Innovative Drug Development for Infertility Therapy

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Para nymphs

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Front cover

Three-dimensional structure of respectively follitropin-β (alpha-subunit in green and beta-subunit in blue) bound to the external domain of the FSH receptor (red), corifollitropin alfa with carbohydrate side chains (purple/yellow) and the decapeptide ganirelix.

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Innovative Drug Development for Infertility Therapy

Innovatieve ontwikkeling van geneesmiddelen ter behandeling van infertiliteit

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
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Introduction to Fertility Drugs, Mechanism of Action and Development History

Chapter 1 Introduction to Fertility Drugs, Mechanism of Action and Development History

General Introduction

Healthcare innovation depends on the development of new drugs which includes drug discovery, lead optimization, large scale production, development of a pharmaceutical formulation, development of a final presentation, pre-clinical pharmacology, toxicology and clinical research. Clinical studies are conducted to evaluate the efficacy and safety of investigational drugs and are performed in accordance with the principles of Good Clinical Practice (GCP). The latter is a standard for the design, conduct, performance, monitoring, auditing, recording analyses and reporting of clinical trials to ensure that the collected data are credible and accurate while the rights, safety and confidentiality of trial subjects are protected. Clinical studies are generally divided into 3 sequential phases. Phase I studies are the first-in-human exposure studies (Human Pharmacology) and are often performed in healthy volunteers unless certain safety aspects require a subgroup specification. Phase I studies provide the first assessments of safety and tolerability, metabolism, and pharmacologic actions following single or/and multiple rising doses of a new drug.

Following a favorable outcome, drug development proceeds to phase II studies (Proof of Concept) in patients to evaluate the effectiveness and establish the dose range of the drug in the target population. Sometimes drugs are first tested in a phase IIa study with a relatively small number of patients to decide on the range of doses to be tested in a phase IIb (dose-finding) study. Relevant biomarkers may be the primary (surrogate) endpoint to allow the testing of several doses during phase II of clinical development. Evaluation of both efficacy and safety should indicate which doses are too low or too high for the intended indication. Dose selection of drugs for further development in phase III studies is based on the outcome of dose-finding studies, and may be supported by modeling and simulation to predict the optimal dose(s) for the intended study population. After final dose selection, additional phase I trials in volunteers are required to document among others the absolute bioavailability, drug-drug interaction, or cardiac safety of the specific selected dose(s).

Phase III studies are large, randomized, comparative trials performed to document the safety and efficacy of a new drug in the target population and to evaluate the overall benefit-risk ratio of the drug and/or new treatment regimen under development. Prospective phase III studies may include several hundred to thousands of patients depending on the intended indication, the desirable primary endpoints, and the size of the target population. Retrospective pooled analyses of individual patient data of phase III studies may provide further safety and efficacy data on patient or treatment subsets of interest.

The clinical development of any innovative drug may take 6 to 10 years and is a considerable financial investment, especially as many compounds may enter clinical research but only very few will make it up to approval by Health Authorities. The initial labeling of registered drugs is usually restricted to the study population, posology and clinical outcome of pivotal clinical studies, which for comparative reasons may deviate from the actual clinical practice.

After launch, routine Pharmacovigilance should carefully monitor the safety of these drugs in Real-World practice. In parallel, phase IV studies may support the use of a new drug in subsets of patients not previously studied, or may extend the posology of the drug, which may lead to an update of the labeling at least if phase IV studies are adequate and conclusive.

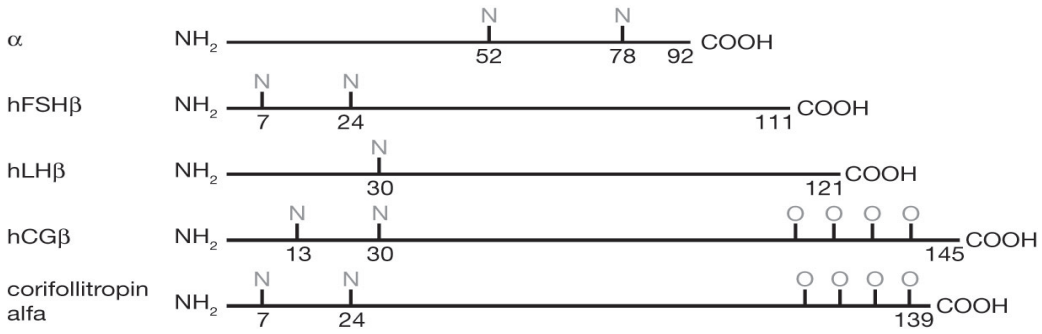
This thesis provides an overview of the sequential development of three fertility drugs, namely follitropin-β (recombinant human follicle-stimulating hormone, rFSH), ganirelix (gonadotropin-releasing hormone (GnRH) antagonist) and corifollitropin alfa (recombinant fusion FSH analog of human origin). Follitropin-β and corifollitropin alfa are both New Biological Entities (NBEs) and ganirelix is a New Chemical Entity (NCE). In contrast to NCEs, NBEs are often complex therapeutic (glyco)proteins of large molecular size with three-dimensional structural elements which are essential for their bioactivity. The next chapters summarize the main outcome of preclinical testing and of clinical research in the different phases I, II and III. They present the main scientific findings per phase for the three mentioned fertility drugs and explain per phase which information was essential in the development strategy and design of clinical trials which finally led to a positive benefit/risk evaluation of each drug by different Health Authorities. The introduction of these fertility drugs has changed clinical practice dramatically over the last 15 years from lengthy and complicated to short and simple treatment regimens. Actually, it is a very good example of how drug development can drive alternative treatment regimens and result in therapeutic approaches with fewer interventions.

Recombinant human FSH, follitropin-β

Follicle-stimulating hormone (FSH) is produced by the pituitary gland and is a member of the glycoprotein hormone family which also includes luteinizing hormone (LH) and chorionic gonadotropin (CG), collectively called gonadotropins. These heterodimeric hormones share a common α-subunit, but differ in their β-subunit which confer biological specificity. Figure 1 shows the common α-subunit and different β-chains of FSH, LH, hCG and corifollitropin alfa [Fauser et al 2009]. The FSH molecule (≈34 kD) contains four asparagine-linked carbohydrate chains of which two are linked to the α-chain of 92 amino acids and two are linked to the β-chain of 111 amino acids. The presence of these carbohydrate chains gives FSH its very characteristic isohormone profile known as microheterogeneity [Pierce and

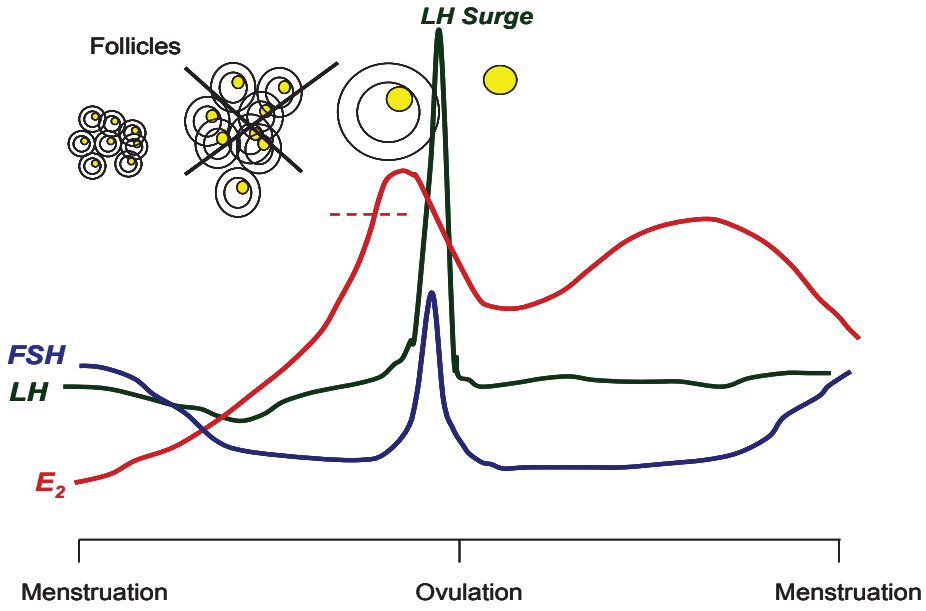
Parsons, 1981]. Natural FSH exists in different isoforms which differ in charge, relative abundance, receptor affinity, bioactivity and plasma half-life [Ulloa-Aquirre et al, 1992; Timossi et al 2000].

Figure 1 **Structure of the glycoprotein hormone gonadotropins: schematic representation of the universal α-subunit and the unique β-subunits showing N- and O-linked glycosylation binding sites.**



In the ovary, FSH is involved in follicle recruitment and follicle maturation through its growth-promoting and steroidogenic effects (see Figure 2). In the early follicular phase of the natural cycle, high FSH levels initiate follicular development, which leads to rising serum estradiol (E_2) levels. Due to negative feedback caused by the rising E_2 levels, FSH levels decline, resulting in atresia of the smaller follicles and selection of a single dominant follicle. Once serum E_2 levels surpass a certain level, a positive feedback loop stimulates the pituitary and results in the pre-ovulatory LH surge. This LH surge is responsible for final oocyte maturation and subsequent ovulation.

Figure 2 **Role of FSH and LH in follicular dynamics during the natural cycle**



In the testis, FSH stimulates proliferation of Sertoli cells during fetal life, sustains final differentiation of Sertoli cells during puberty and maintains normal spermatogenesis in adults. Deficient endogenous production of FSH may cause infertility.

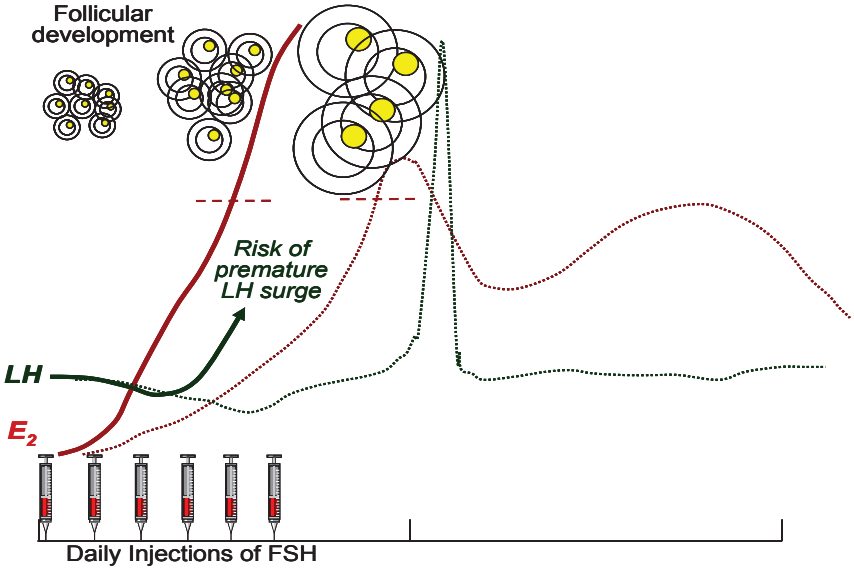
Administration of exogenous gonadotropins may solve infertility, and gonadotropin preparations derived from the urine of postmenopausal women have been on the market for over 30 years [Lunenfeld 2004]. Today's recombinant DNA technology is providing a more attractive source for producing large quantities of FSH. The manufacturing of recombinant DNA-derived FSH is independent of the collection of large volumes of urine and the recombinant product is devoid of contaminants of human origin such as urinary proteins and potential human viruses. For the production of recombinant FSH (rFSH) the application of a mammalian cell-line is required to guarantee proper glycosylation, and thus bioactive FSH [Keene et al, 1989]. For the expression of FSH, a Chinese Hamster Ovary (CHO) cell-line was used as a host cell system as these cells can be easily transfected, can be grown on a large scale, and are able to secrete glycoproteins closely related to those found in man. For the expression of rFSH (INN[1]: follitropin-β. Brandnames: Puregon, Follistim) a CHO cell-line was transfected with plasmids containing one gene encoding the human α-subunit and one gene encoding the human β-subunit of human FSH [Wezenbeek et al 1990]. The nucleotide sequences of the coding regions are identical to those described in the literature human FSH [Fiddes and Goodman 1981; Jameson et al 1988]. After transcription and translation processes, the α- and β-subunit is glycosylated and assembled and the intact FSH dimer is secreted into the culture medium. Like natural FSH, rFSH exists in different isoforms which differ in charge due to difference in the amount and/or composition of the carbohydrate chains, in particular sialic acid residues. The charge heterogeneity of rFSH mainly depends on the choice of the host cell, vector system, fermentation and purification process [Olijve et al 1996; De Leeuw et al 1996] After purification of rFSH from the culture medium, the final pharmaceutical product (follitropin-β) has a high biochemical purity (>99%) and a high specific biological activity (at least 10,000 IU/mg protein) [De Boer and Mannaerts 1990] Moreover, at the protein level, the amino acid composition and amino acid sequence of the individual subunits match those of natural FSH. Olijve et al described how rFSH can be produced in CHO cells and confirmed that the monosaccharide composition of the carbohydrate moiety is highly similar to that of purified urinary FSH [Hård et al 1990; Olijve et al 1996]. This is crucial as the glycosylation pattern of rFSH determines its structural and functional properties, stability, receptor affinity and half-life [Mannaerts et al 1991].

[1] International Nonproprietary Names (INN) facilitate the identification of pharmaceutical substances or active pharmaceutical ingredients. Each INN is a WHO-assigned unique name that is globally recognized and is public property. A nonproprietary name is also known as a generic name

Clinical research of follitropin-β started in 1990 (see Table 1) with the first-in-human exposure studies including male and female volunteers with hypogonadotropic hypogonadism. The first patient study of follitropin-β tested the combination with various long GnRH agonist protocols to prevent premature LH surges during stimulation was performed at the VUB in Brussels [Devroey et al, 1994].

The need to control for premature LH surges, premature luteinization and ovulation during multiple follicular growth is explained in Figure 3. During ovarian stimulation when FSH levels are maintained because of exogenous gonadotropin administration, natural selection of a single dominant follicle does not occur and multiple follicles continue to grow. This increased number of follicles produces higher serum E_2 concentrations and consequently, the serum E_2 level which triggers the pre-ovulatory LH surge is reached prematurely, i.e. when the follicles have not yet fully developed and ovulation becomes imminent.

Figure 3 Role of exogenous FSH and endogenous LH in follicular dynamics during stimulation for induction of multiple follicular development



Note: For reference, dotted lines represent the E_2 profile (red) and the LH profile (black) in a natural cycle

Without intervention by a GnRH analogue, premature luteinization occurs in about 25% of treatment cycles with multiple follicular development, leading to cycle cancellation or compromised treatment outcomes.

Following the phase II study of follitropin-β in combination with different GnRH agonist protocols and controlled randomized phase III studies in IVF patients comparing follitropin-β with urinary FSH in a long GnRH agonist protocol and in patients undergoing classical ovulation induction, the compound was approved for both female indications in 1996. The male indication for treatment of deficient spermatogenesis due to hypogonadotropic hypogonadism was obtained in 2000.

Follitropin-β was first brought to the market in 1996 as a lyophilized powder in vials (50, 75, 100, 150 IU) for reconstitution. The formulation was improved in 1999 to a solution for injection (50, 75, 150, 200, 225 or 250 IU/0.5 mL) in vials and in 2000 to a solution for injection in cartridges (150 IU/0.18 mL, 300 IU/0.36 mL, 600 IU/0.73 mL, 900 IU/1.08 mL) designed to be used in conjunction with a pen injector (Puregon Pen). Follitropin-β is approved in nearly 100 countries and has the global brand name Puregon. In the United States and Japan the brand name is Follistim, whereas the trade name in India, Sri Lanka and Nepal is Recagon.

GnRH antagonist, ganirelix

The synthesis and secretion of FSH (and LH) by the pituitary gland is regulated by GnRH and its receptor. GnRH is a decapeptide secreted by the hypothalamus in a pulsatile manner and via a direct venous portal connection it binds to the GnRH receptors of the pituitary gland. The structure of GnRH was elucidated by Schally and coworkers in 1971 [Baba et al 1971]. GnRH is composed of 10 amino acids with crucial functions at positions 1, 2, 3, 6 and 10. Position 6 is involved in enzymatic cleavage, positions 2 and 3 in gonadotropin release, and positions 1, 6 and 10 are important for the three-dimensional structure.

Synthetic analogues of GnRH with a deletion or substitution of the histidine in position 2 have been shown to be competitive antagonists of the native hormone. Sequential and multiple substitutions at other positions in the molecule have resulted in progressive increases in antagonistic potency [Karten and Rivier, 1986; Conn and Crowley 1994; Huirne and Lambalk 2000].

The synthesis of GnRH receptor antagonists posed greater problems than those associated with the synthesis of GnRH agonists. Problems included insufficient potency and lack of solubility, and first and second-generation antagonistic analogues of GnRH were known to cause hypersensitivity responses due to direct activation of mast cells resulting in the release of various mediators, in particular histamine.

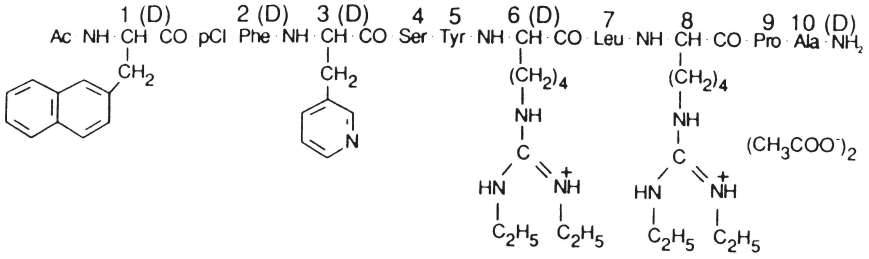
Ganirelix (INN: ganirelix. USAN[2]: ganirelix acetate. Brandname: Orgalutran) is a typical example of a third generation antagonist with high GnRH antagonistic activity and minimal histamine releasing properties. It blocks the GnRH receptor by competitive binding but does not activate the receptor in the pituitary gland [Mannaerts and Gordon 2000].

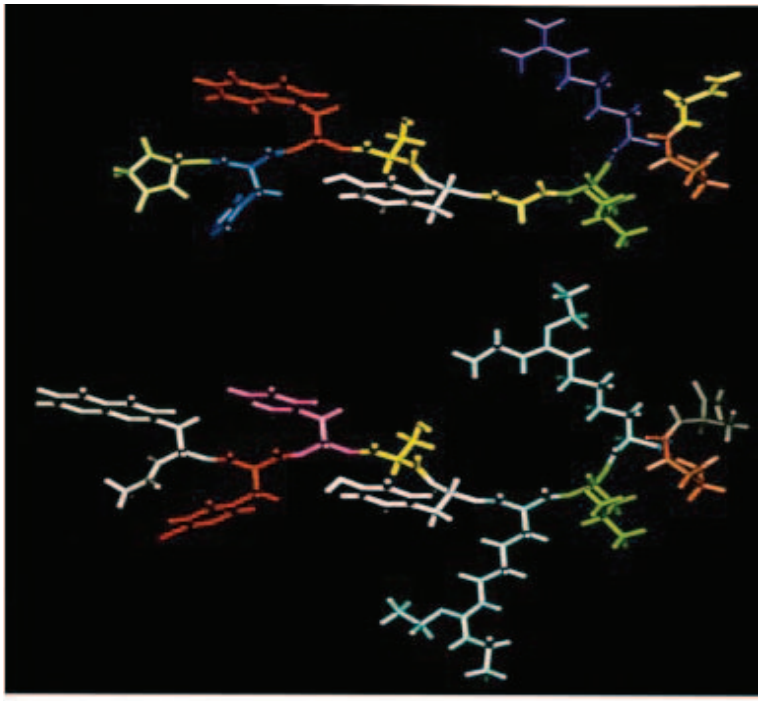
As a result a rapid, profound, reversible suppression of the release of pituitary gonadotropins (LH and FSH), and consequently of gonadal function, occurs. The decapeptide contains several amino acids that are unnatural in stereochemistry and/or in structure (see Figure 4). Its molecular weight is 1570.4 and substitutions may be found at

[2] United States Adopted Names (USAN) are unique nonproprietary names assigned to pharmaceuticals marketed in the United States Each name is assigned by the USAN Council

position 1, 2, 3, 6, 8 and 10. The latter determine its physical-chemical properties which include high aqueous solubility, high stability and high receptor-binding affinity.

Figure 4 Molecular structure of the decapeptide ganirelix. Picture (taken from ORGYN 2000) provides 3-D model of native GnRH (upper figure) and ganirelix (lower figure).





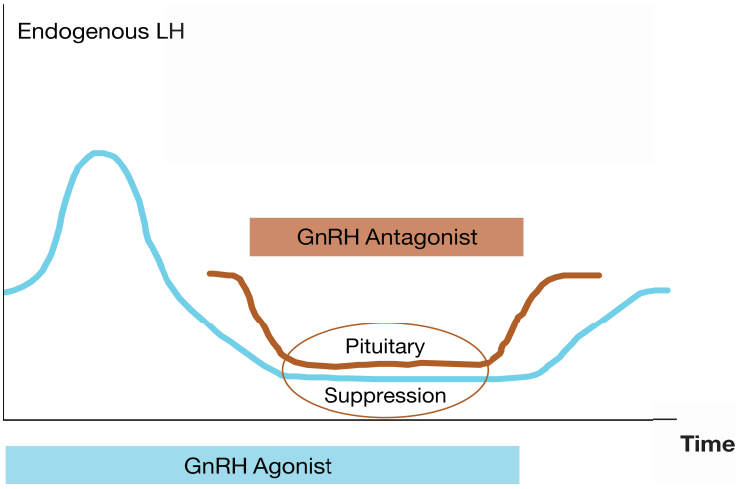
As a result ganirelix has a 9-fold higher rat GnRH-receptor binding affinity (KD = 0.4 nM) than GnRH (KD = 3.6 nM). Additional experiments investigated the specificity of binding of ganirelix to other receptors of the G-protein-coupled-receptor family and other types of receptors. Subsequently, specificity and efficacy of action was demonstrated in *in vivo* animal pharmacology, both with respect to general and reproductive pharmacology. Special attention has been paid to the reversibility of effects of ganirelix on female reproductive function.

Non-clinical pharmacokinetic studies show that the *in vitro* and *in vivo* stability of ganirelix is considerably greater than of native GnRH due to the use of D-amino acids for substitution. There are many conditions for which suppression of endogenous hormones would be helpful for treatment, for example suppression of estradiol concentrations in endometriosis could

lead to reduced menstrual cycle-related pain and such treatment could diminish the growth of sex-hormone dependent malignant neoplasms such as prostatic and breast cancers. Non-clinical research of ganirelix has been performed mainly by Syntex Research (Palo Alto, CA, USA, RS-26306-298) for the long-term treatment of various hormone-dependent disorders such as prostate cancer.

First and second generations of antagonistic analogues of GnRH were suffering from weak activity, depot formation and/or histamine-mediated side effects due to direct activation of mast cells. The third-generation antagonist ganirelix appeared to have significantly reduced histamine-releasing capacity in *in vitro* studies and was well-tolerated by humans [Rabinovici et al, 1992; Nelson et al 1995]. In the framework of non-clinical safety and efficacy testing, ganirelix was compared to second-generation GnRH antagonists such as detirelix in several studies. The compound was developed by NV Organon for the prevention of premature LH surges in women undergoing ovarian stimulation prior to Assisted Reproductive Technology (ART). In contrast to a GnRH agonist, a GnRH antagonist causes immediate and reversible blockage of the GnRH receptor at the pituitary gland and therefore reduces the secretion of FSH and LH within several hours after injection (see Figure 5). In 1999 ganirelix was the first approved GnRH antagonist and today ganirelix is registered in over 70 countries and is known under the brand name Orgalutran in most countries.

Figure 5 **Cartoon explaining the mechanism of action of a GnRH agonist and a GnRH antagonist, the latter causing immediate and reversible blockage of the GnRH receptor.**



Clinical research for this indication started in 1996 with a dose-finding trial in IVF patients (see Table 1) which was followed by 3 randomized controlled phase III studies comparing the efficacy and safety of a daily dose of 0.25 mg ganirelix with long protocols of GnRH agonists i.e. buserelin, triptorelin and leuprolide acetate. An overview on the clinical development of ganirelix including its historical background was first published in Human Fertility [Out and Mannaerts, 2002].

