (7/54)	15.3 (9/59)	0 (0/23)

Table 2.4
Clinical outcome in the six different dose groups for all patients who started Org 37462 treatment (per attempt) unless otherwise indicated. Values are mean 6 SD, unless otherwise indicated. [a]Including one subject who was lost to follow-up after assessment of a vital pregnancy.

Follow-up of the 67 women with a vital pregnancy revealed an additional six miscarriages up to 16 weeks after embryo transfer. In the 0.25 mg group, the ongoing pregnancy rate 12-16 weeks after embryo transfer was 33.8% per attempt and 37.1% per transfer. Interestingly, the group of patients with an ongoing pregnancy included one patient (0.5 mg dose group) with only 84 pg/mL estradiol on the day of hCG.

In total, three ectopic pregnancies occurred in the three lowest dose groups (one in each group) and in total, seven patients treated with 0. 125, 0.25, 0.5 or 1.0 mg Org 37462 per day were reported to have grade II (*n* = 5, including three pregnancies) or III (*n* = 2) ovarian hyperstimulation syndrome (OHSS).

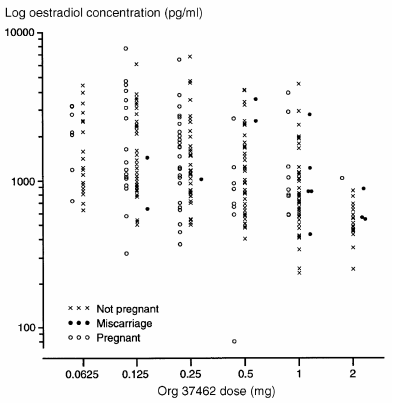

Figure 2.6
Scatter plot of individual serum estradiol concentrations on the day of human chorionic gonadotropin (hCG) of women who became pregnant and those who had a miscarriage or menses.

Safety and tolerance

In total, eight patients (2.4%) were hospitalized because of an ectopic pregnancy (*n* = 3), OHSS (*n* = 2), miscarriage (*n* = 1), fever (*n* = 1) or pelvic inflammation (*n* = 1). Adverse experiences indicated as possibly or probably drug-related were reported for 11 patients and included asthenia, nausea and malaise.

The local tolerance outcome indicated that daily, s.c. administered Org 37462 was well tolerated. The percentage of patients with at least one moderate or severe local tolerance reaction (skin redness, swelling, bruising, pain or itching) occurring I h after Org 37462 injection was 20.5% and 1.2%, respectively. At this time point, skin redness was most frequently observed and increased in a dose-dependent manner, from 3.2% in the 0.0625 mg group to 33% in the 2 mg group (overall 19.6%; 0.25 mg treatment group 17.1%). At 24 h after injection, bruising was the most commonly observed reaction (overall 2.4%). None of the patients had to discontinue Org 37462 treatment because of a hypersensitivity reaction or because of any drug-related adverse experience.

2.1.4 Discussion

This is the first randomized, dose-finding study establishing the minimal effective dose of a GnRH analogue in patients undergoing ovarian stimulation (*Itskovitz-Eldor et al.* 1998). In total, six doses of the GnRH antagonist Org 37462 were tested during ovarian stimulation with recFSH, lacking LH activity. The outcome of this study indicates that 0.25 mg Org 37462 per day was the minimal effective dose with regard to preventing LH surges, and resulted in a good clinical outcome.

Whereas the 0.25 mg Org 37462 per day was effective with respect to the prevention of LH surges, lower daily doses (viz. 0.0625 or 0.125 mg) resulted in LH rises ($\geq$10 IU/L) during Org 37462 treatment. In total, four of 11 women with LH rises after starting Org 37462 treatment became pregnant, indicating that the rises did not reduce the clinical pregnancy rates in the two lowest dose groups.

After daily administration of Org 37462, a steady-state level was reached within 2 days and, on the day of embryo transfer, circulating Org 37462 was virtually absent. Daily Org 37462 compliance appeared to be essential to prevent LH surges, as one forgotten or failed Org 37462 injection resulted in an immediate LH rise, supporting a rapid recovery of pituitary blockade and an unaffected synthesis and storage of endogenous LH (*Felberbaum et al.* 1995).

The median duration of Org 37462 treatment was 4-5 days, up to and including the day of hCG. During this treatment period, serum LH curves showed a transient increase, especially in the lower dose groups. This observation might indicate that, due to increases in serum estradiol concentrations in the late follicular phase, an increased endogenous GnRH secretion occurs (*Filicori et al.* 1986), causing displacement of Org 37462 at the level of the GnRH receptor. This hypothesis supports a deterministic role for endogenous GnRH in eliciting the spontaneous LH surge during ovarian stimulation cycles (*Dubourdieu et al.* 1994; *Karsch et al.* 1997).

In the present study, serum Org 37462 concentrations increased in a linear, dose-proportional manner, while serum LH and estradiol concentrations decreased in a dose-dependent manner. Accordingly, the amount of circulating androstenedione tended to decrease with increasing Org 37462 doses. The present study clearly demonstrates that the remaining endogenous LH concentrations during GnRH antagonist treatment may become critical when pituitary suppression is too profound. In patients treated with 1 or 2 mg Org 37462 per day, serum LH concentrations were mostly $\leq$1 IU/L, which is much lower than in patients undergoing controlled ovarian stimulation with recFSH in combination with a ‘long protocol’ of a GnRH agonist. The latter treatment usually results in serum LH

concentrations between 1 and 2 IU/L depending on the compound and regimen applied (*Devroey et al.* 1994). In five patients with no or minimal follicular growth after starting treatment with 1 or 2 mg Org 37462, treatment cycles were successfully rescued after switching from recFSH to HMG. Whether this reversal was related to the exogenous LH activity and/or increasing estrogen concentrations remains unclear, but if so, this finding would be in contrast to those of previous studies in gonadotropin-deficient women, demonstrating that despite minimal estrogen increases, recFSH may induce normal follicle growth up to pre-ovulatory stages (*Couzinet et al.* 1988; *Schoot et al.* 1992; *Schoot et al.* 1994). In addition, in the present study the number of antral follicles, number of oocytes, fertilization rate, number of (good quality) embryos and the number of transferred embryos were similar in the six dose groups (data not shown). This indicates that an impaired estradiol synthesis does not interfere with normal folliculogenesis, oocyte maturation and fertilization processes. In previous studies with comparable doses of the GnRH antagonist cetrorelix, dose effects on estradiol rises were less apparent, as patients were treated daily with HMG containing LH activity (*Albano et al.* 1996; *Albano et al.* 1997). In contrast, a small comparative study applying 5 mg of the GnRH antagonist Nal-Glu for only 2-3 days, revealed serum LH and peak estradiol concentrations in the GnRH antagonist group which were clearly lower than in the GnRH agonist group (*Minaretzis et al.* 1995a). In view of the current findings, it needs to be assessed whether in women undergoing ovarian stimulation with recFSH (instead of HMG), a single-dose protocol with a relatively high dose of GnRH antagonist to prevent LH surges for several days would be feasible. The lower vital pregnancy rates in the highest dose groups could not be explained by the lower rises of serum estradiol on the day of hCG or oocyte retrieval, but the total numbers of patients per treatment group were small and hamper a final explanation of the outcome among the higher dose groups. Additional research will be required to examine whether Org 37462 has any direct effects on the ovary or endometrium, although recent research seems to indicate that the GnRH-receptor is not expressed in human preovulatory follicles, endometrium or decidua (*Latouche et al.* 1989; *Minaretzis et al.* 1995b; *Brus et al.* 1997; *Ikeda et al.* 1997).

The further development of Org 37462 (0.25 mg/day) for patients undergoing ovarian stimulation may have several advantages over the current practice, namely a ‘long protocol’ of a GnRH agonist. The main advantage will concern patient convenience since the overall duration of treatment with GnRH analogue will be reduced from several weeks to several days. In the present study the amount of recFSH per ovarian stimulation cycle in the 0.25 mg dose group was 22 ampoules (75 IU/ ampoule), which on average was 300-400 IU less than the amount of recFSH used in a ‘long protocol’ of the

GnRH agonist buserelin (*Out et al.* 1995). Using daily 0.25 mg cetrorelix, and the same criteria for giving hCG, Albano et al. (1997) reported that 33 ampoules of HMG were needed when starting ovarian stimulation, with a daily dose of 225 IU HMG. With this higher starting and total dose of gonadotropins, the duration of treatment was similar, but the number of small antral follicles seemed to be larger.

A daily dose of 0.25 mg Org 37462 during ovarian stimulation with recFSH resulted in lower peak serum estradiol concentrations than previously reported for recFSH in a 'long protocol' of intranasal buserelin (median values 1160 pg/mL versus 1602 pg/mL, respectively) (*Out et al.* 1995). In the current regimen, monitoring of estradiol concentrations during ovarian stimulation is only required in case of increased risk for developing OHSS, which reduces the monitoring costs and patient discomfort. It has been suggested previously that GnRH antagonist treatment may play a role in preventing severe OHSS (*de Jong et al.* 1998). Moreover, the immediate reversibility of the hypogonadotrophic state allows the administration of native GnRH or GnRH agonist to induce ovulation for subjects at risk of developing OHSS (*Olivennes et al.* 1996). However, to establish the improved safety and patient convenience of this short Org 37462 regimen, additional clinical studies will be required.

2.1.5 Appendix

In the current study, a statistical subset selection procedure was performed to select the dose group with the best effect estimate, but no statistical tests were carried out to compare the results of the various dose groups. A rationale for this approach is summarized below.

P-values do not depend only on the outcome of a study but also on the sample size. An example may illustrate this. An observed difference in pregnancy rates of 20% versus 25% in two groups of 500 subjects each would yield $P = 0.06$. In the present study, with dose groups of approximately 60, such a difference would yield $P = 0.51$, while a much larger difference, e.g. 15% versus 29%, would be required to obtain a (non-significant) *P*-value of 0.06. The *P*-value is not a measure of clinical significance, but is an indication for the robustness of the outcome; a large trial tends to produce more reliable results (*Freeman* 1993).

Differences of 15% were not expected in the present study, so the outcome of each individual test of significance was a priori

expected to be negative and therefore non-informative (*Altman & Bland* 1995).

On the other hand, many tests of significance could have been carried out as there are six dose groups and many outcome parameters (multiplicity). Also, in such a complicated study, some unexpected and remarkable results are likely to appear for which a test may be carried out (post-hoe testing). Such an approach will inevitably lead to significant results, the validity of which may be low (data dredging). Statistical correction for multiplicity or using confidence intervals would not solve these problems, it would only modify them (*Pocock* 1997).

The methods used in the trial circumvent those problems by looking at the whole pattern, the aggregate outcome for all doses. Conclusions are neither based on nor made about the differences between individual dose groups. The aim of the analysis is modest; it describes the results and suggests a dose for further investigation in Phase III confirmatory trials.

2.2 Follicular development during ovarian stimulation for IVF and various doses of a GnRH antagonist

2.2.1 Introduction

The recent clinical introduction of gonadotropin releasing-hormone (GnRH) antagonists provides the opportunity to develop novel strategies for assisted reproduction. GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotropin release and a rapid recovery of normal pituitary secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (*Ditkoff et al.* 1991). Recent studies have shown GnRH antagonists, either at single or multiple doses, to be effective in preventing a premature LH rise during ovarian stimulation for in vitro fertilization (IVF) (*Frydman et al.* 1991; *Diedrich et al.* 1994; *Olivennes et al.* 1994; *Albano et al.* 1996; *The ganirelix dose-finding study group* 1998). The administration of GnRH antagonists in such a regimen can be limited to the period vulnerable to a premature LH surge (*de Jong et al.* 2000). Under these conditions, ovarian stimulation with FSH can start in the early follicular phase of the undisturbed normal menstrual cycle, without the need for prior pituitary 'down-regulation'.

Estrogens have several important functional roles in the human female reproductive system including induction of the mid-cycle gonadotropin surge, stimulation of cervical mucus production and endometrial proliferation. Under LH stimulation, theca cells convert cholesterol into androstenedione (AD) and testosterone (T) by means of cytochrome p450 side chain cleavage oxidases and 3β-hydroxy-steroid dehydrogenase (*Erickson et al.* 1985). Only when the follicles are at a more advanced stage of development, reaching the dominant stage at a diameter beyond 10 mm (*Pache et al.* 1990; *van Dessel et al.* 1996), does FSH dependent granulosa cell aromatase activity convert the theca derived AD and T to estrone and E_2 (*Hillier et al.* 1981; *van Santbrink et al.* 1995). This so-called 'two-gonadotropin, two-cell' concept emphasizes that sufficient stimulation of both theca cells and granulosa cells by LH and FSH is required for adequate E_2 biosynthesis (*Short* 1962; *Schoot et al.* 1992).

Changes over time in FSH levels during the follicular phase are crucial for determining the growth of either single or multiple dominant follicles (*Fauser et al.* 1999). When follicles are at a more advanced stage of maturation, their requirement for FSH is reduced (*van Santbrink et al.* 1995). Recent *in vivo* evidence demonstrates that dominant follicle development and E_2 production is also

dependent on late follicular phase LH concentrations (*Zeleznik et al.* 1974; *Sullivan et al.* 1999).
In contrast to GnRH agonists, the suppression of LH secretion by GnRH antagonists is more pronounced than that of FSH (*Hall et al.* 1988). However, the effects of GnRH antagonist induced suppression of gonadotropin secretion on follicle dynamics and ovarian E_2 production have not been described. The aims of the present study were to investigate the influence of different dosages of GnRH antagonist on follicular development and to assess the relationship between the follicle diameter, total follicular surface area, number of follicles and serum steroid and gonadotropin levels in patients undergoing ovarian stimulation for IVF. The influence of GnRH antagonist on the 'two-gonadotropin, two-cell' mechanism could thus be explored.

2.2.2 Materials and methods

Patients characteristics
A total of 329 women between 18 and 39 years of age, for whom ovarian stimulation and IVF with or without intracytoplasmic sperm injection (ICSI) was indicated, were included in the present prospective, double-blind, randomized, multi-centre trial. The study protocol was approved by the Local Ethics Review Committees. All women had a normal menstrual cycle history (cycle length 24-35 days), a body weight of 50-75 kg, a body mass index (BMI) of 18-29 kg/m^2, were in good health and had refrained from any hormonal therapy for at least 30 days prior to entering the study. On transvaginal ultrasound, two normal ovaries were observed in all subjects, and a normal follicular hormone status was determined. The subjects were studied during a single IVF treatment cycle. All patients (n = 311) who proceeded to human chorionic gonadotropin (hCG) administration for final oocyte maturation were included in the analysis. Patients (n = 18) with an insufficient response (defined as < 3 follicles of $\geq$ 17 mm) to ovarian stimulation, drug non-compliance, protocol violations related to in- or exclusion criteria, or other reasons for which hCG for final oocyte maturation was not given, were excluded from analysis.

Stimulation regimen and IVF procedure
Prior to ovarian stimulation, subjects were randomly assigned to one of the six treatment groups. Stratified randomization was performed by code numbers. Patients received a fixed dose of 150 IU recombinant FSH (recFSH, Puregon®, NV Organon, Oss, The Netherlands) as a daily s.c. injection, from cycle day (CD) 2 until CD

7. From CD 7 until the day hCG was administered, the recFSH dose could be adjusted according to the individual ovarian response as assessed by daily ultrasound. Participants were co-treated with a GnRH antagonist (ganirelix, Orgalutran®/Antagon™, NV Organon) in a dose of either 0.0625, 0.125, 0.25, 0.5, 1.0, or 2.0 mg/day from CD 7 onwards. When at least 3 follicles $\geq$ 17 mm were observed on transvaginal ultrasound, 10.000 IU hCG (Pregnyl®, NV Organon) was administered by i.m. injection. Oocyte collection took place 36 h after hCG administration. A maximum of 3 embryos were transferred on day 2 to 5 after oocyte retrieval. Luteal phase support (hCG or progestins) was initiated on or before the day of embryo transfer, according to the clinics' routine practice.

Monitoring
Bloodsampling for hormone analysis took place daily, immediately prior to recFSH administration, on the morning of CD 2 until and including CD 6. Thereafter (from CD 7 until and including the day of hCG) daily bloodsamples were withdrawn just before GnRH antagonist administration. During the follicular phase, transvaginal ultrasound (TVS) was applied, according to the clinics' standards, to monitor the number and size of the follicles. Follicle size was categorized into 3 subclasses of follicles: 11-15 mm, 15-17 mm, and $\geq$ 17 mm, respectively (*The ganirelix dose-finding study group* 1998). Follicular surface areas were calculated using the formula: surface area = $4\pi r^2$, where r (the radius of the follicle) was derived from the mean diameter of each subclass. Total follicular surface area per patient was obtained from the sum of the surface area of each follicle. When no pregnancy occurred, the time of onset of the next menstruation was documented. Biochemical pregnancy was defined as a positive urinary hCG test 2 weeks after embryo transfer. Viable pregnancy was defined as a positive intrauterine fetal heart activity observed by TVS 5-6 weeks after embryo transfer.

Hormone assays
Blood samples were centrifuged within 1 h after withdrawal and stored at – 20°C until assayed. Serum LH, FSH, E_2, and P levels were measured by a central laboratory, using a fluoroimmunoassy (Delphia®, Wallac OY, Finland) and AD by a coat-a-count direct radioimmunoassay. Interassay coefficients of variation were less than 4.7, 7.0, 9.9, 9.1 and 9.3 % for FSH, LH, E_2, P and AD respectively. All samples from one subject were run in the same assay.

Statistical analysis
Potential differences between the groups in age, duration of subfertility, total amount of FSH administered, number of follicles, and total follicular surface area were analyzed using the Kruskal-Wallis and Mann-Whitney tests, as were potential differences in LH, FSH, E_2, AD, and P. Potential differences within the same group on different time intervals were analyzed using the Friedman and Wilcoxon matched pair test. Potential differences in cancellation rate and pregnancy rate were analyzed using Fisher's exact test. Univariate regression was used to test the effect of the dose of GnRH antagonist on logarithmic transformed levels of E_2 on the day of hCG. With multiple regression the effect of dose of GnRH antagonist on logarithmic transformed E_2 levels was tested, controlling for logarithmic transformed AD, FSH, LH, and total follicular surface area. In principle, this analysis assesses statistically the effect of dose GnRH antagonist on E_2 levels when corrected for the other variables. Differences were considered to be statistically significant if $P < 0.05$.

2.2.3 Resuls

No significant differences were found between the treatment groups in duration of subfertility or indication for IVF (data not shown). The mean age was 31.6 years and the mean body mass index was 22.7 kg/m^2. Eighteen of the 329 women who began ovarian hyperstimulation for IVF were excluded from analysis. Five were excluded due to premature luteinization, drug non-compliance or imminent ovarian hyperstimulation and thirteen due to insufficient ovarian response. The latter was observed in 2, 1, 4, 2, and 4 patients in the 0.0625, 0.25, 0.5, 1.0, and 2.0 mg GnRH antagonist treatment group, respectively. A total of 311 patients proceeded to hCG administration for final oocyte maturation and were included in the analysis. The total duration of ovarian stimulation, total amount of gonadotropins used, total number of oocytes retrieved, and number of embryos developed after fertilization were not significantly different between the six treatment groups (Table 2.5). Pregnancy and implantation rates were higher in the treatment groups receiving low doses of GnRH antagonist treatment, as shown previously (*The ganirelix dose-finding study group* 1998).

No significant differences in the number of follicles, arbitrarily categorized into 3 size classes, were found on the day of initiation of ovarian stimulation, starting day of GnRH antagonist treatment, one day before hCG, or on the day of hCG (Figure 2.7). The total follicular surface area per patient on the day of hCG was not significantly different between the six treatment groups (data not shown).

	Daily dose of ganirelix (mg)					
	0.0625	0.125	0.25	0.5	1.0	2.0
No. of patients reaching hCG (n)	29	65	66	63	62	26
Age (yrs)	31.6 ± 4.3	31.9 ± 3.8	31.4 ± 2.9	31.2 ± 4.0	31.7 ± 3.8	32.2 ± 4.2
Total dose of recFSH (IU)	1810 ± 1104	1840 ± 826	1831 ± 688	2068 ± 1011	2243 ± 1219	1997 ± 1228
Follicular phase length (days)	9.6 ± 1.7	9.9 ± 1.8	10.0 ± 1.7	10.1 ± 1.9	10.6 ± 2.4	9.3 ± 1.9
Ultrasound (day of hCG)						
No. of follicles ø 11-15 mm	10.6 ± 5.2	10.7 ± 4.8	11.7 ± 4.6	10.2 ± 4.6	10.6 ± 4.4	9.4 ± 3.6
No. of follicles ø 15-17 mm	7.0 ± 2.9	7.3 ± 3.2	7.6 ± 3.1	6.7 ± 2.7	7.0 ± 2.8	6.3 ± 2.4
No. of follicles ø ≥ 17 mm	4.2 ± 1.7	4.6 ± 2.1	4.5 ± 2.0	4.4 ± 2.2	4.3 ± 1.9	3.7 ± 1.2
No. of follicles ø ≥ 11	21.8 ± 8.3	22.6 ± 9.0	23.8 ± 8.5	21.2 ± 8.2	21.9 ± 7.5	19.4 ± 6.2
Total Follicular Surface Area (mm^2)	14770 ± 5244	15446 ± 6052	16048 ± 5760	14489 ± 5553	14857 ± 5009	13115 ± 4082
Endocrinology (day of hCG)						
LH (IU/L)	5.3 ± 5.4*	3.5 ± 3.1*	2.4 ± 2.2*	1.6 ± 1.3*	0.8 ± 0.6*	0.4 ± 0.2*
FSH (IU/L)	11.6 ± 6.1	10.6 ± 5.7	10.4 ± 4.2	11.7 ± 4.9	11.7 ± 5.7	12.0 ± 6.4
E_2 (pg/mL)	1714 ± 905*	1625 ± 1259*	1468 ± 1157*	1099 ± 787*	913 ± 720*	480 ± 225*
AD (ng/mL)	2.9 ± 1.3*	2.8 ± 1.4*	2.8 ± 2.6*	2.2 ± 0.7*	2.1 ± 0.8*	1.6 ± 0.5*
P (ng/mL)	1.1 ± 0.9	0.8 ± 0.5	0.8 ± 0.7	0.7 ± 0.4	0.9 ± 1.3	0.7 ± 0.4
Embryology						
No. of oocytes retrieved	9.6 ± 5.3	9.6 ± 5.4	10.3 ± 5.2	9.8 ± 6.2	9.5 ± 6.0	8.6 ± 4.4
No. of embryos obtained	5.8 ± 3.4	5.9 ± 4.3	5.6 ± 4.4	5.1 ± 4.1	5.4 ± 3.9	4.9 ± 3.7

Table 2.5
Patient characteristics and late follicular phase ultrasound and endocrine assessments (mean ± SD) in 311 women undergoing ovarian hyperstimulation for in vitro fertilization (IVF) using recombinant follicle stimulation hormone (recFSH) and various doses of the gonadotropin releasing hormone (GnRH) antagonist ganirelix.
* Kruskal-Wallis test: $P < 0.0005$, hCH = human chorionic gonadotropin, LH = luteinizing hormone, FSH = follicle stimulating hormone, E_2 = estradiol, AD = androstenedione, P = progesterone.

FSH levels increased throughout the follicular phase in all six treatment groups ($P < 0.005$) but serum FSH concentrations did not differ between the groups at any stage during the follicular phase (Table 2.5). With respect to the serum LH concentrations, no significant differences were observed between the six groups prior to

starting GnRH antagonist treatment. However, a significantly lower LH serum level was found on the day prior to and on the day of hCG administration in the 0.25, 0.5, 1.0, and 2.0 mg GnRH antagonist treatment groups as compared to the 0.0625 and 0.125 mg GnRH antagonist treatment groups, $P < 0.005$ (Figure 2.8).

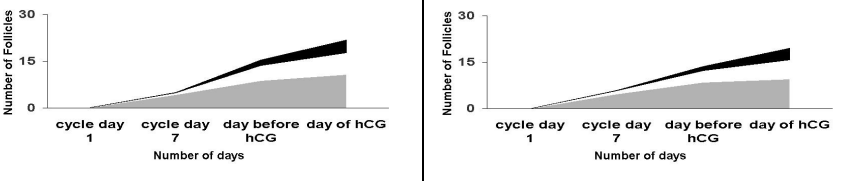

Figure 2.7
Number of follicles (categorized into three different size classes) for the lowest dose (0.0625 mg/d; left panel) and the highest dose (2.0 mg/d; right panel) of the gonadotropin releasing hormone (GnRH) antagonist ganirelix during ovarian stimulation for IVF. Gray area, 11-14 mm; white area, 15-17 mm; black area, ≥ 17 mm.

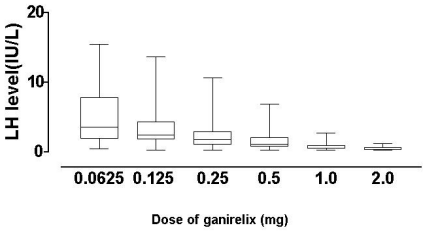

Figure 2.8
Box and whisker plot of serum luteinizing hormone (LH) levels on the day of human chorionic gonadotropin (hCG) in women undergoing ovarian stimulation for in vitro fertilization (IVF) using recombinant follicle stumulating hormone (recFSH) and 2.0 mg per day of the gonadotropin relaesing hormone (GnRH) antagonist ganirelix.

AD levels were significantly lower on the day prior to and on the day of hCG administration in the 1.0 and 2.0 mg treatment groups compared to those receiving the smaller doses, $P < 0.05$ (Table 2.5). The effect of increasing doses of GnRH antagonist on serum E_2 levels was similar to that on LH levels. A significantly lower E_2 level was detected on the day prior to and on the day of hCG in those receiving 0.5, 1.0, and 2.0 mg of GnRH antagonist compared to those receiving 0.0625, 0.125, and 0.25 mg $P < 0.05$ (Table 2.5). Follicular phase progesterone (P) levels did not differ across the 6 treatment groups.