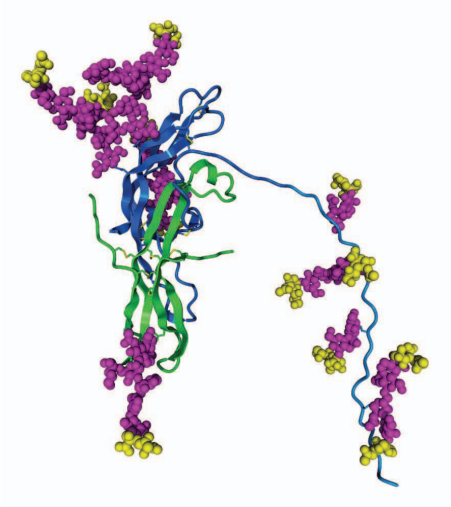
Long-acting recombinant FSH agonist, corifollitropin alfa

The first idea to design a fusion recombinant protein with a longer half-life than wild-type FSH was from Professor Irving Boime of Washington University in St. Louis, while investigating the hormone human Chorionic Gonadotropin (hCG) and comparing it with (natural) LH. Both hormones bind to the same receptor, probably because the first 114 amino acids of their β-subunits share 85% sequence identity. However, hCG-β is distinct from the LH-β subunit due to an additional carboxy-terminal peptide (CTP) consisting of 31 amino acids and 4 serine O-linked oligosaccharides (see Figure 1). This extension is not primarily involved in receptor binding or *in vitro* signal transduction, but provides the hCG molecule a much longer half-life and thus a longer *in vivo* bioactivity [Matzuk et al 1990; Mannaerts et al 1998; Wide et al 2010]. Boime and co-workers applied site-directed mutagenesis and gene transfer techniques [Fares et al 1992] to construct a hybrid gene containing the sequence encoding the CTP of the hCG β-subunit fused to the translated sequence of the human FSH β-subunit. The FSH β-CTP hybrid gene was then transfected with the common glycoprotein α-subunit gene and expressed in Chinese hamster ovary (CHO) cells. The recombinant fusion molecule (see Figure 6) also termed FSH-CTP, had similar *in vitro* receptor binding and steroidogenic activity compared with wild-type FSH but had significantly enhanced *in vivo* activity and plasma half-life [Fares et al 1992]. Further studies showed an approximately 10-fold increase in biopotency for the hybrid molecule compared with wild-type FSH [LaPolt et al 1992]. It was also demonstrated that a single injection of this fusion molecule stimulated sufficient follicular maturation in rats to facilitate ovulation induction 52 h later. In comparison, a single injection of the same dose of wild-type FSH was ineffective in increasing ovarian ovulatory potential. Interestingly, splitting the total dose of wild-type FSH into four injections given 12 h apart was as effective as a single dose of the new recombinant FSH analogue. These results indicated for the first time the importance of sustained FSH bioactivity during follicular maturation.

It was only several years after the studies by LaPolt that researchers at NV Organon generated a new CHO cell-line expressing the genes encoding the same hybrid molecule which finally led to the development of a long-acting FSH analog (INN: corifollitropin alfa. Brandname: Elonva). Since this molecule was produced on a large scale by a different CHO

cell-line, a full pharmacological characterization was performed which appeared to be in line with the early research by LaPolt [Van Schanke et al 2010; Verbost et al 2011]. Interestingly, this recombinant technology has been applied for other recombinant hormones like erythropoietin and has allowed the development of long-acting agents in other therapeutic areas [Fares et al 2007].

Figure 6 **Three-dimensional structure of corifollitropin alfa. In green the α-subunit, in bleu the β-subunit with the carboxyterminal peptide extension, in purple the carbohydrate side chains with sialic acid in yellow (Taken from Pharmaceutisch Weekblad, 21 jan, 2011).**



Early clinical development plans were to assess the suitability of corifollitropin alfa both for monofollicular ovulation in anovulatory patients and for the induction of multiple follicular development prior to ART. Phase II clinical research indicated that corifollitropin alfa is less suitable for the induction of monofollicular growth and development continued for multiple follicular development only. The rationale for development of a long-acting FSH agonist for therapeutic purposes was mainly to reduce the number of injections and to simplify treatment. Therefore, corifollitropin alfa was developed for the induction of multiple follicular development in combination with a GnRH antagonist protocol (see Figure 7) and the optimal dose(s) should replace the first 7 injections of FSH/hMG during stimulation. The phase II dose-finding study of corifollitropin alfa in a GnRH-antagonist protocol was started in 2003 (see Table 1). Two double-blind randomized controlled phase III trials of the 2 selected doses of corifollitropin alfa and one uncontrolled safety study with the highest dose of corifollitropin alfa led to its first registration in Europe in 2010. An overview on the clinical development of corifollitropin alfa including its historical background was first published in Human Reproduction Update [Fauser et al 2009]. At the end of 2012, corifollitropin alfa was registered in 65 countries including the 27 member states of the European Union.

Figure 7 Therapeutic interventions of the corifollitropin alfa regimen with concomitant GnRH antagonist treatment as applied during clinical development

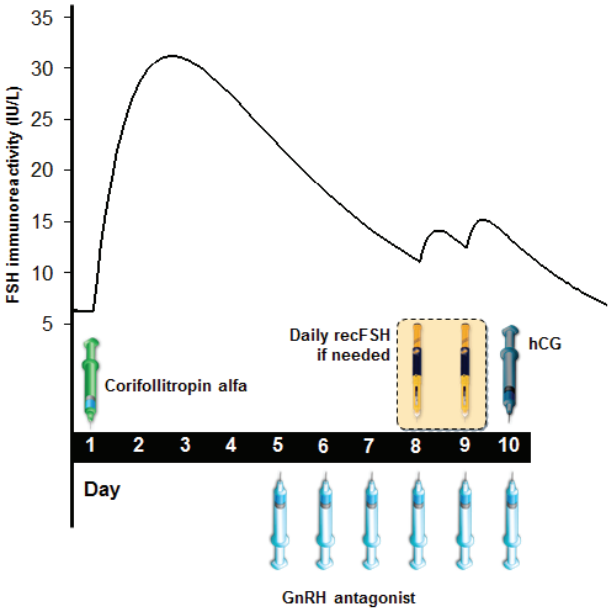


Table 1 Historical overview of the pivotal phase I, II and III intervention trials of follitropin-β, ganirelix and corifollitropin alfa for the approved indications only. First follitropin-β was developed in comparison to urinary FSH in a long GnRH agonist protocol, second the GnRH antagonist ganirelix was developed in comparison to a long GnRH agonist protocol and finally corifollitropin alfa was developed in comparison to follitropin-β using a GnRH antagonist protocol.

	Follitropin-β		Ganirelix		Corifollitropin alfa	
Phase I	P37601 P37602 **1990-1991**	Single and multiple dose studies in male and female volunteers with hypogonadotropic hypogonadism (HH).	First human exposure trials P38604 P38605 **1997-1998**	Performed by Syntex Single-dose and multiple-dose studies in female volunteers of reproductive age.	P38801 **1997-1998** P38802 **2000**	Repeated exposure study in male volunteers with HH. Female healthy volunteers of reproductive age and pituitary suppressed by Lyndiol.
Phase II	P37603 **1991-1992**	Open pilot study of follitropin-β in combination with different GnRH agonist protocols.	P38602 **1996**	Double-blind, randomized dose-finding study of ganirelix in patients undergoing ovarian stimulation with follitropin-β.	P38807 **2001-2002** P38826 **2003-2004**	Pilot study in IVF patients testing corifollitropin alfa using a ganirelix protocol. Dose-finding trial in IVF patients treated with three doses of corifollitropin alfa using a ganirelix protocol.
Phase III	P37608 P37611 P37604 **1992-1993** P37609 **1992-1994** P37618 **1996-1998**	Randomized controlled trials in comparison to urinary FSH using a long protocol of buserelin or triptorelin or in comparison to hMG without any GnRH analog. Randomized controlled trial in comparison to urinary FSH in patients with chronic anovulation (WHO group II). Open, uncontrolled trial in HH men to induce spermatogenesis.	P38607 P38816 P103001 **1997-1998**	Randomized controlled trials in comparison to long protocol of buserelin, triptorelin or leuprolide acetate in patients undergoing ovarian stimulation with follitropin-β.	P38819 P107012 **2006-2007** P38825 **2006-2009** P06029 **2010-2012**	Double-blind randomized controlled trials in comparison to follitropin-β using a ganirelix protocol (≤ 36 yrs). Open, uncontrolled trial of repeated cycle of corifollitropin alfa using a GnRH antagonist protocol (≤ 39 yrs). Double-blind randomized trial in comparison to follitropin-β using a ganirelix protocol (35-42 yrs).
First scientific approval	**1996**		**1999**		**2010**	

Chapter 2

Preclinical research

Chapter 2 Preclinical Research

2.1 Introduction

Evaluation of preclinical pharmacology is essential to estimate the potency of new drugs in first-in-human clinical studies. The biological action of (recombinant) FSH or its analogs is determined by its structure which affects receptor affinity, intrinsic bioactivity and elimination half-life. Preclinical comparative tests and models should be validated and able to capture those characteristics that are predictive for human pharmacology. For the detection, quantification and characterisation of follitropin-β and corifollitropin alfa basically 4 different type of assays have been applied, namely immunoassays, receptor binding assays, *in vitro* bioassays and *in vivo* bioassays [Rose et al 2000]. Comparative experiments were indicated to prove that follitropin-β and corifollitropin alfa bind to the FSH receptor in the same mode as natural FSH, regardless of whether the rat or human FSH receptor was applied. For comparative experiments with follitropin-β, the preferred reference was either a recognized pituitary or urinary FSH standard, or urinary FSH (Metrodin), the anticipated reference in clinical studies. For comparative experiments with corifollitropin alfa, the preferred reference was follitropin-β which rapidly became the standard of care after registration. Parallel dose-response curves allowed the expression of immunoreactivity and *in vitro* bioactivity relative to rFSH. Comparative neutralization of *in vitro* bioactivity by monoclonal antibodies recognizing different epitopes indicates similar binding and affinity, supporting the structural and functional similarity of the tested recombinant and natural molecules.

Characterization by means of immunoassays

The different assays used to characterize follitropin-β and corifollitropin alfa each provide specific information which should be combined to obtain a full appreciation of the preclinical pharmacology. The immunoassay is usually a so-called structurally-specific assay and today's double-antibody immunoassays are based on the detection of two antigenic epitopes on respectively the FSH α- and β-subunits resulting in the recognition of intact FSH molecules only. Immunoassays usually have a high sensitivity, specificity and precision and are routinely applied to measure serum FSH levels. Even when using the same International Standard, there is significant inter-assay heterogeneity for FSH immunoassays, the outcome depending on the assay design and the selected monoclonal antibodies recognizing the different FSH epitopes [Sturgeon and Ellis, 2007]. Ideally, all FSH charge isoforms are equally well-recognized in a sensitive and highly specific double-antibody FSH immunoassay. The consistent use of the same FSH immunoassay allows direct comparison of circulating FSH levels during preclinical and clinical research and, if indicated, a pooled comparative analysis of serum FSH levels from patients of several randomized studies.

In vitro receptor and bioassay studies

The receptor binding assay and *in vitro* bioassay are functionally-specific assays measuring hormone-specific processes in isolated target cells or cell-lines. In general these assays are hormone-specific and sensitive, but they may suffer from a relatively high intra- and inter-assay variation due to culture conditions. The added value of the *in vitro* bioassay in comparison to the receptor binding assay, is that not only the receptor binding but also the intrinsic bioactivity of a sample is measured. The latter is affected by the FSH micro-heterogeneity which is determined by the structure of the carbohydrate side chains and the amount of sialic acid [Simoni et al 1994; Creus et al 2001].

In vitro bioassays are usually based on hormone-specific processes in isolated target cells. Compared with *in vivo* bioassays, they have a high sensitivity and precision. Application of such assays for the quantification of bioactivity diminishes the use of laboratory animals and reduces the amount of gonadotropins required for testing. Like *in vivo* bioassays, *in vitro* bioassays are less species-dependent and therefore may be suitable alternatives to immunoassays when bioactivity is considered more relevant than immunoreactivity. *In vitro* FSH bioassays are essential during the characterization of natural or recombinant FSH (analogues) as they can be applied for several research objectives, namely i) to select the transfected cell-line that expresses the highest FSH bioactivity, ii) to measure *in vitro* FSH bioactivity in FSH preparations or blood samples from clinical trials iii) to determine the specificity and neutralizing activity of monoclonal or polyclonal antibodies [Mannaerts et al 1987].

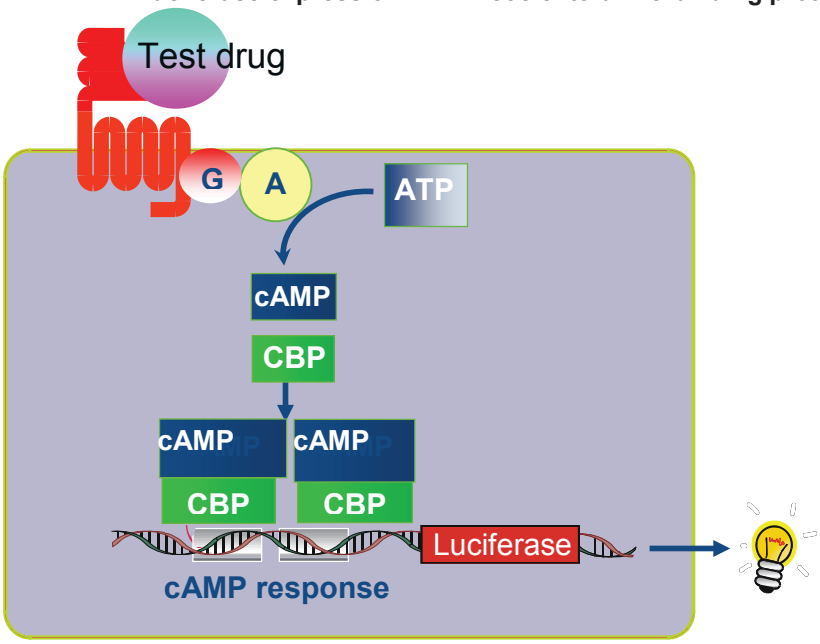
The first *in vitro* FSH bioassays were based on the induction of aromatase activity in either isolated rat Sertoli cells or granulosa cells [Van Damme et al 1979; Ritzen et al 1982; Jia and Hsueh 1986]. While the Sertoli cell assay was less sensitive than the granulosa cell assay, the latter was complicated by interference by other peptide hormones such as LH, hCG, prolactin and GnRH. A sensitive bioassay of LH activity was described earlier using the stimulation of testosterone production in mouse Leydig cells [Van Damme et al, 1974].

Accordingly, the comparative *in vitro* bioactivity of follitropin-β produced by a CHO cell-line was studied and compared to that of natural FSH preparations using an *in vitro* bioassay with cultured Sertoli cells of immature rats [Mannaerts et al 1991]. The specificity, or the lack of LH bioactivity, was documented in the mouse Leydig cell assay. In addition, monoclonal antibodies recognizing different epitopes of the α-unit and β-unit of the intact FSH molecule were incubated with rFSH and natural FSH to compare their neutralising capacity in the *in vitro* FSH bioassay. The *in vitro* efficacy of rFSH in rat Sertoli cells was subsequently confirmed in human granulosa cells isolated from preovulatory follicles in which rFSH

induced dose-dependent increases in the production of estradiol and progesterone [Mason et al 1993].

Today, most research groups use the transfected CHO cell-line expressing the rat or human FSH receptor (CHO-FSHR cell-line) to test the bioactivity of gonadotropin preparations [Albanese et al 1994; Christin-Maitre and Bouchard, 1996]. Such assays may use a CHO cell-line which expresses both the FSH receptor (FSH-R) gene and the fire fly luciferase reporter gene. Binding of FSH to the FSH receptor promotes the conversion of ATP into cAMP. Subsequently, cAMP initiates the cellular responses which cause luciferase production. Thus, the level of luciferase activity is correlated to the FSH bioactivity. Luciferase activity is measured by chemiluminescence caused by an enzymatic conversion of the SteadyLite substrate (see Figure 8).

Figure 8 **CHO cells stably transfected with rat or human FSH receptor and luciferase reporter gene. Activation of the FSH-R leads to cAMP production followed by luciferase expression. CBP= coelenterazine-binding protein.**



In vivo bioassays

Quantification of FSH for clinical use traditionally involves the use of *in vivo* bioassays which reflect both the biological action at the target organ and the clearance of the FSH preparation. The assay developed in 1953 by Steelman and Pohley [Steelman and Pohley 1953] based on the augmentation of ovarian weight due to FSH with hCG has proven to be a robust specific *in vivo* bioassay and is still the basis of Pharmacopeia monographs for the statutory determination of the FSH potency of therapeutic preparations. The major drawbacks of this assay that requires large numbers of laboratory animals are its insensitivity and poor precision. In addition the daily dosing regimen may preclude FSH forms with a short

half-life from exerting a biological effect whereas FSH forms with a very long half-life may provide an over-estimated effect.

The *in vivo* biopotency of follitropin-β as well as of corifollitropin alfa was determined by means of the Steelman and Pohley assay in the earliest stages of preclinical research. However, due to its long half-life the relationship between the dose of corifollitropin alfa and the increase of ovarian weight appeared to be different from that observed with daily FSH preparations. Thus, the calculation of the *in vivo* bioactivity of corifollitropin alfa in terms of the current international FSH standard does not provide a reliable guide to the best therapeutic dose in humans. For highly purified recombinant gonadotropins like follitropin-β and corifollitropin alfa the unitage may also be expressed in mass or protein, if alternative methods to predict the *in vivo* bioactivity are applied, which may include an *in vitro* bioassay combined with physico-chemical tests [Mulders et al 1997; Driebergen and Baer 2003].

Animal pharmacology

Animal pharmacology experiments with follitropin-β were undertaken i) to compare the *in vivo* efficacy of follitropin-β with that of urinary FSH or human menopausal gonadotropins (hMG), ii) to re-examine the two-cell two gonadotropin theory, and iii) to gain further knowledge on the role of FSH and LH activity in follicular growth and atresia and simultaneous uterine development [Mannaerts et al 1994]. For that purpose immature hypophysectomized rats were treated for 4 days with follitropin-β without or with supplementary doses of hCG. Immature rats undergoing hypophysectomy at the age of 21 to 22 days develop only very few small antral follicles in contrast to intact animals. The development of primordial follicles to primary follicles is known to be gonadotropin independent and the development of primary follicles to small antral follicles is known to to be inhibited in the absence of gonadotropins [Baird 1987]. With the availability of rFSH, the induction of follicle growth and atresia could be examined in the complete absence of LH activity, whereas supplementation with exogenous LH activity documented its added value during folliculogenesis. These experiments also provided more definitive evidence in favor of the two-cell two gonadotropin theory, a concept that was proposed several decades earlier following studies in immature hypophysectomized rats treated with purified gonadotropins [Lohstroh and Johnson, 1966]. This theory advocates that both FSH and LH are essential for estrogen biosynthesis, as LH in the theca cells induces androgen production which is the substrate for aromatase cytochrome P450 induced by FSH in the granulosa cells. In 1989 Hodgen suggested reexamination of this theory as normal rising estradiol levels in response to urinary FSH were shown following gonadotropin-suppression. However, following treatment with GnRH-analogues, the remaining endogenous LH secretion as well as ovarian androgens could be sufficient to cause the increases in estradiol.

Animal pharmacology experiments with corifollitropin alfa were undertaken mainly to compare the *in vivo* potency of corifollitropin alfa with follitropin-β, either in a rat superovulation assay or in a rat ovarian weight assay [Verbost et al 2011], as well as to confirm the metabolism of corifollitropin alfa [Van Schanke et al 2010].

2.2 Results

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Comparative *in Vitro* and *in Vivo* Studies on the Biological Characteristics of Recombinant Human Follicle-Stimulating Hormone

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ABSTRACT. The *in vitro* and *in vivo* activities of recombinant human FSH (recFSH) produced by a Chinese hamster ovary cell line were studied and compared with those of natural FSH preparations. The specific FSH activities of recFSH established by immunoassay and *in vivo* bioassay were greater than 10,000 IU/mg protein and considerably higher than the activities of tested urinary FSH references, while the *in vivo* bio/immuno ratios of these preparations were not significantly different. Compared to a highly purified pituitary standard (IS 83/575), recFSH had a comparable high specific *in vivo* bioactivity, but the specific immunoreactivity of IS 83/575 was about 2 times lower.

In receptor displacement and *in vitro* bioassay studies recFSH provided dose-response curves parallel to those of pituitary and urinary FSH references. When equal amounts of immunoreactive FSH were tested, recFSH and urinary and pituitary FSH displayed comparable activities in both assays. The *in vitro* bioactivity of recFSH could be neutralized effectively by each of three monoclonal antibodies raised against recFSH (α-specific), urinary FSH (β-specific), and pituitary FSH ($\alpha\beta$-specific), respectively. Moreover, 50% inhibition of comparable responses induced by recFSH, urinary "pure" FSH, or pituitary FSH was established by the same amount of monoclonal antibody. These results support the structural and functional similarity of recFSH and natural FSH. To test whether recFSH is capable of inducing LH-specific biological responses, the *in vitro* induction of testosterone production in mouse Leydig cells was assessed. At least 16 IU recFSH/ml incubate were needed to increase testosterone production, indicating that the intrinsic LH bioactivity of recFSH is negligible (<0.025 mIU LH/IU FSH). The *in vivo* efficacy of recFSH was examined by treating immature female hypophysectomized rats during 4 days with recFSH only or with recFSH supplemented with hCG. RecFSH only treatment increased ovarian weight and aromatase activity in a dose-dependent manner. When recFSH dosages providing submaximal responses were supplemented with 1 IU hCG, both ovarian weight and aromatase activity were largely augmented. Neither recFSH nor urinary pure FSH, administered in a high dose was able to increase plasma estradiol levels, while ovarian weight and aromatase activity were increased to the same extent. However, when recFSH was supplemented with only 0.1 IU hCG, a 3-fold increase in median plasma estradiol levels was obtained. These findings support the two-cell two-gonadotropin theory, holding that both FSH and LH are required for estrogen biosynthesis, but also reveal that only very small amounts of LH activity are sufficient to increase estrogen secretion up to measurable plasma levels. The increases in ovarian weight, aromatase activity, and plasma estradiol induced by recFSH supplemented with 1 and 10 IU hCG were comparable to those induced by two human menopausal gonadotropin references. In conclusion, the present study demonstrates that the *in vitro* and *in vivo* biological characteristics of recFSH are indistinguishable from those of FSH isolated from natural sources. (*Endocrinology* **129:** 2623–2630, 1991)

FSH PRODUCED by the anterior pituitary gland plays a pivotal role in female and male reproduction by stimulating gonadal differentiation and maturation via its regulatory action on the Sertoli cell in the testis and the granulosa cell in the ovary. The mechanism of FSH action includes binding to FSH-specific plasma membrane receptors and subsequent activation of the adenylate cyclase system, resulting in the *de novo* synthesis of steroidogenic enzymes and various paracrine and endocrine factors (1, 2).

In granulosa cells, FSH enhances the synthesis of aromatase cytochrome P450, resulting in an increased conversion of androgens to estrogens. Since aromatase substrate, mainly androstenedione, is produced in the thecal cell under the influence of LH, both FSH and LH are thought to be essential for estrogen biosynthesis (2, 3). The first evidence for this so-called two-cell two-gonadotropin concept was obtained several decades ago using immature hypophysectomized rats and highly purified gonadotropins (4–6). More recently, it has been suggested to reexamine the two-cell theory (7), since ovarian estradiol biosynthesis in hypogonadotropic subjects is equally well stimulated by human "pure" FSH as by human menopausal gonadotropins (hMG) containing an equal ratio of FSH to LH activity (8–10).

While natural human FSH preparations may still con-

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tain small amounts of contaminating hormones such as LH, recombinant human FSH (recFSH) is guaranteed to be free from such hormones. RecFSH has been produced by a Chinese hamster ovary (CHO) cell line transfected by the genes encoding human FSH (11). Carbohydrate structure analysis has demonstrated a close resemblance between the recombinant and the natural FSH glycans (12). Moreover, recFSH appeared to have the same charge heterogeneity as natural FSH (13). In the present study the *in vitro* and *in vivo* biological properties of recFSH were investigated and compared with those of natural human FSH. For this purpose, receptor displacement and *in vitro* bioassay studies were performed. The *in vivo* efficacy of recFSH alone or in combination with hCG was tested in immature hypophysectomized rats by measuring increases in ovarian weight, ovarian aromatase activity, and plasma estradiol levels.

Materials and Methods

CHO cell line

Stable transfected cell lines producing recFSH have been established by transfection of CHO cells (CHO K1;ATCC CCL 61) with plasmids containing the two subunit genes encoding FSH, *i.e.* hCGα and FSHβ (11). The nucleotide sequences of the coding regions were identical to those described previously (14, 15). Transcription of the α gene is directed from the simian virus-40 promoter, whereas the β gene is transcribed from the human metallothionein-II_A promoter. Stable integration of the plasmid in the chromosomal DNA was verified by Southern blot analyses of total cellular DNA. A single cell clone producing FSH at a constant level was selected and used for large scale production via continuous perfusion.

Hormones and monoclonal antibodies (MCAs)

Highly purified (≥99%) (13) lyophilized recFSH (batch 63, 65 and ML-FSH-95) was supplied by Diosynth (Oss, The Netherlands), and hCG (Pregnyl) was supplied by Organon (Oss, The Netherlands). Purified iodinated pituitary human FSH ([^{125}I]FSH; 3.3–7.4 megabecquerels (MBq)/μg) and [1β-^{3}H]androstenedione (1 TBq/mmol) were purchased from New England Nuclear-DuPont (Boston, MA). The International Standard (IS) of urinary human gonadotropins (code no. 70/45; 54 IU FSH and 46 IU LH per ampoule according to *in vivo* bioassays) and the IS of pituitary human FSH (code no. 83/575; 80 IU highly purified FSH/ampoule according to *in vivo* bioassay) were gifts from the National Institute of Biological Standards and Control (NIBSC, Hertfordshire, United Kingdom). Urinary FSH references were pure FSH (Metrodin, batches 88F02, 88I05, 89E16, 89J30, and 07325089, Serono, Rome, Italy), hMG 3/1 (batch Hu 332, Organon), and hMG 1/1 (Humegon, batches 08–2023–105, 890405–017, 881017–030, and 002025–105, Organon). The declared FSH/LH ratios of these three preparations according to *in vivo* bioassays were 60, 3, and 1, respectively. The FSH and hCG doses applied in *in vivo* experiments refer to their *in vivo* bioactivities as declared by the manufacturers (16, 17), which is in terms of IS 70/45 for recFSH, pure FSH, and hMG and in terms of the Third IS for hCG (code no. 75/537) for hCG. The protein content of FSH preparations was estimated from absorbance measurements at 280 nm of their solutions, assuming that $A^{1\%}_{1cm}$ 280 = 10.0 (18). MCAs (48A and 4B) raised against recFSH and urinary FSH, respectively, were developed by Organon. One MCA (INN-117) raised against pituitary FSH was a gift from Prof. G. Wick (Innsbruck, Austria).