Univariate regression analysis showed the serum level of E_2 on the day of hCG to be related to the dose of GnRH antagonist, LH, FSH, AD and total follicular surface area (all $P < 0.001$). E_2 levels on the day of hCG for the different doses GnRH antagonist, adjusted for AD, FSH, LH, and total follicular surface area, are depicted in Figure 2.9. Multiple regression of E2 levels with daily dose of GnRH antagonist controlling for AD, FSH, LH, and total follicular surface area on the day of hCG, demonstrated a regression coefficient of -1.61 and a standard error of 0.035 ($P < 0.001$).

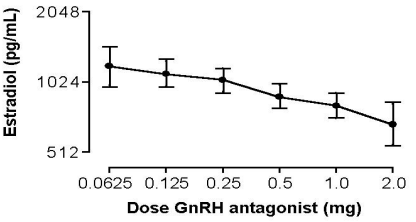

Figure 2.9
Logarithmic transformed E_2 levels on the day of hCG (adjusted for AD, FSH, LH, and total follicular surface area) under different doses of the GnRH antagonist ganirelix during ovarian stimulation for IVF.

2.2.4 Discussion

This study demonstrates that follicle growth dynamics were similar in all treatment groups receiving various daily doses of the GnRH antagonist ganirelix (0.0625 - 2.0 mg/d). Although the multicentre design of the study, the use of different ultrasound machines and measurement criteria may have masked subtle differences in follicle size, clear equivalence in follicle size and total follicular surface area were observed in all groups. In addition, in terms of follicles observed, oocytes retrieved, and embryos obtained, data from the different treatment groups were also camparable. The duration of stimulation and the total amount of gonadotropins administered were independent from the GnRH antagonist dose in women undergoing ovarian stimulation for IVF.

In the present study, serum FSH levels rose during the follicular phase resulting in similar late follicular phase levels in all six treatment groups. FSH serum concentrations reflect the sum of exogenous FSH and endogenous FSH, possibly suppressed by GnRH antagonist (*de Jong et al.* 2000). Minor differences in pituitary FSH release due to the suppressive action of increasing doses of GnRH antagonist were not reflected in changes in serum FSH. Thus, prevention of the physiological decrease in FSH was effected, enabling ongoing growth of multiple dominant follicles. Under FSH

stimulation, low levels of LH are sufficient to induce growth of healthy preovulatory follicles (*Schoot et al.* 1992; *The european recombinant human LH study group* 1998). It may therefore be speculated that the low LH concentrations occuring during high dose GnRH antagonist treatment were sufficient to allow normal follicular growth in the presence of exogenous FSH stimulation.

A clear dose related fall in LH levels on day of hCG was observed, with levels in the highest dose group only 12% of those observed in the lowest dose group (Figure 2.8). LH levels in all treatment groups were significantly lower than observed in the normal menstrual cycle (*Macklon & Fauser* 2000). Caution is required when interpreting these LH data since the sampling frequency may not have fully represented the pulsatile nature of endogenous LH stimulation of follicles. Further, samples for LH analysis were withdrawn prior to GnRH antagonist administration, almost 24 hours following the previous injection, whereas maximum of suppress of LH by GnRH antagonists is normally established after 4 hours (*Oberye et al.* 1999b). Data concerning the recovery of pulsatile LH secretion under different dosages of GnRH antagonist treatment is lacking. In contrast to the case with studies of GnRH agonists, LH levels assessed in the present study do not represent 24 hour exposure of the ovaries to LH. In addition, the magnitude of overestimation of LH may be GnRH antagonist dose related.

Despite similar patterns of follicular growth, E_2 levels showed a dose related response to GnRH antagonist. On the day of hCG, E_2 levels in the 2.0 mg GnRH antagonist group reaching only 33 % of the E_2 levels observed in the 0.25 mg GnRH antagonist group. However, even in these highly suppressed conditions, absolute E_2 concentrations were still elevated compared to late follicular phase levels in the menstrual cycle (*Macklon & Fauser* 2000). The production capacity for E_2 derived from the number of granulosa cells can be quantified by measuring the total follicular surface area per patient (*van Wezel & Rodgers* 1996). In both spontaneous and stimulated cycles, E_2 production per unit total follicular surface area remains relatively constant throughout the late follicular phase (*Eissa et al.* 1986). In the present study, E_2 serum levels per unit total follicular surface area decreased with increasing daily doses of GnRH antagonist. Low E_2 levels in the high dose GnRH antagonist treatment groups despite comparable number of follicles on day of hCG may indicate a decrease in E_2 production by granulosa cells (*McNatty et al.* 1979) which may simply reflect a reduced availability of AD, the substrate for aromatization to E_2. Indeed, a significant regression coefficient was calculated between E_2 and AD serum levels on day of hCG.

Lower serum AD levels on the day of hCG were observed in the two high GnRH antagonist dose groups. This may in turn have

been due to suppressed LH activity. Alternatively, AD levels in serum may not represent the availability in the follicular compartment since a substantial part of circulating AD is produced by the adrenals (*Abraham et al.* 1969).

Consistent with the 'two-gonadotropin, two-cell' concept (*Short* 1962; *Makris & Ryan* 1977; *Sullivan et al.* 1999), FSH, LH, and AD levels were all found to affect E_2 biosynthesis. However, when E_2 levels were adjusted for FSH, LH, and AD levels and total follicular surface area, the daily dose of GnRH antagonist still appeared to exhibit an additional suppressive action on E_2 levels (regression coefficient = -1.61, SD = 0.035, $P < 0.001$). This analysis suggested that GnRH antagonist may be impacting on E_2 levels by means of an additional mechanism not directly related to these other variables. Evidence for the differential effects that GnRH analogs have on granulosa-lutein cell steroidogenesis (*Minaretzis et al.* 1995a) and for the presence of GnRH receptors on human granulosa cells (*Latouche et al.* 1989) would be consistent with an additional agonist/antagonist mediated affect on E_2 production, possibly acting on intra-ovarian modifiers of response to FSH and LH such as vascular endothelial growth factor (VEGF) and insuline-like growth factor (IGF) (*Fauser & Van Heusden* 1997).

Pregnancy and implantation rates were lower in the treatment groups of patients receiving higher doses (1.0 - 2.0 mg/d) of GnRH antagonist compared to those receiving a lower dose (0.0625 - 0.5 mg/d), as reported previously (*The ganirelix dose-finding study group* 1998). It remains doubtful if this observation is of any clinical relevance, since the minimal effective dose of the GnRH antagonist ganirelix was established at 0.25 mg/d (*The ganirelix dose-finding study group* 1998). A proper analysis of pregnancy and implantation rates implies accurate evaluation of the luteal phase, the analysis of follicular phase characteristics is unsuitable for that purpose. However, a large randomized multicentre trial showed similar pregnancy and implantation rates of a GnRH antagonist protocol, utilizing a daily dose of 0.25 mg ganirelix, in comparison to a 'long protocol' (*The european orgalutran study group et al.* 2000).

In conclusion, follicular growth was not influenced by the daily dose of GnRH antagonist in ovarian hyperstimulation protocols for IVF. Despite similar follicular growth patterns, a marked dose related suppression of late follicular phase E_2 levels was observed, secondary to a dose related suppression of LH, and to some extent AD levels. Multivariate analysis showed E_2 levels to be additionally affected by the dose GnRH antagonist itself. These data suggest that lack of circulating androgens and LH may not be the only factors causing low E_2 levels in the high dose GnRH antagonist treatment groups and that high doses of GnRH antagonists may influence

follicular steroidogenesis by an additional, as yet unelucidated mechanism.

2.3 Non-inferiority study of a GnRH antagonist protocol for IVF

2.3.1 Introduction

Ganirelix is the active ingredient of Orgalutran® and Antagon™,a gonadotropin-releasing hormone (GnRH) antagonist preparation developed for the prevention of premature LH surges in women undergoing ovarian stimulation. In comparison with native GnRH, ganirelix has substituted amino acids at positions 1, 2, 3, 6, 8 and 10, which results in a potent antagonist with only minimal histamine-releasing properties (*Rabinovici et al.* 1992; *Nelson et al.* 1995) and high aqueous solubility. The latter property is reflected by the high absolute bioavailability (F) of ganirelix being more than 90% after s.c. injection (*Oberye et al.* 1999a).

In current practice, GnRH agonists are used to suppress endogenous gonadotropins during ovarian stimulation (*Loumaye* 1990). However, agonists initially stimulate the release of gonadotropins (‘flare-up’), and complete pituitary suppression is only achieved after 2–3 weeks pretreatment when pituitary desensitization occurs due to receptor down regulation.

The introduction of a GnRH antagonist such as ganirelix allows a short and simple treatment regimen for IVF patients undergoing ovarian stimulation, since antagonists immediately suppress gonadotropins by blocking the GnRH receptor, and thus treatment may be restricted to those days when a premature LH surge is likely to occur. Studies in healthy female volunteers and IVF patients have shown that steady-state concentrations of ganirelix are reached within 2–3 days of treatment, and that maximal suppression of endogenous LH occurs about 4 h after each injection (*Oberye et al.* 1999b). Moreover, after discontinuation, a rapid recovery of pituitary function (*Gordon et al.* 1990) was observed, also due to the relative short elimination half-life (about 13 h) of ganirelix. The additional anticipated advantages of antagonist treatment in ovarian stimulation programmes are a reduction of the use of gonadotropins, a lower risk for developing ovarian hyperstimulation syndrome (OHSS), and the ability to use a bolus injection of a GnRH agonist to trigger a midcycle LH surge for final follicular maturation (*Olivennes et al.* 1996). Moreover, in cases of multiple follicles and excessively high estradiol concentrations, the use of GnRH agonist instead of human chorionic gonadotropin (hCG) is thought to prevent the clinical manifestation of OHSS (*Itskovitz-Eldor et al.* 1991). If nevertheless, the IVF cycle is cancelled, ganirelix treatment may be continued to prevent spontaneous ovulation as well as signs and symptoms of OHSS (*de Jong et al.* 1998).

The third-generation GnRH antagonists cetrorelix and ganirelix have both been applied in a multiple-dose regimen in women undergoing ovarian stimulation. Clinical research of cetrorelix started off with relatively high daily dosages of 3 and 1 mg (*Diedrich et al.* 1994; *Felberbaum et al.* 1995), but finally the lowest effective daily dose of cetrorelix appeared to be 0.25 mg (*Albano et al.* 1997; *Albano et al.* 1998). To select the minimal effective daily dose of ganirelix, a multicentre, double-blind, randomized, dose-finding study was performed in 333 women including six different dosages ranging between 0.0625 and 2 mg (*Itskovitz-Eldor et al.* 1998; *The ganirelix dose-finding study group* 1998). In this study, patients were treated with a fixed dose of 150 IU recFSH for 5 days before starting ganirelix. The study revealed that a daily dose of 0.25 mg ganirelix prevented LH from rising above 10 IU/L during stimulation, and resulted in a good clinical outcome, i.e. the ongoing pregnancy rate was 34% per attempt (23/68) and 37% per transfer (23/62). Moreover, as in other studies (*Fujimoto et al.* 1997; *Oberye et al.* 1999b), serum ganirelix concentrations increased in a linear dose-proportional manner, and serum LH decreased in a dose-proportional manner, indicating that the degree of pituitary suppression can be adjusted by changing the ganirelix dose.

In the current study the efficacy and safety of a multiple dose regimen administering 0.25 mg ganirelix daily was assessed in a randomized study in women undergoing ovarian stimulation with recFSH for IVF or intracytoplasmic sperm injection (ICSI).

2.3.2 Materials and methods

Patients

A total of 730 patients, for whom ovarian stimulation and IVF or ICSI was indicated, was screened and randomized in this study. In total, 20 IVF centres in 10 European countries participated, and the number of randomized patients per centre ranged from 11 to 60. Main inclusion criteria were: age at least 18 years but not older than 39 years; body mass index (BMI) between 18 and 29 kg/m^2; regular menstrual cycle, ranging from 24 to 35 days.

Study design

This trial was a phase III, multi-centre, open-label, randomized study to assess the efficacy and safety of the GnRH-antagonist ganirelix in women undergoing ovarian stimulation. Eligible patients were randomized by an interactive voice response system (IVRS) to either treatment with ganirelix (Orgalutran®, Org 37462; NV Organon, Oss, The Netherlands) or buserelin (Suprecur®; Hoechst, Frankfurt Am

Main, Germany) in a ratio of 2:1. To improve balance, a minimization method was used for randomizing patients to treatment (*Treasure & MacRae* 1998), stratifying for centre age, for primary or secondary infertility, and for IVF or ICSI.

A diagrammatic representation of the applied treatment regimens is shown in Figure 2.10. Injections of recFSH (Puregon®; NV Organon) and ganirelix were given in the morning. In the ganirelix group, treatment with recFSH was started in patients on day 2 or 3 of the menstrual cycle by a once-daily s.c. injection. After 5 days of recFSH treatment, ganirelix treatment was started by daily s.c. administration in the upper leg. Ganirelix treatment was continued up to and including the day of hCG administration.

In the buserelin reference group, pretreatment with buserelin was started in the midluteal phase (cycle day 21–24) with a daily dose of 0.6 mg intranasally (four puffs per day). Ovarian stimulation was started after 2 weeks if pituitary 'down-regulation' was established (serum estradiol concentration <50 pg/mL or <200 pmol/L). In case 'down-regulation' was not achieved after 2 weeks, stimulation was postponed and the daily dose of buserelin was doubled to 1.2 mg. The dose of buserelin at which 'down-regulation' was established (0.6 or 1.2 mg) was continued up to the day of hCG. If 'down-regulation' with buserelin was not achieved within 4 weeks, treatment discontinued.

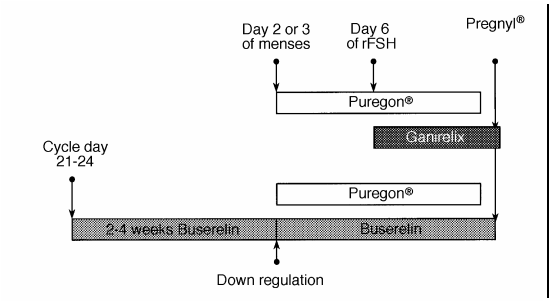

Figure 2.10
Schematic of the treatment regimen with ganirelix (upper part of diagram) and with a 'long protocol' of intranasal buserelin (lower part) in patients undergoing ovarian stimulation with recombinant FSH (rFSH; Puregon®).

In both treatment groups, ovarian stimulation was started with a fixed daily dose of 150 IU recFSH for the first five treatment days. From day 6 onwards, the dose of recFSH was adapted depending on the ovarian response as monitored via ultrasonography. On the day of hCG, recFSH was not administered. hCG (10 000 IU, Pregnyl®; NV Organon) was administered when at least three follicles of $\geq$17 mm diameter were observed, and 30–36 h thereafter oocyte retrieval was performed. Oocyte retrieval was followed by IVF or ICSI, and no more than three embryos were to be replaced 2–5 days thereafter. Luteal

phase support was given according to the clinics' routine practice, and was started no later than the day of embryo transfer.

Assessments

In the ganirelix group prior to the start of recFSH, and in the buserelin group prior to the start of buserelin, an hCG test was to be performed to exclude pregnancy. When bleeding did not occur within 2 weeks after starting buserelin treatment, an additional hCG test was performed. Just before the first injection of recFSH, a blood sample was taken for hormone assessment and ultrasonography (USS) was performed. From recFSH treatment day 6 up to and including hCG administration, the patient returned to the clinic for USS and blood sampling before ganirelix administration once every 2 days. Serum FSH, LH, estradiol and progesterone were assessed by a central laboratory by means of fluoro-immunoassay (Delfia®, Wallac OY, Finland).

Local tolerance was assessed by the patient at 1, 4 and 24 h after each ganirelix injection. The subject was asked to record on a diary card the score (none, mild, moderate or severe) for five different parameters, i.e. bruising, swelling, pain, itching and redness.

Statistical methods

The study was designed as a non-inferiority trial to test whether the combination of efficacy, safety and convenience of ganirelix treatment was clinically equivalent to the current care, i.e. GnRH agonist treatment in a 'long protocol'. As a large previous study with Puregon and intranasal buserelin in a 'long protocol' had an excellent clinical outcome (*Out et al.* 1995), this regimen was selected as the reference treatment in the current study. Data from the intent-to-treat (ITT) group were used for efficacy analysis, and data from the all-subjects-treated (AST) group were used for safety analysis. The ITT group and the AST group consisted of all patients randomized who started treatment. Patients in the ITT group were grouped according to the treatment they should have received by randomization, whereas patients in the AST group were grouped according to the actual treatment they received.

Efficacy analysis

A total of 701 subjects started treatment with recFSH or buserelin and was included in the ITT group. Of the treated subjects, 463 were randomized to the ganirelix group and 238 to the buserelin group. One subject was randomized to buserelin, but was treated with the ganirelix regimen, and one subject was randomized to buserelin and received during buserelin treatment also three injections of ganirelix. Since both subjects were intended to receive buserelin, they were included in the ITT group of buserelin. One subject with a

spontaneous pregnancy who started buserelin treatment was also included in the ITT group. Main efficacy parameters were treatment failure, number of cumulus–oocyte complexes, number of good quality embryos, and ongoing pregnancy rate. For patients treated with both IVF and ICSI, oocyte quality was not analysed.

The estimated difference of ganirelix and buserelin in ongoing pregnancy rate was compared with the margin of –5%. For cumulus–oocyte complexes, the lower one-sided 97.5% confidence limit of the treatment difference was compared with the equivalence margin of –3 oocytes. For continuous efficacy variables, adjusted-for-centre treatment means and their differences were calculated, using a weighted average over the centres based on the Cochran–Whitehead method (*Whitehead & Whitehead* 1991). For the ongoing pregnancy outcome the Cochran–Mantel–Haenszel weights (*Cochran* 1954) were used. Lower one-sided 97.5% confidence limits of the adjusted-for-centre treatment differences between ganirelix and buserelin were calculated for the number of oocytes, good quality embryos, and the ongoing pregnancy rate. For the rate of study medication treatment failure in each group, a one-sided 97.5% confidence limit was calculated based on the binomial distribution.

Freeze–thaw cycles

Embryos were frozen in 18 out of the 20 IVF centres; in two German centres only two-pronuclear (2PN) oocytes were frozen which were not included in this analysis. Data were collected for all patients (*n* = 126) who did not become pregnant after replacement of fresh embryos, and for whom spare embryos were cryopreserved. By June 1999, 53 of these patients had had at least one embryo transfer using thawed embryos. The outcome of these first freeze–thaw cycles is presented.

Safety analysis

Analysis was performed by means of frequency distributions of the incidence of adverse events, local tolerance outcome, clinically significant abnormal laboratory values and vital signs. For patients treated with the ganirelix regimen, analysed data included all days of stimulation, thus also the first 5 days of recFSH treatment when patients were not yet exposed to ganirelix.

2.2.3 Results

The two treatment groups were similar with respect to age, body height, weight and BMI (see Table 2.6). The overall (*n* = 701) mean age, body height, weight and BMI were 31.9 years, 166.6 cm, 63.8 kg and 23 kg/m^2 respectively. The majority (98%) of patients was Caucasian. No relevant differences were found between the treatment groups for the cause of subfertility which was overall 40.1% male factor, 29.2% tubal factor and 15.5% unknown factors. The overall percentage of subjects with primary infertility was 56.5%, and similar in both groups.

Characteristic	Ganirelix *n* = 463	Buserelin *n* = 238
Age (years)[a]	31.9 ± 3.6	31.9 ± 3.8
Body mass index (kg/m^2)[a]	23.0 ± 2.9	23.0 ± 2.7
Duration of subfertility (years)[a]	4.5 ± 2.7	4.4 ± 2.7
Main causes of subfertility (%)		
Male (only)	41.0	38.2
Tubal (only)	29.8	28.2
Unknown	13.8	5.0
Parity (%)		
Primary infertility	56.6	56.3
Secondary infertility	43.4	43.7

Table 2.6
Demographics and subfertility characteristics.
[a] Values are mean ± SD.

Disposition and cancellations
In total, 730 subjects were randomized, 486 patients to the ganirelix group and 244 patients to the buserelin group (ratio~2:1). A total of 701 subjects received recFSH or GnRH analogue treatment. The number of patients per treatment stage is presented in Table 2.7.

In total, 16 subjects (3.5%) in the ganirelix group and 14 subjects (5.9%, including the patient who was randomized to this group but was treated with ganirelix) in the buserelin group had a treatment failure in that they did not receive hCG, or received hCG because of premature luteinization. The overall cancellation rate up to embryo transfer was 13.8 and 12.6% for the ganirelix and buserelin groups respectively. Main reasons for discontinuation of treatment

were insufficient ovarian response (3.2 versus 2.5%) and fertilization failure (6.2 versus 4.1%). Overall, eight patients in the ganirelix group and two patients in the buserelin group had intrauterine insemination instead of oocyte retrieval. In the buserelin group, seven patients (3.0%) were discontinued before starting recFSH due to insufficient 'down-regulation'. In the ganirelix group, two patients (0.4%) were discontinued because of premature luteinization.

	Ganirelix	Buserelin
No. of subjects randomized to group	463	238
Treated with buserelin	-	237 (100)
Treated with recFSH	463 (100)	228 (96)
Treated with ganirelix	460 (99)	2[a]
hCG for triggering ovulation	448 (97)[b]	224 (95)
Oocyte retrieval	440 (95)	221 (93)
Embryo transfer	399 (86)	208 (88)

Table 2.7
Number of patients per treatment stage.
[a] Two patients who were randomized to the buserelin group were treated with ganirelix, one of whom received no buserelin at all (see Materials and methods; Efficacy analysis).
[b] Includes one patient who received HCG because of premature luteinization. Values in parentheses are percentages. hCG = human chorionic gonadotropin.

Duration of GnRH analogue treatment and total dose of recFSH
The median (range) duration of GnRH analogue treatment was 5 (2–14) days in the ganirelix group, and 26 (18–44) days in the buserelin group. The total amount of GnRH analogue administered was 1.25 versus 16.2 mg. The median (range) duration of recFSH treatment was 9 (6–18) and 10 (6–19) days respectively. The total amount of recFSH administered was in total 1500 (900–6400) IU and 1800 (900–6450) IU, and the median daily dose was 150 IU/day and 178 IU/day in the ganirelix and buserelin groups respectively.

LH rises (≥10 IU/L) before and during ganirelix treatment
Early LH rises at day 6 of stimulation, before the first ganirelix administration, were observed in 20 patients (4.3%). In these patients serum LH values ranged between 10.4 and 33.4 IU/L and concomitant rises of serum progesterone >1 ng/mL were observed in seven out of these 20 patients. On day 6 of stimulation, this subset of patients had on average 6.8 follicles ≥11 mm diameter, and their

median serum estradiol concentration was 856 (range 348–1900) pg/mL, indicating that most of these patients were high responders. Due to initiation of ganirelix treatment, endogenous LH rises were effectively cutdown in all 20 patients. Out of the 20 patients, 19 had embryo
transfer, and in three patients an ongoing pregnancy was established.

After the day of the first ganirelix injection, 13 women (2.8%) had an LH value ≥10 IU/L. Peak LH values ranged between 10.1 and 58.1 IU/L, and concomitant rises (>1 ng/mL) of serum progesterone were observed in six patients (1.5%). Seven patients were discontinued before embryo transfer, and none of the other patients became pregnant. In the buserelin group, three patients (1.3%) had an LH rise to ≥10 IU/L during stimulation. Of these, one patient was discontinued and one patient became pregnant. Miscarriage did not occur in any of the patients with an early or late rise of serum LH after confirmation of an early clinical pregnancy.

Follicle growth

The mean (± SD) number of follicles ≥11 mm diameter measured on days 6, 8 and 10 of stimulation for patients with at least 9 days of stimulation are presented in Figure 2.11. The number and size of follicles measured at the day of hCG administration are summarized in Table 2.8.

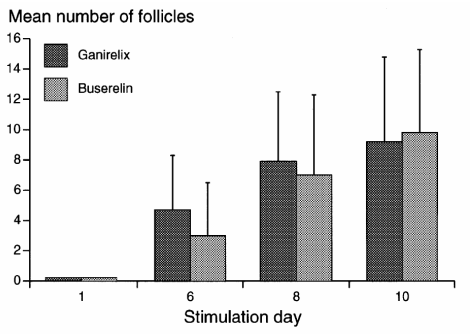

Figure 2.11
Number of follicles ≥ 11 mm in diameter on stimulation days 1, 6, 8, and 10 of recFSH for patients with at least 9 days of stimulation. Values are mean ± SD.

Comparison of the number and size of growing follicles indicates a different follicle growth pattern in the ganirelix group than in the buserelin group. At day 6 of stimulation, after 5 days of 150 IU recFSH per day, absence of follicular growth (no follicles ≥11 mm diameter) was observed in 13.2% of patients treated with the ganirelix regimen, whereas this incidence was 31.5% in the buserelin reference group. Accordingly, initial follicular growth appeared to be more rapid in the ganirelix group than in the buserelin group, as indicated by the mean number of follicles ≥11 mm diameter on day 6

of stimulation (4.7 versus 3.0; Figure 2.11). On day 8 of stimulation, the difference between the treatment groups was less apparent, and on the day of hCG the opposite was observed in that the number of follicles ≥11 mm was smaller in the ganirelix group than in the buserelin group (10.7 versus 11.8). Comparison of follicle sizes between the two treatment groups indicates that this difference was mainly due to fewer small follicles in the ganirelix group (Table 2.8), as also reflected by the comparable mean number of follicles ≥17 mm at the day of hCG administration (4.9 versus 5.2).

	Ganirelix *n* = 448 [a]	Buserelin *n* = 224
Follicles (*n*)		
≥11 mm	10.7 ± 5.3	11.8 ± 5.4
≥15 mm	7.7 ± 4.0	8.3 ± 3.9
≥17 mm	4.9 ± 2.6	5.2 ± 2.3
Hormones		
FSH (IU/L)	7.7 (5.0-14.1)	8.4 (5.4-17.6)
LH (IU/L)	1.6 (<0.6-6.9)	1.5 (<0.6-4.4)
Estradiol (pg/mL)	1190 (373-3105)	1700 (527-4070)
Progesterone (ng/mL)	0.7 (0.4-1.6)	0.7 (0.4-1.6)
Estradiol/follicle (pg/mL)	111	144

Table 2.8
Number and size of follicles (mean ± SD) grouped according to diameter and serum hormone values (median with 5% and 95% percentiles) on the day of hCG administration. Restricted to patients who received an hCG injection.
[a] Includes one patient who received hCG because of premature luteinization.

Serum hormone concentrations
Median serum FSH, LH, estradiol and progesterone concentrations measured at days 1, 6, 8 and 10 of stimulation for patients with at least 9 days of stimulation are presented in Figure 2.12. At the start of recFSH stimulation (day 1), serum hormone concentrations were higher in the ganirelix group than in the buserelin group, and represented normal levels as measured at day 2 to 3 of the menstrual cycle and after pituitary 'down-regulation' respectively. On day 6 of stimulation, serum FSH and LH concentrations had become similar in both treatment groups, probably due to a negative feedback by initial rising estradiol concentrations in the ganirelix group. Median serum estradiol concentrations rose from 38 pg/mL on day 1 to 358 pg/mL on day 6 of stimulation in the ganirelix group, and from 19 pg/mL to 160 pg/mL in the buserelin group. This increase was in accordance with the number of growing follicles in each group. From day 6 to the day of hCG, serum FSH increased by 0.5 IU/L in the ganirelix group and by 1 IU/L in the buserelin group. Predose concentrations of

serum LH in patients treated with ganirelix were comparable with those measured in the buserelin group. After day 6 of stimulation up to the day of hCG, serum estradiol concentrations followed the pattern of follicular growth in that on day 8 the estradiol concentrations were only 128 pg/mL higher in the ganirelix group than in the buserelin group, whereas on the day of hCG (see Table 2.8) the opposite was observed, with serum estradiol concentration being about 500 pg/mL lower in the ganirelix group than in the buserelin group (1190 versus 1700 pg/mL). Finally, serum estradiol content per follicle (≥11 mm) was lower in the ganirelix group than in the buserelin group.

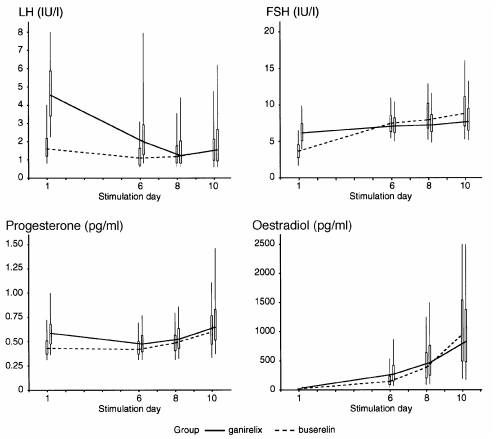

Figure 2.12
Serum hormone concentrations on stimulation days 1, 6, 8 and 10 of recFSH for patients with at least 9 days of stimulation. The boxes indicate the 75% and 25% percentiles, the vertical lines indicate the 95% and 5% percentiles, and median values are connected.

Individual serum LH values measured on day 6 and measured on the day of hCG were plotted in Figure 2.13. Comparison of the predose values in the ganirelix group with LH values measured during 'down-regulation' with buserelin, indicates a larger variability in the ganirelix group. A possible relationship between serum LH and pregnancy outcome (Figure 2.13) or between serum estradiol and pregnancy outcome (data not shown) was not revealed.

Treatment outcome

The mean number of oocytes recovered, their quality and the mean number of embryos obtained and their quality in each treatment group are given in Table 2.9. In comparison with buserelin treatment, ganirelix treatment resulted in one preovulatory follicle less (see above) and, as a consequence, one cumulus–oocyte complex less was recovered at oocyte retrieval. The estimated treatment difference was –1.0 oocyte (lower 97.5% confidence limit –1.8) which was within the equivalence margin of –3 oocytes.

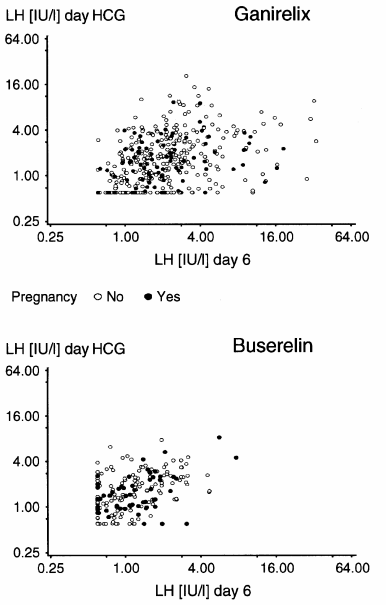

Figure 2.13
Concentrations of serum LH in individual patients on day 6 of stimulation versus serum LH on the day of hCG. Filled poits represent values of patients who became pregnant.

	Ganirelix	Buserelin
Cumulus-oocyte complexes/attempt	*n* = 463 8.7 ± 5.6	*n* = 238 9.7 ± 6.2
Number of oocytes before IVF	*n* = 234 9.6 ± 5.7	*n* = 123 10.3 ± 5.7
Number of oocytes before ICSI	*n* = 196 8.6 ± 4.8	*n* = 95 10.3 ± 5.9
Cumulus-oocyte complexes/attempt	*n* = 440 9.1 ± 5.4	*n* = 221 10.4 ± 5.8
Embryos	*n* = 463	*n* = 238
Total	6.0 ± 4.5	7.1 ± 5.2
Good quality	3.3 ± 3.0	3.5 ± 3.2
Frozen	1.1 ± 2.3	1.3 ± 2.5
Replaced	2.2 ± 0.6	2.2 ± 0.6

Table 2.9
Number of cumulus–oocytes complexes recovered per attempt and per oocyte retrieval, number and quality of embryos obtained, and number of transferred embryos. Values are presented as mean ± SD. ICSI = intracytoplasmic sperm injection.

In total, 357 patients had IVF and 291 patients had ICSI, whereas 10 patients had both IVF and ICSI (1.5 versus 1.3% in the ganirelix and buserelin groups respectively). The mean (± SD) number of metaphase II oocytes recovered in ICSI patients was 83%

in each group, i.e. 7.1 ± 4.2 versus 8.5 ± 5.2, and the overall fertilization rate was 62.1% in each group. In addition, the number of good quality embryos obtained was comparable between the groups, and the estimated treatment difference was only –0.1 embryo (lower 97.5% confidence limit –0.4). At transfer, in each group a mean of 2.2 embryos were replaced, including a mean of 2.0 and 1.9 good quality embryos in the ganirelix and buserelin groups respectively.

The clinical outcome of all patients receiving embryo transfer, expressed per attempt and per embryo transfer, is shown in Table 2.10. Although a similar number of embryos was replaced in each group, the implantation rate (number of gestational sacs divided by the number of replaced embryos) was relatively lower in the ganirelix group (15.7 versus 21.8%), whereas the miscarriage rate per clinical pregnancy was comparable (12.0 versus 13.9%). Accordingly, the vital and ongoing pregnancy rate tended to be lower in the ganirelix group than in the buserelin group. For the ITT group, the ongoing pregnancy rate per attempt was 20.3% in the ganirelix group and 25.7% in the buserelin group (including one spontaneous pregnancy in the buserelin group). The estimated difference for the ongoing pregnancy rate was at the margin of –5%. When comparing the ongoing pregnancy rate per study site, this difference ranged from 26.3% in favour of ganirelix to 28.6% in favour of buserelin. The ongoing pregnancy rate per attempt for patients (*n* = 337) treated at study sites (*n* = 10) that had previous experience with the ganirelix regimen was similar, i.e. 24.2% in the ganirelix group and 23.6% in the buserelin group, whereas this rate was respectively 16.5 and 27.5% for patients (*n* = 363) treated in study sites (*n* = 10) that had applied the ganirelix regimen for the first time.

Overall, 94 and 61 ongoing pregnancies respectively were established at 12–16 weeks after embryo transfer. The multiple pregnancy rate was 23.4% in the ganirelix group and 29.5% in the buserelin group.

Outcome of subsequent freeze–thaw cycles

The mean (± SD) number of embryos frozen was 1.1 ± 2.3 and 1.3 ± 2.5 in the ganirelix and buserelin groups respectively. The first frozen–thawed embryo cycles (*n* = 53) performed within one year after study completion resulted in three miscarriages, one induced abortion and 10 ongoing pregnancies (12–16 weeks after embryo transfer). Seven pregnancies (20.0%) were established in patients previously treated with ganirelix, and three pregnancies (16.7%) were established in patients treated with buserelin. The overall ongoing pregnancy rate was 18.9% (see Table 2.11).

	Ganirelix	Buserelin
No. of patients receiving embryo transfer	399	208
Implantation rate (%)	15.7	21.8
Miscarriage rate (%)	12.0	13.9
Vital pregnancies		
Per attempt (%)	21.8	28.2
Per transfer (%)	25.1	31.7
Ongoing pregnancy rate		
Per attempt (%)	20.3	25.7
Per transfer (%)	23.3	29.0
Ongoing pregnancies		
Singletons (%)	76.2	68.9
Twins (%)	20.2	26.2
Triplets (%)	3.2	3.3

Table 2.10
Clinical outcome as percentage per attempt and per transfer in patients treated with the ganirelix regimen and with a 'long protocol' of buserelin

Safety and tolerance
The number of subjects who experienced at least one adverse experience was 125 (26.9%, i.e. 125/465) in the ganirelix group, and 74 (31.4%, i.e. 74/236) in the buserelin group. The most frequently reported experiences were headache, abdominal pain (gynaecological), OHSS and miscarriages. Treatment discontinuation because of an adverse experience occurred for one patient in the ganirelix group (0.2%) due to the risk for developing OHSS, and for one patient (0.4%) in the buserelin group because of spontaneous ovulation before oocyte retrieval. The number of subjects with possible or probable drug-related experiences was 11 (2.4%) in the ganirelix group, and nine (3.8%) in the buserelin group. In the ganirelix group, 18 patients (3.9%) were hospitalized because of an adverse experience, i.e. ectopic pregnancy (*n* = 4), OHSS (*n* = 4), miscarriage (*n* = 6), threatening abortion (*n* = 1), hyperemesis (*n* = 1), urinary retention (*n* = 1) and abdominal pain (gynaecological) (*n* = 1). In the buserelin group, 11 patients (4.6%) were hospitalized because of ectopic pregnancy (*n* = 1), OHSS (*n* = 6 including 1 case of enteritis) and miscarriage (*n* = 4). All these adverse experiences were indicated as not, or unlikely to be, drug-related.

The incidence of OHSS was two-fold lower in the ganirelix group than in the buserelin group. Eleven subjects (2.4%) in the ganirelix group and 14 subjects in the buserelin group (5.9%) experienced signs and symptoms related to OHSS. For two pregnant patients in the ganirelix group OHSS was graded as severe; all other cases were of moderate or mild intensity.

	Ganirelix	Buserelin	Overall
No. of first attempts	35	18	53
Embryos (mean)			
Good quality	1.6[a]	1.9[a]	1.7
Transferred	2.1[a]	2.4[a]	2.2
No. miscarriages	2	1	3
Induced abortions (*n*)	1	0	1
No. of ongoing pregnancies	7	3	10
Pregnancy rate (%)	20.0	16.7	18.9

Table 2.11
Outcome of 53 first freeze-thaw cycles. [a] Data are missing.

The local tolerance outcome indicated that ganirelix administered daily by the s.c. route was well tolerated. The percentage of patients with at least one moderate or severe local tolerance reaction (skin redness, swelling, bruising, pain or itching) during ganirelix treatment was 16.6, 2.0 and 2.7%, at 1, 4 and 24 h after the injection respectively. Most frequently reported were moderate or severe skin redness (9.5%) or swelling (9.5%) at 1 h after injection, but by 4 h after injection these reactions had mostly disappeared. At 24 h after injection, bruising (moderate or severe) was most frequently reported (2.5%). None of the patients had to discontinue ganirelix treatment because of a hypersensitivity reaction or because of a drug-related adverse experience.

2.3.3 Discussion

The ganirelix regimen is a new treatment option for patients undergoing ovarian stimulation, which largely reduces the duration of GnRH analogue treatment and prevents adverse events related to ‘flare-up’ or ‘down-regulation’ induced by GnRH agonists. In addition

to its convenience, the regimen appeared to be safe and well-tolerated.

Ganirelix is used to prevent premature LH surges occurring during ovarian stimulation. However, in the literature no clear definition on an LH surge has been provided, and therefore data analysis was based primarily on the incidence of LH rises ≥10 IU/L and additionally on concomitant rises of serum progesterone 1 ng/mL, indicating premature luteinization. During ganirelix treatment the overall incidence of LH rises was 2.8%, and only in 1.5% of the cases was a concomitant rise in progesterone concentration observed, demonstrating the effective suppression of endogenous LH during ovarian stimulation.

In this efficacy trial, ganirelix treatment was started on day 6 of stimulation, since the previous dose-finding study (*The ganirelix dose-finding study group* 1998) demonstrated that during the first days of stimulation, median serum LH concentrations decrease and nadir LH concentrations are reached at day 5 of stimulation. This initial suppression of endogenous LH is thought to be established by a negative feedback of rising estradiol concentrations during the first days of stimulation. When follicular growth is progressing and estradiol concentrations become as high as in the late follicular phase of the normal menstrual cycle, then the reverse occurs and the risk for a premature LH surge becomes imminent (*Filicori et al.* 1986). In the current study, early LH rises before the first ganirelix administration occurred in 20 patients who were high responders, i.e. stimulation resulted in more rapid initial follicular growth and in a more pronounced rise of serum estradiol as compared with the overall treatment group. Even though the clinical outcome of this small subset of patients was good, early LH rises may be prevented by starting ganirelix treatment on day 5 instead of day 6 of stimulation. On the other hand, 13.2% of all patients did not show any follicles ≥11 mm diameter at day 6 of stimulation; in these lower responders exposure to ganirelix may be limited by delaying the start of treatment up to the moment of actual follicle growth.

In the current study, duration of treatment was short, i.e. on average 9 days with recFSH including 5 days of ganirelix treatment. Since initial growth of follicles was more rapid and endogenous FSH concentrations were only partly suppressed (during the late follicular phase), the duration of recFSH treatment was one day shorter and the amount of recFSH required was lower in the ganirelix group. Since the number of subjects without any follicles ≥11 mm diameter on day 6 of stimulation was twice as high in the 'long protocol' of buserelin, the ganirelix regimen might be of special benefit for poor-responders, who are frequently treated with a short 'flare-up' protocol of GnRH agonist (*Frydman et al.* 1988a).

Comparison of the number and size of follicles indicated that, in the ganirelix group, initial follicular growth was faster but the final cohort of growing follicles was smaller and produced on average less estradiol, which is explained by the different endocrine status of the patient at the start of stimulation. In view of this different follicular pattern, recFSH dose adjustments in patients treated with the ganirelix regimen should be based on the number and size of growing follicles, rather than on the amount of circulating estradiol. The smaller cohort of follicles and the lower estradiol concentrations are in good agreement with the lower incidence (less than half)
of OHSS in the ganirelix group. The possible direct effect of GnRH antagonists on follicle growth, steroidogenesis, oocyte or embryo quality or implantation is of specific interest, since GnRH receptors have been identified in human granulosa-lutein cells (*Latouche et al.* 1989; *Brus et al.* 1997), and might be present in uterine endometrial tissue (*Raga et al.* 1998), although their function and interaction with GnRH or GnRH analogues is not (yet) understood (*Ikeda et al.* 1997). Recent studies *in vitro* with human granulosa cells have demonstrated that neither ganirelix or cetrorelix exert any significant action on ovarian steroidogenesis (*Verbost et al.* 1999; *Ortmann et al.* 2002), and in the ganirelix dose-finding study no difference was noted in the number or size of follicles of patients treated in the six different dose groups (*The ganirelix dose-finding study group* 1998).

In comparison with buserelin treatment in a 'long protocol', the ganirelix regimen resulted in one preovulatory follicle less and, as a consequence, one cumulus–oocyte complex less was recovered at oocyte retrieval. This difference is within the preset equivalence margin of –3 oocytes, and is thought to be related to the short regimen rather than ganirelix *per se*. The recovery of fewer oocytes is a well-described phenomenon of the short protocol of GnRH agonists in comparison with the 'long protocol' (*Tan et al.* 1992; *Cramer et al.* 1999). Overall, the ganirelix regimen resulted in the recovery of good quality oocytes as reflected by the percentage of metaphase II oocytes in ICSI patients (83% in each group), the high fertilization rate (62.1% in each group), and the number of good quality embryos, which was comparable with the reference group. The latter finding suggests a higher recovery of good quality embryos in the ganirelix regimen than in the buserelin group, which is in good agreement with the outcome of a previous small study which compared the antagonist Nal-Glu (5 mg/day) with the agonist leuprolide acetate (*Minaretzis et al.* 1995a). The recovery of good quality oocytes and embryos is further supported by the good success rates of replaced frozen embryos collected in the dose-finding study of ganirelix (*Kol et al.* 1999), as well as in this study.

In the current trial, on average only 2.2 embryos were replaced, which is in line with the current standard practice in several

European IVF centres not to replace more than two good quality embryos, in order to prevent multiple pregnancies. In comparison with a large previous multi-centre study of Puregon, using the same stimulation regimen with buserelin, the ongoing pregnancy rate per attempt was very similar to the outcome of the ganirelix regimen (*Out et al.* 1995). Although the clinical outcome is considered good (ongoing pregnancy rate per attempt was 20.3%), in the current study the pregnancy rate tended to be higher in the reference group. In view of the limited study power, it cannot be excluded that this tendency is related to chance. However, using the same multiple dose regimen and also intranasal buserelin in a 'long protocol' as control, others (*Felberbaum* 1999) reported a vital pregnancy rate per transfer of 27% (compared with 25% in this study) in patients treated with human menopausal gonadotropin (HMG) and 0.25 mg GnRH antagonist cetrorelix (n = 188) and of 33% (cf. 32% in this study) in patients treated with the agonist (n = 85). Therefore, several other factors that may influence clinical outcome should be considered. For instance, study sites that participated in the previous ganirelix dose-finding study provided a favourable outcome more often for the ganirelix regimen compared with study sites who participated for the first time. This indicates that some clinical experience with the new ganirelix regimen might contribute positively to the success rate. In addition, the regimen applied has not been selected based on prospective research, and further optimization of the regimen might appear beneficial. In the current applied regimen, stimulation was started on day 2 or 3 of the follicular phase; thus serum gonadotropin and steroid concentrations were higher than after 'down-regulation' in the reference group. High concentrations of serum hormones are especially established in patients who receive the GnRH-agonist 'flare-up' protocol, and these were reported to have a lower clinical pregnancy rate, which is thought to be related to a higher estradiol concentration before hCG administration, and a higher production of estradiol per oocyte recovered (*Cramer et al.* 1999). In the ganirelix group, the opposite was shown in that before hCG administration estradiol concentrations were lower, and also the estradiol production per follicle was lower in the ganirelix group than in the buserelin group. Although in the current study neither serum LH nor estradiol concentrations appeared to be predictive factors for pregnancy (*Loumaye et al.* 1997), it cannot be excluded that the hormonal milieu of the current ganirelix regimen is less favourable for a certain subset of patients. If so, patients might benefit from postponing hCG administration and allowing follicles to grow larger, or to pretreat patients with oral contraceptives which would also allow patient scheduling. Thus, further optimization of treatment, as well as clinical experience with the antagonist regimen, might further optimize clinical outcome in the near future. Overall, it may be concluded that ganirelix

introduces a new treatment option for patients undergoing ovarian stimulation for IVF or ICSI which is safe, short and simple. The clinical outcome was good, and the ongoing pregnancy rate was within the range of pregnancy rates of a long-protocol GnRH-agonist, the latter being supported by many years of clinical experience.

2.3 Prevention of imminent OHSS with high doses GnRH antagonist, a case report

2.3.4 Introduction

Ovarian hyperstimulation syndrome (OHSS) is a potentially life-threatening complication of assisted reproduction (*Schenker & Weinstein* 1978). OHSS does not occur in the absence of either the endogenous luteinizing hormone (LH) surge, or surrogate human chorionic gonadotropin (hCG). Conditions which may indicate imminent OHSS include late follicular phase serum estradiol concentrations ≥12675 pmol/L (3500 pg/mL), and the occurrence of >25 small and intermediate sized follicles (*Rizk & Aboulghar* 1991). Under these circumstances ovarian stimulation for in-vitro fertilization (IVF) is usually cancelled by cessation of exogenous gonadotropins, withholding hCG, and continuation of pituitary down regulation by gonadotropin releasing hormone (GnRH) agonists in order to prevent unpredictable changes in release of endogenous gonadotropins. However, it has been reported that the continued administration of GnRH agonists does not affect subsequent ovarian quiescence (*Wada et al.* 1992). This may be related to prolonged suppression of pituitary function due to slow recovery from 'down-regulation' (*Donderwinkel et al.* 1993). Some residual gonadotropin release may remain during GnRH agonist suppression, whereas pituitary release of LH and follicle-stimulating hormone (FSH) may be virtually abolished with the sustained use of a high dose of GnRHantagonist. In this case report we describe an alternative method, using a high dose of a GnRH antagonist which may decrease the risk of a severe OHSS.

2.3.5 Case report

A 33 year old regularly menstruating woman (body mass index 23 kg/m^2) with an subfertility duration of 4.5 years due to tubal pathology underwent IVF in our unit. Before initiation of treatment, endocrine screening and sonographic examination appeared to be normal. As part of a multicentre phase II clinical trial (approved by the local ethics review committee), 150 IU recombinant follicle stimulating hormone (recFSH, Puregon®; NV Organon, Oss, The Netherlands) daily subcutaneous injections were administered, starting on cycle day 2. From cycle day 7 onwards she was co-treated daily with a GnRH antagonist (ganirelix, Org 37 462; NV Organon) (*Nelson et al.* 1995) 0.125 mg subcutaneously. Initially normal follicular growth was

observed and although estradiol concentrations rose steadily, they remained within the limits normally associated with controlled ovarian hyperstimulation for IVF. The moderate starting dose of 150 IU recFSH was therefore continued. However, on cycle day 11 the patient presented with a serum estradiol level of 16 500 pmol/L, a level associated with an increased risk of OHSS (*Rizk & Aboulghar* 1991). Transvaginal ultrasound showed four preovulatory (≥16 mm) follicles, and nine intermediate sized (10–16 mm) follicles. It was decided to cancel the cycle by withholding hCG injection and discontinuing daily recFSH administration. On cycle day 12 ultrasound showed an increase in the number of follicles (six preovulatory and 27 intermediate sized follicles). The patient complained of abdominal discomfort and a pocket of ascites in the pouch of Douglas was demonstrated on ultrasound. The estradiol concentration was 22 315 pmol/L and the right and left ovaries were enlarged, with respective mean diameters of 72 mm and 54 mm. The ganirelix dose was increased to 2 mg/d to prevent a possible LH surge (*Ditkoff et al.* 1991) and to further decrease endogenous LH and FSH secretion. Within 3 days estradiol concentrations returned to levels associated with a normal ovarian response for hyperstimulation in IVF. Moreover, symptoms and ascites disappeared over the following days. In the same period, the mean diameters of the right and left ovary decreased to 65 mm and 47 mm respectively. Daily serum levels of LH, FSH and estradiol are depicted in Figure 2.14.

2.4.3 Discussion

Administration of GnRH antagonists in the late follicular phase has recently been applied effectively in IVF programmes to prevent a premature rise in endogenous LH and subsequent luteinization instead of extended administration of GnRH agonists (*Diedrich et al.* 1994; *Olivennes et al.* 1994; *Albano et al.* 1996; *Felberbaum et al.* 1996; *Albano et al.* 1997). In contrast to GnRH agonists, GnRH antagonists elicit an immediate effect by competitive blockage of GnRH receptors (*Klingmuller et al.* 1993). The likelihood of preventing a premature LH rise and subsequent luteinization is clearly dependent on the dose of the GnRH antagonist (*Ubaldi et al.* 1996). Further evidence for a dose dependent pituitary response is the observation that gonadotropin secretion could be restored by pulsatile GnRH therapy during GnRH antagonist treatment (*Gordon et al.* 1990). Since in the late follicular phase growth of follicles and subsequent estradiol production is dependent on stimulation by both LH and FSH, further development of follicles may be arrested effectively in cases of imminent OHSS through pronounced suppression of pituitary

gonadotropin release by prolonged use of high dose GnRH antagonists. It should, however, be recognized that a spontaneous LH surge may still occur under these circumstances, which could induce OHSS without exogenous hCG. As demonstrated previously (*Gordon et al.* 1990), GnRH can override the inhibitory actions of antagonist. We therefore elected to increase the ganirelix dose. Simply reducing the dose of recFSH is unlikely to have had a significant effect since follicular sensitivity to FSH increases in the advanced stages of development. There is no clear evidence that reducing the dose of FSH at this late stage has a preventative effect on OHSS.

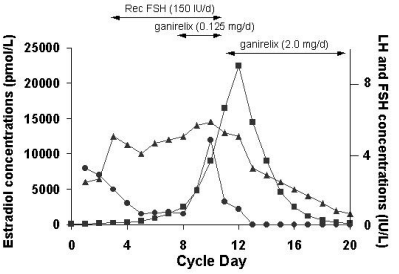

Figure 2.14
Estradiol (■–■), follicle-stimulating hormone (FSH) (▲–▲) and luteinizing hormone (●–●) serum levels during treatment with recombinant FSH and ganirelix for in-vitro fertilization in a patient with imminent ovarian hyperstimulation syndrome

GnRH receptors have been shown to be present in granulosa–lutein cells (*Latouche et al.* 1989; *Minaretzis et al.* 1995b) and some studies suggest that steroidogenic activity of cultured granulosa cells may be affected by GnRH (*Pellicer & Miro* 1990) suggesting that the human ovary could be a target for direct extrapituitary GnRH action in the human.