Animals

Rats and mice were purchased from Harlan CPB (Zeist, The Netherlands) and housed at 21 C (hypophysectomized animals at 25 C), with intervals of 14 h of light and 10 h of darkness. The animals had free access to standard pelleted food (Hope Farms, Woerden, The Netherlands) and tap water.

Immunoassay for FSH

FSH immunoreactivity was measured in a sandwich enzyme immunoassay using a β-directed capturing MCA (4B) and an α-directed horseradish peroxidase-labeled detection MCA (116B). This assay, recognizing only intact dimers, is known to recognize all *in vitro* bioactive recFSH isoforms (13). The assay sensitivity using IS 70/45 was 0.4 IU/liter, and the intra- and interassay coefficients of variation were 7% and 8%, respectively. The cross-reactivities of the FSH immunoassay were less than 0.001% with hCG and less than 0.01% with hLH.

Receptor assay for FSH

This assay was based on the displacement of iodinated pituitary human FSH from calf testicular membrane preparations, by unlabeled FSH preparations.

The procedure of preparing partially purified membrane receptors was adapted from that of Abou-Issa and Reichert (19). In brief, fresh bovine testes from calves (4–5 months of age) were collected on ice and stored at −80 C. The decapsulated testes were homogenized in ice-cold 0.25 M sucrose in 10 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ (3 ml/g tissue) with a Polytron homogenizer (Brinkmann, Westbury, NY) at maximum speed for 60 sec. The homogenate was filtered through two layers of mesh grid and centrifuged (10 min; 200 × *g*; 4 C). The supernatant was further centrifuged at 15,000 × *g* (30 min; 4 C). The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ (1 ml/g starting tissue). Protein content was determined by the method of Bradford (20), using BSA (Sigma, St. Louis, MO) as a standard. Testicular membrane fractions (50 μg protein/200 μl assay buffer; 10 mM Tris-HCl, pH 7.4, supplemented with 5 mM $MgCl_2$ and 1 g/liter BSA) were incubated with [^{125}I]FSH (50,000 cpm/200 μl) and FSH sample (100 μl). After 24 h of incubation at room temperature, 500 μl ice-cold assay buffer were added, and bound and free hormone were separated by centrifugation (15,000 μg; 5 min). Radioactivity in the pellet was measured using a LKB γ-counter (Rockville, MD). Data were expressed as the percentage bound divided by the total added counts.

In vitro bioassays for FSH and LH

In vitro FSH and LH bioassays were based on induction of aromatase activity in immature rat Sertoli cells (21) and induc-

tion of testosterone production in mouse Leydig cells (22), respectively. The procedures of these assays were described previously (23).

In brief, Sertoli cells were collected from 10-day-old Wistar rats and cultured in 24-well plates (Costar, Cambridge, MA) for 3 days at 37 C in a humidified atmosphere of 5% CO_2-95% air. Culture medium consisted of a 1:1 (vol/vol) mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium supplemented with 5 μg/ml bovine insulin (Diosynth) and 5 mg/ml human transferrin (Sigma). After an initial culture period, each well was washed and incubated under the same conditions for 18 h with 1 ml of the above-described culture medium containing 0.2 mM 3-isobutyl-1-methylxanthine (Aldrich-Europe, Beerse, Belgium), 1 g/liter BSA, and FSH test sample. For the testing of MCAs, FSH and MCAs were preincubated for 1 h at room temperature. The FSH dose chosen gave a just maximal response in the absence of MCAs.

Aromatase activity was assessed by measuring the release of ^3H_2O from [1β-^{3}H]androstenedione. Therefore, the culture plates were incubated for 4 h (37 C) with Dulbecco's PBS (Gibco Europe, Breda, The Netherlands) containing 5.6 mM glucose and labeled androstenedione (37 kBq/ml/well; 0.22 μM). Supernatants were extracted with 5 ml chloroform, and the aqueous phase was treated with a suspension of 50 g/liter Norit-A (Sigma) and 5 g/liter Dextran T-70 (Pharmacia, Uppsala, Sweden) in distilled water. The radioactivity of the supernatant fraction was measured by a liquid scintillation counter (Packard, Zurich, Switzerland).

Leydig cells were isolated from the testes of mature Swiss mice (9–13 weeks old). The cells were obtained by sucking each decapsulated testis five times through a glass tube and filtering the suspension through a 30-μm nylon mesh. The cells were suspended in medium 199 supplemented with 4.2 mM $NaHCO_3$, 20 ml/liter fetal calf serum (Gibco-Europe), and 1 g/liter BSA, and 100 μl cell suspension were added to each well of a microtiter plate (Greiner, Nurtingen, Germany) along with 50 μl test sample. Plates were incubated for 4 h at 37 C in a humidified atmosphere of 5% CO_2-95% air and subsequently stored at −20 C until testosterone determination by RIA.

In vivo experiments

Four days after hypophysectomy, immature female Wistar rats (45–50 g; six or seven animals per treatment group) were treated for 4 days by twice daily sc injections of recFSH only (total dose, 5, 10, 20, or 40 IU) or recFSH (total dose, 40 IU) supplemented with various doses of hCG (total doses, 0, 0.1, 1, and 10 IU) or with hCG only (total dose, 1 or 10 IU). In a comparable way, animals were treated with urinary FSH preparations, *viz.* pure FSH, hMG 3/1, or hMG 1/1. Control animals were injected with vehicle solution only, consisting of 43.7 mM NaH_2PO_4, 109.7 mM Na_2HPO_4, 1 g/liter methylhydroxybenzoate, and 1 g/liter gelatin. Animals were killed 18 h after the last injection. After diethyl ether anesthesia, the rats were exsanguinated, and their ovaries were dissected out, weighed, and frozen at −80 C until determinations of aromatase activity. For that purpose, ovaries were thawed, minced by scissors, and homogenized with a Potter-Elvejhem homogenizer in ice-cold 0.1 M potassium phosphate buffer supplemented with 5 mM EDTA. The final tissue concentration was 1 mg/ml. After centrifugation at 300 × *g* for 10 min at 0 C, 5 μl of an ethanolic solution of [1β-^{3}H]androstenedione (37 kBq/incubate; 74 nM) were added to 0.9 ml supernatant, and the incubation was started by adding 0.1 ml of a NADPH-generating system. The final concentrations in the incubate were 2.5 mM NADP, 5 mM glucose-6-phosphate, and 0.525 U/ml glucose-6-phosphate dehydrogenase. The incubation was performed at 37 C for 15 min and terminated by adding 5 ml chloroform and thoroughly mixing. The ^3H_2O content in the aqueous phase was assessed as described above.

For histological examination, ovaries were fixed in formal sublimate, dehydrated, and embedded in paraffin. Serial sections (10 μm) were stained with hemalum and eosin.

Steroid assessments

Testosterone was established in a direct RIA kit (Farmos Diagnostica, Oulunsalo, Finland), using a calibration curve of standard testosterone doses in Leydig cell culture medium. Estradiol in rat plasma samples was assayed in an 17β-estradiol kit (ICN Biomedicals, Inc., Carson, CA) after solid phase extraction using octadecyl columns (Baker, Deventer, The Netherlands). Androstenedione in supernatants of rat ovarian homogenates was determined using a direct androstenedione kit (Diagnostic Systems Laboratories, Inc., Webster, TX).

Statistical analysis

Potencies relative to the standard were calculated after testing for linearity and parallelism of the dose-response curves, as described by Finney (24).

Statistical analysis of responses in hypophysectomized rats was performed according to a complete randomized design, and significance was defined as $P < 0.05$. Responses of ovarian weight and aromatase activity were analyzed by one-way variance analysis after log transformation of the original data. Median estradiol levels were tested in the Wilcoxon sum of ranks test.

Results

Specific FSH activity

Specific FSH activities (international units per mg protein) of recFSH, pituitary FSH (IS 83/575), urinary pure FSH (Metrodin), and hMG 1/1 (Humegon) according to immunoassay and *in vivo* bioassay were calculated after estimation of their protein contents from absorbance measurements at 280 nm. The calculated activities, expressed in terms of IS 70/45, are presented in Table 1. The specific *in vivo* bio- and immunoactivities of recFSH were considerably higher than those of pure FSH (Metrodin) and hMG 1/1 (Humegon), but the *in vivo* bio/immuno ratios of these preparations were not significantly different. When compared to the highly purified pituitary standard IS 83/575, recFSH and IS 83/575 had comparable high specific *in vivo* bioactivities, but the specific immunoreactivity of IS 83/575 was about 2 times lower, resulting in a significantly increased *in vivo*/immuno ratio.

TABLE 1. Specific FSH activity (international units per mg protein; mean with 95% confidence limits) of recFSH and natural FSH references according to *in vivo* bioassay and immunoassay in terms of the urinary IS 70/45

Preparation	n	*In vivo* bioactivity	Immunoreactivity	*In vivo*/immuno ratio
RecFSH	3	14,000 (12,100–15,900)	12,100 (11,100–14,900)	1.2 (0.8–1.4)
IS 83/575	1	14,100 (12,500–15,800)	5,600 (5,400–5,900)	2.5 (2.1–2.9)
Pure FSH	2	221[a] (177–276)	180 (168–192)	1.2 (0.9–1.6)
hMG 1/1	3	65[a] (52–81)	42 (40–43)	1.5 (1.2–2.0)
IS 70/45	1	54[b]		

n represents the number of batches tested.
[a] Assuming 75 (60–94) IU *in vivo* bioactive FSH/ampoule, as stated by the manufacturers.
[b] Taken from Storring *et al.* (25).

In vitro receptor binding and bioactivity

The receptor affinity and *in vitro* bioactivity of recFSH were examined in receptor displacement studies using calf testicular membranes and in bioassay studies using immature rat Sertoli cells. In these experiments recFSH provided dose-response curves parallel to those of pituitary and urinary FSH references.

When equal amounts of immunoreactive FSH were tested, recFSH, urinary pure FSH (Metrodin), hMG 1/1 (Humegon), and pituitary FSH (IS 83/575) inhibited the receptor binding of pituitary [^{125}I]FSH in a dose-dependent manner and in the same dose range.

RecFSH, urinary FSH (IS 70/45), and pituitary FSH (IS 83/575) increased the aromatase activity in rat Sertoli cells in a dose-dependent manner (Fig. 1, *lower panel*). Like receptor-binding potencies, *in vitro* bioactivities of recFSH and natural FSH references were comparable. Also, recFSH and natural FSH induced comparable maximal responses in all experiments.

In vitro neutralization of bioactivity

The capacity of anti-FSH antibodies to inhibit the *in vitro* bioactivity of recFSH was investigated by means of three MCAs raised against urinary FSH (MCA 4β; β-specific), pituitary FSH (MCA INN-117; $\alpha\beta$-specific), or recFSH (MCA 48A; α-specific). In the Sertoli cell bioassay, each MCA inhibited the aromatase activity induced by recFSH. Moreover, the amounts of MCA needed to neutralize the *in vitro* bioactivity of pure FSH, pituitary FSH (IS 83/575) and recFSH were comparable. To obtain 50% inhibition of responses induced by immunoreactive FSH doses of 6.4 IU recFSH, 7.9 IU pure FSH, or 7.8 IU pituitary FSH (IS 83/575), 4 nM MCA 4B, 0.1 nM MCA INN-117, and 0.08 nM MCA 48A in total were required. As a typical example, the results of one neutralization experiment with MCA 48A are shown in Fig. 2.

Intrinsic LH activity

To establish whether recFSH is capable of inducing LH-specific biological responses, an *in vitro* mouse Leydig cell testosterone bioassay was applied. Figure 3 shows the induction of testosterone synthesis by urinary IS 70/45, urinary pure FSH, and recFSH. While 0.2–6 IU pure FSH induced dose responses parallel to those of IS 70/45, 16–64 IU recFSH were needed to increase testosterone production. The calculated *in vitro* LH activity in

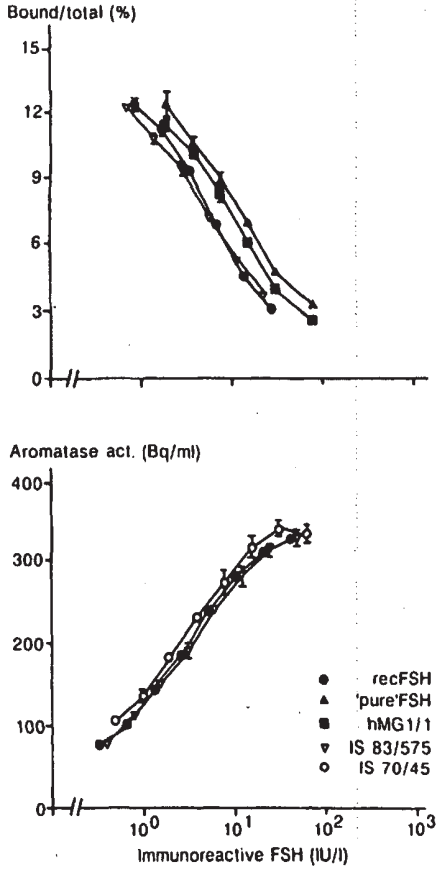

FIG. 1. Dose-dependent displacement of labeled human pituitary [^{125}I] FSH binding to calf testicular membranes (*upper panel*) and stimulation of aromatase activity in immature rat Sertoli cells (*lower panel*) by recFSH produced by CHO cells and natural FSH references. Responses represent the mean of triplicates ± SD.

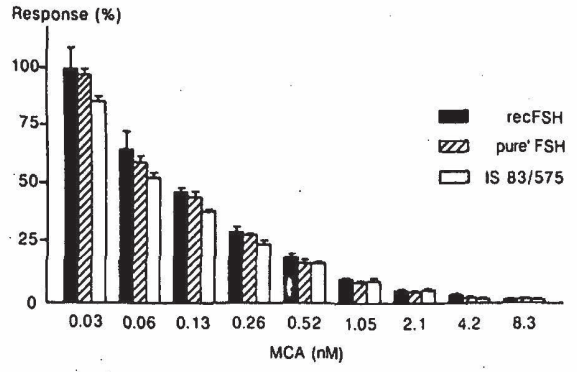

FIG. 2. Inhibition of FSH-induced aromatase activity in immature rat Sertoli cells. Increasing doses of MCA 48A were preincubated for 1 h at room temperature with one FSH dose, causing nearly maximal aromatase activity in the absence of MCA. Responses represent the mean of triplicates ± SD.

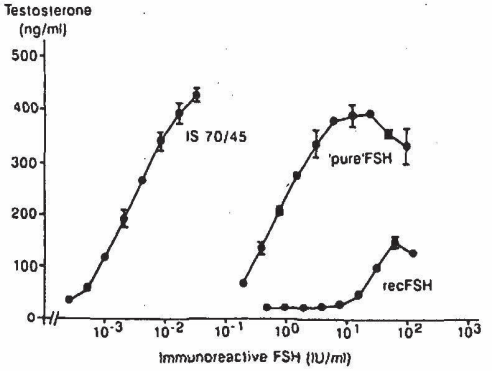

FIG. 3. Stimulation of testosterone production in mouse Leydig cells by increasing doses of recFSH and urinary FSH preparations. Responses represent the mean of quadruplicates ± SD.

terms of IS 70/45 per IU *in vivo* bioactive FSH was 2.4 (2.2–2.7) mIU LH/IU FSH for pure FSH (Metrodin batch 88F02) and less than 0.025 mIU LH/IU FSH for recFSH.

In vivo bioactivities

The *in vivo* efficacy of recFSH was established by treating immature hypophysectomized female rats for 4 days with either recFSH only or recFSH supplemented with 1 IU hCG. The FSH and hCG dosages applied in these animal experiments represent their *in vivo* bioactivities.

Total dosages of 5, 10, 20, and 40 IU recFSH increased ovarian weight and ovarian aromatase activity in a dose-dependent manner (Fig. 4). Both responses were significantly ($P < 0.05$) increased by a total dose of 5 IU recFSH, while at least 20 IU were needed to reach maximal responses. Gross histological examination of ovaries after treatment with 10 IU recFSH revealed the presence of large antral follicles, while in vehicle-injected animals, only primordial and primary follicles were observed. When the various recFSH dosages were supplemented with 1 IU hCG, both ovarian weight and aromatase activity were further augmented ($P < 0.05$), especially at recFSH dosages that produced submaximal responses. Treatment with 1 IU hCG alone did not affect these parameters.

In the absence of hCG, as much as 40 IU recFSH was unable to increase plasma estradiol levels, while recFSH supplemented with 1 IU hCG largely increased median plasma estradiol levels in a FSH dose-dependent manner (Table 2). Animals treated with 1 IU hCG only had unchanged basal estradiol levels.

To estimate how much LH activity would be needed to increase plasma estradiol levels in the above-described experimental model, animals were treated with recFSH in addition to 0, 0.1, 1, or 10 IU hCG, and pure FSH, hMG 3/1, and hMG 1/1 were used as references. All animals received 40 IU *in vivo* bioactive FSH, since this dose of recFSH was known to induce maximal aromatase activity. Effects of endogenous androstenedione levels in rat ovaries on the assessment of aromatase were negligible, even after treatment with the highest dose hCG or hMG 1/1, since these levels were less than 5% of the total amount of substrate used.

Increases in ovarian weight and aromatase activity induced by recFSH only and urinary pure FSH were not significantly different (Fig. 5). When recFSH was supplemented with 1 and 10 IU hCG, increases in ovarian weight and aromatase were further augmented ($P < 0.05$) and comparable to those induced by hMG 3/1 and hMG 1/1, respectively. In comparison to controls, animals treated with 10 IU hCG only had slightly increased ($P < 0.05$) ovarian weights and aromatase activity (data not shown).

Like recFSH, urinary pure FSH was unable to increase circulating estradiol levels (Table 2). However, when recFSH was supplemented with hCG, recFSH largely increased circulating estradiol levels in a hCG dose-dependent manner. As little as 0.1 IU hCG produced a 3-fold increase in median estradiol levels, while these levels remained unchanged after treatment with 10 IU hCG alone. The estradiol levels induced by recFSH supplemented with 1 and 10 IU hCG compared well with those induced by hMG 3/1 and hMG 1/1, respectively.

Discussion

In the present study the *in vitro* and *in vivo* biological properties of recFSH produced by CHO cells were compared to those of natural FSH preparations. In this study recFSH was the only preparation tested with a biochemical purity of at least 99% (13); all natural FSH preparations were contaminated with small or large amounts

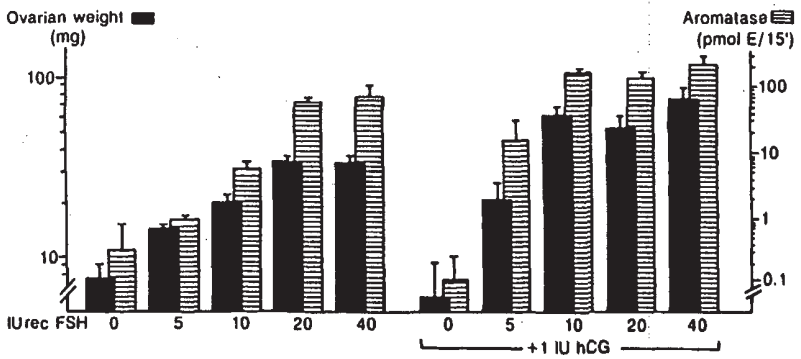

FIG. 4. Ovarian weight and ovarian aromatase activity in immature hypophysectomized rats (n = 6–7) after treatment with increasing doses of recFSH alone or in combination with 1 IU hCG. Responses represent the geometric mean ± SEM.

TABLE 2. Median plasma 17β-estradiol levels in immature hypophysectomized rats after FSH treatment

Treatment	Total dose (IU)		Estradiol (pg/ml)
	FSH	LH/hCG	
RecFSH only	0		8.9
	5		10.4
	10		9.6
	20		10.0
	40		10.8
RecFSH + hCG	0	1	3.6
	5	1	93.8
	10	1	95.7
	20	1	200
	40	1	454
RecFSH+ hCG	40	0	10.9
	40	0.1	34.3
	40	1	674
	40	10	1090
	0	10	5.2
Pure FSH	40	≤0.7	9.0
hMG 3/1	40	13	527
hMG 1/1	40	40	1440

Four days after hypophysectomy, rats (six or seven animals per treatment group) were treated for 4 days by twice daily sc injections of recFSH only and recFSH supplemented with hCG, urinary pure FSH, hMG 3/1, and hMG 1/1.

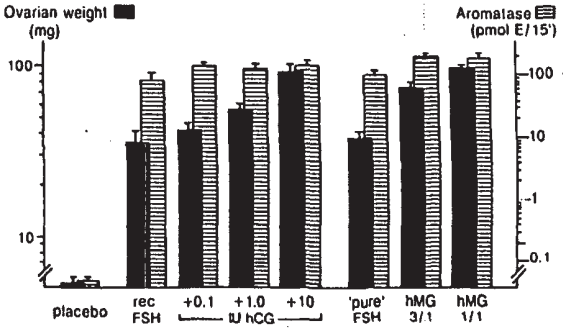

FIG. 5. Ovarian weight and aromatase activity in immature hypophysectomized rats (n = 6–7) after treatment with a total of 40 IU FSH or recFSH alone or in combination with hCG (0.1, 1, or 10 IU), urinary pure FSH (batch 89E16), hMG 3/1, and hMG 1/1. Responses represent the geometric mean ± SEM.

of other proteins from pituitary or urinary origin. For this reason, a comparison of preparations based on the amount of FSH protein was not feasible; dose uniformity of the various FSH preparations was obtained by their calibration in terms of IS 70/45 in immunoassay and *in vivo* bioassay for the purpose of comparative *in vitro* and *in vivo* experiments, respectively.

The high specific activities (>10,000 IU/mg protein) of recFSH, determined by immunoassay and *in vivo* bioassay, support the high purity of the preparation tested. In the present study all estimations of protein content were based on the absorbance of FSH solutions at 280 nm. When applying protein assays based on different principles of protein recognition and/or using various protein standards, the specific activity of recFSH ranged between 10.000–40.000 IU/mg protein (data not shown).

Measurement of specific *in vivo* bioactivity and immunoreactivity revealed that the *in vivo*/immuno ratios of recFSH and urinary FSH were not different, indicating that the isohormone profiles of these two types of preparations are very similar (13). The *in vivo*/immuno ratio of the highly purified IS 83/575 appeared to be about 2 times higher than those of the other tested FSH preparations. This finding is in good agreement with the previous reported discrepancy of the declared *in vivo* bioactivity (80 IU/ampoule) and the much lower actual immunoreactivity of this IS (18). In general, the acidic FSH isoforms contribute far more to the *in vivo* bioactivity than to the *in vitro* bioactivity or immunoreactivity (26). Therefore, these data suggest that IS 83/575 contains relatively more acidic isoforms than the other tested FSH preparations. Since one would expect pituitary FSH to be less acidic than urinary FSH, it might well be that this IS 83/575 has lost considerable amounts of basic isoforms during purification.

The data of receptor displacement assays and *in vitro* bioassays revealed that recFSH and the tested natural

FSH references have similar *in vitro* activities, meaning that the immuno/*in vitro* ratios of these preparations are similar. Previous reports on *in vitro* bioactive recFSH produced by CHO cells were based on induction of aromatase activity in rat granulosa cells (27, 28). In the present study the functional effect of recFSH was established by means of an *in vitro* Sertoli cell aromatase bioassay. Like the granulosa cell bioassay, this assay has demonstrated its suitability for FSH quantification of gonadotropin preparations and serum samples (23, 29–31).

The *in vitro* induction of aromatase activity could be inhibited by preincubation of recFSH or urinary or pituitary FSH with MCAs raised against either of these FSH preparations. The almost identical neutralization profiles of recombinant and natural FSH in various experiments using MCAs directed against three different FSH epitopes supports the structural and functional similarity of both types of molecules.

One of the obvious advantages of recFSH is that it does not contain other hormones, such as LH. By means of the Leydig cell bioassay, it was demonstrated that the intrinsic LH bioactivity of recFSH is negligible; the amounts of recFSH needed to increase *in vitro* testosterone production were extremely high and clearly supraphysiological.

The first report (4) supporting the two-cell two-gonadotropin theory was made about 50 yr ago. It demonstrated that highly purified FSH could increase ovarian growth and follicular development in immature hypophysectomized rats without stimulating the release of estrogen, since uterine weights remained unchanged. These findings were confirmed by some investigators (5, 6), while others (32) reported increases in both ovarian and uterine weights. Since increases in uterine weight are thought to reflect both FSH and LH activities and may also be due to nonspecific factors (17), previous studies using natural FSH were not conclusive.

The present study provides more definite evidence in favor of the two-cell theory, confirming that FSH alone can stimulate follicular growth and that LH activity is required to increase estradiol secretion up to measurable plasma levels. Like recFSH, urinary pure FSH was unable to increase circulating estradiol, while a combination of recFSH and 0.1 IU hCG (<2.5 mIU hCG/IU FSH) provided a 3-fold increase in median plasma estradiol levels. Obviously, the remaining LH activity in pure FSH (2–3 mIU LH/IU FSH) was insufficient to increase circulating estradiol, which could be due to the much shorter elimination half-life of LH than of hCG (33). Our findings in hypophysectomized rats seem to be in disagreement with those in higher species demonstrating that gonadotropin-suppressed subjects may have normal rising estradiol levels in response to urinary pure FSH (7). However, up to now, neither GnRH agonists nor antagonists have been shown to establish complete pituitary suppression (34), and remaining endogenous LH secretion as well as ovarian androgens might be sufficient to cause increases in estradiol.

The ability of recFSH to induce follicular growth in hypophysectomized rats has recently also been demonstrated by Galway *et al.* (35). Moreover, their experiments indicate that recFSH alone is able to induce ovulation. This suggests that FSH-induced synthesis of ovarian estradiol and/or growth factors is sufficient for complete follicle maturation, although the quality of oocytes and their progeny remains to be investigated. Our study also demonstrated that recFSH supplemented with hCG causes much higher increases in ovarian weight and aromatase activity than treatment with recFSH only when using recFSH doses that provided submaximal responses. The effect of LH activity on ovarian aromatase is known to be mediated via testosterone and 5α-reduced androgens, causing amplification of cAMP-mediated FSH responses (36). Thus, while LH might not play a crucial role in follicular maturation, it remains obvious that LH supports and augments the regulatory function of FSH during folliculogenesis.

In summary, the present study shows by means of various *in vitro* and *in vivo* models that the biological properties of recFSH produced by CHO cells are very similar, if not identical, to those of natural FSH preparations.

Acknowledgments

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Folliculogenesis in Hypophysectomized Rats after Treatment with Recombinant Human Follicle-Stimulating Hormone

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ABSTRACT

To examine the role of FSH and LH in follicular growth and atresia, immature hypophysectomized (hypox) rats were treated twice daily for four days with a total dose either of 2.5 to 40 IU recombinant human FSH (recFSH; Org 32489) or of 8 IU recFSH supplemented with 0.2 to 5 IU hCG.

RecFSH alone caused dose-dependent increases in ovarian weight and intraovarian estradiol (E_2) but was unable to elevate circulating E_2 levels. The number of antral follicles was also increased in a recFSH dose-dependent manner, and a gradual shift of small antral follicles to large preovulatory follicles was noted. The latter ovulated after a single bolus injection of 10 IU hCG. In comparison with follicles from hypox vehicle-treated animals, these follicles showed a diminished incidence of atresia, especially in the smallest size class of antral follicles. A total dose of $\geq$ 10 IU recFSH increased uterine weight accompanied by endometrium proliferation.

When 8 IU recFSH was supplemented with 0.2 to 5 IU hCG, ovarian weight was augmented in an hCG dose-dependent fashion, but no further increases in total number of antral follicles were noted except with the highest hCG dose given. Nevertheless, addition of relatively low doses of hCG caused considerable shifts of small follicles to large, preovulatory follicles. Furthermore, supplementation with hCG, especially low dosages of hCG (0.2 and 0.5 IU), reduced the incidence of atresia in antral follicles of all size classes.

These data suggest that in the complete absence of LH activity, recFSH induces follicular growth up to the stage of mature preovulatory follicles and induces ovarian estradiol production and endometrium proliferation. The addition of small amounts of LH activity increases the percentage of healthy follicles.

INTRODUCTION

The relative contributions of FSH and LH in the control of folliculogenesis have been under investigation for many years; and several models for follicular selection and development, resulting in single or multiple ovulation, have been proposed [1–3]. The conversion of primordial into primary follicles is thought to be gonadotropin-independent, since this process continues normally after hypophysectomy. The further development of growing follicles up to the antral stage can occur in the absence of gonadotropins, although quantitatively this process will be strongly inhibited. Whenever a follicle reaches the antral stage and the level of FSH exceeds a certain threshold value, the follicle continues its development to the preovulatory stage. However, if the antral stage is reached and FSH levels are insufficient, follicles become atretic. The total number of selected follicles would depend on the (species-dependent) degree of synchrony by which follicular growth is initiated as well as the time period during which the level of FSH remains above this threshold value.

The exact role of LH during folliculogenesis is less well understood, since granulosa cells of developing follicles acquire their LH receptors only in the antral stage in response to FSH and estradiol (E_2) [4]. Only recently, it has been demonstrated that recombinant human FSH (recFSH), devoid of LH activity, stimulates multiple follicular growth up to the preovulatory stage in immature, hypophysectomized (hypox) rats, whereas circulating levels of E_2 remain low at baseline [5]. The latter finding is in agreement with the two-cell, two-gonadotropin theory holding that both FSH and LH activity are required for estrogen biosynthesis [4]. Studies in gonadotropin-deficient women using recFSH or urinary FSH have confirmed that FSH administered alone can induce follicular growth but that small amounts of LH are essential for adequate steroidogenesis and subsequent endometrial proliferation [6–8]. On the other hand, excessive amounts of LH activity are thought to exert detrimental effects on the maturation of ovulatory follicles, causing atresia and premature resumption of meiosis [1].

The current study was undertaken to gain further knowledge about the role of FSH and LH activity in follicular growth and atresia and simultaneous uterine development. For this purpose, immature hypox rats were treated with recFSH or with recFSH supplemented with hCG. Effects on ovaries were evaluated by assessment of ovarian weight, ovarian E_2 and androstenedione production, and the number of healthy and atretic antral follicles of the various size classes [9]. In addition, the uterus was weighed and histologically examined.

MATERIALS AND METHODS

Animals

Immature female Wistar rats (45–50 g, 21 days old) were purchased from Harlan CPB (Zeist, The Netherlands) and

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TABLE 1. Effect of recFSH in immature hypophysectomized rats treated twice daily for 4 days.

Treatment total dose (IU)	N[a]	Ovarian weight (mg)	Ovarian estradiol (pg/ovary)	Plasma estradiol (pg/ml)	Uterine weight (mg)
0	6	7.6 ± 0.7	4.2 ± 0.5	4.0 ± 1.1	15.8 ± 0.8
2.5	5	11.0 ± 1.2*	7.1 ± 2.7	1.9 ± 0.4	18.3 ± 3.5
5	5	12.8 ± 1.6*	13.3 ± 3.9*	2.8 ± 1.0	17.9 ± 2.6
10	5	28.8 ± 3.1*	24.8 ± 7.0*	2.5 ± 0.4	34.2 ± 15.7*
20	4	28.2 ± 3.2*	32.3 ± 7.4*	1.9 ± 0.3	50.4 ± 14.8*
40	6	35.3 ± 2.4*	72.0 ± 16.9*	4.2 ± 1.4	68.1 ± 11.6*

[a]N represents the number of animals per treatment group.
*Significantly ($p < 0.05$) increased in comparison to vehicle treatment only.

housed in a temperature-controlled room, at 21°C before and 25°C after hypophysectomy, with a light cycle of 14L:10D. The animals had free access to standard pelleted food and tap water.

Gonadotropins

Highly purified (> 99%) lyophilized recFSH (Org 32489, batch 65) was supplied by Diosynth (Oss, The Netherlands), and hCG (Pregnyl, batch O5–2135) was supplied by Organon International b.v. (Oss, The Netherlands). The specific in vivo bioactivity of the recFSH preparation was 13 100 (12 100–14 300) IU/mg protein [5]. The in vivo bioactivity of the hCG preparation was 520 IU/ampule. For injection, gonadotropins were dissolved in a buffer (pH 7.2) consisting of 43.7 mM NaH_2PO_4, 109.7 mM Na_2HPO_4, 0.1% methylhydroxybenzoate, and 0.1% gelatin.

Experiments

The procedure for these experiments has been described previously [5]. Animals were hypophysectomized at 22 days of age; four days later, if animals had not gained weight, treatment was started by means of twice daily s.c. injections of recFSH (total doses: 2.5, 5, 10, 20, 40 IU) or recFSH supplemented with hCG (total doses: 0.2, 0.5, 2, 5 IU). In experiments with hCG supplementation, a total submaximal dose of 8 IU recFSH was used. Control animals were treated with vehicle solution only or with hCG only (total dose, 5 IU). After four days of treatment, 18 h after the last injection, animals were administered diethylether anesthesia and were exsanguinated by drawing blood from the abdominal aorta. Animals with pituitary remnants in the sella turcica were excluded. Ovaries and uterus were dissected out and weighed. From each animal, one ovary was fixed in Bouin's fluid for histological examination and one ovary was frozen at −80°C until determination of levels of E_2 and androstenedione. Uteri of animals treated with vehicle only or with 40 IU recFSH were also fixed in Bouin's fluid for histological examination. In one of the experiments described above, animals treated with 20 or 40 IU recFSH only were given a bolus injection of 10 IU hCG to induce ovulation. HCG was administered simultaneously with the last injection of recFSH. Eighteen hours thereafter, tubes were inspected for the presence of oocytes. If animals had ovulated, ovaries were examined histologically for the presence of CL.

Steroid Assessments

Intraovarian and plasma E_2 was measured in a 17β-E_2 kit (detection limit 10 pg/ml; ICN Biomedicals, Inc. Carson, CA), and intraovarian androstenedione was measured in an androstenedione kit (detection limit 40 pg/ml; ICN Biomedicals).

The intra- and interassay coefficients of variation were 6.4% (higher detection limit) to 10.6% (lower detection limit)

TABLE 2. Effect of recFSH alone on numbers of healthy and atretic follicles in immature hypophysectomized rats

Total dose (IU) recFSH	N[a]	Total number of follicles (>275 μm)	Number of atretic follicles (>275 μm)			Percentage of atretic follicles (>275 μm)
			Early atretic[b]	Late atretic[c]	Other[d]	
0	6	11 ± 4	6.5 ± 2.0	1.0 ± 0.5	—	69 ± 15
2.5	5	36 ± 4*	13.2 ± 2.1	3.0 ± 1.2	—	45 ± 8
5	4	52 ± 12*	17.8 ± 3.8	4.3 ± 1.7	—	44 ± 9
10	5	99 ± 6*	35.6 ± 2.7	8.0 ± 3.3	—	44 ± 3
20	4	85 ± 14*	27.0 ± 11.0	0.5 ± 0.5	—	31 ± 9
40	6	80 ± 7*	0.5 ± 0.2	3.8 ± 1.7	26 ± 3	39 ± 2

[a]N represents the number of animals per treatment group.
[b]Early atretic: stage 1a and 1b.
[c]Late atretic: stage 2a and 2b.
[d]Other: follicles with loosely arranged granulosa cells.
*Significantly ($p < 0.05$) increased in comparison to vehicle treatment only.

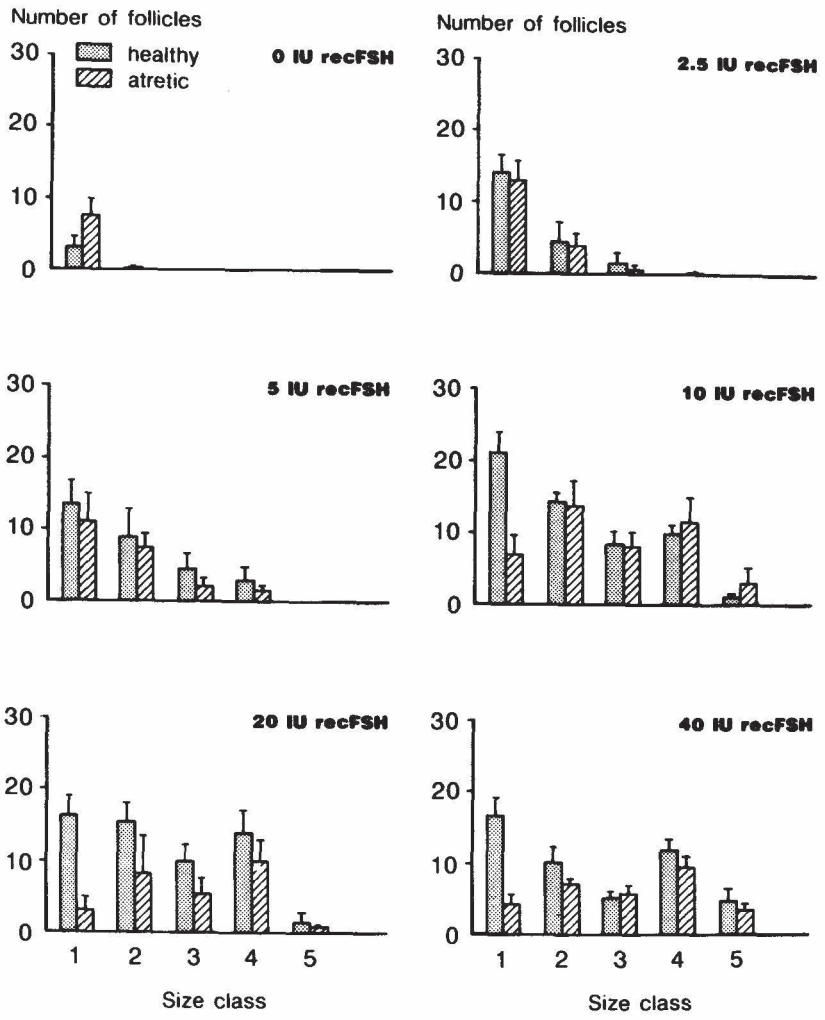

FIG. 1. Number of healthy and atretic antral follicles in various size classes (mean diameter class 1:275–350; class 2:351–400; class 3:401–450; class 4:451–575; class 5: ≥576 μm) present in one ovary after treatment of immature, hypox rats with 0 to 40 IU recFSH. "Other" follicles with loosely arranged granulosa cells (40 IU recFSH, size classes 1 to 5) were included as atretic follicles.

and 5.9% (higher detection limit) to 11.9% (lower detection limit) for E_2 and < 5% and < 6% for androstenedione, respectively. In the E_2 assay, the cross-reactivity with estrone was 20%; in the androstenedione assay, the cross-reactivity with androsterone was 0.03%.

Before analysis, plasma samples were extracted with methanol using octadecyl columns (Baker, Deventer, The Netherlands). After evaporation to dryness, the residues were dissolved in assay buffer. During this step, samples were concentrated at maximum five times, increasing the sensitivity of the E_2 assay from approximately 10 to 2 pg/ml.

For the measurement of intraovarian E_2 and androstenedione, ovaries were homogenized and extracted three times with 1 ml of methanol. After evaporation to dryness, the ovarian residues were dissolved in 1 ml charcoal-treated human serum with E_2 and androstenedione levels below the detection limit of the respective assay. The tissue extracts were assayed after methanol extraction using octadecyl columns. During extraction, samples were concentrated at maximum five times, increasing the sensitivity of the E_2 and androstenedione assay from approximately 10 to 2 pg/ml and from 40 to 8 pg/ml, respectively.

Histology

For histological examination, fixed ovaries and uterine material were embedded in paraffin, and serial sections (6 to 10 μm) were stained with hematoxylin and eosin. Differential follicle counts were made according to the method described by Osman [9]. In one ovary from each animal, follicles of a mean diameter > 275 μm were counted. Five follicle size classes were distinguished: class 1 (275–350 μm), class 2 (351–400 μm), class 3 (401–450 μm), class 4 (451–575 μm), and class 5 (≥ 576 μm). Follicles of these sizes were all antral follicles. The mean follicle diameter was calculated from the two perpendicular diameters in the section containing the oocyte nucleolus. Degenerative changes

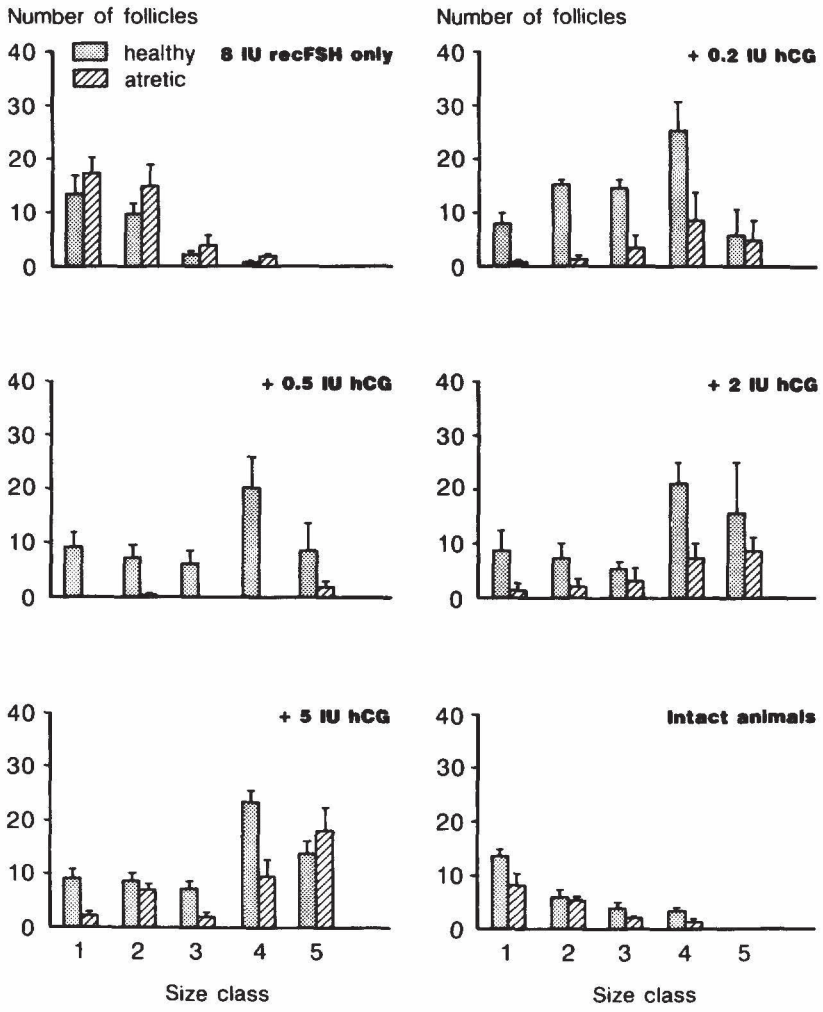

FIG. 2. Number of healthy and atretic antral follicles in various size classes (as specified in legend to Figure 1) present in one ovary after treatment of immature, hypox rats with 8 IU recFSH supplemented with 0 to 5 IU hCG. "Other" follicles with loosely arranged granulosa cells (8 IU recFSH with 2 or 5 IU hCG; size classes 4 to 5) were included as atretic follicles.

detectable by light microscopy were used as criteria for atresia in each counted follicle. In principle, two stages of atresia were distinguished, i.e., early and late atresia. Follicles with early atresia included those in which the granulosa cell wall showed cell shrinkage and only a few pyknotic cells were present (stage Ia [9]), as well as those in which the whole granulosa cell wall was affected by degeneration and pyknotic cells were present in the periphery of the antrum (stage Ib [9]). Follicles with late atresia contained oocytes showing resumption of meiosis—though still surrounded by granulosa cells (stage IIa [9]), or "naked" oocytes showing resumption of meiosis (stage IIb [9]).

Statistical Analysis

Statistical analysis of responses was performed according to a randomized design, and significance was defined as $p < 0.05$. Ovarian and uterus weight, ovarian E_2, and plasma E_2 were expressed as geometric means with SEM and were compared, after log transformation of the original data, by a one-way variance analysis with pairwise t-tests (for comparison of unknowns with control). Statistical analysis of follicle counts, which were expressed as means with SEM, was performed by means of Wilcoxon's test.

RESULTS

RecFSH Alone

The effect of recFSH on ovarian weight, uterine weight, ovarian E_2, and plasma E_2 in immature, hypox rats is presented in Table 1. Total dosages of 2.5, 5, 10, 20, and 40 IU recFSH induced dose-dependent increases in ovarian weight from 7.6 ± 0.7 mg in vehicle-treated animals (controls) to 35.3 ± 2.4 mg in animals treated with 40 IU recFSH. Compared to the value for controls, a significant ($p < 0.05$) increase was noted with the lowest dose of 2.5 IU recFSH, while a maximal response was reached at 10 IU recFSH. In

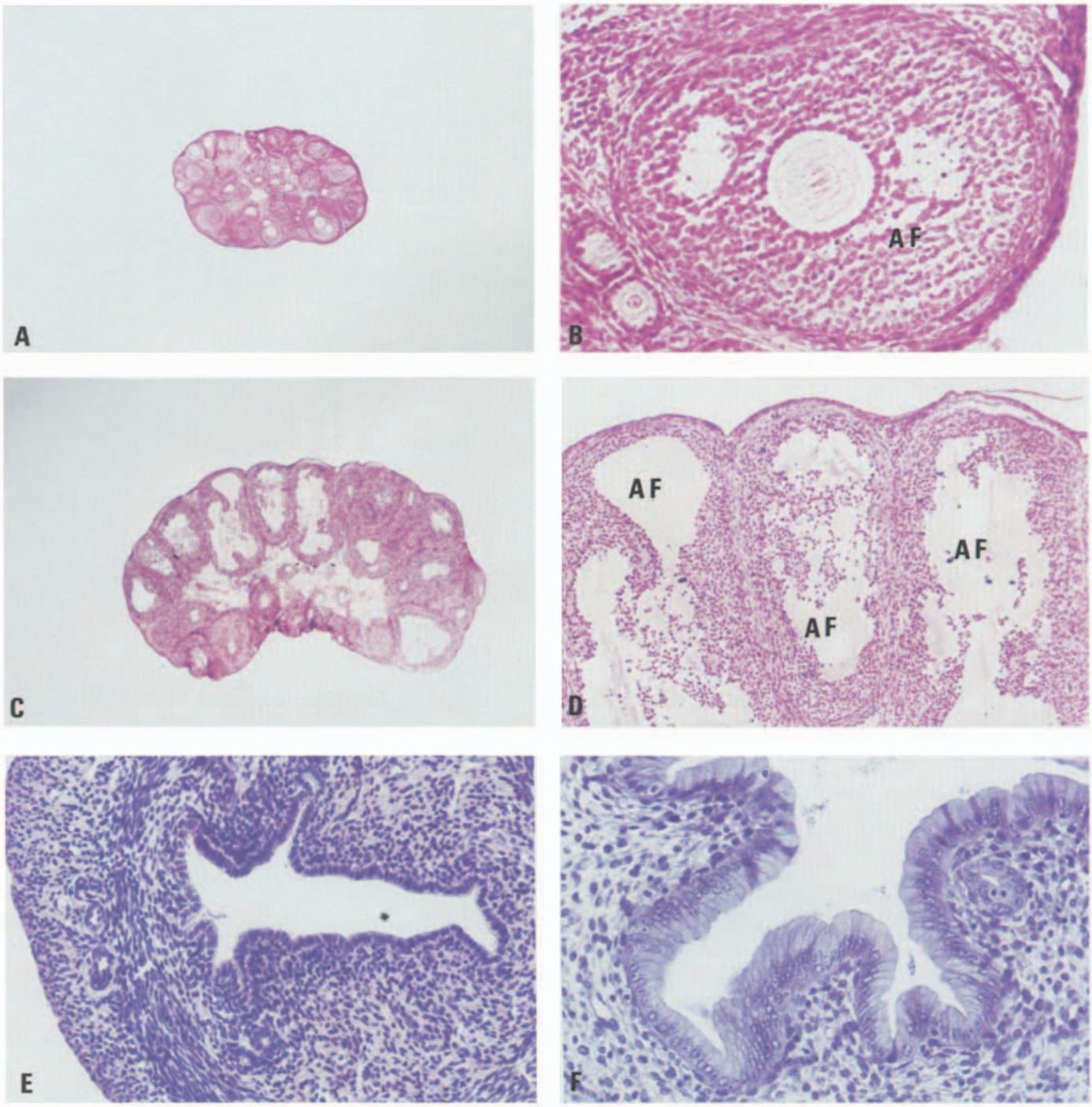

FIG. 3. A and B) Sections of ovaries after treatment with vehicle solution at magnification ×2.5 and ×25, respectively. Only a limited number of small antral follicles (size class 1), mainly early atretic, are perceptible.

C and D) Sections of ovaries after treatment with 40 IU recFSH at magnification ×2.5 and ×10, respectively. Atretic antral follicles with dispersion of granulosa cells, a thin granulosa cell layer, and an atrophic interstitium are apparent.