For reasons of safety, we considered it mandatory to cancel ovarian stimulation in this IVF patient presenting with clear signs of imminent OHSS. Since spontaneous LH surges may occur after a short period of GnRH antagonist treatment (*Ditkoff et al.* 1991), sustained administration of a high dose GnRH antagonist could potentially reduce the risk of severe OHSS. Indeed, in-vitro studies demonstrate that chronic administration of GnRH antagonist virtually abolishes GnRH induced LH release from the pituitary (*Pinski et al.* 1996).

The present case confirms the previously reported efficacy of high dose GnRH antagonist in achieving rapid suppression of endogenous gonadotropin release even when LH levels have started to rise (*Dubourdieu et al.* 1994), and in eliciting subsequent ovarian quiescence. It is yet to be determined whether GnRH antagonists act

solely through suppression of pituitary function, or whether direct actions at the ovarian level may also be involved. The clinical role played by GnRH antagonist in this case remains uncertain since progression to OHSS may have been prevented by discontinuation of recFSH and withholding hCG. Controlled studies are required to assess the extent to which GnRH antagonist contributes to the early resolution of ovarian hyperstimulation syndrome.

Chapter 3

Mild ovarian stimulation for in vitro fertilization

3.1 The added value of cryopreservation of embryos is limited: no justification for maximal ovarian stimulation for IVF

3.1.1 Introduction

The first reports of a clinical pregnancy (*Trounson & Mohr* 1983) and birth (*Zeilmaker et al.* 1984) from frozen thawed embryos introduced the clinical application of cryobiology into in-vitro fertilization (IVF) programs. Since the recognition of cryopreservation of preimplantation embryos as an established clinical procedure (*The american fertility society* 1990), its application in IVF programs has steadily increased, becoming integral to the service provided by the majority of IVF centres (*Fugger* 1989).

Ovarian stimulation of multiple dominant follicle development has considerably improved the clinical outcome of assisted reproductive techniques (*Quigley et al.* 1982; *MacDougall et al.* 1994). At present, the most commonly practised ovarian stimulation regimen for IVF includes pituitary down regulation with a gonadotropin-releasing hormone (GnRH) agonist for at least two weeks followed by the co-administration of high dose exogenous follicle stimulating-hormone (FSH). This so called 'long protocol' aims to induce ongoing growth of large numbers of follicles in normo-ovulatory women, resulting in large quantities of oocytes (and resultant embryos) obtained from a single oocyte retrieval (*Tan et al.* 1992). The 'long protocol' for ovarian hyperstimulation has been shown to be effective in providing large numbers of embryos for transfer or cryopreservation, improved pregnancy rates, and lower cancellation rates compared to other stimulation protocols for IVF (*Hughes et al.* 1992).

The transfer of all available embryos dramatically increases the risk of high order multiple pregnancies and the associated obstetrical and perinatal complications. As a result, many IVF centres have adopted the policy of limiting the number of embryos transferred and of cryopreserving surplus embryos in an attempt to reduce the risk on multiple pregnancies and births (*Schieve et al.* 1999). Indeed, the availability of cryopreservation programs is frequently cited as a justification for maximal stimulation of large quantities of follicles in a single IVF cycle (*Mandelbaum et al.* 1998). The risks associated with this approach has provoked increasing concern (*Edwards et al.* 1996; *Olivennes & Frydman* 1998; *Fauser et al.* 1999). The obligatory intensive conventional ovarian stimulation and extended oocyte retrieval procedures levy a high degree of physical and emotional

stress on patients undergoing IVF (*Fauser et al.* 1999). This is reflected by the high drop-out rate seen in conventional IVF. If no pregnancy occurs in the first IVF attempt, many patients (around 25%) fail to attend second attempt, even in countries where IVF is covered by health insurance (*De Vries et al.* 1999; *Osmanagaoglu et al.* 1999).

Cryopreservation of supranumerical embryos and transfer of thawed cryopreserved embryos in a subsequent cycle is now generally accepted. However, this approach is not free of legal, moral and ethical issues (*Fasouliotis & Schenker* 1996; *Schenker* 1997). The added benefit of cryopreservation programs to IVF remains uncertain (*Jones et al.* 1995). A significant portion of frozen preimplantation embryos will not survive the thawing process (*Van der Elst J. et al.* 1995). Moreover, embryo transfer of thawed cryopreserved preimplantation embryos is less successful, in terms of pregnancy and implantation rates, when compared to transfer of fresh embryos (*Van Steirteghem et al.* 1994).

We carried out an observational longitudinal study to calculate the benefit of a cryopreservation program to the overall cumulative ongoing clinical pregnancy rate over a maximum of three consecutive IVF cycles.

3.1.2 Materials and methods

Study population

All couples with an indication for IVF (*Macklon et al.* 2001) who began their first IVF treatment, with or without intracytoplasmic sperm injection (ICSI), in the period between January 1995 to December 1999 were evaluated. In the Netherlands, the obligatory health insurance covers three IVF cycles, independent of the indication for IVF. In order to reduce possible bias related to socio-economical status, a maximum of three IVF cycles, including subsequent cryopreservation of supranumerical embryos, were analyzed. Women who began pituitary down regulation in our routinely applied 'long protocol' and agreed on cryopreservation of the surplus of preimplantation embryos were included in the study (n = 1792). Couples progressed to a second or third stimulation IVF cycle only when all cryopreserved embryos had been thawed for intended transfer. Couples undergoing oocyte donation, who had undergone previous IVF treatments, or who had decided to cancel treatment were excluded from analysis. The study was performed according to the standards outlined in the Declaration of Helsinki.

IVF procedure

All patients underwent pituitary down regulation by daily subcutaneous injections of 0.1 mg of the GnRH agonist triptorelin (Decapeptyl®, Ferring GmbH, Kiel, Germany) for at least two weeks prior to ovarian stimulation. Ovarian stimulation was initiated by daily injections (150 IU to 450 IU) of human menopausal gonadotropin (HMG; Humegon®, NV Organon, Oss, The Netherlands) i.m. or recombinant FSH (RecFSH; Puregon®, NV Organon) s.c. When at least 3 follicles $\geq$ 18 mm were observed on transvaginal sonography, all medication was stopped and 10.000 IU human chorionic gonadotropin (hCG; Pregnyl®, NV Organon) was administered by i.m. injection in order to complete oocyte maturation. Oocyte retrieval took place 35 h after hCG administration. A maximum of 2 embryos were transferred on day 3 to 5 after oocyte retrieval according to our previously published protocol (*Huisman et al.* 2000; *Hunault et al.* 2002). Surplus embryos of adequate quality were subsequently cryopreserved. Luteal phase support was initiated on the day of embryo transfer by daily vaginal administration of 600 mg progesterone (Progestan®, NV Organon), continued until menstruation or a positive urinary hCG test. Biochemical pregnancy was defined as a positive urinary hCG test 2 weeks after embryo transfer. Ongoing pregnancy was defined as a positive intrauterine fetal heart activity observed by transvaginal sonography 10 weeks after embryo transfer.

Cryopreservation and thawing procedure

Embryos remaining after transfer and meeting previously published quality criteria (*Veeck* 1998; *Huisman et al.* 2000) were frozen. The cryopreservation procedure was performed using a slow freezing 1.5 M DMSO (British Drug House Ltd, Poole, UK) protocol (*Van der Elst J. et al.* 1995) in a programmed freezer (Planer Kryo 10-III; Planer Products, Sunbury-on-Thames, UK). The medium for cryopreservation consisted of Earle's balanced salt solution and Hams F10 medium (Life Technologies, Paisley, UK), supplemented with a pasteurized plasma protein solution and 1.5 M DMSO. Embryos were placed in medium for cryopreservation and subsequently transferred into cryostraws and equilibrated for 30 minutes at room temperature. Cryostraws were then placed in the freezing apparatus, preset at 0 °C and frozen as follows: The embryos were cooled to -4 °C at a rate of -2 °C/min and subsequently cooled further at a rate of -1 °C/min until a temperature of -6 °C was reached. Seeding was performed manually with liquid nitrogen (LN_2)-cooled sponge forceps. Further cooling was performed at a rate of -0.3 °C/min to -40 °C and then at -40 °C/min to -120 °C., at which temperature the cryostraw was immersed in LN_2. Thawing was performed by removing the cryostraw from the LN_2, followed by

placing in ice water. The embryos were removed from the cryostraw and placed in diluted freezing medium of consecutively 1.2 M, 0.9 M, 0.6 M, 0.3 M DMSO for 5 minutes, respectively. Embryos were subsequently rinsed in embryo culture medium and transferred to a 25 µL droplet in a culture dish followed by culturing before transfer. Post thaw survival of cryo preserved embryos was defined as $\leq$ 50 % blastomere loss. A maximum of 2 selected thawed embryos were transferred in the natural cycle after ovulation timed by urinary LH tests or monitoring by transvaginal sonography. The same transfer technique was used as in fresh transfer cycles.

Data analysis

For the purpose of measuring the ongoing pregnancy rate of IVF treatment from both fresh and cryopreserved embryos, pregnancies arising from cryopreservation cycles were considered to contribute to the pregnancy rate of the associated stimulation cycle. The 'patient-specific concept' proposed by Jones et al. (1995) is characterized by considering pregnancies arising from the cryopreservation cycle as augmentation only among patients who did not achieve an ongoing pregnancy from the fresh embryos transfer cycle. We applied this concept to evaluate the contribution of cryopreservation programs to the cumulative pregnancy rate of a maximum of three IVF cycles. The 'projected cryoaugmented pregnancy rates', which assume that pregnancy rates of patients with unthawed cryopreserved embryos are comparable to those who had an embryo transfer of thawed cryopreserved embryos, were calculated (*Jones et al.* 1997a). Cumulative ongoing pregnancy rates calculated by life table analysis assume that patients who drop out from the study have the same probability of achieving an ongoing pregnancy as patients who continue treatment. In order to test this assumption, differences in patient characteristics which may affect the probability of achieving an ongoing pregnancy between patients who continue treatment and those who drop out from the study were compared using Mann-Whitney U test and Fisher's exact test. Clinical outcome in terms of ongoing pregnancy rates was analyzed by Markov chain analysis (*Muenz & Rubinstein* 1985) to estimate the added value of the cryopreservation program. Differences were considered significant if $P < 0.05$.

3.1.3 Results

Patients
In the period January 1995 to December 1999, 1251 couples met the inclusion criteria for analysis. The age of the women at the start of treatment ranged from 21 to 44 years. 77.8 % of the study population were treated for primary infertility. Indications for IVF were respectively male factor (44.4 %), tubal factor (22.5 %), idiopathic (21.6 %), anovulation (4.8 %), cervical hostility (4.0 %), endometriosis (2.4 %), and other (0.2 %). Fertilization of the oocytes by ICSI was performed in 12.1 % of the couples.

Couples who had children prior to IVF treatment and couples in which the IVF procedure did not result in an embryo transfer were more likely to stop treatment following an unsuccessful cycle (respective odds ratios 1.5 (95% C.I. 1.1 – 2.0) and 3.1 (95% C.I. 1.9 – 5.1)). These couples were considered as non-informative drop-outs, since pregnancy rates in those patients who did continue were comparable to those of the rest of the study population.

Treatment outcome
In the 1251 couples analyzed, a total of 2367 IVF cycles and 392 thawed cryopreserved embryo cycles were initiated. An embryo transfer of at least one embryo was performed in 71.8 % of the started IVF cycles. In 25.0 % of the initiated IVF cycles surplus embryos were available for cryopreservation. The post thaw survival rate of cryopreserved embryos was 24.9 % per thawed embryo. An embryo transfer was performed in 41.3 % of the initiated thawed cryopreserved embryo cycles. Pregnancy and ongoing pregnancy rates per initiated cycle were 21.6 % and 17.6 % for the fresh embryo transfer cycles and 5.9 % and 4.8 % for thawed cryopreserved embryo cycles, respectively. Corresponding rates per embryo transfer were 30.0 % and 24.5 % for the fresh embryo transfer cycles and 14.2 % and 11.7 % for the thawed cryopreserved embryo cycles, respectively (Figure 3.1).

Of the 1251 women beginning their first IVF stimulation cycle, 864 underwent fresh embryo transfer and 227 achieved an ongoing pregnancy. Of the 1024 women who did not conceive, 335 had embryos cryopreserved. Of these, 217 women had their embryos thawed, resulting in embryo transfer in 94 women with 14 achieving an ongoing pregnancy. For 118 women unthawed embryos are still available for thawing and subsequent transfer. For these women a total of 8 projected pregnancies was calculated (25) (Table 3.1).

A total of 741 women proceeded to a second and 375 to a third IVF stimulation cycle, of whom respectively 557 and 280 underwent fresh embryo transfer, with 137 and 52 achieving an ongoing pregnancy, respectively. In the second cycle, embryos from

167 non-conceiving women were cryopreserved, of whom 112 had their embryos thawed, resulting in an embryo transfer for 45 women and subsequently 4 ongoing pregnancies. In the third cycle embryos of 90 non-conceiving women were cryopreserved, 63 had their embryos thawed, resulting in an embryo transfer for 23 women and subsequently 1 ongoing pregnancy. Of the non conceiving women who had their embryos cryopreserved in the second and third cycle, a total of 55 and 27 women have cryopreserved embryos available for thawing, respectively. For these women a total of 3 ongoing pregnancies could be calculated (25) (Table 3.1).

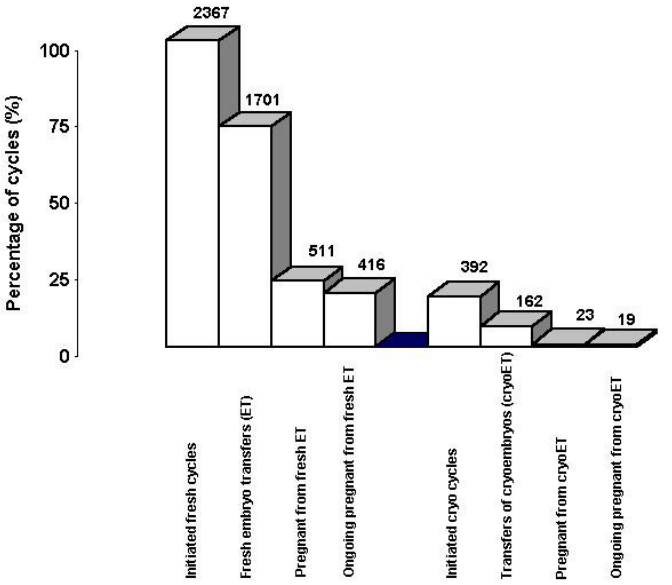

Figure 3.1
Treatment outcome of a total of initiated 2367 IVF cycles in 1251 patients. ET = embryo transfer.

	Cycle 1	Cycle 2	Cycle 3
Number of patients who started IVF	1251	741	375
Fresh embryo transfer (Fresh ET)	864	557	280
Pregnant after Fresh ET	227	137	52
Not pregnant after Fresh ET and no cryo embryos available	916	574	285
Not pregnant after Fresh ET and cryo embryos available	335	167	90
Cryopreserved embryos thawed	217	112	63
Embryo transfer of thawed cryopreserved embryos (Cryo ET)	94	45	23
Pregnant after Cryo ET	14	4	1
Unthawed cryopreserved embryos	118	55	27
Projected pregnancies	8	3	0

Table 3.1
Clinical outcome of 3 consecutive IVF cycles of 1251 patients starting IVF in the period 1995-1999. Values represent number of patients

The cumulative ongoing pregnancy rate per started cycle for 3 successive fresh IVF cycles was 42.5 %. When pregnancies arising from transfer of thawed cryopreserved embryos were added, the cumulative pregnancy of consecutive 3 IVF cycles was 43.8 %. When projected pregnancies resulting from unthawed embryos were included, the cumulative ongoing pregnancy rate was 44.5 % (Figure 3.2).

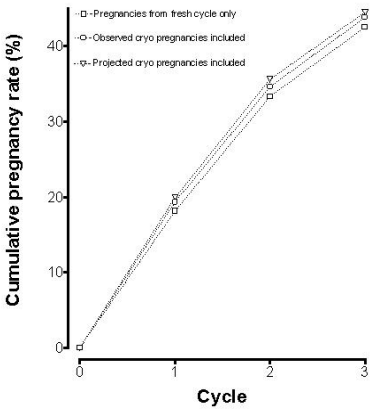

Figure 3.2
Cumulative pregnancy rate of 3 consecutive IVF cycles, without embryo cryopreservation (□-□), including pregnancies resulting from the transfer of thawed cryopreserved embryos (○-○), and including projected pregnancies (▽-▽).

In the 227 women achieving an ongoing pregnancy arising from the fresh ET in their first IVF attempt, 32 had a subsequent transfer of thawed cryopreserved embryos. This resulted in 3 ongoing pregnancies (9.4%). In the women achieving an ongoing pregnancy arising from the fresh ET in second and third IVF attempt, in both attempts 9 had a subsequent transfer of thawed cryopreserved embryos, none of which resulted in an ongoing pregnancy.

3.1.4 Discussion

The demographic and subfertility characteristics of the study population were representative of those in other European centres (*The ganirelix dose-finding study group* 1998; *The european orgalutran study group et al.* 2000). The ongoing pregnancy rate per initiated IVF cycle excluding thawed cryopreserved embryos (16.7%), and the ongoing pregnancy rate per transfer of thawed cryopreserved embryos (11.8 %) was also comparable with that reported by other European centres (*Mandelbaum et al.* 1998; *Nygren & Andersen* 2001). Comparisons of outcomes from embryo cryopreservation are often complicated by difficulties in controlling for differences in the populations of embryos being assessed (*Ludwig et al.* 1998; *Testart* 1998). This may partially explain the relatively lower pregnancy rates per thawed embryo transfer we observed when compared to those reported in the USA IVF registries during the same period (*society for assited reproductive technology (SART)* 2000).

By adopting the novel approach of analysing outcomes in terms of cumulative pregnancy rate for 3 IVF cycles, we have revealed the limited added value of a representative embryo cryopreservation program. The additional contribution of embryo cryopreservation to cumulative pregnancy rates over three cycles of IVF was just 2% (42.5% vs. 44.5%). This meant that, of the 1251 women who started IVF treatment in the 5 year study period, only 19 had a direct benefit from the cryopreservation program in terms of the 'patient specific concept', with an additional 11 expected to benefit. Given the considerable day-to-day effort and commitment required to maintain a cryopreservation program, these results were disappointing.

The low post thaw survival rate of cryopreserved embryos may explain these findings. Despite the application of a widely used and recommended cryopreservation protocol utilizing DMSO (*Van der Elst J. et al.* 1995; *Van den Abbeel & Van Steirteghem* 2000), and plastic cryostraws as the freezing container (*Van den Abbeel & Van Steirteghem* 2000), the post thaw survival rate of cryopreserved embryos in our study appeared to be lower (24.9 %) when compared

to cohorts described previously (*Jones et al.* 1997b; *Mandelbaum et al.* 1998). This discrepancy may be due in part to differences in the definition of survival. When post thaw survival of cryopreserved a embryo is defined as ≤ 50 % blastomere loss, as in our center, the relatively low percentage survival we observed is comparable with other reports where similarly defined post-thaw survival rates of 27% have been described (*Van der Elst et al.* 1996). More recently, thus defined post-thaw survival rates of up to 78% have been reported (*Edgar et al.* 2000). However, these data were limited to early cleavage stage (day 2) embryos. In the present study, results from cryopreserved day 3,4 and 5 embryos were analyzed. The possible effect on outcome of the stage of development at which embryos were cryopreserved was not delineated. Possible differences in post thaw survival rate between 8-cell, morula and blastocyst stage embryos, and their impact on the overall outcome cannot therefore be ruled out. The relatively complex structure of blastocysts and their fluid filled cavity may render them more difficult to freeze successfully when compared to the morula or 8-cell embryo (*Fauser et al.* 2002a). Therefore, the blastocyst may require a different freezing procedure or freezing medium than those normally applied to less mature embryos. However, published data in this area remain contradictory (*Troup et al.* 1991; *Kaufman et al.* 1995).

The severe damage which can be sustained by an embryo during the cryopreservation and thawing procedure is well documented (*Fauser et al.* 2002a), and the chance of implanting appears to be directly related to the degree of blastomere loss following thawing (*Edgar et al.* 2000). Ongoing improvements in cryopreservation techniques are suggested by recent data from the United States where a 5.9% increase in live birth rates arising from cryopreserved embryo cycles was observed in one year (*Society for assited reproductive technology (SART)* 2002). Moreover, it has been recently demonstrated that when cryopreserved embryos show no blastomere loss, implantation rates are similar to fresh embryos (*Edgar et al.* 2000). Further improvements in selection of embryos for cryo-preservation and cryo-thaw techniques which reduce the rate of blastomere loss will be required in order to further improve outcomes of cryopreservation.

The legal, moral and ethical issues surrounding the cryopreservation of supranumerical embryos (*Shannon* 1990; *Eugster & Vingerhoets* 1999) continue to command attention. The practice of holding embryos cryopreserved for many years, even when the couples to which the embryos belong are lost to contact is also a matter of concern, as illustrated by the vocal public protests aroused when embryo banks are destroyed. Given this background, and the IVF-burden imposed on patients in order to produce sufficient

embryos for cryopreservation, the benefits of crypreservation services should be clear and substantial.

Based on the current findings we conclude that profound ovarian stimulation aimed at producing supranumerical embryos for cryostorage using present techniques is poorly justified.

The demands made on our patients by current stimulation protocols for IVF is illustrated by the substantial number of patients in our study who were unwilling to undergo a second (n = 269) or a third (n = 225) IVF attempt. This issue has been raised previously (*Fauser et al.* 1999) but received little attention. Apparently, a substantial proportion of patients refrain from subsequent IVF attempts, therefore reducing the overall success rate per started IVF treatment. Those couples who already had children were more likely to halt their treatment, confirming a previously described phenomenon (*De Vries et al.* 1999). The need to conceive at least one child may urge couples to accept the continued inconvenience, emotional and physical stress, and mask the effects of the 'IVF burden' from their clinicians (*Shannon* 1990; *Eugster & Vingerhoets* 1999).

Milder ovarian stimulation protocols aimed at a producing a limited number of oocytes for fertilization *in vitro* with less burden for the patient and fewer risks have been proposed (*de Jong et al.* 2000; *Macklon & Fauser* 2001). There are indications that patients favour shorter, more convenient ovarian stimulation protocols even when the chance of conceiving in a given cycle is slightly reduced (*Hojgaard et al.* 2001). However, providing milder stimulation protocols may actually increase cumulative pregnancy rates, since a higher proportion of patients may elect to continue treatment if the first cycle is unsuccessful. Our findings suggest that creating supernumery embryos for cryopreservation is a weak justification for profound ovarian stimulation. We conclude that any reduction in embryos available for cryopreservation arising from the institution of mild ovarian hyperstimulation protocols will have little negative impact on cumulative pregnancy rates.

3.2 Mild ovarian stimulation for IVF – a concept

3.2.1 Introduction

At present, the most commonly practised ovarian stimulation regimen for in-vitro fertilization (IVF) includes pituitary down regulation with a gonadotropin-releasing hormone (GnRH) agonist for at least two weeks followed by the co-administration of high dose exogenous gonadotropins. This so-called 'long protocol' aims to induce ongoing growth of multiple dominant follicles in normo-ovulatory women. The presence of many pre-ovulatory follicles allows for lower efficacy at oocyte pick-up, fertilization in-vitro, embryo culture and implantation. Despite intensive monitoring, a concomitant risk of complications remains, introducing the need for intense ovarian response monitoring. Short-term risks include ovarian hyperstimulation syndrome (OHSS) and higher order multiple pregnancies arising from the transfer of multiple embryos. Potential detrimental effects of stimulating large numbers of follicles on oocyte quality and fertilization rates cannot be ruled out. Moreover, high serum estradiol (E_2) concentrations, which occur as a result of these regimens, may reduce embryo implantation rates (*Simon et al.* 1998). With regard to long term complications, epidemiological studies have yet to clarify a possible association between ovarian hyperstimulation and ovarian cancer (*Shoham* 1994; *Bristow & Karlan* 1996).

No agreement exists regarding the optimal number of oocytes required for IVF. Individual responses to standard treatment vary greatly, but in most cases between 10 and 20 oocytes are obtained. Cryopreservation of supranumerical embryos and transfer in subsequent (unstimulated) cycles is often considered to justify the stimulation of a large number of follicles. Certainly, repeated ovarian stimulation and painful oocyte pick-up procedures may be prevented using cryopreserved embryos. The added value of cryopreservation programs remains debatable however (*Jones et al.* 1997b) and the resulting increase in specific pregnancy rates (i.e.: increased chance of an ongoing pregnancy from a cryo-transfer after a failed transfer) is less than generally perceived. Additionally, cryopreservation of excess embryos gives rise to complex ethical, religious and legal considerations. The possibility of cryopreserving supranumerical oocytes rather than embryos has recently been proposed (*Porcu et al.* 1998) as a means of circumventing these issues.

Given these concerns, current strategies for ovarian hyperstimulation for IVF have been questioned (*Edwards et al.* 1996; *Olivennes & Frydman* 1998; *Fauser et al.* 1999) and novel approaches to ovarian stimulation for IVF deserve consideration.

3.2.2 Natural cycle IVF

In recent years outcomes of IVF using the natural menstrual cycle have improved. Data from several series indicate cancellation rates of 10-30%, egg recovery rates of 75-90%, fertilization rates of 60-80%, implantation rates of between 20-30% and ongoing pregnancy rates per started cycle of 5-15% (*Garcia* 1989; *Lenton et al.* 1992; *Lenton & Woodward* 1993; *Seibel* 1994; *Society for assited reproductive technology (SART)* 1996). While less invasive and less expensive, cancellation rates remain high. As in standard IVF protocols, prevention of the luteinizing hormone (LH) surge may simplify treatment and reduce cancellation rates. The administration of a GnRH antagonist in the late follicular phase may have clinical use in this regard (*Olivennes et al.* 1995). However, intense monitoring still is required and this regimen could only be applied under specific conditions.

3.2.3 The normal menstrual cycle as basis for mild ovarian stimulation for IVF

Healthy, early antral follicles measuring 2-5 mm in diameter are present throughout the entire menstrual cycle (*McNatty et al.* 1983). These follicles will only continue to grow when sufficiently stimulated by rising follicle-stimulating hormone (FSH) concentrations (*Baird* 1990). Due to the demise of the corpus luteum and subsequent decreased estrogen output (*le Nestour et al.* 1993) FSH levels rise at the end of the luteal phase (*Hall et al.* 1992). During the subsequent follicular phase, FSH levels plateau during initial days and are gradually suppressed thereafter by ovarian steroid and inhibin negative feedback. Only those follicles that happen to be at a more advanced stage of maturation gain gonadotropin dependence and will continue to grow when the 'FSH threshold' (*Brown* 1978) is surpassed. The cohort size of healthy early antral follicles recruited during the luteo-follicular transition is around 5 to 10 per ovary (*Pache et al.* 1990; *Gougeon* 1996). The duration of FSH elevation above the threshold, referred to as the 'FSH window', emphasizes the importance of duration of elevated FSH rather than the actual concentration (*Baird* 1987; *Fauser & Van Heusden* 1997). Decremental follicular phase FSH levels restrict the 'FSH window' for which FSH levels remain above the threshold, and appear to be crucial for selection of a single dominant follicle from the recruited

cohort (*van Santbrink et al.* 1995). Single dominant follicle selection can be disrupted by the administration of low doses of exogenous FSH during the mid- to late-follicular phase, effectively preventing a decrease in serum FSH, widening the 'FSH window' and therefore enabling multifollicular development (*Lolis et al.* 1995; *Schipper et al.* 1998).

The median day of appearance of the dominant follicle in normo-ovulatory women is cycle day 7 (*Pache et al.* 1990; *van Santbrink et al.* 1995). Interference with the process of single dominant follicle selection immediately prior to this point may form the basis for a simplified stimulation regimen for IVF. The clinical introduction of GnRH antagonists provides novel opportunities regarding this approach. GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotropin release and a rapid recovery of normal secretion of endogenous LH and FSH (*Ditkoff et al.* 1991). Recent studies have shown that GnRH antagonists, either at single or multiple doses, are effective in preventing a premature LH rise during ovarian stimulation for IVF (*Frydman et al.* 1991; *Diedrich et al.* 1994; *Olivennes et al.* 1994; *Albano et al.* 1996; *The ganirelix dose-finding study group* 1998). Thus a treatment cycle can commence with an undisturbed menstrual cycle and recruitment of a normal cohort of about 10 follicles. Exogenous FSH may therefore be used during the mid- to late-follicular phase to prolong the growth of these follicles up to the preovulatory stage and a GnRH antagonist may be used to avoid a premature LH rise. By making optimal use of endogenous FSH, the amount of exogenous FSH required should be substantially reduced.

In a recent IVF pilot study, such an intervention in the midfollicular phase -designed to extend the duration of the 'FSH window'- resulted in multifollicular development (*de Jong et al.* 1999b). As expected, the number of follicles > 15 mm observed at day of hCG was administered was smaller (3 $^1/_2$ follicles) compared to that observed in 'long protocol' regimens for maximum ovarian stimulation for IVF (7 $^1/_2$ follicles) (*Out et al.* 1996). However, the number of oocytes retrieved was larger (median 6-9) and sufficient embryos were obtained to enable embryo transfer. The total amount of RecFSH administered in this study (700 – 1200 IU) was substantially less than the amount of exogenous FSH usually administered in conventional ovarian stimulation regimens for IVF (around 2200 IU) (*Out et al.* 1996). With a minimal ovarian stimulation regimen described in this study it was possible to obtain pregnancies.

3.2.4 Possible benefits arising from mild stimulation protocols for IVF

While at present still experimental, the described minimal stimulation protocol deserves attention for the following reasons. In addition to a significantly reduced duration of stimulation and amount of exogenous FSH used, less monitoring would be required and the chances of long and short-term risk should be reduced. While fewer oocytes will be available for IVF than in conventional stimulation protocols, the smaller cohort of growing follicles may be better synchronized which may carry advantages in terms of better oocyte quality and capacity to be fertilized and form good quality embryos. In addition, the reduced stimulation may help to avoid adverse effects of hyperstimulation on corpus luteum function and endometrial receptivity (*Simon et al.* 1998).

Following GnRH agonist co-treatment during the follicular phase, luteolysis is initiated earlier due to slow recovery of endogenous pituitary secretion of gonadotropins (*Smitz et al.* 1988b; *Donderwinkel et al.* 1993). Therefore, provision of luteal support is common practice following ovarian stimulation for IVF (*Soliman et al.* 1994). A rapid recovery of pituitary LH-release after cessation of GnRH antagonist administration (*Ditkoff et al.* 1991) might permit abandoning additional luteal support and further simplifying IVF treatment.

However, although in our recent pilot study employing a minimal hyperstimulation protocol pregnancies were obtained without provision of exogenous luteal support (*de Jong et al.* 1999b), the luteal phase differed from that of the normal menstrual cycle. The treated subjects showed supra-physiological progesterone and E_2 concentrations (3 to 4 times higher) and decreased FSH and LH levels (4 to 5 times lower) compared with the normal menstrual cycle controls. It appears unlikely that reduced LH and FSH concentrations in the luteal phase were caused by an absent pituitary recovery from GnRH antagonist treatment. Other non-physiological mechanisms could be involved. Since hCG has a longer half-life than native LH (*Mannaerts et al.* 1998) it may have prolonged effect on the early- to mid-luteal phase, suggesting that hCG itself or an indirect effect of hCG (supraphysiological steroid levels) may suppress pituitary secretion of gonadotropins in the luteal phase. Moreover, it has been demonstrated that activation of LH receptors by hCG abolished pulsatile GnRH release from hypothalamic cells in-vitro (*Mores et al.* 1996).

With the use of GnRH antagonists during the late follicular phase, final stages of oocyte meiotic maturation can be induced by cessation of the GnRH antagonist, recombinant LH (*The european recombinant human LH study group* 1998), native GnRH (*Gordon et*

al. 1990) or GnRH agonist instead of hCG (*Gonen et al.* 1990; *Itskovitz-Eldor et al.* 1991; *Olivennes et al.* 1996). These approaches are at present under investigation and may reduce the risk of OHSS.

Milder forms of ovarian stimulation for IVF are likely to generate fewer embryos. However, improvements in culture conditions is enabling up to 50% of embryos to reach the blastocyst stage, whereby implantation rates per embryo of 30-50% have been reported (*Gardner & Lane* 1998). Transfer of 1 or 2 rapidly developing embryos at the blastocyst stage allows the incidence of multiple pregnancy to be reduced without causing a fall in overall pregnancy rates (*Scholtes & Zeilmaker* 1996). Indeed, the transfer of just one good quality embryo may now be considered a realistic approach (*Vilska et al.* 1998).

In conclusion, the prospect of minimal ovarian stimulation protocols for IVF offers women the possibility of 'softer', less expensive and more user friendly IVF treatments, low complication rates, and perhaps most importantly, high singleton pregnancy rates while avoiding multiple gestations and presumably reduced complication rates. However, further studies are required to establish its clinical efficacy.

3.3 Mild ovarian stimulation for IVF, a pilot study

3.3.1 Introduction

The concept of the 'FSH window', emphasizes the importance of duration of elevated FSH rather than the actual concentration for follicle recruitment. The follicular phase decrease in serum FSH levels, which secures single dominant follicle selection in normo-ovulatory women, can be prevented by the administration of low dose exogenous FSH in the mid to late follicular phase (*van Santbrink et al.* 1995; *Fauser & Van Heusden* 1997). Such an intervention in normo-ovulatory women results in ongoing growth of multiple dominant follicles (*Schipper et al.* 1998). The recent availability of GnRH antagonists for acute suppression of a premature rise in luteinizing hormone (LH) enables this concept to be tested clinically in IVF. The use of GnRH antagonists allows normal undisturbed initiation of the menstrual cycle for IVF.

In this pilot-study we investigated whether it is possible to induce multiple dominant follicle development by instituting low doses of exogenous FSH from the mid-follicular phase onwards and whether such a regimen (including GnRH antagonist administration) might result in pregnancies in the absence of subsequent exogenous luteal support.

3.3.2 Materials and methods

Approval of the study protocol was given by the Local Ethics Review Committee. Informed consent was obtained from all participants. A total of 15 normo-ovulatory women under 38 years of age enrolled in our IVF program, were included in this prospective randomized pilot-study. Subjects were studied during a single IVF treatment cycle. In 3 patients with an insufficient response (defined as < 3 follicles $\geq$ 15 mm) to ovarian stimulation were excluded from analysis. Data obtained from 40 regular cycling women, as published previously (*Fauser & Van Heusden* 1997), served as controls.

Prior to ovarian stimulation, subjects were randomly (sealed envelopes) assigned to one of the two stimulation regimens. Patients received either 100 IU (group A) or 150 IU (group B) of recombinant FSH (RecFSH, Puregon®, N.V. Organon, Oss, The Netherlands) as a daily s.c. injection, from cycle day (CD) 5 onwards until the day human chorionic gonadotropin (hCG) was administered. Participants in both groups were co-treated with a GnRH antagonist (cetrorelix,

Cetrotide®, Asta Medica AG, Frankfurt, Germany) 0.25 mg s.c./day from CD 8 onwards when at least one follicle of 13 mm was present. Otherwise, Cetrorelix administration was postponed. When at least one follicle ≥ 18 mm and 3 follicles ≥ 15 mm were observed on transvaginal ultrasound (TVS), 10.000 IU hCG (Pregnyl®, N.V. Organon) was administered by i.m. injection to trigger final stages of oocyte maturation. No luteal phase support was provided.

Oocyte collection took place 36 hours after hCG administration. A maximum of 2 embryos was transferred on day 3 to 5 after oocyte retrieval. Repeated bloodwithdrawal took place during the entire cycle. When no pregnancy occurred, the time of onset of the next menstruation was documented. Viable pregnancy was defined as a positive intrauterine fetal heart activity observed by TVS 5-6 weeks after embryo transfer.

Blood samples were centrifuged within 2 h after withdrawal and stored at – 20°C until assayed. Luteinizing hormone (LH) and FSH levels were measured by IRMA. Progesterone (P) and E_2 levels were assessed by RIA. Intra- and interassay coefficients of variation were less than 3% and 8% for FSH, less than 5% and 15% for LH, less than 16% and 17% for P, and less than 15% and 18% for E_2, respectively. All samples from one subject were run in the same assay.

Potential differences between groups in patients age, total amount of FSH administered, number of follicles at day of hCG, number of oocytes retrieved, and number of embryos obtained were analyzed using the Mann-Whitney test, as were differences in LH, FSH, E_2, and P. Potential differences in cancellation rate and pregnancy rate were analyzed using Fisher's exact test. Differences were considered to be statistically significant if $P < 0.05$.

3.3.3 Results

Patient characteristics, endocrine assessments, and treatment outcome of patients during the intervention cycle are shown in Table 3.2. Three patients in the 100 IU group (exhibiting less than 3 follicles of ≥ 15 mm) were excluded from data analysis. Oocytes were retrieved from all remaining patients (n = 12). No embryos were obtained in 3 subjects. Three patients achieved a viable pregnancy. The total amount of RecFSH administered in the 100 IU group was significantly less as compared to the 150 IU group (700 vs. 1200, $p < 0.005$).

	IVF patients		Controls
	100 IU RecFSH/day	150 IU RecFSH/day	
No. of subjects undergoing ovum pick-up	5	7	40
Age (years)	34 (25-37)	30 (27-37)	28 (20-36)
Follicular phase			
Duration follicular phase (days)	12 (12-14)	14 (12-16)	15 (10-20)
Total dose of RecFSH (IU)	700 (700-900)	1200 (900-1500)	
FSH 1 day pre hCG (IU/L)	5.7 (2.7-6.6)	7.6 (5.7-11.1)	3.8 (1.8-6.3) [a]
LH 1 day pre hCG (IU/L)	2.4 (0.6-3.0)	1.5 (1.2-2.6)	4.8 (1.3-10.3) [a]
E_2 day hCG (pmol/L)	3248 (1257-4773)	2899 (1378-8476)	820 (370-1525) [b]
No. of follicles > 10 mm on day hCG administration	7 (5-11)	10 (3-19)	
Clinical outcome			
No. of oocytes retrieved per person	6 (3-10)	9 (1-19)	
No. embryos obtained per person	3 (2-8) [c]	3 (1-13) [d]	
No. of pregnancies	2 (2/4) [e]	1 (1/5) [f]	
Luteal phase			
Duration luteal phase (days)	12 (11-14) [g]	13 (11-21) [h]	12 (5-16)
E_2 5-7 days post hCG (pmol/L)	1822 (939-3094) [i]	1641 (898-3400) [i]	449 (253-940)
P 5-7 days post hCG (nmol/L)	221.1 (96.3-409.2) [i]	179.0 (65.8-340.0) [i]	43.7 (11.7-74.9)
FSH 5-7 days post hCG (IU/L)	0.4 (0.3-1.6) [i]	1.1 (0.5-2.2) [i]	2.5 (0.7-7.1)
LH concentration 5-7 days post hCG (IU/L)	0.7 (0.2-0.7) [i]	1.0 (0.1-2.7) [i]	3.7 (0.2-12.1)

Table 3.2
Patient characteristics, steroid and gonadotropin levels, and treatment outcome of 12 IVF patients during minimal ovarian stimulation and normo-ovulatory controls.
Values are median (range), unless otherwise indicated. [a] One day before the spontaneous LH surge, [b] at day of the spontaneous LH surge, [c] no embryos obtained in 1 subject, [d] no embryos obtained in 2 subjects, [e] 2 out of 4 patients who underwent embryo transfer conceived, [f] 1 out of 5 patients who underwent embryo transfer conceived, [g]duration of the luteal phase for all subjects who did not conceive, including one without an embryo transfer (n = 3), [h] duration of the luteal phase for all subjects who did not conceive, including those without an embryo transfer (n = 6), and [i] in non-conceiving patients.

No statistical difference was found in LH and FSH concentrations between the low and high dose group (data not shown). In both groups, LH serum concentrations fell to low levels during GnRH antagonist exposure compared to controls (< 2.0 IU/L), while FSH serum concentrations remained steady (± 6 IU/L) during the follicular phase. Treated subjects of both groups combined showed elevated FSH and suppressed LH levels 1 day before hCG compared to the natural cycle (6.5 and 1.9 vs 3.8 and 4.8 IU/L, p <

0.0001, respectively). There were no significant differences in follicle growth between the low and high dose group.

Significantly lower luteal phase LH and FSH concentrations were observed 5-7 days after hCG administration compared to the natural cycle (0.7 and 0.8 vs. 3.7 and 2.5 IU/L, $p < 0.0001$, respectively). No statistical difference was found in serum E_2 and P concentration between the low and high dose group at any stage during this regimen. However, when compared to the control group, treated subjects of both groups combined showed elevated E_2 serum levels on day of hCG and 5-7 days post hCG (2959, 1641 vs. 847, 449 pmol/L, $p < 0.0001$, respectively), and elevated P serum levels 5-7 days post hCG (180 vs. 44 nmol/L, $p < 0.05$).

The median duration of the luteal phase (number of days between day of hCG and onset of menstruation) in the patients who did not conceive was 12 days (10-20 days) for the treated patients compared to 12 days (5-16 days) for the controls.

3.3.4 Discussion

The aim of the present study was to determine if a minimal intervention during the mid- to late-follicular phase - designed to extend the duration of the 'FSH window'- results in multiple dominant follicle development sufficient for IVF.

While the mean number of follicles > 15 mm observed on the day of hCG administration seems smaller than that observed in the standard long GnRH agonist protocols for IVF, a median number of 6-9 oocytes were retrieved and a mean of 3 embryos were generated. In 3 patients receiving 100 IU/d RecFSH, cycles were cancelled due to low response, suggesting that the FSH 'threshold' of remaining cohort follicles was not surpassed in these patients. In the remaining 5 subjects receiving 100 IU/d, ovarian response was similar compared to the 150 IU/d group. The total amount of RecFSH administered in this study (700 – 1200 IU) was substantially less than the amount of exogenous FSH usually administered in conventional ovarian stimulation regimens for IVF (around 2200 IU).

Late follicular phase E_2 concentrations were lower compared to conventional ovarian stimulation regimens for IVF, but $3^1/_2$ times higher compared to the natural cycle consistent with multiple dominant follicular development. During the luteal phase E_2 and P levels were supraphysiological while LH and FSH levels were extremely low. The GnRH antagonist was an unlikely causative factor for the observed luteal gonadotropin suppression since its cessation in the follicular phase leads to a rapid recovery of the pituitary-gonadal axis (*Ditkoff et al.* 1991). Other possibilities include ovarian

hyperstimulation per sé or profound negative feedback due to high luteal phase steroid levels. The duration of the luteal phase in the treated patients was comparable with the controls despite that no luteal support was provided, suggesting that the use of GnRH antagonists during ovarian stimulation for IVF may not compromise subsequent corpus luteum function (*Ditkoff et al.* 1991).

Using the described minimal ovarian stimulation regimen it was possible to obtain pregnancies despite withholding luteal support. However, the number of patients included in this pilot study is too small for meaningful conclusions regarding clinical outcome. This study suggests that a distinct simplification of ovarian stimulation for IVF is feasible. This alternative approach deserves further attention with the focus towards overall clinical outcome versus patient dyscomfort, risks, and cost.

Chapter 4

GnRH agonist for induction of final oocyte maturation

4.1 GnRH agonist treatment for triggering the final stages of oocyte maturation in IVF, a case report

4.1.1 Introduction

In the normal menstrual cycle, the endogenous midcycle luteinizing hormone (LH) surge induces resumption of oocyte meiosis and dissociation of the cumulus which leads to rupture of the follicular wall and ovulation of the oocyte from the Graafian follicle (*Hoff et al.* 1983).

In conventional in vitro fertilization (IVF) programs, gonadotropin-releasing hormone (GnRH) agonists are used for pituitary 'down-regulation' in order to prevent a premature LH surge. Because of its LH-like properties, human chorionic gonadotropin (hCG) is given to induce final oocyte maturation. However, the increased carbohydrate content of hCG in comparison to LH results in a longer circulating half-life and a longer biological effect (*Moyle et al.* 1975).

In contrast to GnRH agonists, GnRH antagonists induce an immediate suppression of pituitary gonadotropin release and a rapid recovery of normal secretion of endogenous LH and follicle stimulating hormone (FSH) (*Ditkoff et al.* 1991). These analogues have recently been developed for the prevention of premature LH surges during ovarian stimulation for assisted reproduction (*Albano et al.* 1997; *Olivennes et al.* 1998a; *The ganirelix dose-finding study group* 1998; *de Jong et al.* 2000). A GnRH antagonist regimen offers the opportunity to trigger the final maturation of the oocyte with pulsatile native GnRH administration (*Gordon et al.* 1990) or with a single dose of a GnRH agonist (*Gonen et al.* 1990). Indeed, it has been reported that the midcycle FSH and LH surge could thus be simulated in patients co-treated with a GnRH antagonist, undergoing ovarian hyperstimulation for intra-uterine insemination (*Olivennes et al.* 1996). Therefore, triggering of final stages of oocyte maturation may be induced by administration of a GnRH agonist in patients undergoing ovarian stimulation and co-treated with a GnRH antagonist to prevent a possible LH surge for IVF.

This case report describes the characteristics of a woman who underwent ovarian stimulation for intracytoplasmic sperm injection (ICSI) co-treated with a GnRH antagonist and a single bolus of GnRH agonist for triggering the final stages of oocyte maturation, resulting in the birth of a healthy girl.

4.1.2 Case Report

A 28-year old normo-ovulatory woman (body mass index 27.5 kg/m^2) with an subfertility duration of 4^1/$_2$ years due to male factor underwent ovarian stimulation for ICSI in our unit. Before initiation of treatment, endocrine screening and sonographic examination appeared to be normal. The patient participated in a multi-centre trial, approved by the local ethics review committee. Ovarian stimulation using 150 IU recFSH (Puregon®, N.V. Organon, Oss, the Netherlands) daily subcutaneous injections was started on cycle day (CD) 2. From CD 7 onwards, daily subcutaneous injections of 0.25 mg GnRH antagonist (ganirelix; Orgalutran®/Antagon™, N.V. Organon) (*Nelson et al.* 1995) were started. On CD 12 this patient showed 3 preovulatory ($\geq$ 17 mm), and 9 intermediate sized (10 - 16 mm) follicles on transvaginal ultrasound sonography. According to the protocol, after randomization, a bolus injection of 0.5 mg GnRH agonist leuprorelin (Lupron®, Abbott Laboratories, Montreal, Canada) was administered subcutaneously. Subsequently, blood samples were taken frequently after the GnRH agonist was administered, on the day of oocyte pick-up, on the day of embryo transfer (ET), and 7 and 14 days thereafter.

Serum concentrations of LH, FSH, estradiol (E_2), and progesterone (P) are depicted in Figure 4.1. In the late follicular phase LH serum levels were 0.6 IU/L, whereas FSH serum levels remained steady at 5 IU/L. During the follicular phase, increasing E_2 (reaching 588 pg/mL) and steady P levels of 0.5 ng/mL were observed. After administration of the GnRH agonist leuprorelin an increase in gonadotropin levels was observed, reaching a maximum of 103 and 12.2 IU/L for LH and FSH, respectively, at 8 hours after GnRH agonist administration.

Ovum pick-up (OPU) resulted in 11 metaphase II oocytes and 1 germinal vesicle. Subsequently, the metaphase II oocytes were fertilized by ICSI. As per the center's current practice, embryo culture was performed to day 5 after ovum pick-up (*Huisman et al.* 2000). Two blastocyst-stage embryos were transferred and 3 were cryopreserved. Daily intramuscular injections of 50 mg P were provided as luteal phase support from the day the oocytes were transferred. Analyses of blood samples drawn in the luteal phase up to 14 days post-ET resulted in decreasing FSH and LH serum to levels which were below the limit of detection for their assays (< 0.6 and < 1.0 IU/L, respectively). From OPU up to 7 days post-ET, a decrease in E_2 levels (from 161 pg/mL to 45.4 ng/mL) was observed, followed by an increase to 126 pg/mL 14 days after ET. P levels increased from 2.61 at OPU to 36.7 ng/mL at 7 days post-ET, followed by a decrease to 16 ng/mL at 14 days after ET.

A viable intra-uterine twin-pregnancy was observed at 8 and 12 weeks amenorrhoea on ultrasound, which spontaneously reduced to

a singleton pregnancy at 14 weeks amenorrhoea. After a non-complicated pregnancy and spontaneous vaginal delivery, this patient gave birth to a healthy girl at 40 weeks amenorrhoea.

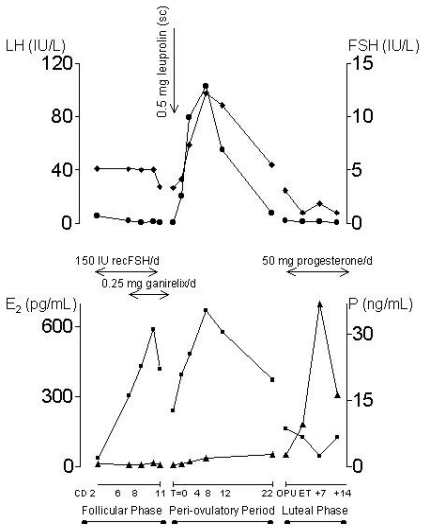

Figure 4.1
LH (●-●), FSH (◆-◆) (top panel), E_2 (■-■), and P (▲-▲) (bottom panel) serum levels in a single patient undergoing ovarian stimulation for IVF, using the GnRH antagonist ganirelix to prevent a premature LH surge. Recombinant FSH (recFSH) was initiated on cycle day (CD) 2, GnRH antagonist on CD7. Final stages of oocyte maturation were induced by a single bolus of GnRH agonist on CD12 (t=0h). Monitoring during the peri-ovulatory period was performed at 2, 4, 8, 12, and 20-24 hours after the GnRH antagonist was administered. Luteal phase blood samples were withdrawn on the day of ovum pick-up (OPU), on the day of ET, 7 days after ET, and 14 days after ET.

4.1.3 Discussion

The routinely applied 'long protocol' for IVF, characterized by pituitary 'down-regulation' by a GnRH agonist and ovarian hyperstimulation by exogeneously administered gonadotropins, may lead to undesired complications such as the development of ovarian hyperstimulation syndrome (OHSS) (*Itskovitz-Eldor et al.* 1991). Alternative strategies for ovarian stimulation for IVF are needed (*Edwards et al.* 1996). These include the application of GnRH antagonists for avoiding a premature LH rise and the use GnRH agonists, native GnRH, and recombinant LH for induction of final stages of oocyte maturation (*Fauser et al.* 1999).

The availability of GnRH antagonists may offer alternative

strategies for countering imminent OHSS (*de Jong et al.* 1998). Replacing hCG with a single bolus of GnRH agonist for final oocyte maturation may prevent OHSS and permit continuation of the treatment cycle (*Itskovitz-Eldor et al.* 1991). This possibility arises because the pituitary is not 'down-regulated' and the GnRH receptors are still present. The efficacy of triggering final oocyte maturation and ovulation with GnRH agonists has been demonstrated in women undergoing ovarian stimulation with (*Olivennes et al.* 1996) or without (*Romeu et al.* 1997) a GnRH antagonist followed by intra uterine insemination. The present case describes the successful treatment of a woman who underwent ovarian stimulation for ICSI using a GnRH antagonist protocol and a single bolus of a GnRH agonist to trigger the final stages of oocyte maturation. A healthy baby was delivered at 40 weeks amenorrhoea.

The use of GnRH agonists for triggering final stages of oocyte maturation instead of hCG may further reduce the incidence of OHSS, reduce cancellation rates, and result in a safe treatment of high responders. Further controlled studies are required to assess whether such a regimen should be applied in daily routine practice.