E and F) Uterine sections after treatment with vehicle only and after treatment with 40 IU recFSH at magnification ×25 and ×25, respectively. Folding of the endometrial layer, height and number of epithelial cells, glandular cells, and endometrial stroma are clearly increased due to recFSH treatment.

contrast to circulating E_2 levels, which remained low at baseline level, intraovarian E_2 and uterine weight increased with the dose of recFSH given. Total dosages of 0 to 40 IU recFSH induced increases in intraovarian E_2 from 4.2 ± 0.5 pg/ovary (control) to 72.0 ± 16.9 pg/ovary and in uterine weight from 15.8 ± 0.8 mg (control) to 68.1 ± 11.6 mg. In comparison to controls, significant ($p < 0.05$) increases were noted at a total dose of ≥ 5 IU recFSH for ovarian E_2 and

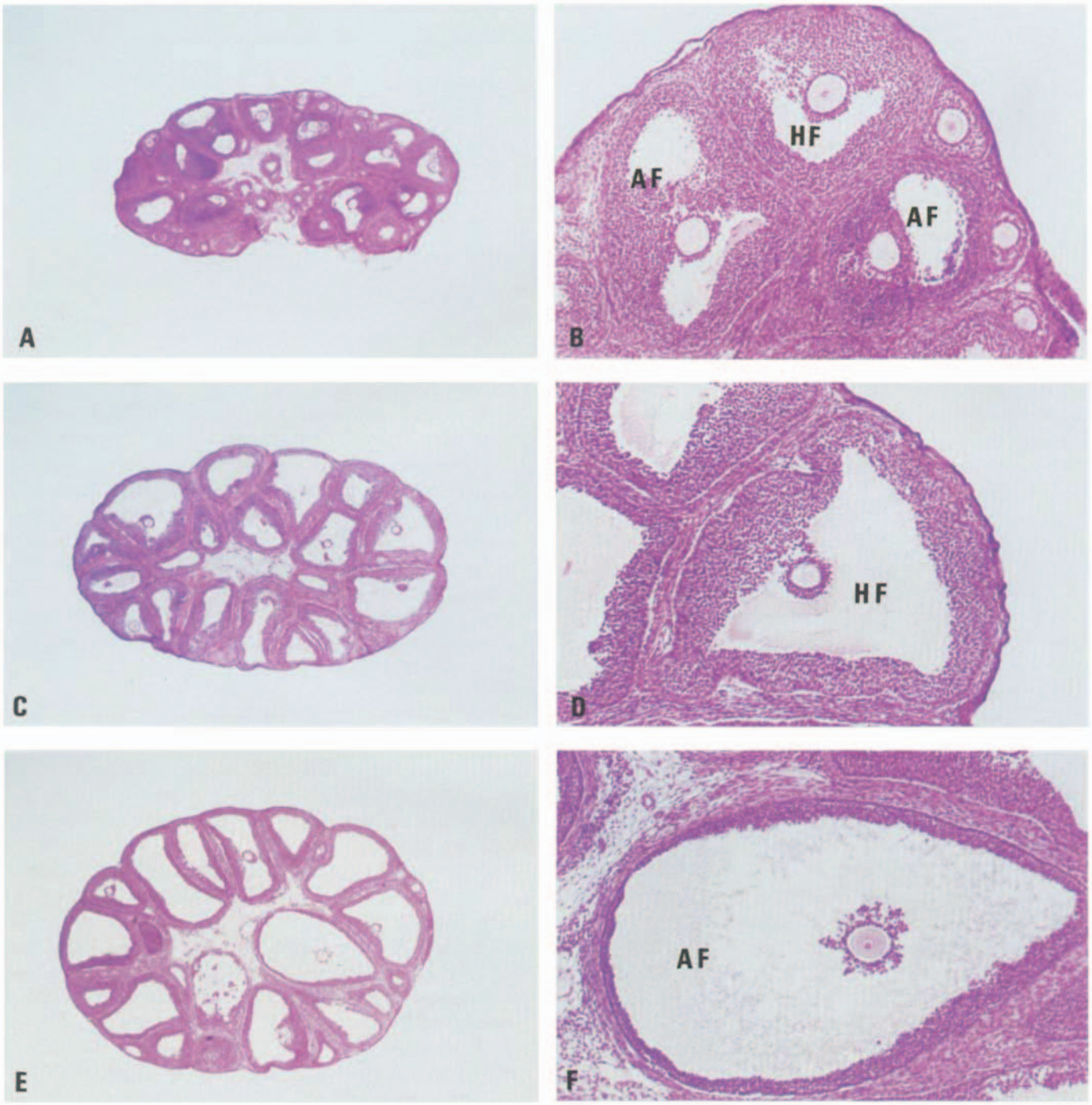

FIG. 4. A and B) Sections of ovaries after treatment with 8 IU recFSH at magnification ×2.5 and ×10, respectively. Antral follicles of size classes 1 to 4 are present. The highest magnification shows one healthy follicle (HF) and two atretic follicles (AF) of size class 2.

C and D) Sections of ovaries after treatment with 8 IU recFSH and 0.2 IU hCG at magnification ×2.5 and ×10, respectively. Antral follicles of size classes 1 to 5 are present. The highest magnification shows a healthy follicle of size class 4.

E and F) Sections of ovaries after treatment with 8 IU recFSH and 2 IU hCG at magnification ×2.5 and ×10, respectively. Atretic antral follicles with dispersion of cumulus cells and a thin granulosa cell layer are apparent. The highest magnification shows a typical example of a size class 5 follicle without resumption of meiosis.

at a total dose of ≥ 10 IU for uterine weight. Levels of ovarian E_2 induced by the highest dose of recFSH were comparable to those of intact, immature animals (72.0 ± 16.9 and 79.4 ± 6.3 pg/ovary, respectively). In contrast, ovarian androstenedione was undetectable (< 8 pg/ovary) both in intact animals and in recFSH-treated hypox animals.

TABLE 3. Effect of recFSH alone, recFSH supplemented with hCG and hCG alone in immature hypophysectomized rats treated twice daily for 4 days.

Total dose (IU)				
recFSH	hCG	N[a]	Ovarian weight (mg)	Uterine weight (mg)
8	0	4	18.3 ± 2.8	19.5 ± 1.0
8	0.2	3	43.2 ± 9.3*	106.3 ± 5.2*
8	0.5	4	32.1 ± 2.7*	114.3 ± 5.3*
8	2	3	59.2 ± 7.4*	118.8 ± 5.6*
8	5	5	75.4 ± 5.8*	123.7 ± 3.2*
0	5	3	8.1 ± 1.0	16.0 ± 0.3

[a]N represents the number of animals per treatment group.
*Significantly ($p < 0.05$) increased in comparison to treatment with 8 IU FSH only.

The total number of healthy and atretic antral follicles and their distribution over the various size classes are depicted in Table 2 and Figure 1, respectively. Typical histological illustrations of ovarian and uterine sections are presented in Figure 3.

The total number of antral follicles was increased in a dose-dependent manner from 11 ± 4 per ovary in controls to 80 ± 7 per ovary in animals treated with 40 IU recFSH. As with the increases in ovarian weight, a significant ($p < 0.05$) rise in number of follicles was induced by doses ≥ 2.5 IU recFSH and maximal response was reached at 10 IU recFSH (Table 2). Antral follicles of controls were 69% atretic (Table 2, Fig. 3, A and B). The incidence of follicular atresia decreased due to recFSH treatment from 69% in controls to 31% in animals treated with 20 IU recFSH. This decrease in atresia was most apparent and most dependent on the dose in size class 1 follicles (Fig. 1). Treatment with increasing doses of recFSH caused a gradual shift in follicle counts from size class 1 (0 IU) via size classes 2 and 3 (2.5 IU) to size classes 4 and 5 (≥ 5 IU).

After treatment with 40 IU recFSH, 39% of all antral follicles were classified as atretic, i.e., 6% as early and late atretic and 33% as "other" (see Table 2). These latter follicles exhibited loosely arranged granulosa cells around the oocyte and an antral layer without pyknosis and a large number of mitotic figures (Fig. 3, C and D). The appearance of these follicles was very similar to that seen in proestrous animals several hours after the LH surge, but in contrast, no germinal vesicle breakdown was observed and the interstitium had a somewhat atrophic appearance. Although these follicles were not included in the classification of Osman [9], they were regarded in the present study as atretic follicles, especially since continuation of treatment with this highest dose of recFSH for another four days induced both early and late atretic follicles with pyknosis and macrophage invasion (data not shown).

When hypox animals were treated for four days with recFSH only, no CL were observed. The subsequent administration of 10 IU hCG to animals treated with 20 or 40 IU recFSH resulted in ovulation in 7 of 8 and 8 of 8 animals, respectively. The median (range) number of CL was 3 (0–28) and 6 (0–26) per ovary, respectively. In addition to CL, large follicles with loosely arranged granulosa cells and germinal vesicle breakdown were present, but large healthy (size class 5) follicles were absent.

Histological examination of uterine endothelium originating from animals treated with vehicle only or with 40 IU recFSH revealed that the height and number of epithelial cells and the folding of the endometrial layer were markedly increased (Fig. 3, E and F). Although the number of epithelial cells per micrometer was unchanged, the number of cells lining the uterine cavity was increased fourfold (data not shown). As seen in Figure 3, E and F, the height of the epithelial glandular cells in the endometrial stroma and the thickness of the latter layer were increased after treatment with 40 IU recFSH.

RecFSH with hCG

To evaluate the additional effect of LH activity, animals were treated with 8 IU recFSH supplemented with 0, 0.2, 0.5, 2, or 5 IU hCG. Controls were treated with 5 IU hCG only. Effects on ovarian and uterine weight are presented in Table 3.

Addition of hCG augmented ovarian weight significantly ($p < 0.05$) in a dose-dependent fashion, from 18.3 ± 2.8 mg in animals treated with 8 IU recFSH alone up to 75.4 ± 5.8 mg in those receiving the highest dose of hCG (5 IU). In contrast, even the lowest dose of hCG caused a maximal increase ($p < 0.05$) in uterine weight, from 19.5 ± 1.0 mg after treatment with 8 IU recFSH alone to 106.3 ± 5.2 mg after treatment with 8 IU recFSH and 0.2 IU hCG. Ovarian and uterine weights of animals treated with 5 IU hCG alone were similar to those of vehicle-treated animals.

The total number of healthy and atretic antral follicles and their distribution over the various size classes are depicted in Table 4 and Figure 2, respectively. Typical histological illustrations of ovarian sections are presented in Figure 4. In contrast to the effect on ovarian weight, the total number of antral follicles was further increased ($p < 0.05$) by only the highest dose of hCG, i.e., from 65 ± 8 per ovary after treatment with 8 IU recFSH alone to 105 ± 11 per ovary after treatment with 8 IU recFSH plus 5 IU hCG (Table 4). After treatment with 5 IU hCG alone, no antral follicles larger than 275 μm were present. In intact rats of the

TABLE 4. Effect of recFSH supplemented with hCG on the number of healthy and atretic follicles in immature hypophysectomized rats.

Treatment total dose (IU)			Total number of follicles	Number of atretic follicles			Percentage of atretic follicles
recFSH	hCG	N[a]	(275 μm)	Early atretic[b]	Late atretic[c]	Other[d]	(>275 μm)
8	0	4	65 ± 8	29 ± 5	9 ± 3	—	58 ± 2
8	0.2	3	89 ± 15	19 ± 11	—	—	20 ± 8
8	0.5	3	54 ± 14	2 ± 1	—	—	2 ± 8
8	2	3	67 ± 12	1 ± 1	4 ± 4	18 ± 6	31 ± 9
8	5	5	105 ± 11*	—	4 ± 2	25 ± 8	33 ± 4
0	5	5	—	—	—	—	—
intact	(day 30)	6	42 ± 4	6 ± 1	10 ± 2	—	39 ± 3

[a]N represents the number of animals per treatment group.
[b]Early atretic: stage 1a and 1b.
[c]Late atretic: stage 2a and 2b.
[d]Other: follicles with loosely arranged granulosa cells.
*Significantly ($p < 0.05$) increased in comparison to treatment with 8 IU FSH only.

same age (30 days), the number of antral follicles was 42 ± 4 per ovary (Table 4), which is fourfold higher than in hypox vehicle-treated rats and about twofold lower than in hypox rats treated with at least 10 IU recFSH alone (Table 2). In contrast to these latter rats, intact animals had only a few class 4 and no class 5 follicles.

In comparison to follicles in animals treated with 8 IU recFSH only, addition of as little as 0.2 IU hCG caused a pronounced shift from small (class 1 and 2) to large (class 4 and 5) antral follicles (Fig. 2). Higher doses of hCG provided comparable size distributions.

In animals treated with 8 IU recFSH only, the incidence of atresia was 58% vs. 39% in intact animals (Table 4; Fig. 4, A and B). This was due to a higher incidence of early atretic follicles (29 vs. 6). Supplementation with the lowest hCG doses, i.e., 0.2 and 0.5 IU, largely diminished the incidence of atresia to 20% and 2%, respectively (Table 4; Fig. 4, C and D). Addition of 2 and 5 IU hCG induced class 4 and 5 follicles with a thin granulosa cell layer and loosely arranged granulosa cells (Fig. 4, E and F), as did treatment with 40 IU recFSH only, resulting in 31% and 33% atretic follicles, respectively (Table 4).

DISCUSSION

The very first experiments in immature hypox rats, showing the synergistic effect of FSH and LH, date from the 1940s [10, 11]. Thereafter, many comparable experiments were published (reviewed in [12]), sometimes with conflicting results due to the testing of FSH preparations with varying degrees of residual LH activity. Through the expression of FSH in mammalian host cells, pure human FSH devoid of LH has become available and allows definite elucidation of the specific action of FSH during follicular growth and steroidogenesis.

The current study in immature, hypox rats demonstrates that recFSH, in the complete absence of LH, increases the number of antral follicles and diminishes the incidence of atresia in these follicles. The effect on atresia was most apparent in the smallest follicle size class, indicating that FSH induces multiple follicular development by preventing small antral follicles from undergoing atresia. Furthermore, recFSH alone induced the growth of follicles to preovulatory stages, and subsequent administration of hCG resulted in ovulation of healthy follicles only, since large follicles with loosely arranged granulosa cells were still present after the induction of ovulation.

The above-described growth of ovulatory follicles confirms previous findings in hypox rodents treated with human FSH produced by recombinant DNA technology [13, 14]. In the study by Galway et al. [14] with immature hypox rats, recFSH treatment without subsequent hCG administration could also induce ovulation. In these animals, however, a diethylstilbestrol capsule was implanted to prevent atresia after the withdrawal of gonadotropins. In our immature hypox rats, circulating E_2 remained at baseline. Although intraovarian E_2 increased in a recFSH dose-dependent manner, these levels were relatively low and were clearly suppressed due to the lack of LH activity since the highest dose of recFSH caused concentrations of E_2 comparable to those measurable in ovaries of intact immature animals, whereas the number of antral follicles was twice as high and these follicles were mainly of large size classes. Neither in hypox nor in intact animals was ovarian E_2 production sufficient to increase its concentration in the circulation.

In the current hypox model, ovarian androstenedione was undetectable, indicating that any available androgen substrate was immediately converted to estrogens. Production of androgens by ovarian theca-interstitial cells might have occurred because of factors other than LH. For instance, in vitro studies on the control of androgen synthesis by thecal cells from rat and human ovaries have suggested that granulosa cell-derived inhibin enhances androgen production [15, 16]. Furthermore, ovarian androgen biosynthesis is known to be promoted by insulin-like growth fac-

tor-1 of granulosa cell origin [17], which is also capable of augmenting FSH-induced estrogen biosynthesis [18].

Interestingly, uterine weight increased during recFSH treatment while plasma E_2 remained low at baseline, indicating that uterine weight is a more sensitive parameter for ovarian E_2 production than levels of serum E_2. So far, reports on uterine weight after recFSH treatment have been contradictory. After two days of recFSH treatment (total dose, 72 IU/hypox rat), Whitelaw and coworkers [19] found that uterine weights were not different from those of controls. However, in our study, treatment for four days with at least 10 IU recFSH per animal significantly increased uterine weight and caused endometrial proliferative growth. Increases of uterine weight have also been reported in adult hypox mice after recFSH treatment [13]. Previous measurements of intrauterine E_2 showed that recFSH-induced increases in uterine and ovarian E_2 are comparable [20], suggesting that estrogens produced by the ovaries bind to a uterine protein to cause local accumulation.

Supplementation of recFSH with hCG augmented ovarian weight, but except in the case of the highest hCG dose (5 IU), no further increases in the total number of follicles were observed. However, addition of only 0.2 to 0.5 IU hCG (doses sufficient to cause considerable increases in circulating E_2 and to augment ovarian aromatase activity [5]) caused a large shift of small follicles to preovulatory follicles. Also, addition of 0.5 IU hCG caused a large reduction in atresia in the various follicle size classes. Together these findings demonstrate that the number and quality of follicles are strongly determined by the FSH/LH ratio of gonadotropins used to induce superovulation. For administering hCG in the hypox rat, a ratio of 16:1 (8 IU FSH:0.5 IU hCG) might be most favorable, although additional dose-finding and time-course experiments would be required to substantiate these data.

The present study also demonstrated that doses of FSH and hCG that are too high are detrimental for normal follicular development. After treatment with 40 IU (200 IU/kg/day) of recFSH alone or after treatment with 8 IU recFSH and 2 to 5 IU hCG, 20 to 25% of antral follicles had lost their compact structure with respect to the granulosa cell layer and showed loosely arranged cumulus cells, suggesting secretion of an excess of follicular fluid. Most likely, these follicles represent an abnormal stage of atresia as indicated by the results after prolonged treatment with recFSH. The follicles with a thin layer of granulosa cells present after combined treatment with 8 IU recFSH and 2 or 5 IU hCG probably develop to cystic follicles as described by Bogovich [21], who treated immature hypox rats with ovine FSH and hCG for 2 wk. Obviously these findings are of interest for understanding ovarian pathophysiological processes, but the doses required to induce these processes were extremely high—much higher than those applied to induce superovulation in animals or humans.

In summary, recFSH alone is able to induce follicle growth up to the preovulatory stage, mainly by preventing small antral follicles from undergoing atresia; but small amounts of LH activity, either exogenous or endogenous, are beneficial during recFSH-induced multiple follicular growth in that the number of healthy follicles is enlarged. In the complete absence of LH activity, recFSH increases uterine weight by stimulating proliferative growth of the endometrium.

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human reproduction update

Advances in recombinant DNA technology: corifollitropin alfa, a hybrid molecule with sustained follicle-stimulating activity and reduced injection frequency

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BACKGROUND: Recombinant DNA technologies have been used to develop longer-acting therapeutic proteins. One approach is to introduce sequences containing additional glycosylation sites. Using this technique, a new chimeric gene has been developed containing the coding sequences of the FSH β-subunit and the C-terminal peptide of the hCG β-subunit, which bears four O-linked oligosaccharide binding sites. Co-expression of the α-subunit and the chimeric FSH β-subunit produces a new recombinant molecule, named corifollitropin alfa, with a prolonged elimination half-life and enhanced *in vivo* bioactivity compared with wild-type FSH.

METHODS: Medline searches by subject and additional searching by hand.

RESULTS: Initial studies in pituitary suppressed female volunteers confirmed the extended half-life of the compound. Phase II studies have shown that corifollitropin alfa is able to induce and sustain multi-follicular growth for an entire week in women undergoing ovarian stimulation using GnRH antagonist co-treatment for IVF. Corifollitropin alfa regimens have been developed with dosages of 100 and 150 μg, for patients with body weight $\leq$60 and $>$60 kg, respectively.

CONCLUSIONS: Corifollitropin alfa is the first long-acting hybrid molecule with sustained follicle-stimulating activity developed for the induction of multi-follicular growth along with GnRH antagonist co-treatment for IVF. This new treatment option may be simpler and more convenient for patients compared with conventional long protocols of daily FSH injections in combination with GnRH agonist co-treatment. The safety and efficacy of such regimens is currently being evaluated in large comparative phase III clinical trials. The development of corifollitropin alfa is the first step towards a new generation of recombinant gonadotrophins.

Key words: corifollitropin alfa / ovarian stimulation / FSH / IVF

Introduction

Gonadotrophin therapy in various forms has been used to restore ovulation since the 1930s. Only after the introduction of IVF, gonadotrophins have been applied to stimulate multiple follicle development (Edwards and Steptoe, 1975; Lunenfeld, 2004). The latter treatment overrides the physiologic selection of a single dominant follicle by extending the time during which serum follicle-stimulating hormone (FSH) concentrations remain above the threshold level required for follicular recruitment and ongoing maturation (Brown, 1978; Baird, 1987; Fauser and van Heusden, 1997). The availability of a number of mature oocytes suitable for IVF procedures improves the likelihood of achieving fertilization, of generating good quality embryos, and of a successful pregnancy (Macklon *et al*., 2006).

The relatively short elimination half-life ($t_{1/2}$) and rapid metabolic clearance of current FSH preparations requires that daily injections are administered to maintain steady state FSH levels above the threshold level during ovarian stimulation (Fauser and van Heusden, 1997). Frequent injections may increase stress, error rates and the treatment burden experienced by IVF patients. Therefore, it has previously been examined whether intermittent administration of recombinant FSH (rFSH) by increasing the loading dose could result in similar outcomes as daily FSH injections (Crooke *et al*., 1963; Balasch *et al*., 2001; Scholtes *et al*., 2004). However, the development of FSH analogues with a longer terminal $t_{1/2}$ and a slower absorption to peak serum levels may be more helpful to render an extended injection-free period than to increase the initial dose of current FSH preparations.

Many therapeutic areas have seen a shift in drug delivery regimens in recent years, with a move towards reduced frequency of administration. For example, once-a-year zoledronate for the prevention and treatment of osteoporosis (Black *et al*., 2007), long-acting injectable risperidone for the treatment of schizophrenia (Möller, 2007) and contraceptive implants, such as etonogestrel, that provide sustained hormone release over a number of years (Croxatto *et al*., 1999). The main advantages of less frequent dosing are an increase in patient convenience, fewer chances for mistakes during drug administration and improved compliance (Richter *et al*., 2003), which is particularly relevant during long-term treatment. An additional benefit is that long-acting agents induce more stable serum levels of a drug compared with repeated dosing using a short-acting agent (Darney, 2000; Eerdekens *et al*., 2004).

This review addresses developments in gonadotrophin therapy for ovarian stimulation for IVF and discusses the latest advances in the production of longer-acting compounds with FSH bioactivity, including the development of corifollitropin alfa, a new hybrid molecule with sustained follicle-stimulating activity.

Methods

Searches were initially performed using the Medline database with the search terms 'gonadotrophin therapy', 'ovarian stimulation', 'fertility', 'infertility treatment', 'follicle-stimulating hormone'. Where appropriate, further searches for references cited in the literature derived from the initial Medline search were performed manually or using Medline as appropriate. However, this was not a systematic review of the literature and inclusion of specific discussion topics was the subjective decision of the authors.

Native gonadotrophins: structure and function

FSH is a member of the glycoprotein hormone family, which also includes LH, hCG and thyroid-stimulating hormone. The glycoprotein hormones are cysteine-rich dimeric proteins made up of two non-identical, non-covalently linked α- and β-subunits. The α-subunit is common to all family members, whereas the β-subunit is unique to each hormone and confers biologic specificity (Ryan *et al*., 1988) (Fig. 1).

The glycoprotein hormones are biosynthesized as arrays of isoforms that differ from each other mainly in the structure of oligosaccharides covalently linked to the protein molecules. Post-translational modification of the primary protein structure results in differential glycosylation, which produces molecules with different isoelectric properties, molecular weights and bioactivity (Chappel, 1995). The main determinants of FSH polymorphism appear to be glycan complexity and sialic acid content (Creus *et al*., 2001). Heavy sialylation produces acidic isoforms with longer half-lives *in vivo* than more basic isoforms. This effect is largely due to the pharmacokinetic (PK) properties of the acidic isoforms, as their *in vitro* potency tends to be similar to or lower than that of less sialylated isoforms (D'Antonio *et al*., 1999; Barrios-de-Tomasi *et al*., 2002).

The carbohydrate moieties on the FSH molecule serve a number of functions, including correct protein folding, assembly and secretion of the gonadotrophins and signal transduction (Stockell-Hartree and Renwick, 1992). Additional carbohydrate moieties may reduce metabolic clearance due to increases in molecular size and charge. Increased sialylation increases metabolic stability by decreasing glomerular filtration within the kidney (Wide, 1986; D'Antonio *et al*., 1999), and through protection against clearance by asialoglycoprotein receptors in the liver (Gottschalk *et al*., 1960; Morell *et al*., 1971; van Lenten and Ashwell, 1972). The most heavily glycosylated of the mammalian pituitary and placental glycoproteins is pregnant mares' serum gonadotrophin (PMSG), which has multiple N- and O-linked glycosylation sites on both subunits (Murphy and Martinuk, 1991). PMSG glycosylation composition has a high sialic acid content that confers a significantly extended half-life compared with other glycoprotein hormones (Christakos and Bahl, 1979).

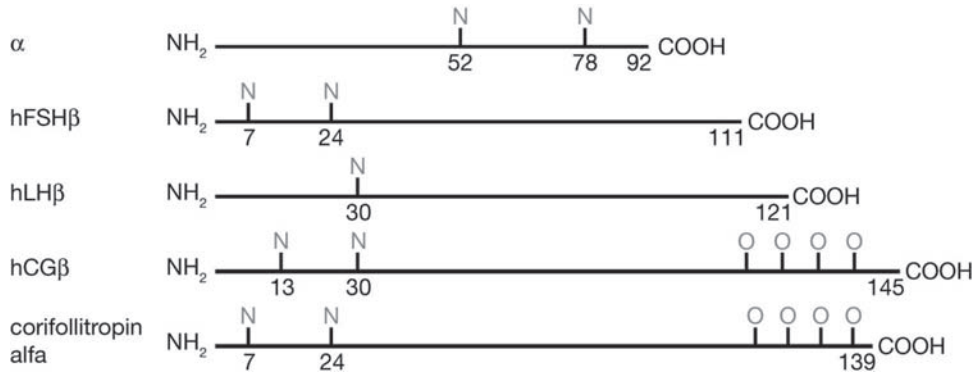

Figure 1 Structure of the glycoprotein hormone gonadotrophins: schematic representation of the universal α-subunit and the unique β-subunits showing N- and O-linked glycosylation binding sites.-N, site of N-linked carbohydrate; -O, site of O-linked carbohydrate.

With the exception of hCG, the human glycoprotein hormones have relatively short terminal half-lives *in vivo* (Amin and Hunter, 1970; Sowers *et al*., 1979). HCG exhibits a nearly 10-fold increase in plasma half-life compared with LH (Kohler *et al*., 1968; Rizkallah *et al*., 1969), despite a high level of amino acid sequence homology between the two hormones (Stockell-Hartree and Renwick, 1992). The main structural difference between the two molecules is an additional 31 amino acids forming the so-called C-terminal peptide (CTP) of the hCG β-subunit. The hCG-CTP includes four additional O-linked carbohydrate side chains (Birken and Canfield, 1977), each of which has two terminal sialic acid residues (Kessler *et al*., 1979) (Fig. 1). Deletion of the CTP has been shown to decrease the *in vivo* activity of the hCG molecule 3-fold compared with wild-type in a rat ovulation assay (Matzuk *et al*., 1990).

Ovarian stimulation for infertility treatment: how it all started

It is nearly 100 years since ablation experiments in dogs provided the first empiric evidence of a role for the pituitary gland in the regulation of gonadal function (Crowe *et al*., 1910). Animal pituitary extracts with both FSH- and LH-like activities such as PMSG were used until the late 1960s for ovulation induction in women with gonadotrophin insufficiency. These products were eventually withdrawn from clinical use due to their antigenic potential, although PMSG is still used experimentally in laboratory animals and for ovulation induction in cattle (Lunenfeld, 2004).

The first successful induction of ovulation with FSH derived from human pituitary glands was described by Gemzell *et al*. in 1958. Subsequent research focused on extracting and purifying gonadotrophins from human sources. Human pituitary gonadotrophins (hPG) were isolated at autopsy from lyophilized human pituitary glands (Gemzell *et al*., 1958), and were used successfully for over three decades for ovulation induction in anovulatory women. However, the supply of human pituitary glands was limited, and concerns over the risk of prion diseases from human brain tissue products led to the withdrawal of hPG in the early 1990s.

Human urinary gonadotrophins

An alternative human source of gonadotrophins was found in the urine of post-menopausal women (Lunenfeld, 2004). Human menopausal gonadotrophin (HMG) preparations were <5% pure FSH, contained much LH bioactivity and were contaminated with potentially immunogenic urinary proteins. HMG was successfully used to induce ovulation in hypogonadotrophic anovulatory women (Lunenfeld, 1963). The lack of purity and the limited batch-to-batch consistency may have negatively affected clinical results. It is now known that excessive LH levels during the early or late follicular phase (Hillier, 1994) may have a negative impact on subsequent fertilization, implantation and embryo survival rates (Kolibianakis *et al*., 2004).

Increasing efforts were made to separate and purify the individual components of urinary gonadotrophin preparations by various physical, chemical and immunologic means (Lunenfeld, 2004). In the early 1990s, monoclonal antibodies were used to produce highly purified urinary FSH (FSH-HP) from bulk HMG. The purity of FSH-HP was ~95%, compared with 1–2% for HMG (le Cotonnec *et al*., 1993). This increased purity reduced the total amount of injected protein and allowed for the first time for s.c. administration. Product consistency also enabled PK and pharmacodynamic (PD) analysis, and facilitated more patient-specific treatment plans adjusted to individual responses (Lunenfeld, 2004).

The world's first IVF baby, Louise Brown, was born in 1978 following oocyte recovery in a natural cycle. However, it was subsequently shown that IVF success rates could be improved significantly by the use of exogenous gonadotrophins to stimulate multi-follicular development (Laufer *et al*., 1983; for review see Macklon *et al*., 2006). This new technology led to an exponential growth in the worldwide demand for gonadotrophin preparations and doubts regarding quality control, both in donor recruitment and in product purity and safety (Lunenfeld, 2004).

Recombinant FSH

The problem of the short supply of high-quality gonadotrophins was solved by the advent of recombinant DNA technology, which

permitted the large-scale production of pure recombinant human gonadotrophin preparations. Following the sequencing (Rathnam and Saxena, 1975; Saxena and Rathnam, 1976) and subsequent cloning (Keene *et al*., 1989) of the gene encoding the human FSH molecule, the first human rFSH preparations became commercially available in 1996 (De Leeuw *et al*., 1996; Howles, 1996; Olijve *et al*., 1996). These recombinant molecules are structurally very similar to native pituitary FSH and the final product is highly purified (>99%), with a high specific *in vivo* bioactivity. The availability of the recombinant molecule allowed investigators to study the role of FSH in the complete absence of LH for the first time. Studies of rFSH in hypophysectomized animals (Mannaerts *et al*., 1994) and gonadotrophin-deficient women (Schoot *et al*., 1992, 1994) confirmed for the first time that growth of multiple follicles to pre-ovulatory sizes was possible in the complete absence of LH activity.

The FSH threshold/window concept and follicle development

At the same time as DNA technology was facilitating the development of recombinant gonadotrophins, the mechanism of action of FSH was further elucidated. In a normal menstrual cycle, the degeneration of the corpus luteum in the late luteal phase leads to a reduction in serum levels of estradiol (E_2), progesterone and inhibin-A. These endocrine changes give rise to reduced inhibition of hypothalamic GnRH production. The subsequent increased frequency of pulsatile GnRH secretions stimulates a rise in FSH at the end of the luteal phase (Hall *et al*., 1992). FSH continues to rise gradually during the early follicular phase, exceeding the so-called threshold level required for continued follicular development (Brown, 1978; Baird, 1987; Fauser and van Heusden, 1997). This process is currently referred to as secondary or cyclic follicle recruitment.

FSH levels subsequently stabilize and plateau, before falling again due to negative feedback from inhibin-B and E_2 produced by the growing follicles (Schipper *et al*., 1998a). Only follicles that happen to be at a more advanced stage of development during the inter-cycle FSH rise are able to respond to FSH (Zeleznik and Kubik, 1986). As serum FSH concentrations fall below the threshold level, all but the dominant follicle loss the stimulus to develop further and enter atresia (Zeleznik and Kubik, 1986; Van Santbrink *et al*., 1995). Hence, the physiologic FSH window for follicle development is quite narrow and in normo-ovulatory cycles only a single (dominant) follicle escapes from atresia due to decreased dependency on FSH.

Ovarian stimulation prior to IVF or ICSI involves the application of relatively high doses of exogenous FSH in a timely manner to maintain serum FSH concentration above the threshold, necessary to support multi-follicular growth (Macklon *et al*., 2006) (Fig. 2). The prolonged period above this threshold (effectively extending the FSH window) facilitates the development of more than one follicle, thus increasing the number of oocytes available for subsequent IVF procedures (Lolis *et al*., 1995; Schipper *et al*., 1998b; Hohmann *et al*., 2001). Due to the relatively short $t_{1/2}$ of rFSH of about 30 h (Mannaerts *et al*., 1993), daily FSH injections are needed during the stimulation period to prevent serum FSH levels from dropping below the threshold and subsequent follicular growth arrest. After each injection, peak serum FSH levels are reached within 10–12 h and then decline

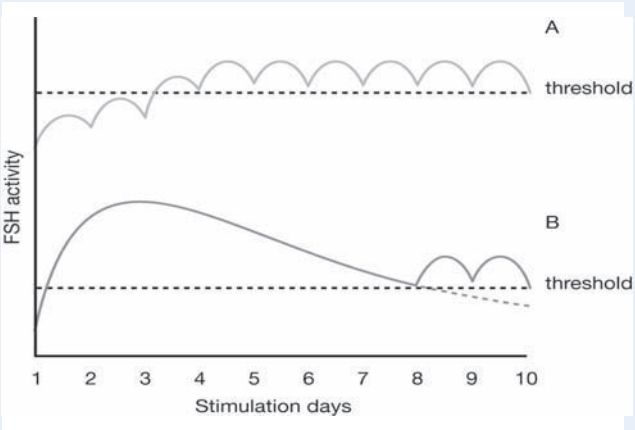

Figure 2 The FSH threshold/window concept using (**A**) daily FSH treatment or (**B**) a single injection of corifollitropin alfa to induce and sustain multi-follicular development during the first week of stimulation. The FSH threshold represents the FSH concentration above which follicle development is stimulated. The FSH window represents the number of days where FSH levels are above the threshold (See also Fauser *et al*., 2005).

until the next injection. Steady state levels are reached only after 3–5 days of treatment, thus dose adjustments before stimulation Day 5 are not advised. The single dose and multiple dose PK properties of rFSH have been described for follitrophin-alpha and follitrophin-beta and are included in Table I (Mannaerts *et al*., 1993; le Cotonnec *et al*., 1994a, b). Clearly, these properties are influenced by gender, route of administration, and dose and frequency of injections, rather than by the specific rFSH preparation injected (Mannaerts *et al*., 1996).

GnRH analogue co-treatment during ovarian stimulation prior to IVF or ICSI

Despite our understanding of the mechanism of FSH action during ovarian stimulation, one of the problems associated with stand-alone gonadotrophin therapy was the relatively high proportion of cycles exposed to a premature rise in LH concentrations (Pelinck *et al*., 2002). Developing follicles exposed to inappropriately high concentrations of LH ('ceiling level') may enter follicular growth arrest, premature luteinization and even ovulation prior to oocyte retrieval (Stanger and Yovich, 1985; Loumaye, 1990; Hillier, 1994).

The problem of premature luteinization could be overcome by the use of GnRH agonists causing suppression of pituitary gonadotrophins via GnRH receptor down-regulation and desensitization (Fleming *et al*., 1982; Porter *et al*., 1984). This discovery resulted in the widespread use of adjuvant GnRH agonist co-treatment during ovarian stimulation. However, due to the initial release of endogenous FSH and LH ('flare-up'), the introduction of GnRH agonists required the development of the so-called 'long protocol', with agonist treatment beginning 2–3 weeks prior to the start of ovarian stimulation. One drawback of this approach is that pituitary suppression due to the

Table I Pharmacokinetic properties of recombinant FSH in male and female gonadotrophin-deficient or pituitary-suppressed volunteers

References	Route/subjects	Dose	C_{max} (ng/ml)	t_{max} (h)	$AUC_{0-\infty}$ (ng.h/ml)	$t_{½}$ (h)
Mannaerts *et al.* (1993)	i.m.; 8 women	single; 300 IU	4.3 ± 1.7	27 ± 5	339 ± 105	44 ± 14
	i.m.; 7 men	single; 300 IU	7.4 ± 2.8	14 ± 8	452 ± 183	32 ± 12
le Cotonnec *et al.* (1994a)	i.v.; 12 women	single; 300 IU	62 ± 18	–	575 ± 114	17 ± 4
le Cotonnec *et al.* (1994b)	s.c.; 12 women	single; 150 IU	3 ± 1	16 ± 10	235 ± 144	37 ± 28
	s.c.; 12 women	multiple; 150 IU/day	9 ± 3	8 ± 6	187 ± 61	24 ± 8

C_{max}, peak serum concentration at any time; t_{max}, time at which serum peak FSH concentration is reached; $t_{½}$, elimination (terminal) half-life; $AUC_{0-\infty}$, area under the serum FSH concentration curve. Values are presented as means ± SD.

administration of GnRH agonist results in an increased requirement for exogenous FSH. A further potential problem is that GnRH agonists are usually started in the mid-luteal phase of the pre-stimulation cycle aiming to reduce chances of ovarian cysts, which carries a small risk of agonist administration in the presence of an early pregnancy.

GnRH antagonists circumvent many of the problems associated with GnRH agonist co-treatment. Unlike GnRH agonists, GnRH antagonists act by competitively blocking the receptor preventing the binding of endogenous GnRH (Klingmüller *et al.*, 1993). Thus, inhibition of endogenous gonadotrophin release is induced within a few hours after GnRH antagonist injection and does not involve an initial 'flare-up' of gonadotrophins (Fauser and Devroey, 2005; Tarlatzis *et al.*, 2006).

One benefit of the rapid action of GnRH antagonists is that administration is needed only when a premature LH rise may occur, usually during the mid- to late-follicular phase of the stimulation cycle (Diedrich *et al.*, 1994). Another advantage is that higher levels of endogenous gonadotrophins are present at the start of stimulation, reducing the requirement for exogenous FSH in GnRH antagonist protocols compared with long agonist co-treatment regimens (Hohmann *et al.*, 2003). Also, due to a shorter duration of stimulation with fewer intermediate-sized follicles and lower E_2 levels, there is a reduced risk of developing ovarian hyperstimulation syndrome (OHSS) (Kolibianakis *et al.*, 2006). Fewer side effects, and less patient discomfort per cycle have been reported (Heijnen *et al.*, 2007). The rapid action and reversibility of GnRH antagonists also allow for more treatment cycles to be performed within a given time period (Heijnen *et al.*, 2004, 2007; Eijkemans *et al.*, 2006). Moreover, improved tolerability of IVF treatment may well lead to a reduction in drop-out rates (Verberg *et al.*, 2008). Therefore, the overall pregnancy rate per treatment started may be similar or increased compared with conventional GnRH agonist protocols.

Although GnRH antagonist regimens offer clear advantages compared with long GnRH agonist protocols, initial clinical uptake has been slower than expected (Fauser and Devroey, 2005; Tarlatzis *et al.*, 2006), due mainly to concerns in regard to reduced pregnancy rates per cycle compared with conventional regimens. Some combined analyses suggested a small but significant reduction in pregnancy rates (Al-Inany *et al.*, 2006). However, a recent systematic review has shown that the probability of a live birth after IVF does not depend on the type of GnRH analogue used (Kolibianakis *et al.*, 2006). Outcomes from IVF cycles using GnRH antagonist protocols have no doubt benefited from increasing clinical experience with these products. As a result, the use of GnRH antagonists is now becoming more widespread in general IVF practice for ovarian stimulation in normal responders.

Concepts of long-acting molecules with FSH bioactivity

A number of technologic approaches have been used to develop longer-acting FSH molecules, most of which have involved altering the structure of the FSH molecule itself. It was hypothesized that one way of extending the $t_{1/2}$ of FSH would be to reduce glomerular filtration by increasing the molecular weight and charge of the molecule via the introduction of additional glycosylation. Increasing the number of N- or O-linked carbohydrate moieties extends $t_{1/2}$ by as much as 100% compared with wild-type rFSH, but there appears to be a maximum plasma half-life beyond which further increases cannot be achieved by additional glycosylation (Weenen *et al.*, 2004).

A unique approach was chosen by Boime and co-workers, who attached the CTP of the hCG β-subunit to the FSH β-subunit using site-directed mutagenesis and gene transfer techniques (Fares *et al.*, 1992). They constructed a chimeric gene containing the sequence encoding the CTP of the hCG β-subunit fused to the translated sequence of the human FSH β-subunit (Fig. 1). The FSH β-CTP chimera was then transfected with the common glycoprotein α-subunit and expressed in Chinese hamster ovary (CHO) cells. It was found that the presence of the CTP sequence did not significantly affect assembly or secretion of the intact dimer by stable cell lines.

The chimeric recombinant molecule had similar *in vitro* receptor binding and steroidogenic activity compared with wild-type FSH but had significantly enhanced *in vivo* activity and plasma half-life (Fares *et al.*, 1992). Further studies showed an ~10-fold increase in biopotency for the chimeric molecule compared with wild-type FSH (LaPolt *et al.*, 1992). It was also demonstrated that a single injection of this fusion molecule stimulated follicular maturation in rats sufficiently to facilitate ovulation induction 52 h later. In comparison, a single injection of the same dose of wild-type FSH was ineffective in increasing ovarian ovulatory potential. Interestingly, splitting the total dose of wild-type FSH into four injections given 12 h apart was as effective as chimeric FSH. These results indicate the importance of sustained plasma levels of FSH rather than total dose, for stimulating follicular maturation (LaPolt *et al.*, 1992).

The generation of a new CHO cell line expressing the FSH hybrid molecule has led to the development of corifollitropin alfa, a new gonadotrophin preparation with increased *in vivo* FSH bioactivity. Interestingly, the linkage of CTP to recombinant hormones like erythropoietin has also led to the development of long-acting agents in other therapeutic areas (Fares *et al*., 2007).

Other investigators have generated longer-acting FSH molecules by introducing additional sequences containing potential glycosylation sites at the N-terminus of the FSH α-subunit (Perlman *et al*., 2003), or by creating a contiguous, single-chain, covalently-bound fusion protein containing the common α- and FSH β-subunits separated by the hCG β-CTP (Ben-Menahem and Boime, 1996; Sugahara *et al*., 1996; Klein *et al*., 2002). Using the single-chain platform, an FSH analogue with additional N-linked carbohydrates has also been constructed (Klein *et al*., 2003). All these approaches have yielded similar results to corifollitropin alfa in terms of extending the *in vivo* half-life of the molecules.

An alternative approach to producing long-acting molecules involves fusion with the constant region fragment (Fc) domain of immunoglobulin G1. A naturally occurring Fc receptor is expressed in the lungs and has been shown to transport high-molecular weight Fc fusion protein molecules non-invasively by the pulmonary route in non-human primates (Bitonti *et al*., 2004) and humans (Dumont *et al*., 2005). Two forms of FSH were created: Fc fusion protein (a single-chain configuration, with α- and FSH β-subunits fused sequentially to each arm of an Fc dimer, producing a molecule dimeric for both Fc and FSH) and a heterodimer format, with a single α-subunit fused to one arm of an Fc dimer and a single FSH β-subunit fused to the other arm (producing a structure monomeric for the FSH molecule) (Low *et al*., 2005). Both forms demonstrated increased stability in the blood with a $t_{1/2}$ of 55–210 h after pulmonary delivery in non-human primates, compared with ~24–30 h for rFSH given intravenously in humans and intramuscularly in non-human primates (le Cotonnec *et al*., 1994; Weinbauer *et al*., 1994). Both Fc–FSH fusion protein forms were more effective than rFSH at stimulating ovarian response as measured by ovarian weight gain in rats and serum inhibin levels in cynomolgus monkeys, with the Fc heterodimer–FSH monomer being more potent than the single-chain (Low *et al*., 2005). This was possibly due to improved transmucosal transport in the lung with decreasing size and charge or to the increased stability conferred on the non-covalently linked heterodimeric FSH molecule by fusion of the α- and; β-subunits to the dimeric Fc moiety (Dumont *et al*., 2006). This monomeric FSH:Fc molecule is the subject of further investigation, but is not currently in clinical development.

Corifollitropin alfa: a new rFSH analogue

Corifollitropin alfa is a new gonadotrophin preparation currently in development for the stimulation of multi-follicular development in women undergoing ovarian stimulation for IVF or ICSI. The active compound is a chimeric recombinant molecule composed of FSH and the CTP of the hCG β-subunit (Fig. 1). Like wild-type FSH, corifollitropin alfa interacts only with the FSH-receptor and lacks LH activity (Lapolt *et al*., 1992). However, corifollitropin alfa has a longer $t_{1/2}$ and an extended time-interval (t_{max}) to peak serum levels (C_{max}) (Duijkers *et al*., 2002).

In previous phase II trials, a single dose of corifollitropin alfa was able to induce and sustain multi-follicular growth during the first week of stimulation. Accordingly, the proposed class name for this new gonadotrophin is sustained follicular stimulants. Corifollitropin alfa has the same pharmacological activity as pure FSH preparations. However, a single dose of corifollitropin alfa is able to keep circulating FSH activity above the threshold necessary to support multi-follicular growth for an entire week (Fig. 2). As such, one injection of corifollitropin alfa replaces the first seven daily injections of rFSH. Thereafter, stimulation may be continued with daily FSH injections until the criteria for final oocyte maturation have been reached. In the context of improving treatment simplicity and reducing the burden of IVF treatment, corifollitropin alfa has been developed in combination with GnRH antagonist co-treatment.

To date, corifollitropin alfa has been tested in over 400 women in phase I and II clinical trials, in doses ranging from 7.5 to 240 μg. Phase I started with one trial in male hypogonadotrophic hypogonadal volunteers (Bouloux *et al*., 2001), who received four repeated s.c. injections of 15 μg corifollitropin alfa to examine the safety and possible immunogenicity of corifollitropin alfa. In the next phase I study, the PK of a single dose of 30–120 μg corifollitropin alfa and the ovarian response to this dose were investigated in pituitary-suppressed female volunteers (Duijkers *et al*., 2002). Two phase II studies of corifollitropin alfa have been conducted in patients undergoing ovarian stimulation for IVF or ICSI using single doses of 120–240 μg (Devroey *et al*., 2004) and of 60–180 μg (The Corifollitropin Alfa Dose-finding Study Group, 2008).

To explore whether corifollitropin alfa could also be useful in classical ovulation induction in anovulatory patients diagnosed with polycystic ovary syndrome (PCOS), a first feasibility study of administration of low dosages (7.5–60 μg) of corifollitropin alfa was performed in anovulatory women (Balen *et al*., 2004). Distinct individual response differences regardless of dose were observed.

Pharmacokinetics

Exposure after injection of corifollitropin alfa can be measured most reliably by means of a specific corifollitropin alfa enzyme immunoassay, which does not cross-react with native or rFSH (Devroey *et al*., 2004). Although corifollitropin alfa may cross-react in a linear dose-related fashion in commercial FSH kits as demonstrated for the FSH Delfia assay (AutoDELFIA, Wallac Oy, Finland), the immunoreactivity of corifollitropin alfa in such assays cannot be translated into international units, since the monoclonal antibodies raised against FSH have a different affinity for corifollitropin alfa.

The main calculated PK parameters for corifollitropin alfa in IVF patients are shown in Table II. The results of phase I and phase II trials in pituitary-suppressed volunteers and patients, respectively, show that the mean $t_{1/2}$ of corifollitropin alfa is ~65 h for all doses tested between 60 and 240 μg, compared with ~35 h for rFSH (Duijkers *et al*., 2002; Balen *et al*., 2004; Devroey *et al*., 2004; The Corifollitropin Alfa Dose-finding Study Group, 2008). Dose-normalized (dn) area under the curve (AUC) and dn C_{max} are similar across all doses, indicating that the PK parameters of corifollitropin alfa are dose-proportional over this range. Median C_{max} of

Table II Pharmacokinetic properties of corifollitropin alfa in IVF patients

Dose (μg)	*n*	$t_{½}$ (h)	t_{max} (h)	C_{max} (ng/ml)	dn-C_{max} [(ng/ml)/μg]	$AUC_{0-\infty}$ (ng.h/ml)	dn-$AUC_{0-\infty}$ [(ng.h/ml)/μg]
60	75‡	65.7	41.9	1.90	0.0317	275	4.58
120	24†	64.1	24.6	4.26	0.0355	511.7	4.26
	75‡	65.3	41.2	3.71	0.0309	534	4.45
180	24†	65.6	24.8	6.61	0.0367	815.2	4.53
	76‡	66.0	44.1	5.53	0.0307	827	4.59
240	25†	64.9	24.7	8.85	0.0369	1080	4.50

Data taken from †Devroey *et al.* (2004) and ‡The Corifollitropin Alfa Dose-finding Study Group, (2008). All values are means, unless otherwise stated. $AUC_{0-\infty}$, median area under the serum corifollitropin alfa concentration curve; C_{max}, peak serum concentration at any time; dn, dose-normalized; $t_{½}$, elimination (terminal) half-life; t_{max}, time at which serum peak corifollitropin alfa concentration is reached.

Table III Efficacy of corifollitropin alfa for ovarian stimulation in two phase II IVF trials

	Devroey *et al.* (2004)			Corifollitropin alfa dose-finding Study Group (2008)		
	120 μg (*n* = 25)	180 μg (*n* = 24)	240 μg (*n* = 25)	60 μg (*n* = 77)	120 μg (*n* = 77)	180 μg (*n* = 79)
Median duration of additional stimulation from cycle Day 8 (days)	3	3	3	4	3	2
Median total dose rFSH From cycle Day 8 (IU)	450	450	450	600	450	300
No. of follicles on day of hCG						
>11 mm	12.7 ± 6.8	13.5 ± 7.1	15.5 ± 8.3	11.4 ± 5.3	13.5 ± 6.5	16.4 ± 7.2
>15 mm	5.9 ± 2.5	6.6 ± 3.1	7.8 ± 3.1	6.3 ± 2.9	8.1 ± 4.0	9.4 ± 4.4
>17 mm	3.3 ± 0.9	3.5 ± 1.2	4.0 ± 2.0	3.7 ± 1.3	4.2 ± 1.7	4.6 ± 2.1
No. of cumulus–oocyte complexes retrieved/started cycle	11.0 ± 7.1	11.1 ± 7.5	12.0 ± 7.3	5.2 ± 5.5	10.3 ± 6.3	12.5 ± 8.0
No. of metaphase II oocytes[a]	10.9 ± 6.9	8.5 ± 6.3	9.1 ± 5.5	7.7 ± 5.5	10.1 ± 6.0	11.6 ± 6.6
Fertilization rate (%)	73 ± 27	68 ± 31	67 ± 31	61 ± 27	65 ± 24	60 ± 23
No. of embryos obtained						
Total	8.5 ± 5.5	6.6 ± 4.9	7.3 ± 5.9	4.9 ± 3.3	7.1 ± 4.1	8.2 ± 6.5
Good-quality	4.8 ± 5.0	3.8 ± 3.3	3.9 ± 4.1	2.2 ± 2.0	3.5 ± 2.7	3.5 ± 3.4
Transferred	2.0 ± 0.2	2.0 ± 0.5	1.9 ± 0.5	1.3 ± 0.7	1.4 ± 0.7	1.3 ± 0.7
No. of ongoing pregnancies/started cycle	4/25	5/24	6/25	12/78	12/77	11/79

[a]Restricted to subjects with IVF or ICSI rFSH, recombinant follicle-stimulating hormone. All data represent mean ± SD, unless otherwise specified.

corifollitropin alfa is reached between 25 and 45 h after injection. No differences were observed between the PK in volunteers pituitary-suppressed by oral contraceptives (Duijkers *et al.*, 2002) and non-suppressed patients undergoing ovarian stimulation in a GnRH antagonist protocol. Elimination of corifollitropin alfa is not largely affected by body weight, but exposure is inversely correlated to body weight, exhibiting a linear relationship to both serum clearance and volume of distribution (The Corifollitropin Alfa Dose-finding Study Group, 2008).

In summary, the single-dose PK of corifollitropin alfa are characterized by a slow absorption resulting in peak levels within 2 days after injection. Thereafter, serum corifollitropin alfa levels decrease steadily, though the FSH activity may be retained above the FSH threshold for an entire week if the administered dose of corifollitropin alfa is sufficiently high.

Efficacy

The PK profile of corifollitropin alfa after a single injection implies the highest FSH activity during the first 2 days of stimulation, followed by decreasing FSH activity until treatment with daily FSH is started. As such, the profile mimics rather the high FSH starting dose and if needed FSH step-down as practiced in North America instead of the low starting dose and if needed FSH step-up as practiced in Europe (Macklon *et al.*, 2006). Injection of corifollitropin alfa in the early follicular phase of the menstrual cycle results in the ongoing stimulation of the recruited cohort of antral follicles. Therefore,

corifollitropin alfa is effective in the stimulation of multi-follicular growth for IVF, but seems far less suitable for the induction of mono-follicular growth, as documented in a first feasibility study in anovulatory women (Balen *et al*., 2004). In this small trial, in several cases corifollitropin alfa induced multi-follicular growth even though much lower dosages were tested. To date, there are insufficient data to support the application of this compound for ovulation induction in anovulatory patients or in intrauterine insemination patients.