4.1 GnRH agonist treatment for triggering the final stages of oocyte maturation in IVF, a randomized controlled trial

4.1.1 Introduction

Human chorionic gonadotropin (hCG) represents the standard of care for the substitution of the endogenous luteinizing hormone (LH) surge to induce final stages of oocyte maturation in ovarian hyperstimulation protocols for in vitro fertilization (IVF). Unfortunately, hCG is also believed to contribute to the occurrence of the ovarian hyperstimulation syndrome (OHSS), a potentially life threatening complication (*Elchalal & Schenker* 1997; *Fauser et al.* 1999; *Whelan & Vlahos* 2000). Exogenous hCG is implicated in the development of multiple corpora lutea and sustained luteotropic effects due to its prolonged circulating half-life as compared to native LH (*Damewood et al.* 1989; *Gonen et al.* 1990; *Beckers et al.* 2000). Moreover, the occurrence of OHSS is associated with continued late luteal phase hCG production in case of pregnancy. An alternative to exogenous hCG could be the administration of a gonadotropin-releasing hormone (GnRH) agonist inducing an endogenous rise in both LH and follicle-stimulating hormone (FSH) levels due to the initial flare effect (*Gonen et al.* 1990; *Itskovitz-Eldor et al.* 1991). The capacity of GnRH agonists to trigger ovulation following gonadotropin treatment of anovulatory women (*Emperaire & Ruffie* 1991; *Kulikowski et al.* 1995) or to induce final stages of oocyte maturation after ovarian hyperstimulation for IVF (*Gonen et al.* 1990; *Itskovitz-Eldor et al.* 1991; *Imoedemhe et al.* 1991; *Segal & Casper* 1992; *van der Meer S. et al.* 1993) has been well established. The induction of an endogenous LH (and FSH) surge is more physiological compared to the administration of exogenous hCG and may reduce the risk of OHSS due to the much shorter half-life of LH (*Imoedemhe et al.* 1991; *Shoham et al.* 1995; *Kol et al.* 1996). Moreover, under these conditions luteal phase steroid levels seem closer to normo-ovulatory cycles (*Lanzone et al.* 1994) which may improve endometrial receptivity (*Forman et al.* 1988; *Simon et al.* 1998). Effects on oocyte quality and chances for subsequent fertilization remain uncertain at this stage.

For obvious reasons, GnRH agonist to induce oocyte maturation can only be used when GnRH agonist has not been applied for pituitary down regulation to prevent a premature LH rise during ovarian stimulation. Since the GnRH agonist 'long protocol' has been the standard of care for over a decade, alternative approaches to induce oocyte maturation has received little attention in

recent years. The mid to late follicular phase administration of a GnRH antagonist may also be used to prevent a premature LH rise during ovarian hyperstimulation. The recently introduced GnRH antagonist ganirelix (Orgalutran®, Antagon™) competes with native GnRH in binding to its receptor resulting in the immediate and dose-dependent suppression of endogenous gonadotropin release (*Oberye et al.* 1999a; *Oberye et al.* 1999b). In previous trials, the daily administration of 0.25 mg ganirelix has been shown to be safe and effective (*The ganirelix dose-finding study group* 1998) requiring on average 5 days of treatment and a reduced total dose of recombinant FSH compared to a long GnRH agonist protocol (*Bouchard & Fauser* 2000; *The european orgalutran study group et al.* 2000; *The european and middle east orgalutran study group* 2001; *The north american ganirelix study group* 2001). hCG has been used to trigger final oocyte maturation in these first studies applying GnRH antagonist. However, in contrast to GnRH agonist, the suppressive effect of the GnRH antagonist can be reversed immediately by administering native GnRH or GnRH agonist, resulting in a surge of endogenous LH and FSH. This new concept of triggering final oocyte maturation after GnRH antagonist treatment was successfully tested in 5 patients undergoing ovarian hyperstimulation for intra uterine insemination using a single dose of 0.1 mg triptorelin (*Olivennes et al.* 1996). No information on the maturity of oocytes could be obtained in this first pilot study. In addition, a single dose of 0.2 mg triptorelin was effective in triggering an endogenous LH surge and final oocyte maturation in 8 high responder IVF patients co-treated with ganirelix (*Itskovitz-Eldor et al.* 2000). None of these patients developed OHSS.

The current randomized study was designed to examine whether, following daily late follicular phase treatment with 0.25 mg ganirelix, administration of a single dose of GnRH agonist is at least as effective compared to hCG in inducing final oocyte maturation in patients undergoing ovarian hyperstimulation for IVF. Here we report on the endocrine profile and clinical outcome of the first subset of patients in whom bloodsampling was performed at regular intervals after the administration of triptorelin, leuprolide, or hCG.

4.1.2 Materials and methods

Subjects

The first 57 out of 200 subjects participating in this comparative randomized trial signed informed consent which included hospitalization to monitor hormonal changes after GnRH agonist was given to trigger final oocyte maturation. All subjects underwent ovarian hyperstimulation with recombinant FSH for IVF plus

intracytoplasmatic sperm injection (ICSI) allowing for the assessment of oocyte maturation (metaphase II stage). Subjects had a regular indication for ICSI, were between 18 and 39 years of age, had a regular menstrual cycle (24 – 35 days) and body weight was normal (body mass index 18-29 kg/m^2).

Study Design

Six international centers participated in this study and the protocol was approved by the local ethics review committees. This study was an open-label randomized three-arm trial in subjects who started ovarian hyperstimulation with recombinant FSH (Puregon®, NV Organon, The Netherlands) starting at cycle day 2 or 3 and continued treatment until the day of inducing final oocyte maturation (Figure 4.2). The FSH dose was 150 or 225 IU/d s.c. for the first 5 treatment days. Thereafter, the daily dose could be adjusted based on the follicular growth as observed by ultrasound. To prevent premature LH surges, ganirelix (Orgalutran® / Antagon™; NV Organon) (0.25 mg in 0.5 mL daily) was administered s.c. starting on day 6 of FSH stimulation until and including the day of triggering final oocyte maturation. On that day, randomization was performed by means of an interactive telephone randomization system which stratified for age, primary or secondary infertility and number of follicles at least 11 mm in diameter. Subjects were randomized in a ratio of 1:1:1 to treatment with either 0.2 mg triptorelin (Decapeptyl®, Ferring, Germany), or 0.5 mg leuprorelin (Lupron®, TAP Pharmaceuticals Inc., USA), or 10,000 IU hCG (Pregnyl®, NV Organon). Subjects were only randomized if at least 3 follicles ≥ 17 mm were observed. High responders (defined as having more than 25 follicles beyond 11 mm) were considered drop outs from the study. Approximately 30 to 36 h after triggering of final oocyte maturation, oocyte retrieval was performed followed by ICSI. No more than 3 embryos were transferred at 2 to 5 days after oocyte retrieval. Exogenous P was administered intramuscularly for luteal support (Progestine®, NV Organon) (50 mg in 2 mL daily) from the day of embryo transfer for at least two weeks or until menses.

Assessments

Prior to the start of ovarian stimulation, pregnancy was excluded by means of an hCG test, a blood sample was taken for hormone assessments and ultrasound was performed. The subject returned to the clinic at least once every two days for ultrasound investigation and blood sampling from day 6 of stimulation until the day of triggering of final oocyte maturation. Blood sampling was performed prior to ganirelix and FSH administration. On the day of triggering final oocyte maturation, blood samples were taken just prior to GnRH agonist or hCG administration, at 2, 4, 8, 12 and 24 hours after GnRH agonist

injection and at 12 and 24 hours after hCG injection. Additional blood samples of all subjects were taken on the day of oocyte pick-up, embryo transfer (ET) and at 1 week and 2 weeks after ET. HCG concentrations were not assessed at 4 and 8 hours since previous pharmacokinetic studies (*Mannaerts et al.* 1998) demonstrated a t_{max} at 20 hours.

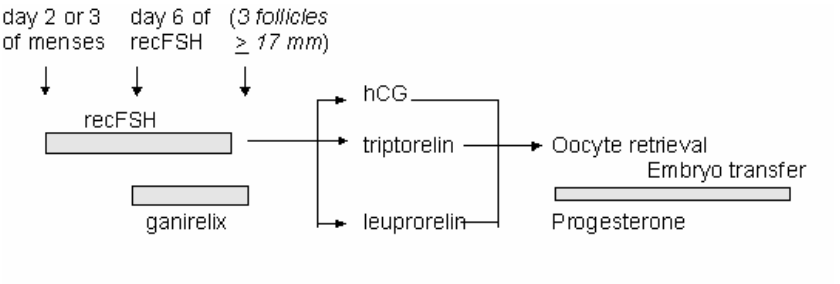

Figure 4.2
Schematic representation of different treatment regimens for ovarian hyperstimulation for IVF using recombinant FSH and ganirelix to prevent a premature rise in LH followed by the triggering of final oocyte maturation by a single dose of two GnRH agonists (triptorelin or leuprorelin) or hCG.

Serum FSH, LH, E_2, hCG, and P levels were assessed by a central laboratory using fluoroimmunoassays (Delfia®, Wallac OY, Finland). Detection limits of these assays were < 1 IU/L for FSH, < 0.6 IU/L for LH, < 14 pg/mL for E_2, < 2.0 IU/L for hCG, and < 0.3 ng/mL for P. Intra- en interassay coefficients of variation were 1.6 and 4.1 % for FSH, 11.0 and 13.0 % for LH, 7.8 and 10.3 % for E_2, 6.9 and 9.3% for hCG, and 5.0 and 6.9 % for P, respectively.

Embryo quality was assessed as; grade 1 (excellent embryos with absent fragmentation), grade 2 (1-20% fragmentation), grade 3 (21-50% fragmentation) or grade 4 otherwise.

Statistical Methods
The primary objective of this study was to compare endocrine characteristics of both GnRH agonist groups versus hCG to induce final oocyte maturation. Relevant data of leuprorelin, triptorelin and hCG have been statistically compared using analysis of covariance (ANCOVA) with center and treatment as factors and endocrine or ultrasound features as covariate. In case the overall treatment factor was statistically significant (i.e. the three treatment groups differ statistically significant) pairwise comparisons were made between hCG and leuprorelin or triptorelin, respectively. These pairwise comparisons were performed using the same analysis of covariance.

Individual areas under the curve (AUC) were calculated using the linear trapezoidal rule. For LH and FSH the concentrations obtained on the day of triggering final oocyte maturation up to 24 h after GnRH agonist or hCG injection were included in the AUC. For E_2 and P the applied timeframe was from the day of triggering of final oocyte maturation until 2 weeks after ET. To correct for possible differences in the timeframes each AUC value was divided by the actual timeframe, resulting in individual estimates of the mean hormone concentration over time. Descriptive statistics were calculated for these derived concentrations per treatment (Table 4.1) and a one way ANCOVA was performed to compare the treatment effects of both leuprorelin and triptorelin versus hCG. In case the ANCOVA showed statistically significant differences between the three treatment groups, two sample t tests were performed to compare both leuprorelin and triptorelin versus hCG, respectively.

Preliminary clinical outcome parameters are described for all randomized patients (using descriptive statistics unadjusted for center) showing no major differences (Table 4.2). Definitive statistical evaluation will be performed separately on the extended series of women (as described earlier under subjects).

P values less then 0.05 were considered to represent statistically significant differences.

4.2.3 Results

Disposition and cancellations

A total of 57 subjects started treatment with FSH of whom 47 were randomized. Eight subjects (14%) were not randomized due to insufficient ovarian response to stimulation. Two subjects were not randomized due to high response. One subject in the hCG group did not undergo ET due to a fertilization failure.

Characteristics of randomized subjects

The overall mean age was 30.4 ± 4.2 years, height 1.67 ± 0.07 m, weight 64.6 ± 7.7 kg, and body mass index 23.3 ± 2.5 kg/m^2. The vast majority of subjects (98%) participating in this study were Caucasian. The three treatment groups were similar with respect to age, height, weight, and body mass index (data not shown).

The total amount of exogenous FSH required (1,579±395, 1,665±455, 1,605±544 IU [mean ± SD] for triptorelin, leuprorelin and hCG, respectively), the duration of FSH stimulation (8.6±1.1, 9.2±1.6, 9.3±2.0 days), the duration of ganirelix treatment (4.6±1.1, 5.1±1.7, 5.3±2.0 days) were similar (*P* = NS, for all these pre-randomization parameters) in the three treatment groups. The overall median

(range) duration of stimulation was 9 (6-14) days and the median total amount of exogenous FSH administered was 1,500 (900-2,625) IU. The median duration of ganirelix treatment was 5 days (range 2-10). The mean number of follicles ≥ 11 mm per treatment group assessed on day 1, 6, and 8 of stimulation, and on the day of randomization for induction of final oocyte maturation are presented in Figure 4.3. On the day of the last ultrasound investigation before triggering of final oocyte maturation the mean number of follicles ≥ 17 mm was 4.8 ± 2.2, 4.6 ± 1.8, and 4.3 ± 1.4 (*P* = NS) for the triptorelin, the leuprolide, and the hCG group, respectively.

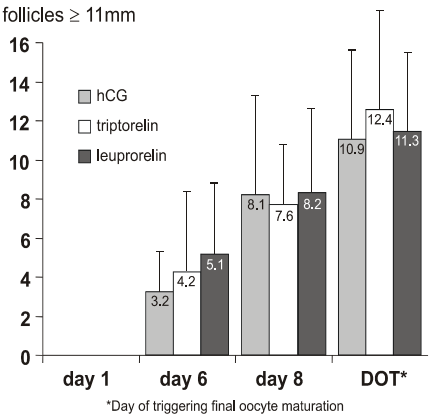

Figure 4.3
Number of follicles ≥ 11 mm (mean ± SD) assessed on stimulation day 1, 6, 8, and on the day of triggering of final oocyte maturation (by either triptorelin, leuprorelin or hCG) during ovarian hyperstimulation for IVF with exogenous FSH.

Serum hormone levels

Serum concentrations of LH, FSH, E_2 and P measured at regular intervals after triggering final oocyte maturation up to 2 weeks after embryo transfer are presented in Figure 4.4. An endogenous LH rise was observed in all subjects who received either triptorelin or leuprorelin. In both treatment groups, serum LH levels rapidly increased and reached peak levels at 4 hrs after GnRH agonist administration. Mean (± SD) LH levels increased from 0.9 ± 0.4 IU/L prior to triggering oocyte maturation to peak levels of 130 ± 60 IU/L after triptorelin administration (P < 0.001) and from 0.9 ± 0.8 to 107 ± 55 IU/L after leuprorelin administration (P < 0.001). On the day of oocyte pick-up LH levels returned to baseline; 4.8 ± 2.5 in the triptorelin group and 2.6 ± 0.4 IU/L in the leuprorelin group. In subjects randomized to hCG treatment, serum hCG levels increased gradually and reached peak levels 240 ± 101 IU/L at 24 hrs after administration. Thereafter, serum hCG levels declined to 5.0 ± 1.6

IU/L one week after ET. Following hCG administration, serum LH remained low or undetectable during the entire luteal phase. In contrast to hCG treated subjects, FSH levels increased after GnRH agonist administration. FSH levels increased from 5.8 ± 1.6 IU/L prior to induction of final oocyte maturation to 19.2 ± 5.2 IU/L at 8 hrs after GnRH agonist administration in the triptorelin group. In the leuprorelin group these levels increased from 5.2 ± 1.6 to 19.7 ± 5.1 IU/L ($P < 0.001$ for both groups), whereas in the hCG group serum FSH levels declined after hCG administration from 5.8 ± 1.6 IU/L to 3.4 ± 0.8 IU/L ($P < 0.001$) on the day of oocyte pick up, reflecting the clearance of exogenous FSH. The individual AUCs for LH and FSH within the first 24 hours are depicted in Fig 4.4, confirming the major difference comparing both GnRH agonist groups versus hCG.

Serum E_2 and P levels were comparable from the time of triggering ovulation until oocyte pick-up (see Figure 4.4). Thereafter, serum E_2 and P levels remained higher in hCG treated subjects compared to both GnRH agonist groups. Respective mean (±SEM) E_2 levels were 279±48, 204±30, and 609±115 pg/mL on the day of embryo transfer ($P_{ANCOVA} = 0.0002$; $P_{triptorelin\ vs\ hCG} = 0.0006$; $P_{leuprorelin\ vs\ hCG} = 0.0001$), and 46±4, 45±9, and 490±145 pg/mL on week thereafter ($P_{ANCOVA} = 0.0001$; $P_{triptorelin\ vs\ hCG} = 0.0001$; $P_{leuprorelin\ vs\ hCG} = 0.0001$) in triptorelin, leuprorelin and hCG treated subjects, respectively. Mean P levels were 7.2 ± 1.7, 8.0 ± 1.5, and 58.6 ± 9.6 ng/mL on the day of transfer ($P_{ANCOVA} = 0.0001$; $P_{triptorelin\ vs\ hCG} = 0.0001$; $P_{leuprorelin\ vs\ hCG} = 0.0001$) and 18.0 ± 3.6, 23.2 ± 3.7, 45.9 ± 11.2 ng/mL one week later ($P_{ANCOVA} = 0.0006$; $P_{triptorelin\ vs\ hCG} = 0.0002$; $P_{leuprorelin\ vs\ hCG} = 0.0054$). At the end of the luteal phase, levels of both hormones were comparable in the three treatment groups. The luteal phase AUCs (Table 4.1) confirmed elevated E_2 and P levels in the hCG group.

Clinical outcome

The clinical outcome (number of oocytes retrieved, percentage of metaphase II oocytes, fertilization rates, embryo quality and implantation rates) is presented in Table 4.2. The number of miscarriages within the first 12 weeks after transfer was 3, 2 and 3 in the triptorelin, leuprorelin and hCG treated subjects, respectively.

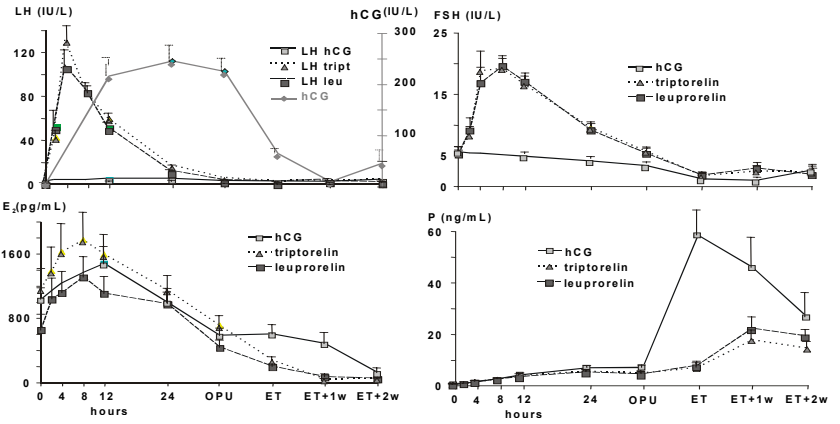

Figure 4.4
Serum concentrations of LH (hCG), FSH, E_2 and P (mean ± SEM) during triggering of final stages of oocyte maturation (0, 4, 8, 12, and 24h) with two GnRH agonist (triptorelin, leuprorelin) or hCG following ovarian hyperstimulation for IVF and during the subsequent luteal phase (day of ovum pick up [OPU], embryo transfer [ET], and 1 and 2 weeks following embryo transfer).

	Triptorelin (n=17)	Leuprolin (n=15)	hCG (n=15)
$LH_{(0\text{-}24h)}$ (IU/L)[a]	59±14[b]	53±14[b]	2.7±2.2
$FSH_{(0\text{-}24h)}$ (IU/L)[a]	14.5±3.8[b]	14.3±3.7[b]	5.0±1.2
$E_{2\ (0\text{-}2\ wks)}$ (pg/mL)[a]	252±154[b]	196±111[c]	515±286
$P_{(0\text{-}2\ wks)}$ (ng/mL)[a]	12.5±7.7[b]	15.2±7.1[b]	37.8±17.1

Table 4.1
Area under the curve divided by the actual time period for serum hormone concentrations (mean + SD) during triggering of final oocyte maturation with the GnRH agonists (triptorelin and leuprorelin) or hCG following ovarian hyperstimulation for IVF (0-24h for LH and FSH) and during the subsequent luteal phase (0-2 weeks for E_2 and P).
[a] $P_{ANCOVA} < 0.001$ comparing all three groups.[b] $P = 0.0001$ vs hCG group (paired t test).[c] $P = 0.0006$ vs hCG group (paired t test).

	Triptorelin (n=17)	Leuprolin (n=15)	hCG[b] (n=15)
Number of oocytes/subject	9.8±5.4	8.7±4.5	8.3±3.3
Proportion of metaphase II oocytes	72±18%	85±17%	86±17%
Fertilization rate	61±30%	62±23%	56±18%
Number of embryos obtained/subject			
Good quality (grade 1)	0.5±0.9	1.6±1.8	0.8±0.9
Good quality (grade 2)	1.9±1.8	2.7±2.5	1.7±1.9
Grade 1 and 2 pooled	2.7±1.9	3.2±2.6	3.3±2.0
Implantation rate	15±34%	18±37%	17±14%
Ongoing pregnancy rate[a]	18%	20%	13%

Table 4.2
Clinical outcome (mean ± SD) of a randomized comparison between two GnRH agonists (triptorelin and leuprorelin) versus hCG in inducing final oocyte maturation after ovarian hyperstimulation with FSH and co-treatment with ganirelix in 47 IVF patients. [a] Defined as a pregnancy per IVF cycle, confirmed by detection of positive heart beat on ultrasound between 12 and 16 weeks of gestation. [b] P=NS for all features comparing the triptorelin or the leuprorelin group versus women receiving hCG.

4.3.4 Discussion

In the normal menstrual cycle, the midcycle LH and FSH surge (lasting for approximately 48 hours) is a complex and carefully orchestrated event elicited in the late follicular phase by persistently elevated estrogen concentrations in combination with a small but distinct rise in P (*Hoff et al.* 1983). Exposure to a transient massive stimulation by LH elicits the resumption of meiotic maturation of the oocyte, rupture of the dominant follicle resulting in the release of the oocyte and luteinization of theca and granulosa cells resulting in the formation of the corpus luteum. Timing of the endogenous LH surge is frequently disrupted if exogenous FSH is administered either for ovarian hyperstimulation for IVF or for the treatment of anovulation. Therefore, exogenous hCG (which activates the LH receptor due to structural and biological similarities to LH and which is easy to extract from urine of pregnant women) has been used for decades to replace endogenous LH during FSH stimulation protocols. HCG has been shown to effectively induce ovulation, final oocyte maturation and

corpus luteum formation. However, hCG has an extended metabolic clearance rate (*Damewood et al.* 1989; *Mannaerts et al.* 1998) and therefore hCG can still be detected in the serum 10 days after the preovulatory bolus injection. Consequently, continued support of the corpus luteum by hCG elicits supra-physiological luteal phase steroid concentrations. Moreover, hCG in the late follicular phase may stimulate growth of medium sized follicles (*Sullivan et al.* 1999; *Loumaye et al.* 1999; *Whelan & Vlahos* 2000) which may subsequently ovulate. A mid-cycle bolus dose of hCG induces elevated follicular fluid progesterone levels suggesting changes in the microenvironment of the oocyte just before ovulation compared to the endogenous LH surge (*Yding Andersen C. et al.* 1993).

Next to a hyperresponse of the ovary to stimulation (i.e. large late follicular phase number of follicles and very high E_2 levels), both exogenous and endogenous hCG has clearly been associated with OHSS (*Lyons et al.* 1994; *Enskog et al.* 1999; *Mathur et al.* 2000). The induction and stimulation of multiple corpora lutea may represent a key phenomenon in this regard. Although luteal phase support by repeated hCG injections has been clearly shown to be beneficial in IVF (*Soliman et al.* 1994), randomized comparative trials have also convincingly demonstrated a higher incidence of OHSS in these patients (*Belaisch-Allart et al.* 1990; *Mochtar et al.* 1996). Therefore, luteal function in IVF cycles is currently supplemented by exogenous P. Moreover, it has been shown that the late and most severe form of OHSS occurs especially in multiple pregnancies (coinciding with elevated hCG production) (*Lyons et al.* 1994; *Mathur et al.* 2000). Indeed, withholding hCG along with discontinuation of ovarian stimulation effectively prevents ovulation, pregnancy and the development of OHSS in women presenting with an excessive ovarian response. Other approaches for the prevention of OHSS include withholding gonadotropins for some days during the late follicular phase (also referred to as 'coasting'), reducing the hCG bolus dose, replacing hCG by GnRH agonist as addressed in the current study, the administration of glucocorticoids or albumen, the avoidance of luteal support by hCG, and cryopreservation of embryos and transfer in subsequent cycles.

It has been clearly shown in several animal models that the mid-cycle gonadotropin surge is accompanied by a major rise in endogenous GnRH in the portal circulation (*Caraty et al.* 1995). Indeed, several initial studies in IVF showed that a mid-cycle endogenous LH rise could be induced by the late follicular phase administration of exogenous GnRH or GnRH agonist. Induction of final oocyte maturation with GnRH agonists in patients undergoing ovarian hyperstimulation is believed to be more physiological and of special benefit to high responders with an increased risk of developing OHSS. In the current study, very high hCG levels (median

> 200 IU/L) lasted for 24 hrs (from 12 hrs to 36 hrs after triggering of oocyte maturation) and clearance of the 10,000 IU hCG required around 10 days.

Although effective, the approach to induce oocyte maturation by the mid-cycle GnRH agonist administration lost interest during the nineties since ovarian hyperstimulation protocols also included GnRH agonist co-administration to suppress a premature rise in LH during the follicular phase. This renders the pituitary in a state of desensitization precluding stimulation of the endogenous LH surge. Several recent studies have shown that short-term GnRH antagonist administration during the late follicular phase is also effective in preventing a premature LH rise. This blockage of GnRH receptors on the gonadotrophic cells by the antagonist can be reversed by GnRH stimulation, allowing reevaluation of stimulating a mid-cycle rise in endogenous LH. The primary purpose of the current study was to investigate the dynamics of the mid-cycle gonadotropin surge under these circumstances. The current study also demonstrates for the first time that final maturation of oocytes can be triggered with GnRH agonists rather than hCG, following ovarian hyperstimulation using ganirelix to prevent a premature LH rise. An adequate pituitary response in terms of a rise in endogenous LH and FSH was observed after the administration of either 0.2 mg triptorelin or 0.5 mg leuprorelin and luteal phase steroid levels were found to be closer to the physiological range.