In IVF, the first case report of a pregnancy and live birth following corifollitropin alfa administration was reported 5 years ago (Beckers *et al*., 2003) in a patient participating in the first phase II clinical trial (Devroey *et al*., 2004). In this feasibility trial (see Table III), dosages of 120, 180 and 240 μg corifollitropin alfa were tested in 25 patients per dose group, but no significant dose–response relationship was observed either in terms of the total dose of FSH required from Day 8 onwards or in the number of oocytes retrieved. This suggested that the lowest effective dose might be lower than those tested. Nevertheless, the study confirmed for the first time the hypothesis that a single injection of corifollitropin alfa could induce and sustain multi-follicular development for an entire week and thus could replace the first seven injections of daily FSH.

Accordingly, the most recent dose-finding study investigated lower dosages of corifollitropin alfa, with initiation of GnRH antagonist fixed at stimulation Day 5 (The Corifollitropin Alfa Dose-finding Study Group, 2008). A total of 325 patients were randomized to receive corifollitropin alfa 60, 120 or 180 μg, or daily fixed 150 IU rFSH (see Table III). A statistically significant increase in the number of oocytes retrieved over this dose range was observed. However, the high cancellation rate in the 60 μg dose group (44%) indicated that this dose was too low to support the first 7 days of ovarian stimulation. When comparing the number of follicles ≥11 mm on stimulation Day 8 or on the day of hCG administration, a similar dose-related increase was noted (Fig. 3). Rises of serum inhibin-B and E_2 levels were very similar between all treatment groups during the first 5 days of stimulation (Fig. 4). From Day 6 onwards, inhibin-B levels continued to increase in the 180 μg group and daily rFSH group, whereas inhibin-B reached a plateau from Day 6 to 8 in the 120 μg group and decreased in the 60 μg group. Median serum E_2 levels declined from Day 6 onwards in the 60 μg group, reached a plateau in the 120 μg group, and continued to increase in the 180 μg group. For patients who received hCG, serum E_2 levels were similar in the 60, 120 μg and rFSH groups, but ~1.5-fold higher in the 180 μg group (Fig. 4).

From these initial trials, it could be concluded that the optimum dose of corifollitropin alfa to sustain follicular development for one week was greater than 60 μg and lower than 180 μg. In order to select the final doses for phase III development, mathematical modelling was applied using historic PK and PD data from corifollitropin alfa, rFSH and GnRH antagonist (ganirelix) trials, which included more than 3000 subjects overall (De Greef *et al*., 2007). Using this approach, modelling revealed that, in the desired one week regimen, 150 μg is the most appropriate dose for achieving an optimal treatment outcome in terms of the number of oocytes or fertilized two-pronucleate oocytes for patients with a body weight >60 kg. However, because of the inverse relationship between body weight and exposure, in women weighing ≤60 kg a lower dose of 100 μg was shown to result in a relevant decrease in exposure without compromising the follicular support during the first week of stimulation. A phase III programme using these single doses of corifollitropin alfa (100 and 150 μg) is ongoing, including follow-up studies of frozen-thawed embryos, pregnant women and their offspring.

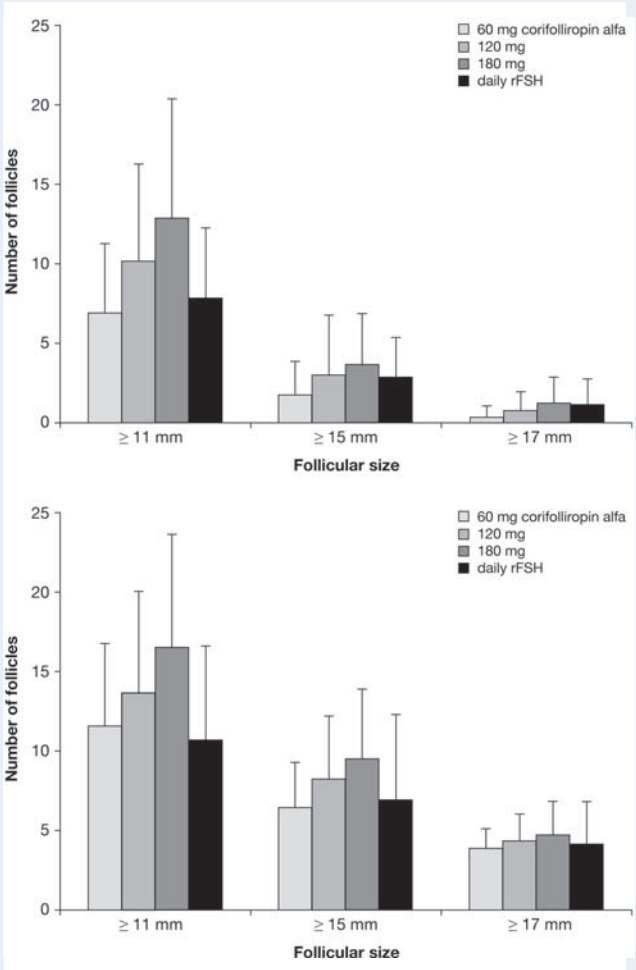

Figure 3 The number of follicles at least 11 mm measured on Day 8 of ovarian stimulation (upper panel) and on the day of hCG administration (lower panel) in all subjects who received hCG for triggering final ooycte maturation (The Corifollitropin Alfa Dose-finding Study Group, 2008).

Safety

Because of the extended action of long-acting FSH-like preparations, in theory safety could be of concern. Chances for OHSS could be augmented due to extended stimulation of the development of multiple follicles. Since the initial corifollitropin alfa dose has been given, it is not possible—as in daily injections—to reduce the dose in case signs of ovarian stimulation are observed during the mid-follicular phase. One week after the initial injection, daily FSH doses can be either reduced or withheld.

Corifollitropin alfa has proved to be well tolerated in all the studies conducted to date, with a safety profile comparable to that of daily rFSH. No antibody formation has been observed, even after repeated dosing (Bouloux *et al*., 2001). In addition,

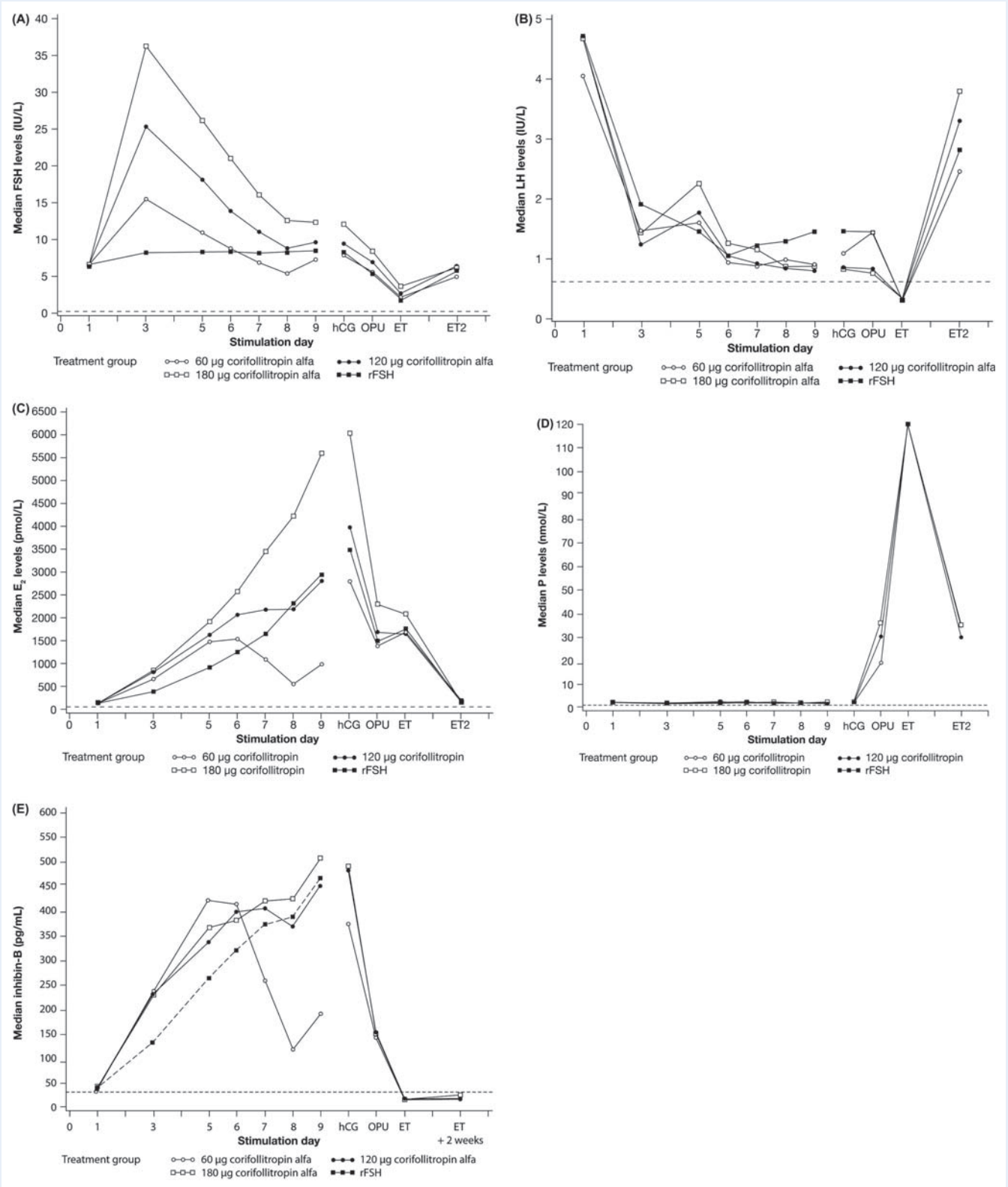

Figure 4 Median serum hormone concentrations measured during stimulation and during the luteal phase in subjects who received hCG.

(**A**) FSH; (**B**) LH; (**C**) estradiol (E_2); (**D**) progesterone (P); (**E**) inhibin-B (The Corifollitropin Alfa Dose-finding Study Group,2008). OPU, oocyte pick-up;ET, embryo transfer, ET2, two weeks after embryo transfer.

measurement of local tolerance demonstrated that s.c. administration of corifollitropin alfa is well tolerated, as no moderate or severe local reactions occurred. Further, no increase in intensity of injection-site responses was observed after repeated exposure (Bouloux *et al*., 2001). The most commonly reported adverse events across all trials to date were headache and nausea, followed by abortion and pelvic pain. Signs and symptoms of OHSS were reported in 15 out of 307 patients treated for IVF or ICSI with 60–240 μg corifollitropin alfa, and 6 of these patients (2%) required hospitalization. This was similar to the incidence of OHSS reported in the rFSH group—four cases in 106 subjects, three of them (3%) requiring hospitalization (Devroey *et al*., 2004; The Corifollitropin Alfa Dose-finding Study Group, 2008).

Benefit-risk profile

One single s.c. injection of corifollitropin alfa is able to initiate and sustain multiple follicular growth for an entire week. Accordingly, the compound has been developed for the induction of multiple follicles in IVF patients. Corifollitropin alfa tested at low dosages has been shown to be less eligible for classical ovulation induction, since its PK profile with relatively high exposure during the first days after administration may not favour mono-follicular development.

In ovarian stimulation prior to assisted reproduction treatment, a single injection of corifollitropin alfa replaces the first seven injections of daily rFSH. As such, corifollitropin alfa may reduce the treatment burden during the first week of stimulation. Corifollitropin alfa may be injected at home by self-administration or at the IVF unit by the medical staff. The reduced frequency of dosing brings an obvious benefit to those that fear needles and also brings an anticipated increase in patient convenience, fewer chances for mistakes during treatment and improved compliance. From stimulation Day 8 onwards, patients may continue treatment with a fixed daily dose of rFSH, depending on their ovarian response. Patients who reach the criteria of triggering final oocyte maturation prior to Day 8 of stimulation do not need any daily FSH to be administered.

The benefits of the corifollitropin alfa regimen should be weighed against the potential risks, which will be finally determined in phase III trials. Corifollitropin alfa is being developed in two dosages, i.e. 100 and 150 μg which, based on simulations, will provide the same exposure and the same degree of ovarian stimulation in the recommended body weight groups. Overall, this may be slightly higher compared with daily rFSH. Although the ovarian response induced by corifollitropin alfa may decrease with the patient's age and ovarian reserve, the dose of corifollitropin alfa cannot be reduced to obtain milder stimulation as the indicated doses are required to cover the one week treatment interval. After corifollitropin alfa injection, serum FSH activity declines from stimulation Day 3 (C_{max}) onwards. Dose reductions during the first week of stimulation cannot be made in case of hyper-response. Therefore, corifollitropin alfa may be less suitable for patients with known risk factors for a hyper-response, such as patients with a history of hyper-response to medication, OHSS or patients with PCOS. Finally, for eligible patients, it remains to be confirmed in a large controlled phase III trial that the pregnancy rate/live-birth rate of this new treatment regimen is comparable to that of daily rFSH/GnRH antagonist protocols.

Future directions

Clinical research with gonadotrophin molecules that have been modified in their PK or PD properties from native or wild-type recombinant gonadotrophins may give further insight into factors which may affect ovarian response. The first compound which has been tested clinically is corifollitropin alfa. This compound introduces a new treatment regimen using a single injection in the early follicular phase followed by daily rFSH injections after 1 week of stimulation. A phase III trial programme is underway to study whether the relatively high exposure to FSH activity during the first days of stimulation affects follicular recruitment, steroid production, follicular and/or oocyte gene expression or endometrial receptivity.

The first phase III trials using corifollitropin alfa in combination with a fixed daily GnRH antagonist co-treatment protocol have recently been completed. This regimen will further simplify treatment and may reduce the treatment burden of IVF for patients. Future trials concerning corifollitropin alfa will need to compare clinical outcomes using GnRH antagonist co-treatment with those achieved using long GnRH agonist protocols.

Consistent with other therapeutic areas, novel drug development in the infertility field is likely to concentrate on less invasive delivery methods, such as the use of long-acting compounds or different routes of administration that may include transdermal, inhaled or oral agents. On the horizon is the development of orally active, low-molecular weight gonadotrophins, for which a first proof-of-concept study has been reported in female volunteers (Mannaerts, 2005).

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2.3 Discussion

The preclinically-defined characteristics of follitropin-β and corifollitropin alfa were helpful in designing phase I and II trials with respect to the anticipated effective dose and treatment interval. Preclinical experiments of follitropin-β indicated that the *in vitro* and *in vivo* bioactivity were indistinguishable from FSH isolated from natural sources. *In vitro* and *in vivo* dose-response curves were very comparable and parallel to those induced by pituitary and urinary FSH preparations and standards with different levels of purity. Also the neutralization of the *in vitro* bioactivity by different monoclonal antibodies recognizing different epitopes was very comparable between follitropin-β and natural FSH preparations, and testing of follitropin-β in the *in vitro* Leydig cell bioassay demonstrated the virtual absence of LH activity. Based on these pharmacology data, no efficacy differences were anticipated between follitropin-β and urinary FSH and no dose-finding studies in either phase I or II trials were indicated. The therapeutic dose was calibrated in International Units (IU) by the Steelman and Pohley bioassay and the main difference for patients was its high purity resulting in high specific activity (> 10,000 IU/mg protein) as determined by immunoassay and *in vivo* bioassay. This purity allowed the route of administration to be changed from intramuscular (IM) to subcutaneous (SC), which is suitable for self-administration.

Thanks to the development of rFSH, the mechanism of action of FSH was further elucidated, initially in animal models. Experiments in immature hypophysectomized rats demonstrated, in the complete absence of LH, that follitropin-β induced normal follicular growth up to pre-ovulatory stage, whereas circulating estradiol remained low at baseline. Ovarian weight, ovarian aromatase, and the number of antral follicles increased in a dose-dependent manner and prevented antral follicles from becoming atretic. The latter effect was most apparent in the smallest follicle size class which showed a 50% reduction in the incidence of atresia. This indicates that follitropin-β induces multiple follicular development by preventing small antral follicles from becoming atretic.

Animal pharmacology of follitropin-β also provided stronger evidence in favor of the two-cell two gonadotropin theory, at least in confirming that LH activity is required to increase estradiol secretion up to measurable plasma levels. Although intra-ovarian estradiol increased in a dose-dependent manner, these levels were relatively low and clearly suppressed due to the lack of LH activity. The highest dose of follitropin-β led to concentrations of estradiol comparable to those measurable in ovaries of intact immature animals whereas the number of antral follicles was twice as high and mainly of large size classes.

Supplementation of follitropin-β with very low doses of hCG increased serum estradiol in a dose-dependent manner, whereas treatment with hCG alone did not increase the estradiol from baseline. When follitropin-β was given in doses providing a submaximal response, low doses of hCG increased ovarian aromatase, ovarian weight and uterine weight. With low doses of hCG, more small antral follicles shifted to large antral follicles, whereas higher doses of hCG reversed that effect. Follitropin-β induced the growth of follicles to preovulatory stages and subsequent administration of hCG resulted in ovulation of healthy follicles only, since large atretic follicles remained in the ovary after induction of ovulation. Interestingly, follitropin-β not only increased ovarian aromatase and ovarian weight, but also uterine weight in a dose-dependent manner, whereas ovarian estradiol remained relatively low and circulating estradiol remained unchanged from baseline. These experiments provided more definitive evidence in favor of the two-cell two gonadotropin theory [Hodgen 1989] confirming that FSH alone can induce (multi)follicular growth and that LH activity is required to increase estradiol secretion up to measurable circulating levels.

In contrast to follitropin-β, the preclinical characteristics of corifollitropin alfa [Verbost et al 2011] were less easy to translate into an anticipated potency in humans. The ability of corifollitropin alfa to activate the FSH receptor upon binding was tested using a cAMP-based luciferase luminescence reporter assay. A slightly (1.8-fold) lower potency to induce luciferase activity was observed with corifollitropin alfa compared with follitropin-β. On the other hand, corifollitropin alfa demonstrated a 2- to 4-fold increase of bioactivity across all *in vivo* parameters assessed. Ovarian weights rose more sharply with corifollitropin alfa than with follitropin-β and the half maximal effective concentration (EC50) of corifollitropin alfa for this parameter (ovarian weight) was 2.1-fold lower than that of follitropin-β. This difference between the *in vitro* and *in vivo* bioactivity is explained by the difference in the circulating half-life of corifollitropin alfa in rats and dogs which was calculated to be 1.5- to 2-fold longer than for follitropin-β. Thus, when corifollitropin alfa entered first-in-human clinical studies, the relationship between a single dose of corifollitropin alfa and the duration/magnitude of ovarian response was uncertain and thus human PK/PD and dose-finding studies were indicated.

Chapter 3

Phase I First-in-Human Trials

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3.1 Introduction

Phase I studies in volunteers are usually first-in-human studies performed to assess the safety, tolerance and exposure of a new pharmaceutical compound in subjects following single and multiple rising doses. Pharmacokinetic properties are calculated based on exposure and a first insight into the pharmacodynamic properties may be obtained. Phase I studies of drugs in volunteers are also performed during phase II or III studies, for instance to perform additional, more specific safety studies, or studies to assess the pharmacokinetics or absolute bioavailability of previous selected doses [Mannaerts et al 1998].

Study population and safety assessments

During first-in-human studies, single and multiple rising doses of the investigational drug are usually administered to healthy male or female volunteers, but depending on the preclinical data, the objectives of the study and the potential clinical indications, the preferred study population should be carefully considered. Previous clinical research of new gonadotropin preparations or devices in volunteers included healthy male or female volunteers with or without pituitary-suppression by contraceptives or GnRH analogue treatment, sterilized female volunteers, healthy postmenopausal volunteers, and male or female volunteers with gonadotropin deficiency or hypogonadotropic hypogonadism.

A general concern of recombinant glycoproteins is their potential immunogenicity. Such a concern may be reduced if the peptide backbone of the natural and the recombinant molecule are known to be identical, but may increase if the amino acid sequence deviates or fusion molecules are tested. Therapeutic proteins produced by recombinant DNA technology may also have an increased potential to induce immune responses when the protein is administered in multiple doses over an extended period of time. The same applies to potential host-cell originating contaminations accompanying these proteins, like CHO-proteins. Through binding with a particular epitope of a therapeutic protein, generated antibodies may directly interact with the receptor binding site, or influence the molecule by altering its tertiary structure. This may lead to altered clearance, and/or loss of receptor site recognition or signal transduction, and thus to neutralization of the pharmacological effect [Schellekens, 2002].

It is very difficult to predict the immunogenicity of a new therapeutic protein like follitropin-β or corifollitropin alfa by preclinical models as the presence or absence of antibodies in animal species is not predictive for humans. Although the structure of follitropin-β was shown to be very similar to natural human FSH, it was thought, prior to first-in-human studies, that treatment of normogonadotropic volunteers with follitropin-β could be accompanied with a

potential risk of developing auto-immunity resulting in untreatable infertility. Therefore only gonadotropin deficient male and female volunteers were included in first-in-human studies including a single dose of 300 IU [Mannaerts et al 1993] and multiple rising doses of 75 to 225 IU follitropin-β [Schoot et al 1992 and 1994:Mannaerts et al 1996a]. First-in-human studies of corifollitropin alfa were initially performed with male gonadotropin deficient volunteers since the magnitude of its pharmacological effect in women was still unknown and the potential immunogenicity of this fusion molecule was to be examined first [Bouloux et al 2001]. The study was designed as a repeated exposure study with a low dose of 15 μg corifollitropin alfa and a wash-out interval of 4 weeks. Only after this study, a single rising dose study was carried out in pituitary-suppressed female volunteers who received a single dose between 15 and 120 μg corifollitropin alfa [Duijkers et al 2002].

In contrast to follitropin-β and corifollitropin alfa, the risk of a small decapeptide like ganirelix being immunogenic in humans was considered low, although not excluded. Safety concerns were mainly focused on the potential immediate hypersensitivity or anaphylactic reactions of these compounds which may bind to the GnRH receptor of cutaneous mast cells causing histamine release. During phase I studies of ganirelix performed by Syntex, more than 100 healthy male and female volunteers were treated SC with ganirelix up to 28 days and in doses up to 12 mg. No antibody formation against ganirelix was detected in any of these volunteers.

Exposure and Pharmacokinetics

Calculation of drug pharmacokinetics starts with a reliable assessment of drug exposure from first drug administration up to complete clearance. Assessment of the pharmacokinetic properties requires consideration of the frequency and timing of blood sample collection which should always include predose samples.

As endogenous FSH and follitropin-β cannot be distinguished, exposure following single or multiple doses of follitropin-β should be estimated without interference by endogenous FSH, thus pharmacokinetic studies in subjects with profound pituitary suppression or with hypogonadotropic hypogonadism are ideal [Mannaerts et al 1996b]. If both male and female volunteers may participate, and different routes of administration may be applied, potential gender differences and the bioavailability should be assessed at an early phase of clinical research. Exposure may be expressed as immunoactivity or *in vitro* bioactivity, and ratios of both units may reflect the changing biopotency of circulating FSH isoforms over time [Matikainen et al 1994].

Estimation of corifollitropin alfa exposure is not hampered by interference by endogenous FSH, if a specific (radio)immunoassay is applied which does not crossreact with endogenous

FSH or LH. In the absence of endogenous gonadotropins, exposure can also be estimated with a FSH immunoassay which crossreacts with corifollitropin alfa.

Additional pharmacokinetic studies with the selected dose of 0.25 mg ganirelix were required to assess single dose and multiple dose pharmacokinetics but did not require any specific limitation on the volunteers to be included [Oberyé et al 1999a; Oberyé et al 1999b].

Biomarkers and Pharmacodynamics

Phase I studies are the earliest trials that may provide insight into the association between drug exposure and the pharmacodynamic effects. The response size effect depends on the dose or doses administered, the elimination half-life, and the responsiveness of the volunteer. Thus baseline hormones should be collected just prior to treatment in order to estimate the size and specificity of the treatment effect. Hormonal biomarkers and time points for their assessment are carefully considered at the design of the study. The most specific and sensitive biomarker to monitor the bioactivity of either follitropin-β and corifollitropin alfa is known to be inhibin-B, which is LH-independent and induced by FSH in Sertoli cells and granulosa cells of men and women, respectively. In women, rises of serum estradiol also reflect mono- or multifollicular development but absolute levels are known to be LH-dependent and are usually impaired in pituitary-suppressed subjects. [Mannaerts et al 1993; Schoot et al 1994; Mannaerts et al 1996a].

Biomarkers of GnRH analogues are primarily endogenous FSH and LH levels. Whereas a single injection of a GnRH agonist causes a flare-up of these hormones, a single injection of a GnRH antagonist causes temporary FSH and LH suppression. In contrast to the final suppressive effect of GnRH agonists, the magnitude and duration of the suppressive effect of GnRH antagonists are dose-dependent. The pharmacodynamic effect of drugs intended for daily administration can be best assessed after the steady state has been established [Oberyé et al 1999b].

3.2 Results

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Single-dose pharmacokinetics and pharmacodynamics of recombinant human follicle-stimulating hormone (Org 32489*) in gonadotropin-deficient volunteers†‡

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Objective: To assess safety, pharmacokinetic, and pharmacodynamic properties of recombinant human follicle-stimulating hormone (FSH; Org 32489, Organon International, Oss, the Netherlands) after a single intramuscular injection in the buttock.