Endogenous LH and FSH surges in the current study were comparable to those described after triggering of oocyte maturation using GnRH agonists in non-suppressed subjects undergoing ovarian stimulation for IVF (*Gonen et al.* 1990; *Itskovitz-Eldor et al.* 1991). Thus, in the current study the doses of GnRH agonist administered 12 hours after the last antagonist injection were sufficient to displace ganirelix from the GnRH receptors. The maximum LH and FSH levels were comparable to circulating mid-cycle LH and FSH levels in natural cycles (*Hoff et al.* 1983). In a recent preliminary study (also applying 0.2 mg triptorelin for triggering of oocyte maturation in 8 high responder patients treated with exogenous FSH and 0.25 mg ganirelix) higher maximum LH concentrations compared to the current study were reported (*Itskovitz-Eldor et al.* 2000). This may indicate that the magnitude of the LH surge is in part determined by the endocrine status of the patient since high response patients were excluded from the current study. In the current study the duration of the LH surge appeared to be shorter compared to the natural cycle (24 hrs versus 36-48 hours, respectively). This phenomenon might be explained by the immediate pituitary desensitization after the initial flare effect, since two doses of buserelin 12 h apart did result in similar LH profiles (*Itskovitz-Eldor et al.* 1991). Despite the shorter duration compared to physiological conditions, the induced LH surge

effectively stimulated final oocyte maturation as reflected by the high percentage of metaphase II oocytes (72-85%) as well as good fertilization and embryo implantation rates.

An alternative means of mimiking the mid-cycle gonadotropin surge is the administration of a high dose of recombinant LH (*Imthurn et al.* 1996). However, the relatively short half-life of this compound (*Porchet et al.* 1995) suggests that very high or multiple doses should be given. A recently published large randomized comparative trial showed the complete absence of OHSS in women receiving a single dose of recombinant LH in doses up to 30,000 IU (*The european recombinant human LH study group* 1998). Extensive studies in the monkey using different doses of LH or hCG suggest that the duration of the midcycle LH surge is critical for inducing a normally functioning corpus luteum (*Zelinski-Wooten et al.* 1992; *Chandrasekher et al.* 1994). A relatively short LH surge resulted in normal oocyte maturation and ovulation, whereas luteal phase length was reduced implying that luteal support is required under these conditions. In the current study, serum E_2 and P levels were comparable for all treatment groups up to the day of oocyte retrieval. However, thereafter both the E_2 and P levels were higher in hCG treated subjects. This difference can be explained by the prolonged half-life of hCG as compared to LH, responsible for continued support of the corpus luteum. These luteal phase steroid levels far above the physiological range may suppress the release of endogenous gonadotropins required for corpus luteum support (*Gibson et al.* 1991) and may exhibit a negative impact on endometrial receptivity (*Forman et al.* 1988; *Simon et al.* 1998). In the current study luteal phase supplementation was applied by daily progestin administration. Therefore, the dynamics of mid-cycle LH requirements for subsequent normal luteal function in the human requires further investigation.

The physiological role of the mid-cycle FSH surge that occurs in the natural cycle has not been elucidated so far, but recent studies suggest that FSH may play a role in the process of nuclear maturation by actively promoting resumption of meiosis (*Zelinski-Wooten et al.* 1995; *Yding Andersen C. et al.* 1999). Moreover, studies in the rat have demonstrated that a high dose of FSH alone is capable of inducing ovulation (*Galway et al.* 1990). On the other hand, a midcycle FSH surge is not mandatory since normal oocyte maturation and ovulation occurs after the administration of hCG. The potential favorable impact of a GnRH-agonist induced FSH surge is unknown at present.

In summary, the application of a single dose of GnRH agonist was shown to be effective in inducing a gonadotropin surge and triggering final oocyte maturation in normal responder patients following ovarian hyperstimulation for IVF co-treated with GnRH antagonist. Effects on the dynamics of the pituitary – ovarian axis

during the luteal phase along with its capacity to prevent OHSS requires further evaluation. This more physiological approach to inducing oocyte maturation may provide a successful and safer alternative for patients undergoing IVF.

Chapter 5

General discussion

General Discussion

The introduction of GnRH agonists to IVF was initially viewed as a major step towards improving IVF outcome. However, the complications arising from the use of these compounds have provoked increasing concerns (*Edwards et al.* 1996; *Fauser et al.* 2002a). The 'long protocol', characterized by pituitary 'down-regulation' using a GnRH agonist for at least two weeks followed by co-administration of daily 150-450 IU FSH, is nowadays the most commonly practized strategy of ovarian stimulation for IVF. These protocols are complex, expensive, time-consuming and associated with a certain degree of risk (*Fauser et al.* 1999). These risks include OHSS, an increased incidence of (higher order) multiple pregnancies and (possibly) ovarian cancer (*Shoham* 1994; *Tarlatzis et al.* 1995; *Bristow & Karlan* 1996). Cryopreservation of excess embryos followed by transfer in a subsequent (unstimulated) cycle is often used as justification for profound ovarian stimulation for IVF. However, a large proportion of cryopreserved embryos will not survive the thawing process and even when the embryos survive the thawing process the supplementary benefit to cumulative pregnancy rates is disappointing, as shown in this thesis (*de Jong et al.* 2002). Therefore alternative strategies for ovarian stimulation for IVF need to be considered (*Edwards et al.* 1996; *Olivennes & Frydman* 1998; *Bouchard & Fauser* 2000; *Fauser et al.* 2002a).

GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotropin release and a rapid recovery of normal secretion of endogenous LH and FSH after cessation (*Hall* 1993). Their arrival in the clinical scene has not been easy. Due to problems with solubility and histamine releasing properties (*Kiesel & Runnebaum* 1992), the development of these compounds has taken a few decades. The previously mentioned problems have been dealt with by the development of the third generation of GnRH antagonists. The potential benefits of these third generation compounds may be considered as substantial, as outlined in this thesis. Before introducing these GnRH antagonists into general clinical practice a mandatory dose-finding study was carried out and described in this thesis. The minimal effective dose of the GnRH antagonists ganirelix and cetrorelix for suppressing a premature LH rise during ovarian stimulation for IVF was established at 0.25 mg per day for both compounds (*Albano et al.* 1997; *The ganirelix dose-finding study group* 1998). The daily dose of the GnRH antagonist had no detectable influence on late follicle development during ovarian stimulation for IVF. However, in the follicular phase the observed low steroid serum concentrations in patients receiving a high daily dose of the GnRH antagonist ganirelix (1.0 and 2.0 mg/day) could only be

partially attributed to the low LH levels (*de Jong et al.* 2001a). In the group receiving 0.25 mg/day of the GnRH antagonist, similar steroid serum concentrations were found when compared to the 'long protocol' (*The european orgalutran study group et al.* 2000). The subsequent non-inferiorty study outlined in this thesis comparing the 'long protocol' with an ovarian stimulation strategy for IVF with co-administration of a GnRH antagonist showed no significant differences in clinical outcome (*The european orgalutran study group et al.* 2000; *Albano et al.* 2000; *The european and middle east orgalutran study group* 2001). The incidence of OHSS may be lower in the patients receiving a GnRH antagonist compared to the 'long protocol' (*Ludwig et al.* 2000; *Olivennes et al.* 2002). But this could not be confirmed in a recent meta-analysis (*Al-Inany & Aboulghar* 2002).However, when the chance on severe OHHS is imminent in patients undergoing ovarian stimulation for IVF and co-treated with a GnRH antagonist, cancellation of the cycle and treatment with a high dose GnRH antagonist might be considered (*de Jong et al.* 1998).

The clinical introduction of GnRH has made feasible the development of new paradigms for ovarian stimulation for IVF (*Bouchard & Fauser* 2000). Due to the competitive-binding properties of GnRH antagonists, the pituitary is not 'down-regulated', providing the opportunity to use a GnRH agonist for inducing an LH and FSH surge for induction of the final stages of oocyte maturation, resulting in pregnancy and birth (*de Jong et al.* 2001b). This approach, described in this thesis, showed comparable quality and fertilization rates of recovered oocytes while luteal phase steroid levels were closer to the physiological range when compared to hCG (*Fauser et al.* 2002b). In addition, due to the shorter half-life of native LH when compared to hCG, this strategy may contribute to a lower incidence of OHSS (*Itskovitz-Eldor et al.* 2000; *Kol & Itskovitz-Eldor* 2000).

Due to the immediate suppressive action of pituitary gonadotropin release by GnRH antagonists application of these compounds could be started from the mid-follicular phase onwards, allowing initiation of a normal menstrual cycle with normal early follicular phase recruitment of a cohort of follicles. Extending the 'FSH window' (*Schipper et al.* 1998; *de Jong et al.* 1999a), aiming at less preovulatory follicles may further decrease the chance on complications. With initiation of ovarian stimulation from the midfollicular phase onwards with 150 IU FSH per day a marked reduction in amount of gonadotropin used could be established (*de Jong et al.* 2000). Moreover, a normal luteal phase was observed and pregnancies occurred in the absence of luteal support in this small pilot study. This was in contrast to other observations showing a short luteal phase without the provision of luteal support (*Albano et al.* 1998; *Tavaniotou et al.* 2001).

With respect to current cryopreservation protocols of excess embryos a choise needs to be made. Either to modify the cryopreservation protocol aiming at higher post thaw survival and implantation rates of thawed cryopreserved embryos (*Valdez et al.* 1992; *Vajta et al.* 1998; *Arav et al.* 2002) or to aim at minimal ovarian stimulation for IVF resulting in less excess embryos (*Rongieres-Bertrand et al.* 1999; *de Jong et al.* 2000). For this thesis we choose the second option. The final goal is to create single embryo transfer as a realistic option. Therefore, selection criteria need to be established for assessing embryo quality (*Hunault et al.* 2002). In addition, the prospect of analyzing the single embryo on chromosomal competence, i.e. aneuploidy screening by FISH, may play a role in designing an effective single embryo transfer IVF strategy (*Martini et al.,* ongoing research). The first steps are taken in an effort to simplify ovarian stimulation strategies for IVF, aiming at less embryos and to reduce the chance on complications (*Heinen et al.*, ongoing research)

Many questions remain to be answered. The cycle day for starting ovarian stimulation for IVF is under investigation. All studies using a GnRH antagonist for avoiding a premature LH rise, ovarian stimulation is initiated on cycle day 3 (*Albano et al.* 1997; *The european orgalutran study group et al.* 2000; *Albano et al.* 2000; *The european and middle east orgalutran study group* 2001). In an attempt to determine the optimal cycle day for initiation of ovarian stimulation initiatives are in progress comparing ovarian stimulation for cycle day 3 vs. cycle day 5 (*Hohmann et al.* 2002). Secondly, the need for luteal support is inconclusive after ovarian stimulation for IVF and co-treatment with a GnRH antagonist (*Albano et al.* 1998; *de Jong et al.* 2000; *Tavaniotou et al.* 2001). Preliminary data from a study comparing recombinant hCG, recombinant LH, and a GnRH antagonist for induction of final oocyte maturation after ovarian stimulation for IVF and co-treatment with an experimental GnRH antagonist (Antide) without the provision of luteal support have been presented recently (*Beckers et al.* 2002). Other initiatives include the comparison in terms of health economics and clinical outcome of 3 cycles profound ovarian stimulation for IVF vs. 4 cycles minimal ovarian stimualtion for IVF. Other trials are needed to determine the effects of GnRH antagonists on endometrial parameters. For simplification of ovarian stimulation for IVF, clinical trials are underway using long-acting FSH. By adding the carboxy-terminal peptide (CTP) of the hCG β-subunit to the FSH β-subunit (FSH-CTP) the circulating half-life up is increased up to 4 days (*Lapolt et al.* 1992; *Fares et al.* 1992) possibly leading to administration regimes of once every 4 days and increasing patient's convenience.

In conclusion, the arrival of GnRH antagonists in clinical practice offers the opportunity for designing flexible, short, efficient

and patient friendly IVF strategies. A reduction in short and long term risks of ovarian stimulation for IVF might be possible without seriously affecting pregnancy rates, although it has been showed recently that pregnancy rates of IVF with application of GnRH antagonists may be slightly reduced in comparison with the 'long protocol' (*Al-Inany & Aboulghar* 2002). However, since clinicans are in the beginning of learning curve using GnRH antagonists, pregnancy rates may improve with increasing clinical experience. In addition, the development of the 'long protocol', currently considered as gold standard, took a decade. Moreover, data derived from the non-inferiority study of a GnRH antagonist protocol for IVF (Chapter 2.3) demonstrated that centres which also participated in the dose-finding study (Chapter 2.1), achieved higher or similar pregnancy rates in the GnRH antagonist arm compared to GnRH agonist arm. This was in contrast to the centres not participating in the dose-finding study showing reversed results. Therefore, to explore the challenging possibilities GnRH antagonists offer to revolutionize IVF, additional studies and gaining clinical experience remain mandatory.

Chapter 6

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Chapter 7

Summary

Summary

Chapter 1

The use of ovarian stimulation has improved the clinical outcome of IVF considerably. However, without the prevention of a possible premature LH surge in IVF cycles a significant portion of the cycles need to be cancelled. Soon after the elucidation of the chemical structure of GnRH, GnRH agonists were developed and used (empirically) for prevention of a premature LH rise in IVF cycles. The development of GnRH antagonists, available for clinicians, lasted a couple of decades. In this chapter, a comparison is made between the use of GnRH agonists and GnRH antagonists. In addition, potential benefits arising from the use of GnRH antagonists is highlighted.

Chapter 2

Section 2.1 A multicentre, double-blind, randomized dose-finding study of Org 37462 (ganirelix) was conducted in 333 women undergoing ovarian stimulation with recombinant follicle stimulating hormone (recFSH; Puregon) to establish the minimal effective dose preventing premature luteinizing hormone (LH) surges during ovarian stimulation. For ovarian stimulation, recFSH was given in a fixed daily dose of 150 IU for 5 days from days 2 to 6 of the menstrual cycle. From cycle day 7 onward, up to and including the day of human chorionic gonadotropin (hCG), Org 37462 (dosages 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/0.5 ml) was administered once daily by s.c. injection, and the recFSH dose was adjusted depending on ovarian response. The lowest (0.0625 mg) and highest (2.0 mg) dose groups were terminated prematurely on the advice of an external independent advisory committee. Serum Org 37462 concentrations increased in a linear dose-proportional manner, whereas serum LH and increases of estradiol fell with increasing Org 37462 dose. During Org 37462 treatment, serum LH concentrations $\geq$10 IU/L were observed in the lowest dose groups with incidences of 16% (0.0625 mg), 9% (0.125 mg) and 1.4 % (0.25 mg). On the day of hCG, the number of follicles $\geq$11, $\geq$15 and $\geq$17 mm were similar in the six dose groups, whereas serum estradiol concentrations were highest in the 0.0625 mg group (1475 pg/mL) and lowest in the 2 mg group (430 pg/mL). The median daily dose of recFSH was between 150 and 183 IU and the overall median duration of Org 37462 treatment was ~ 5 days in the six treatment groups. Overall, Org 37462 treatment

appeared to be safe and well tolerated. The mean number of recovered oocytes and good-quality embryos was similar in all dose groups and ranged from 8.6 to 10.0 and 2.5 to 3.8, respectively. The mean number of replaced embryos in the different dose groups ranged from 2.3 to 2.7. The implantation rate was highest in the 0.25 mg group (21.9%) and lowest in the 2 mg group (1.5%). The early miscarriage rates (first 6 weeks after embryo transfer) were 11.9 and 13% in the 1 and 2 mg group respectively, whereas in the other dose groups this incidence was 0% (0.0625 mg) up to a maximum of 3.7% (0.5 mg). The vital pregnancy rate (with heart activity) at 5-6 weeks after embryo transfer was highest in the 0.25 mg group, i.e. 36.8% per attempt and 40.3% per transfer, and resulted in an ongoing pregnancy rate 12-16 weeks after embryo transfer of 33.8% per attempt and 37.1% per transfer. In conclusion, a daily dose of 0.25 mg Org 37462 prevented LH surges during ovarian stimulation and resulted in a good clinical outcome.

Section 2.2 This randomized controlled multicentre trial was initiated to investigate relations between dose of gonadotropin releasing hormone (GnRH) antagonist and follicular phase characteristics. A total of 311 patients with an indication for IVF were analyzed. Ovarian stimulation for in vitro fertilzation (IVF) with recombinant follicle stimulating hormone (recFSH) was initiated starting on cycle day 2. From cycle day 7 onwards they were co-treated with 0.0625, 0.125, 0.25, 0.5, 1.0, or 2.0 mg/day of the GnRH antagonist ganirelix. The number of follicles, total follicular surface area, gonadotropin, and steroid serum concentrations were measured. In all patients, similar follicular growth was observed comparing all treatment groups. FSH levels increased during the follicular phase. Late follicular phase luteinizing (LH), androstenedione (AD) and estradiol (E_2) levels showed a GnRH antagonist dose related decrease ($P < 0.05$). Late follicular phase E_2 levels correlated with total follicular surface area, AD, LH and FSH (all $P < 0.001$). Increasing GnRH antagonist doses exhibit additional suppressive action on E_2 levels. Based on these findings it was concluded that follicular growth was unaffected by the dose of GnRH antagonist. A rise in follicular phase FSH serum concentrations during the follicular phase, largely related to exogenous FSH, enabled ongoing follicular growth in all treatment groups. Increasing GnRH antagonist doses resulted in lower late follicular phase E_2, AD, and LH levels. The effect of GnRH antagonist on late follicular phase E_2 levels could not be exclusively attributed to LH.

Section 2.3 A multicentre, open-label, randomized study of the gonadotropin-releasing hormone (GnRH) antagonist ganirelix (Orgalutran/Antagon) was performed in women undergoing ovarian

stimulation with recombinant FSH (recFSH;Puregon). The study was designed as a non-inferiority study using a 'long protocol' of buserelin (intranasal) and recFSH as a reference treatment. A total of 730 subjects was randomized in a treatment ratio of 2:1 (ganirelix:buserelin) using an interactive voice response system which stratified for age, type of subfertility and planned fertilization procedure [IVF or intracytoplasmic sperm injection (ICSI)]. The median duration of GnRH analogue treatment was 5 days in the ganirelix group and 26 days in the buserelin group, whereas the median total recFSH dose was 1500 IU and 1800 IU respectively. In addition, in the ganirelix group the mean duration of stimulation was 1 day shorter. During ganirelix treatment the incidence of LH rises (LH $\geq$ 10 IU/L) was 2.8% versus 1.3% during recFSH stimulation in the buserelin group. On the day of triggering ovulation by human chorionic gonadotropin (hCG), the mean number of follicles $\geq$ 11 mm diameter was 10.7 and 11.8, and the median serum estradiol concentrations were 1190 pg/mL and 1700 pg/mL in the ganirelix and buserelin groups respectively. The mean number of oocytes per retrieval was 9.1 and 10.4 respectively, whereas the mean number of good quality embryos was 3.3 and 3.5 respectively. The fertilization rate was equal in both groups (62.1%), and the same mean number of embryos (2.2) was replaced. The mean implantation rates were 15.7% and 21.8%, and the ongoing pregnancy rates per attempt were 20.3% and 25.7% in the ganirelix and buserelin groups respectively. Evaluation of all safety data indicated that the ganirelix regimen was safe and well tolerated. The overall incidence of ovarian hyperstimulation syndrome was 2.4% in the ganirelix group and 5.9% in the reference group. The results of this study support a safe, short and convenient treatment regimen of ganirelix, resulting in a good clinical outcome for patients undergoing ovarian stimulation for IVF or ICSI.

Section 2.4 This case report describes the first attempt to treat imminent ovarian hyperstimulation syndrome (OHSS) by using a gonadotropin releasing hormone (GnRH) antagonist. A 33 year old, normo-ovulatory woman undergoing in-vitro fertilization received daily subcutaneous injections of 150 IU of recombinant follicle-stimulating hormone (recFSH) from cycle day 2, together with GnRH antagonist (ganirelix) 0.125 mg from cycle day 7 onwards. On cycle day 10 the patient was found to have a serum estradiol concentration of 16 500 pmol/L and, on ultrasound examination, four preovulatory (>16 mm) and nine intermediate sized (10–16 mm) follicles. RecFSH injections were discontinued, human chorionic gonadotropin (hCG) withheld, whereas the ganirelix dose was increased to 2 mg/d. This regimen led to a rapid decrease in serum estradiol concentrations and the decrease in ovarian size on ultrasound. Since GnRH antagonists are

clinically available for in vitro fertilization programmes this suggested regimen might have a role in preventing severe OHSS.

Chapter 3

Section 3.1 This observational longitudinal study was carried out to calculate the added benefit of a cryopreservation program to the cumulative ongoing pregnancy rate over a maximum of three consecutive IVF cycles. One thousand two hundred and fifty one couples with an indication for IVF who began their first treatment between January 1995 and December 1999 were evaluated. A maximum of three cycles per couple, including subsequent cycles in which cryopreserved of supranumerical embryos were thawed, were analyzed. Pregnancies arising from the cryopreservation cycle were considered augment the cumulative pregnancy rate among patients who did not achieve an ongoing pregnancy from the fresh embryos transfer cycle. The 'projected cryoaugmented pregnancy rates', which assume that pregnancy rates of patients with unthawed cryopreserved embryos are comparable to those who had an embryo transfer of thawed cryopreserved embryos, were calculated. Clinical outcome in terms of ongoing pregnancy rates was analyzed by Markov chain analysis to estimate the added value of the cryopreservation program. A total of 2367 IVF stimulation cycles and 329 cycles in which cryopreserved embryos were thawed for transfer were initiated. The cumulative ongoing pregnancy rate following 3 successive started fresh IVF cycles was 42.5%. When pregnancies arising from the transfer of thawed cryopreserved embryos were included the cumulative ongoing pregnancy rate increased to 43.8%. When the data was extrapolated to include pregnancies which could be expect to result from as yet unthawed embryos, a further increase was observed to 44.8%. When analyzed in these terms the supplementary benefit of cryopreserving supranumerical embryos appears limited. It is be proposed that a reduction in embryos available for cryopreservation which may result from milder ovarian stimulation protocols will have little negative impact on cumulative ongoing pregnancy rates.

Section 3.2 Based on the previous section it may be concluded that the true added value of the cryopreservation program for excess embryos is limited. The possibility of cryopreserving excess embryos as a justification for aiming at a maximum number of oocytes during ovarian stimulation for IVF is poorly justified. Therefore it is legitimate to design ovarian stimulation for IVF protocols aiming at less follicles, with an opportunity to reduce the chance on complications. This

section deals with mild ovarian stimulation for IVF. A minimal intervention in the mid follicular phase of the normal menstrual cycle could result in less follicles and thus less oocytes without endangering to chance to conceive.

Section 3.3 This pilot study was performed to study if a minimal interference in the process of single dominant follicle selection may serve as the basis for a simplified ovarian stimulation regimen for IVF. The normal menstrual cycle served as reference. Total number of dominant follicles on the day of hCG, and endocrine cycle characteristics were primary outcome measurements. Fifteen normo-ovulatory patients with a regular indication for IVF underwent ovarian stimulation for IVF with 100 or 150 IU per day recombinant FSH starting on cycle day 5. From cycle day 8 or later patients were co-treated with 0.25 mg/d GnRH antagonist. No luteal support was provided. Multiple follicle development occurred in 5 out of 8 patients in the 100 IU group and in all 7 women in the 150 IU group. Follicular phase luteal phase length were normal compared with the normal menstrual cycle, but the endocrine profile was abnormal. Pregnancies were obtained. In conclusion, treatment with a fixed daily dose of 150 IU recombinant FSH starting in the mid-follicular phase resulted in ongoing growth of a restricted number of dominant follicles and oocytes retrieved, adequate to lead to embryo transfer. A marked reduction in the total amount of gonadotropins administered compared to standard treatment can be achieved. Withholding luteal support does not exclude pregnancies.

Chapter 4

Section 4.1 This case report describes to our knowledge the first pregnancy and birth after induction of final oocyte maturation with a GnRH agonist in a woman undergoing ovarian hyperstimulation for IVF while co-treated with the GnRH antagonist Orgalutran. A 28-year old, normo-ovulatory woman undergoing in-vitro fertilization received daily subcutaneous injections of 150 IU of recombinant follicle-stimulating hormone (recFSH) from cycle day 2, and GnRH antagonist (Orgalutran) 0.25 mg from cycle day 7 onwards. On cycle day 11 the patient showed 12 and 3 follicles $\geq$ 11 and $\geq$ 17 mm in diameter on ultrasound, respectively. RecFSH injections were discontinued and 0.5 mg leuprorelin was administered for final oocyte maturation. On the day of oocyte pick-up 12 oocytes were retrieved, 11 metaphase II oocytes and 1 germinal vesicle. On day 5 after oocyte pick-up, 2 blastocysts were transferred and 3 cryopreserved. A viable intra-uterine twin pregnancy was observed on ultrasound 6

weeks after embryo transfer, which spontaneously reduced to a singleton pregnancy. At 40 weeks amenorrhoea the patient delivered a healthy daughter. In conclusion, triggering final stages of oocyte maturation with a GnRH agonist may offer an effective and safe option for patients undergoing ovarian stimulation for IVF, using a GnRH antagonist for the prevention of a premature LH surge.

Section 4.2 In a randomized multi-center study, the efficacy of two different gonadotropin-releasing hormone (GnRH) agonists was compared with human chorionic gonadotropin (hCG) for triggering final stages of oocyte maturation after ovarian hyperstimulation for in vitro fertilization (IVF). Ovarian stimulation was conducted by recombinant follicle stimulating hormone (RecFSH) (Puregon) and the GnRH antagonist ganirelix (Orgalutran) was co-administered for the prevention of a premature luteinizing hormone (LH) rise. Luteal support was provided by daily progestin administration. Frequent blood sampling was performed at midcycle in the first 47 eligible subjects included in the current study which were randomized for a single dose of either 0.2 mg triptorelin (n=17), 0.5 mg leuprorelin (n=15) or 10,000 IU hCG (n=15). Serum concentrations of LH, follicle-stimulating hormone (FSH), estradiol (E_2) and progesterone (P) were assessed at variable intervals. LH peaked at 4 hours after both triptorelin and leuprorelin administration with median LH levels of 130 IU/L and 107 IU/L ($P < 0.001$), respectively. LH levels returned to baseline after 24 hrs. Subjects receiving hCG showed peak levels of 240 IU/L hCG 24 hrs after administration. A rise in FSH to 19 IU/L ($P < 0.001$) was noted in both GnRH agonist groups 8 hrs after injection. Within 24 hrs the AUC for LH and FSH was significantly higher ($P < 0.001$) in both GnRH agonist groups compared to hCG. E_2 and P levels were similar for all groups up to the day of oocyte retrieval. Luteal phase AUC's for P and E_2 were significantly elevated ($P < 0.001$) in the hCG group. The mean ($\pm$ SD) number of oocytes retrieved was 9.8 ± 5.4, 8.7 ± 4.5 and 8.3 ± 3.3, the percentage of metaphase II oocytes was 72%, 85% and 86%, and fertilization rates were 61, 62, and 56% in the triptorelin, leuprorelin and hCG group respectively (P = NS for all three comparisons). These findings support the effective induction of final oocyte maturation in both GnRH agonist groups. In summary, after treatment with the GnRH antagonist ganirelix for the prevention of premature LH surges, triggering of final stages of oocyte maturation can be induced effectively by a single bolus injection of GnRH agonist as demonstrated by the induced endogenous LH and FSH surge and the quality and fertilization rate of recovered oocytes. Moreover, corpus luteum formation is induced by GnRH agonists with luteal phase steroid levels closer to the physiological range compared to hCG. This more physiological approach for inducing oocyte maturation may

represent a successful, and safer alternative for IVF patients undergoing ovarian hyperstimulation.

Chapter 5

This chapter summarises the conclusions which could be drawn form the work presented in the current thesis. Realizing that clinical experience with the application of GnRH antagonists is fairly limited, new paths should be explored to enhance the potentials of this application. Therefore, directions for future research are presented.

Chapter 7

Samenvatting (Dutch)

Samenvatting (Dutch)

Hoofdstuk 1

De klinische uitkomsten van de IVF behandeling zijn door het gebruik van hyperstimulantia voor het ovarium aanzienlijk verbeterd. Echter zonder het gebruik van middelen, welke een voortijdige eisprong verhinderen, zou een significant deel van de pogingen tot IVF gestaakt dienen te worden. Spoedig na de opheldering van de chemische structuur van GnRH werden GnRH agonisten ontwikkeld en later ook (empirisch) gebruikt in de kliniek voor het verhinderen van een voortijdige LH piek tijdens een IVF behandeling. De ontwikkeling van GnRH antagonisten, welke bruikbaar bleken in de kliniek, duurde echter nog enige decennia. Een vergelijking tussen het gebruik van GnRH agonisten en antagonisten wordt in dit hoofdstuk gemaakt, tevens worden de potentiële voordelen van het gebruik van GnRH antagonisten tijdens een IVF behandeling aangehaald.

Hoofdstuk 2

Paragraaf 2.1 Een dubbel blind gerandomiseerd multi-centrisch onderzoek werd uitgevoerd voor het vinden van de minimale effectieve dosering van Org 37462 (GnRH antagonist, ganirelix) voor het verhinderen van een premature LH piek. Bij 333 vrouwen werd ovariële hyperstimulatie bewerkstelligd middels recFSH en de GnRH antagonist ganirelix werd gebruikt voor het verhinderen van een voortijdige LH piek. RecFSH werd toegediend voor ovariële hyperstimulatie in een gefixeerde dosering van 150 IU/dag gedurende 5 dagen (vanaf cyclus dag 2 tot en met 6). Vanaf cyclus dag 7 en verder (tot en met de dag dat hCG werd toegediend) werd een GnRH antagonist (dosering 0.0625, 0.125, 0.25, 0.5, 1.0 en 2.0) dagelijks s.c. geïnjecteerd, terwijl de dosering recFSH werd aangepast aan de ovariële reactie. De toepassing van de laagste (0.0625 mg) en hoogste (2.0 mg) dosering GnRH antagonist werd voortijdig gestaakt op het advies van een extern onafhankelijk advies commissie. De serum concentratie van GnRH antagonist nam lineair toe terwijl de serum concentratie van LH en oestradiol afnam bij het gebruik van hogere doseringen GnRH antagonist. Serum LH concentraties van $\geq$ 10 IU/L werden waargenomen tijdens het gebruik van relatief lage doseringen met incidenties van 16% (0.0625 mg), 9% (0.125 mg), en 1.4% (0.25 mg). Op de dag van hCG toediening waren het aantal follikels $\geq$11, $\geq$15 en $\geq$17 mm vergelijkbaar in de zes

behandelgroepen, terwijl de serum oestradiol concentraties het hoogst was in de groep van 0.0625 mg en het laagst in de groep van 2.0 mg GnRH antagonist (1475 en 430 pg/mL, respectievelijk). De mediaan van de dagelijkse dosering recFSH lag tussen de 150 en 183 IU en de mediaan van de duur met de GnRH antagonist was ~ 5 dagen in alle 6 de onderzoeks groepen. In het algemeen bleek de GnRH antagonist behandeling veilig en werd goed verdragen. In de zes behandelgroepen was het gemiddelde aantal eicellen dat gewonnen werd en het aantal embryo's van goede kwaliteit dat gekweekt kon worden vergelijkbaar (variërend van 8.6 tot 10.0 en 2.5 tot 3.6, respectievelijk). Voor de behandelgroepen lag het gemiddelde aantal embryo's dat teruggeplaatst werd tussen de 2.3 en 2.7. Het implantatie percentage was het hoogst in de 0.25 mg GnRH antagonist behandelgroep (21.9%), het laagst in de 2 mg behandelgroep (1.5%). Het percentage vroege spontane abortussen (binnen 6 weken na embryo transfer) was 11.9 en 13% in de 1.0 en 2.0 mg groep respectievelijk. Ter vergelijking, in de andere groepen varieerde dit percentage van 0% (0.0625 mg) tot maximaal 3.7% (0.5 mg). Het percentage echografische vitale zwangerschappen (hartactie 5-6 weken na embryo terugplaatsing) was het hoogst in de 0.25 mg groep (36.8% per poging en 40.3% per terugplaatsing), resulterend in het doorgaande zwangerschapspercentage (12-16 weken na terugplaatsing) van 33.8% per poging en 37.1% per terugplaatsing. Concluderend, de dagelijkse dosering van 0.25 mg Org 37462 GnRH antagonist voorkomt een LH piek tijdens ovariële hyperstimulatie en resulteert in een goede klinische uitkomst.

Paragraaf 2.2 Deze gerandomiseerde gecontroleerde multi-centrische studie werd uitgevoerd om relaties tussen de dosis GnRH antagonist en karakteristieken van de folliculaire fase van de cyclus te bestuderen. Een totaal aantal van 311 patiënten werd geanalyseerd. Begonnen werd met ovariële hyperstimulatie met recFSH ten behoeve van IVF vanaf cyclus dag 2. Hiernaast kregen de deelnemers vanaf cyclus dag 7 een dagelijkse gift GnRH antagonist in de dosering 0.0625, 0.125, 0.25, 0.5, 1.0 of 2.0 mg. Het aantal follikels, de totale folliculaire oppervlakte, gonadotrofine en steroïd concentraties werden gemeten. Voor alle patiënten werd een vergelijkbare groei van follikels waargenomen voor alle behandelgroepen. FSH serum concentraties namen toe gedurende de folliculaire fase. Serum concentraties van LH, AD en E_2 vertoonde een GnRH dosis gerelateerde afname in de late folliculaire fase (P < 0.05). E_2 concentraties correleerden met de totale folliculaire oppervlakte, AD, LH en FSH (allen P < 0.001). Hogere GnRH antagonist doseringen hadden een additioneel onderdrukkend effect op E_2 concentraties. Geconcludeerd wordt, op basis van deze bevindingen, dat groei van follikels niet door de dosering GnRH

antagonist te beïnvloeden is. De toename van FSH serum concentraties, hoofdzakelijk bepaald door het exogene FSH, maakt groei van follikels mogelijk in alle behandel groepen. Hogere doseringen van GnRH antagonisten hebben een lagere serum concentratie van E_2, AD en LH tot gevolg. Het effect van de GnRH antagonist op E_2 serum concentraties in de laat folliculaire fase berust derhalve niet uitsluitend LH serum concentraties.

Paragraaf 2.3 Dit 'open label' gerandomiseerd multi-centrisch onderzoek met de GnRH antagonist ganirelix werd uitgevoerd bij vrouwen die ovariële hyperstimulatie met recFSH ondergingen. Het onderzoek was als een 'non-inferiority' studie opgezet waarbij het 'lange protocol' (busereline en recFSH) als referentie diende. Een totaal aantal van 730 patiënten werd gerandomiseerd in een ratio van 2:1 (ganirelix:busereline) waarbij gebruik gemaakt werd van een interactief telefoon systeem waarbij gestratificeerd werd voor leeftijd, oorzaak van subfertiliteit en voorgenomen fertilisatie procedure (IVF of ICSI). Bij de duur van de GnRH analoog behandeling was de mediaan 5 dagen in de ganirelix groep en 26 dagen in de busereline groep, terwijl voor de totaal gebruikte hoeveelheid recFSH de mediaan respectievelijk 1500 en 1800 IU was. Ter aanvulling, de gemiddelde duur van ovariële hyperstimulatie was 1 dag korter. Gedurende de ganirelix behandeling was de incidentie van een LH stijging (LH $\geq$ 10 IU/L) 2.8% tegenover 1.3% in de busereline groep. Op de dag dat hCG gegeven werd voor het in gang zetten van de ovulatie was het gemiddeld aantal follikels $\geq$ 11 mm in diameter 10.7 en 11.8, terwijl de mediane oestradiol serum concentraties 1190 en 1700 pg/mL bedroeg in respectievelijk de ganirelix en busereline groep. Het gemiddeld aantal eicellen gewonnen per punctie was 9.1 in de ganirelix en 10.4 in de busereline groep, met een gemiddeld aantal embryo's van goede kwaliteit van respectievelijk 3.3 en 3.5. Het fertilisatiepercentage was vergelijkbaar in beide groepen (62.1%), evenals het gemiddeld aantal embryo's dat werd teruggeplaatst (2.2). Het gemiddeld implantatiepercentage was 15.7 en 21.8%, met het doorgaande zwangerschapspercentage van 20.3 en 25.7% in de ganirelix en busereline groep respectievelijk. Evaluatie van alle data aangaande de veiligheid van ganirelix duidde er op dat ganirelix veilig was en goed verdragen werd. De incidentie van het ovarieel hyperstimulatie syndroom bedroeg 2.4 % in de ganirelix groep tegenover 5.9% in de referentie groep. Uit de resultaten van deze studie kan geconcludeerd worden, dat de behandeling met ganirelix ter verhindering van een LH piek, een veilige, korte en patiënt vriendelijk methode is welke resulteert in een goede klinische uitkomst bij patiënten die ovariële hyperstimulatie ondergaan ten behoeve van IVF of ICSI.

Paragraaf 2.4 Deze casus bespreking beschrijft de eerste poging voor het behandelen van een patiënte met een imminent ovarieel hyperstimulatie syndroom (OHSS) middels een GnRH antagonist. Een 33 jarige normo ovulatoire vrouw onderging een IVF behandeling. Ovariële hyperstimulatie werd bewerkstelligd middels dagelijkse injecties van 150 IU recFSH vanaf cyclus dag 2. Vanaf de 7de cyclus dag kreeg ze hiernaast ook dagelijkse injecties van 0.25 mg GnRH antagonist ganirelix. Op cyclus dag 10 presenteerde de patiënte zich met een oestradiol serum concentratie van 16500 pmol/L en op transvaginale sonografie waren 4 Graafse follikels (diameter > 16 mm) en 9 follikels tussen de 10 en 16 mm in diameter zichtbaar. De recFSH injecties werden gestopt en de dosis GnRH antagonist opgehoogd tot 2.0 mg per dag. Deze behandelwijze leidde tot een snelle afname van de oestradiol concentratie en een echografisch waarneembare afname van ovarieel volume. Daar GnRH antagonisten reeds beschikbaar zijn voor de kliniek zou deze manier van behandelen een rol kunnen gaan spelen in de behandeling van ernstige vormen van OHSS.

Hoofdstuk 3

Paragraaf 3.1 Deze observationele longitudinale studie werd uitgevoerd om de toegevoegde waarde van een cryopreservatie programma aan het cumulatief doorgaande zwangerschapspercentage voor maximaal drie opeenvolgende IVF cycli te berekenen. Twaalfhonderdeenenvijftig paren met een indicatie voor IVF welke hun eerste behandeling begonnen tussen januari 1994 en december 1999 werden geëvalueerd. Maximaal drie cycli per paar, inclusief de eventueel daarop volgende cycli waarin ingevroren embryo's werden ontdooid om terug te plaatsen, werden geanalyseerd. Zwangerschappen voortkomend uit een cyclus waarbij ontdooide cryo embryo's werden teruggeplaatst nadat de voorgaande verse cyclus geen zwangerschap opleverde, worden als additionele waarde beschouwt. De zogenaamde 'geprojecteerde' toegevoegde cryo zwangerschapspercentages, waarbij de resultaten van de ontdooide cryo embryo's werden geëxtrapoleerd naar de nog in de vriezer aanwezige embryo's, werden berekend. De klinische uitkomst in de vorm van doorgaande zwangerschapspercentages werden berekend met behulp van een zogenaamde Markov Chain analyse. Een totaal aantal van 2367 IVF stimulatie cycli en 329 cycli, waarbij ingevroren embryo's werden ontdooid voor terugplaatsing, werden geïnitieerd. Het cumulatief zwangerschapspercentage volgend uit 3 gestarte verse IVF stimulatie cycli was 42.5%. Wanneer zwangerschappen volgend uit de terugplaatsing van ontdooide

embryo's werden toegevoegd dan steeg het cumulatief zwangerschapspercentage tot 43.8%. Inclusief de geëxtrapoleerde data van de nog in de vriezer aanwezige embryo's deed dit percentage stijgen tot 44.8%. Concluderend kan gezegd worden dat de toegevoegde waarde van het cryopreservatie programma van overtollige embryo's zeer beperkt is. Een eventuele reductie van beschikbare embryo's voor cryopreservatie als resultaat van mildere ovariële stimulatie protocollen zal weinig invloed hebben op het cumulatief doorgaande zwangerschapspercentage.

Paragraaf 3.2 In de voorafgaande paragraaf werd geconcludeerd dat de toegevoegde waarde van het cryopreservatie programma van beschikbare overtollige embryo's zeer beperkt is. De mogelijkheid van het invriezen van overtollige embryo's als rechtvaardiging voor maximale ovariële stimulatie is daarom matig onderbouwd. Vanwege de hiervoor genoemde redenen is het legitiem om protocollen te ontwikkelen voor ovariële hyperstimulatie ten behoeve van IVF waarbij gedoeld wordt op minder follikels met daarbij de mogelijkheid tot het verlagen van het complicatie risico van de behandeling. Deze paragraaf introduceert het begrip van milde ovariële stimulatie ten behoeve van IVF. Een minimale interventie tijdens de mid folliculaire fase van de spontane menstruele cyclus zou in minder follikels en oöcyten kunnen resulteren, zonder de kans op zwangerschap in gevaar te brengen.

Paragraaf 3.3 Deze 'pilot studie' werd uitgevoerd ten bewijze of een minimale interventie gedurende het proces van dominante follikel selectie kan dienen als basis voor een eenvoudiger ovarieel stimulatie schema ten behoeve van IVF. De normale cyclus diende als referentie. Het totaal aantal dominante follikels aanwezig op de dag dat hCG werd toegediend en de endocrinologische cyclus karakteristieken waren de belangrijkste parameters voor analyse. Vijftien normo ovulatoire vrouwen met een indicatie voor IVF ondergingen ovariële hyperstimulatie voor IVF behandeling middels 100 of 150 IU recFSH per dag, beginnend op cyclusdag 5. Vanaf cyclusdag 8 of later ontvingen ze hiernaast ook 0.25 mg GnRH antagonist per dag. Van luteale ondersteuning werd afgezien. Multipele dominante follikels werden waargenomen bij 5 van de 8 patiënten in de 100 IU groep en bij alle 7 vrouwen in de 150 IU groep. De lengte van de folliculaire en luteale fase was overeenkomstig de normale menstruele cyclus, echter de endocrinologische karakteristieken bleken afwijkend. Zwangerschappen werden gezien. Concluderend, behandeling met een gefixeerde dosis van 150 IU recFSH beginnend in de mid folliculaire fase van de cyclus resulteerde in doorgaande groei van een beperkt aantal dominante follikels en gewonnen oöcyten, afdoende voor embryo transfer. Een

aanzienlijke reductie in gonadotrofinen gebruik was bewerkstelligd. Het afzien van luteale ondersteuning sluit zwangerschappen niet uit.

Hoofdstuk 4

Paragraaf 4.1 Deze casus beschrijft bij een IVF patiënte, naar onze mening voor de eerste keer, zwangerschap en geboorte na het induceren van de laatste fase van oöcyt maturatie met een GnRH agonist na ovariële hyperstimulatie en GnRH antagonist behandeling (ter preventie van een premature LH piek). Een 28 jarige normo ovulatoire vrouw onderging IVF. Dagelijks subcutane injecties met 150 IU recFSH vanaf cyclus dag 2 werden toegediend, met daarbij vanaf de 7^{de} cyclus dag, dagelijks 0.25 mg GnRH antagonist. Op cyclus dag 11 werden middels echografie 12 en 3 follikels van respectievelijk $\geq$ 11 mm en $\geq$ 17 mm in diameter waargenomen. De recFSH injecties werden gestaakt en 0.5 mg leuproline werd toegediend voor de laatste fase van oöcyt maturatie. Op de dag van punctie werden 12 oöcyten gewonnen, waarvan 11 in metafase II en 1 germinal vesicle. Vijf dagen na punctie werden 2 blastocysten teruggeplaatst. Aanvankelijk werd een intacte tweeling zwangerschap aangetoond, echter deze reduceerde zich spontaan tot een 1-ling zwangerschap. Bij een amenorroe duur van 40 weken werd een gezonde dochter bij de patiënte geboren. Concluderend, het opwekken van de laatste fase van oöcyt maturatie met een GnRH agonist kan een veilige optie zijn voor patiënten die ovariële hyperstimulatie ondergaan, gecombineerd met een GnRH antagonist ter preventie van een premature LH piek.

Paragraaf 4.2 Deze gerandomiseerde multi-centrische studie vergelijkt, na ovariële hyperstimulatie voor IVF, de effectiviteit van twee verschillende GnRH agonisten met hCG voor het opwekken van de laatste fase van oöcyt maturatie. Ovariële hyperstimulatie werd bewerkstelligd middels recFSH en de GnRH antagonist Orgalutran werd gebruikt voor het verhinderen van een voortijdige LH piek. Luteale ondersteuning vond plaats middels dagelijkse progestin injecties. Mid cyclisch werd bij de eerste 47 geïncludeerde patiënten van deze studie frequent bloed afgenomen. Deze patiënten kregen na randomisatie een enkele dosis van 0.2 mg triptoreline (n=17), 0.5 mg leuproline (n=15), of 10,000 IU hCG (n=15). Serum LH, FSH, E_2 en P concentraties werden bepaald op verschillende tijdstippen. Een LH piek werd waargenomen 4 uur na zowel de toediening van triptoreline als leuproline met mediane LH concentraties van respectievelijk 130 IU/L en 107 IU/L ($P < 0.001$). LH waarden daalden naar de basis na 24 uur. Patiënten die hCG kregen toegediend vertoonden een LH

piek 24 uur na toediening. Een toename van FSH tot 19 IU/L (P < 0.001) werd na 8 uur in beide GnRH antagonist groepen waargenomen. Binnen 24 uur was de AUC voor LH en FSH significant hoger (P < 0.001) in de beide GnRH antagonist groepen in vergelijking tot de hCG groep. E_2 en P concentraties waren vergelijkbaar in alle groepen tot aan de dag van punctie. De AUC voor P en E_2 in de luteale fase was significant verhoogd in de hCG groep (P < 0.001). Het gemiddelde (± SD) aantal oöcyten dat gewonnen werd was 9.8 ± 5.4, 8.7 ± 4.5 en 8.3 ± 3.3, het percentage metafase II oöcyten was 72, 85 en 86%, en fertilisatiepercentages waren 61, 62, en 56% in de triptoreline, leuproline en hCG groep respectievelijk (P = NS alle drie de vergelijkingen). Deze bevindingen ondersteunen een effectieve inductie van de laatste fase van oöcyt maturatie in beide GnRH agonist groepen. Concluderend, na behandeling met de GnRH antagonist voor de verhindering van een premature LH piek kan de laatste fase van oöcyt maturatie effectief opgewekt worden middels een enkele bolus injectie met een GnRH agonist, aangetoond door zowel de geïnduceerde endogene LH en FSH piek als de kwaliteit en het fertilisatiepercentage van de gewonnen oöcyten. Er aan toegevoegd, de vorming van het corpus luteum, geïnduceerd door GnRH agonisten, gaat gepaard met steroïd concentraties in de luteale fase, die dichter in de buurt van de fysiologische waarden liggen in vergelijking tot hCG. Deze meer fysiologische aanpak voor de inductie van oöcyt maturatie kan een succesvol veilig alternatief bieden voor IVF patiënten die ovariële hyperstimulatie ondergaan.

Hoofdstuk 5

Dit hoofdstuk geeft een opsomming van conclusies welke uit het werk, gepresenteerd in dit proefschrift, getrokken kunnen worden. Gerealiseerd hebbende dat de klininische ervaring met de toepassing van GnRH antagonisten nog beperkt is, zullen nieuwe wegen bewandeld moeten worden om deze toepassing te optimaliseren. Daarom worden er in dit hoofdstuk mogelijke suggesties voor verder onderzoek gegeven.

Chapter 8

Publications and presentations

Publications and presentations

Publications included in the present thesis (Numbers in italics refer to chapter sections)

1.2 de Jong D., Macklon N.S., Fauser B.C. Current status of gnrh antagonists in art. In: Allahbadia G (ed), *Manual of induction of ovulation*. pp. 199-209. Rotunda India, Mumbai, India. 2001.

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2.2 de Jong D., Macklon N.S., Eijkemans M.J., Mannaerts B.M., Coelingh Bennink H.J., Fauser B.C. for the Ganirelix dose-finding study group. Dynamics of the development of multiple follicles during ovarian stimulation for in vitro fertilization using recombinant follicle-stimulating hormone (Puregon) and various doses of the gonadotropin-releasing hormone antagonist ganirelix (Orgalutran/Antagon). *Fertil.Steril.* **75** (4) 688-93. 2001.

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hormone window'. In: Jansen R and Mortimer D (eds), *Towards reproductive certainty: fertility & genetics beyond 1999*, pp 195-9. Parthenon Publishing, New York. 1999.

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Publications related to the present thesis

Fauser B.C., Laven J.S., de Jong D., Macklon N.S. Gonadotropine-"releasing"-hormoonantagonisten: toepassing bij ovarium-stimulering en geslachtssteroid afhankelijke aandoeningen. *Ned. Tijschr. Geneeskd.* **144**, 370-374. 2000.

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Presentations related to the present thesis

de Jong D., Macklon N.S., Fauser B.C. Minimal ovarian stimulation without luteal support: initiation of low dose recombinant FSH in the mid follicular phase and prevention of the LH surge with a GnRH antagonist (cetrorelix). Presented at the 11th *World Congress on In Vitro Fertilization & Human Reproductive Genetics*, Sydney, May 1999: abstr S-002.

Curriculum vitae auctoris

Curriculum vitae auctoris

De auteur van dit proefschrift, Diederick de Jong, werd geboren op 13 Januari 1968 te Alkmaar. Hij behaalde in 1987 het Atheneum-B diploma aan het Bisschop Beckers College te Eindhoven. De studie Geneeskunde werd in 1987 begonnen aan de Vrije Universiteit te Brussel maar afgemaakt aan de Erasmus Universiteit te Rotterdam. Het doctoraal examen behaalde hij in mei 1994, gevolgd door het arts examen in september 1996. Al vroeg had patient gerelateerd onderzoek zijn aandacht, in 1992 hielp hij gedurende een half jaar mee met obsterisch onderzoek op de afdeling obstetrie en gyneacologie van het Catharina Ziekenhuis te Eindhoven (hoofd: dr. P.A. de Jong). In 1993 en 1994 was hij werkzaam als onderzoeker op de afdeling inwendige geneeskunde I (begeleiders: dr. F.H. Derkx en prof.dr. M.A.D.H. Schalekamp). In 1996 werd een onderzoeksstage gevolgd aan de Royal Infirmary en Centre for Reproductive Biology (hoofd: prof.dr.ir. S.G. Hillier). In september 1996 werd de kans benut om als arts onderzoeker in dienst te komen bij de afdeling gynaecologie en verloskunde van het Academisch Ziekenhuis Dijkzigt te Rotterdam. Hier werden de eerste stappen tot het tot stand komen van dit proefschrift gezet, onder begeleiding van prof.dr. B.C.J.M. Fauser werkte hij gedurende 4 jaar aan het verzamelen van gegevens en schrijven van artikelen. De wens om klinisch werkzaam te zijn werd verhoord, tussen september 2000 en september 2002 was de promovendus werkzaam als arts-assistent in opleiding op de afdeling gynaecologie en verloskunde van het MCRZ locatie Clara (opleider: dr. J.A. Wijnen). Thans is hij werkzaam arts-assistent in opleiding op de afdeling gynaecologie en verloskunde van het Academisch Ziekenhuis Dijkzigt (opleider: prof.dr. Th.J.M. Helmerhorst).

Dankwoord

Dankwoord

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Salut!

Rotterdam,

1 november 2002

Seems that somebody's just opened the door
to the book of life, or is it death!
Is there ever any way out?

Steve Harris
- 1998 -