Design: In a prospective study, safety variables, serum FSH, luteinizing hormone, inhibin, estradiol (females only), and testosterone (males only) were evaluated up to a maximum of 11 days after injection of 300 IU recombinant FSH.

Setting: Four specialist Reproductive Endocrinology and Infertility units.

Volunteers: Fifteen men and women exhibiting all pituitary gonadotropin deficiency.

Result(s): A single bolus of 300 IU recombinant FSH was well tolerated, and no drug-related adverse effects were noted. Comparison of before and after treatment safety variables, including serum antirecombinant FSH antibodies, showed no changes of clinical relevance. Analysis of serum FSH levels revealed comparable elimination half-lives of 44 ± 14 (mean ± SD) and 32 ± 12 hours in women and men volunteers, respectively. In contrast, peak FSH concentrations were significantly lower in women than in men volunteers (4.3 ± 1.7 versus 7.4 ± 2.8 IU/L), and the time required to reach peak levels of FSH was significantly longer in women than in men (27 ± 5 versus 14 ± 8 hours). The area under the serum level versus time curve tended to be smaller in women than in men volunteers (339 ± 105 versus 452 ± 183 IU/L × hours), but the difference did not reach statistical significance. Together these data suggest that recombinant FSH is absorbed from its intramuscular depot to a lower rate and extent in women than in men. In both sexes a relationship between serum FSH levels and body weight was apparent. During the experimental period, other hormones remained low at baseline levels or were only slightly increased.

Conclusion(s): Our findings indicate that recombinant FSH is well tolerated and that it is absorbed from its intramuscular depot to a higher rate and extent in men than in women. After intramuscular administration, its half-life is in good agreement with that previously reported for natural FSH. (Fertil Steril® 1993;59:108–14. ©1993 by American Society for Reproductive Medicine.)

Key Words: Recombinant human FSH, single-dose pharmacokinetics

Human follicle-stimulating hormone (FSH) is a gonadotropic hormone produced by the anterior pituitary gland, whose primary function is regulation of follicular growth in females and of spermatogenesis in males. Hormonal response is accomplished via specific membrane receptors on granulosa cells and Sertoli cells, causing adenylate cyclase activation and thereby secretion and/or synthesis of various factors essential for target cell differentiation and gamete maturation (1, 2).

The FSH molecule has a dimeric structure of which both subunits are glycoproteins in nature. The 92-amino acid α-chain and the 111-amino acid β-chain have each two N-linked oligosaccharide chains presented as complex heterogeneous multiantennary structures (3). The variable degree of glycosylation, especially of sialylation, creates a spectrum of FSH isoforms with differences in charge, bioactivities, and elimination half-lives (4, 5).

The expression of human FSH in Chinese hamster ovary cells transfected with both subunit genes (6, 7) resulted in the synthesis of intact human FSH (recombinant FSH). The polypeptide backbone of recombinant FSH is identical to that of natural FSH, whereas recombinant and natural carbohydrate structures are closely related (8). The charge heterogeneity and bioactivity of recombinant FSH were confirmed by chromatofocusing, receptor displacement, and by in vitro and in vivo animal studies (9–11). In comparison with commercially available urinary gonadotropin preparations, highly pure (99.9%) recombinant FSH appeared to lack intrinsic luteinizing hormone (LH) activity and to exhibit a very high specific bioactivity (>10,000 IU/mg protein). These properties prompted the further development of recombinant FSH by means of clinical studies examining its pharmacokinetic and antigenic properties in humans. This first human exposure study was performed in gonadotropin-deficient male and female volunteers to assess the safety and tolerance and the pharmacokinetic and pharmacodynamic properties of recombinant FSH after a single intramuscular injection.

MATERIALS AND METHODS

Subjects and Study Design

Fifteen gonadotropin-deficient, but otherwise healthy, volunteers (8 women and 7 men) participated in this four-center study. The study protocol was approved by the local ethics review committees, and written informed consent was obtained from all volunteers. Nine subjects had panhypopituitarism, either primary (n = 3) or secondary (n = 6), due to surgical removal of a nonmalignant pituitary tumor. Five volunteers suffered from congenital isolated gonadotropin deficiency, and one volunteer was diagnosed as weight-loss–related hypothalamic hypogonadism. Autoimmunity was excluded by antinuclear and specific antirecombinant FSH antibody assays. Subjects receiving estrogen/androgen replacement refrained from this therapy, which started 1 week (oral therapy) or 3 weeks (intramuscular substitution) before injection up to 1 week after injection while appropriate thyroid and glucocorticoid therapy was continued. With the exception of one male volunteer, all subjects had a history of proven normal gonadal function; seven out of eight women had one or more deliveries, and one woman and six men subjects responded well to previous hormonal therapy.

Subjects received a single intramuscular injection of 300 IU recombinant FSH (Org 32489, CP 90073; Organon International, Oss, the Netherlands) in 2 mL solvent in the upper quadrant of the buttock. Blood samples were taken just before injection and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 16 (optional), 24, 48, 72, 96, 120 (optional), 168, 216 (optional), and 264 hours (11 days) after injection. Blood samples were centrifuged, and serum was stored in 0.5-mL serovials at −20°C until analysis. Serum was assayed for immunoactive FSH, immunoactive and bioactive LH, testosterone (T), estradiol (E_2), and inhibin.

Safety Parameters

Safety analysis included clinical observations, i.e., blood pressure, heart rate, and body temperature, as well as laboratory assessments like routine urinalysis (pH, protein, acetone, glucose, hemoglobin), blood biochemistry (sodium, potassium, chloride, bicarbonate, phosphorus, calcium, glucose, urea, creatinin, uric acid, alkaline phosphatase, alanine and aspartate aminotransferase, lactic dehydrogenase, bilirubin, protein, albumin), and hematology (hemoglobin, hematocrit, erythrocytes, differentiated leucocytes, thrombocytes).

Serum samples were analyzed for the presence of antirecombinant FSH antibodies using a sensitive radioimmunoprecipitation assay and ^{125}I-recombinant FSH as a tracer. When testing a mixture of two mouse monoclonal antibodies (MCAs) raised against recombinant FSH and recognizing an α- and β-specific epitopes, the sensitivity of the assay was 0.5 pmol/L and the intra-assay and interassay coefficients of variation (CV) ranged from 4.3% to 9.6% and from 0.8% to 2.7%, respectively. The induction of antirecombinant FSH antibodies after recombinant FSH treatment was judged by comparing before and after treatment samples according to criteria, allowing a probability of a false-positive result of <0.1%. All serum samples were tested in duplicate, and the MCA mixture was used as a positive control in all experiments.

Hormone Assays

Immunoreactive FSH and LH was measured by an immunofluorometric assay using the time-resolved fluoroimmunoassay technique and reagent kits 1244-017 for human FSH and 1244-31 for human LH (Delfia, Pharmacia, Woerden, the Netherlands). These two-site assays use a β-directed capturing MCA and an α-directed europium-labeled detection MCA. The assays were performed as described by the manufacturer using the Delfia instrumentation system and MultiCalc software (Pharmacia). Follicle-stimulating hormone and LH immunoreactivity was expressed in terms of the 2nd International Reference Preparation (IRP) of pituitary FSH (code no. 78/549) and the 2nd International Standard for pituitary LH (code no. 80/552). The sensitivity of immunofluorometric assay was 0.05 IU/L for both gonadotropins, and the intra-assay and interassay CV were below 4.8% and 4.3% for FSH and 4.7% and 7.5% for LH, respectively. The cross-reactivity of the FSH kit with LH was <0.08% and of the LH kit with FSH <0.01%.

Serum bioactive LH was measured in an in vitro mouse Leydig cell bioassay as described in detail previously (10). The sensitivity of this assay for serum samples using 2nd International Standard 80/552 as the standard was 2 IU/L.

Serum T and E_2 were assessed by radioimmunoassay (RIA) using a coat-a-count T RIA (reagent kit TKTT1 DPC, detection limit 0.27 nmol/L; Diagnostic Products Corpora-

TABLE 1

Clinical characteristics of volunteers.*

	Age	Weight	Height	BMI	FSH	LH
	y	*kg*	*cm*	*kg/m²*	*IU/L*	*IU/L*
Females (n = 8)	36 ± 3	67 ± 13	162 ± 12	26.1 ± 4.3†	0.54 ± 0.34	<0.17
Males (n = 7)	31 ± 7	62 ± 11	171 ± 13	21.3 ± 1.7	0.63 ± 0.57	<0.36

* Values are means ± SD.

† Significantly higher in females than in males

tion, Los Angeles, CA) and a double antibody E_2 RIA (reagent kit KE2D1 DPC, detection limit 11.6 pmol/L; Diagnostic Products Corporation). The intra-assay and interassay CV were <9% and 13% for the T assay and <4% and 5% for the E_2 assay, respectively.

Serum inhibin levels were measured by RIA using an antiserum (no. 1989) raised against purified bovine 31-kd inhibin (12). Purified bovine 31-kd inhibin iodinated by the lactoperoxidase method was used as a tracer. The standard was a pool of human follicular fluid (FF; 280 U/mL) that was calibrated against a rete testis standard preparation of defined bioactivity. The immunoactivity of 1 mU FF was equipotent of 0.121 pg recombinant human inhibin (Biotech Australia, specific in vitro bioactivity 51,060 U/μg protein using World Health Organization [WHO] standard 86/690 as the standard). The recombinant α-subunit of human inhibin exhibited complete cross-reactivity in this assay system. The standard pool, which was diluted in plasma from castrated subjects, provided dose responses parallel to the plasma dilution curves. The sensitivity of the assay was 28 U/L and the intra-assay and interassay CV were <10%.

Data Analysis

The peak recombinant FSH concentration (C_{max}) and the time of its occurrence (T_{max}) were taken from measured serum level data. The area under the serum level versus time curve (AUC) after a single dose of 300 IU was determined by means of the trapezoidal rule from zero time up to infinity ($AUC_{0-\infty}$) under subtraction of the baseline. The elimination half-life ($t_{½}$) was calculated after baseline correction on the basis of increases of FSH concentrations measured between 72 and 264 hours after injection, using log-linear regression. Data are presented as mean ± SD unless stated otherwise. Curvefit coefficients (r) represent Pearson correlation coefficients. Comparison of age, height, weight, and body mass index (BMI) between male and female volunteers was performed by means of the Wilcoxon's test. Gender differences of bioequivalence were tested in a one-way analysis of variance (ANOVA). Differences were considered to be statistically significant if $P \leq 0.05$.

RESULTS

Volunteers

The mean age, weight, height, BMI, and serum gonadotropin levels at screening are listed in Table 1. No significant differences between male and female volunteers with respect to age, height, or weight were found, whereas BMI values were higher ($P = 0.04$) for the female volunteers. During screening, serum FSH levels ranged between 0.12 and 1.08 IU/L in males and between 0.11 and 1.63 IU/L in females. Serum LH levels were either undetectable (<0.05 IU/L) or very low, resulting in individual levels between <0.05 and 0.40 IU/L in females and between <0.05 and 1.13 IU/L in males.

Safety and Tolerance

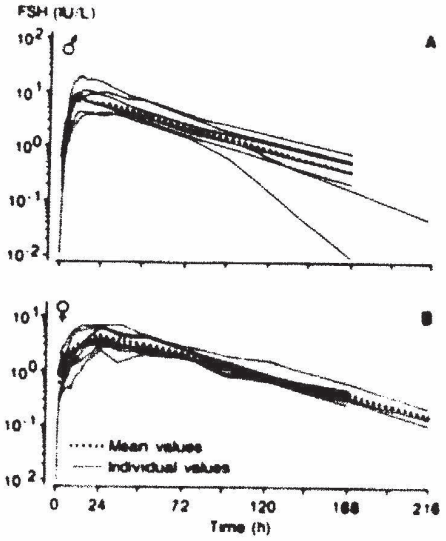
A single injection of 300 IU recombinant FSH was well tolerated, and no drug-related adverse experiences were noted. Neither pain nor skin redness was observed at the site of drug injection. Comparison of before and after treatment safety variables, i.e., blood pressure, heart rate, body temperature, blood biochemistry, hematology, and urinalysis, revealed no changes of clinical significance. When screening volunteers for the possible induction of antirecombinant FSH antibodies, no post-treatment increases in ^{125}I-FSH binding were observed.

Pharmacokinetic Analysis

Individual and mean delta increases of serum FSH after intramuscular injection of 300 IU recombinant FSH in seven male and eight female volunteers are shown in Figure 1. In all subjects, serum FSH levels were raised at 30 minutes after injection, and 13 out of 15 volunteers returned to baseline values 264 hours after injection. Individual pharmacokinetic parameters, AUC, C_{max}, T_{max}, and $t_{½}$, are presented in Table 2. Within the group of male volunteers, one statistical outlier was identified by means of the Dixon test. This subject (M2), with an extremely low body weight (42 kg) and extremely high AUC value (876 IU/L × hours), was not included in mean values and was excluded from further statistical analysis. The mean $t_{½}$ of recombinant FSH was not significantly different between sexes (44 ± 14 versus 32 ± 12 hours). In contrast, C_{max} values were significantly

FIGURE 1

Individual and mean delta increases of serum immunoreactive FSH after intramuscular injection of 300 IU recombinant FSH in seven men (A) and eight women (B) with hypogonadotropic hypogonadism.



(P = 0.0072) lower in female than in male volunteers (4.3 ± 1.7 versus 7.4 ± 2.8 IU/L) and T_{max} was also significantly (P = 0.0004) longer in females than in males (27 ± 5 versus 14 ± 8 hours). The mean AUC after administration of 300 IU recombinant FSH was 339 ± 105 IU/L × hours in females and 452 ± 183 IU/L × hours in males and thus tended to be lower in females, although the difference was not statistically significant (P = 0.058).

Relationship Between Body Weight and Serum FSH Levels

Comparison of body weight and serum FSH levels revealed a negative relationship in both men and women. Although the number of subjects studied are limited, the data suggest that there is a linear relationship between body weight and C_{max} values (males r = 0.85; females r = 0.83) and between body weight and AUC values (males r = 0.89; females r = 0.86). Scatter plots illustrating these associations are presented in Figure 2. The apparent linear relationships between BMI and C_{max} values (males r = 0.48; females r = 0.61) and between BMI and AUC values (males r = 0.51; females r = 0.65) were less strong (data not shown).

Other Hormones

Serum LH levels were assessed during screening (see Table 1), just before injection, and at 1 and 3 days thereafter. Mean immunoreactive before and after treatment LH levels were below 0.4 IU/L for all subjects. Serum samples of five female and five male volunteers were also tested in the in vitro LH bioassay, but serum bioactive LH was below the detection limit of the assay (<2 IU/L) in all cases.

In female volunteers, E_2 was detectable (>9.9 pmol/L) in only three women; 2 days after injection, two women showed a slight increase in E_2 (24 and 33 pmol/L, respectively). Serum inhibin was either undetectable (<30 U/L) or very low in female volunteers, whereas six out of seven males had detectable levels of inhibin. In comparison with baseline values, the mean inhibin of these six men was doubled (238 ± 91 versus 124 ± 66 U/L) 3 days after recombinant FSH injection. Serum T was either undetectable (<0.27 nmol/L) in two men or low (<10 nmol/L) in others, and no changes of any significance were noted (data not shown).

DISCUSSION

For nearly 30 years, infertility treatment with gonadotropins has been based on the application of crude urinary gonadotropin preparations, which have been proven safe and effective. The future of gonadotropin therapy, however, is likely to lie with highly pure recombinant human FSH preparations, devoid of other gonadotropins or inactive contaminants. Furthermore, FSH production by means of recombinant DNA technology is thought to guarantee an improved batch to batch consistency.

TABLE 2

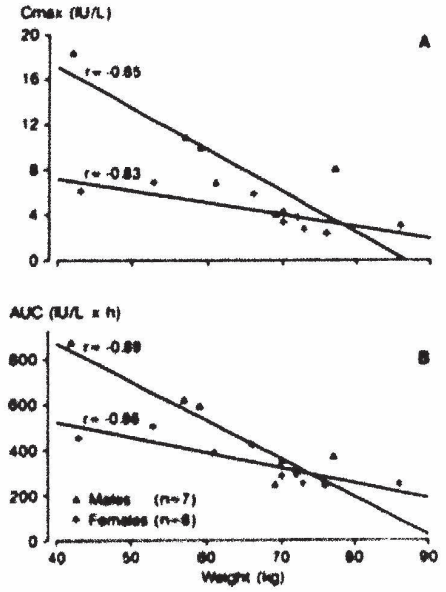
Individual and mean pharmacokinetic parameters of recombinant FSH after one single intramuscular injection in the gluteal area in females (F) and males (M).

	AUC	C_{max}	T_{max}	$t_{½}$
	IU/L × hours	*IU/L/h*	*h*	*h*
F1	506	6.9	36	43
F2	255	2.8	35	35
F3	244	2.4	24	69
F4	252	3.1	24	40
F5	287	3.4	24	63
F6	292	3.8	24	33
F7	454	6.1	24	37
F8	425	5.9	24	31
Mean ± SD	399 ± 105	4.3 ± 1.7*	27 ± 5†	44 ± 14
M1	744	10.0	24	42
M2‡	876	18.4	10	40
M3	367	8.1	9	34
M4	599	10.9	10	44
M5	355	4.4	9	38
M6	250	4.1	24	12
M7	393	6.9	10	25
Mean ± SD	452 ± 183	7.4 ± 2.8	14 ± 8	32 ± 12

* Significantly lower in females than in males.
† Significantly longer in females than in males.
‡ Statistical outlier not included in mean values.

FIGURE 2

Correlation of C_{max} (A) and AUC (B) with body weight in seven men and eight women with hypogonadotropic hypogonadism.



A general concern of recombinant glycoproteins is their potential immunogenicity. Because the peptide backbone structures of the natural and recombinant FSH were known to be identical, this concern was limited to possible minor differences in tertiary structure due to host cell processing. This first clinical study with recombinant FSH was performed in gonadotropin-deficient volunteers to minimize possible hazards in case of an antirecombinant FSH immune response and to prevent interference with endogenous gonadotropins. To date, many patients have been treated successfully with other recombinant glycoproteins, like erythropoietin, without developing specific antibodies (13). In the present study, no serum antirecombinant FSH antibodies were detected, but further studies will be required to demonstrate the safety of recombinant FSH during repeated administrations and long-term infertility therapy.

After intramuscular injection, the release rate of gonadotropins may depend on the formulation, injection depth, site, and volume (14). After intramuscular injection in the buttock, the absorption of immunoreactive recombinant FSH was very slow and even significantly slower in women than in men. Analysis of serum samples taken up to 72 hours after injection revealed that immunoreactive FSH levels were in good agreement with circulating bioactive FSH measured by an in vitro granulosa cell bioassay (Huhtaniemi I, personal communication).

Pharmacokinetic studies with urinary FSH and human menopausal gonadotropin (hMG) preparations administered via the intramuscular route have been limited but demonstrated previously that serum FSH levels depend on both the absorption and excretion rate of the drug. Diczfalusy and Harlin (15) reported that $t_{½}$ of hMG after intramuscular administration (>40 hours) is about four times longer than after intravenous injection. Daily injection of 150 or 225 IU urinary FSH in three women with isolated gonadotropin deficiency revealed a mean $t_{½}$ of 36 ± 16 hours, whereas $t_{½}$ varied between 33 and 59 hours in normal men after single intramuscular administration (16). The $t_{½}$ of recombinant FSH in the present study seems to be in good agreement with those reported for urinary FSH/hMG, although this is the first report on different release rates of FSH in men and women. Pharmacokinetic parameters like T_{max} and C_{max} are defined by the release of the drug from the intramuscular depot and by its $t_{½}$. However, the latter was not significantly different between the sexes; seeming differences may be attributed to the large intersubject variability. Sex differences in drug absorption and bioavailability after injection of aqueous solutions in the gluteus maximus, rather than in the vastus lateralis or deltoid, have been described previously and are thought to be related to the gluteal fat thickness, which is known to be greater in women than in men (14). Consequently, women may receive part of the drug in their subcutaneous adipose layer rather than intramuscular, leading to a less rapid absorption. Whether the latter also results in a lower absolute bioavailability, as indicated by the relative small mean AUC of the female volunteers, remains to be assessed.

Various studies support the hypothesis that body weight is a major determining factor on the dose and length of gonadotropin stimulation for induction of ovulation and for in vitro fertilization (17, 18). Follicle-stimulating hormone doses to initiate ovarian response may vary largely between individuals (19), and also thereafter major differences in ovarian response require close treatment monitoring. The present study revealed a strong negative correlation between body weight and serum FSH levels after recombinant FSH administration, thus suggesting that adjustment of doses of FSH in relation to body weight could reduce ovarian response variability.

In the current study, all volunteers, one man excepted, had previous proof of normal gonadal function. In view of its slow disappearance rate after intramuscular injection, a single injection of recombinant FSH might have been sufficient to induce temporarily gonadal response, influencing directly or indirectly the synthesis of other hormonal factors. During the experimental period, circulating immunoactive and bioactive LH levels were extremely low or undetectable, as previously reported for patients with hypogonadotropic hypogonadism (20). Accordingly, serum T and E_2 levels were very low in male and female volunteers, respectively, and only two out of eight women showed a very small E_2 rise 2

days after injection. Follicle-stimulating hormone-induced E_2 synthesis, however, is known to be impaired in hypogonadotropic subjects (21–23), most likely because the minute amounts of residual LH are too low to support E_2 biosynthesis adequately. Interestingly, six out of seven men volunteers showed slightly increased serum inhibin levels 3 days after recombinant FSH injection. Using antiserum against bovine 31-kd inhibin, comparable levels of inhibin in men with hypogonadotropic hypogonadism were reported by others (24). The fact that no inhibin increases were observed in the female volunteers might be related to the relatively lower availability of recombinant FSH in these subjects.

In conclusion, the data of this first human exposure study with recombinant FSH (Org 32489, Organon International) suggest that it is a safe drug with pharmacokinetic properties comparable with those previously reported for natural FSH. Further clinical data will be required to confirm the safety and efficacy of recombinant FSH in infertile patients treated for induction of ovulation or for induction of controlled ovarian superovulation.

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Pharmacokinetic and pharmacodynamic characteristics of ganirelix (Antagon/Orgalutran*). Part II. Dose-proportionality and gonadotropin suppression after multiple doses of ganirelix in healthy female volunteers

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Objective: To assess the dose-proportionality and pharmacodynamic properties of multiple doses of ganirelix (Antagon/Orgalutran; NV Organon, Oss, the Netherlands).

Design: Randomized, parallel, pharmacokinetic, and pharmacodynamic study.

Setting: Phase I clinical research unit.

Patient(s): Three groups of 15 healthy female volunteers of reproductive age.

Intervention(s): Subcutaneous injections of 0.125 mg, 0.25 mg, or 0.50 mg of ganirelix were given once daily for 7 days. Blood samples were taken to assess serum ganirelix, LH, FSH, and E_2 concentrations.

Main Outcome Measure(s): Pharmacokinetic parameters and hormone suppression.

Result(s): Steady-state levels were reached between days 2 and 3. Peak concentrations, which occurred approximately 1 hour after dosing, increased in a dose-proportional manner and averaged 5.2 ng/mL, 11.2 ng/mL, and 22.2 ng/mL for the 0.125-mg, 0.25-mg, and 0.50-mg doses, respectively. Corresponding mean values for the area under the curve over one dosing interval (24 hours) were 33 ng · h/mL, 77.1 ng · h/mL, and 137.8 ng · h/mL, respectively. After the last 0.25-mg dose of ganirelix, serum LH, FSH, and E_2 concentrations were maximally decreased (by 74%, 32%, and 25% at 4 hours, 16 hours, and 16 hours after injection, respectively). Serum hormone levels returned to pretreatment values within 2 days after the last injection.

Conclusion(s): The pharmacokinetics of ganirelix were dose-proportional within the dose range studied. Multiple injections resulted in immediate suppression of gonadotropins, which was rapidly reversed after treatment discontinuation. (Fertil Steril® 1999;72:1006–12. ©1999 by American Society for Reproductive Medicine.)

Key Words: GnRH antagonist, ganirelix, pharmacokinetics, dose-proportionality, pharmacodynamics, FSH, LH

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The GnRH antagonist ganirelix (Antagon/Orgalutran; NV Organon, Oss, the Netherlands) is a decapeptide derived from native GnRH with substitutions of amino acids at positions 1, 2, 3, 6, 8, and 10. It blocks the GnRH receptor by binding to the receptor without activating it. When sufficient amounts are present in the circulation, native GnRH is displaced from the receptor, and as a consequence, the release of LH and FSH is decreased. Thus, ganirelix, and GnRH antagonists in general, have an advantage over GnRH agonists in that they lack the initial flare-up and therefore have a shorter treatment period. Compared with GnRH antagonists of the previous generation, ganirelix has been shown to have only minimal histamine-releasing properties (1–3). Therefore, it holds promise for the treatment of a variety of disorders for which GnRH agonists currently are prescribed (4).

The single-dose pharmacokinetics of gani-

relix after IV and SC administration were reported in part I of this article (5). The concentration-time profiles and pharmacokinetic parameters were comparable, resulting in a high absolute bioavailability (>90%), and the elimination half-lives averaged 13 hours for both routes of administration.

However, ganirelix was developed for the prevention of premature LH surges in women undergoing controlled ovarian hyperstimulation. On the basis of the results of a previous dose-finding study (1), a daily SC dose of 0.25 mg was selected for further development (1). The present multiple-dose study, in which we assessed the dose-proportionality of ganirelix pharmacokinetics within a dose range that included the selected therapeutic dose, was a better reflection of the intended clinical application. Further, this study provided insight into the degree of pituitary suppression achieved in the absence of exogenous gonadotropins.

MATERIALS AND METHODS

Volunteers

A total of 45 healthy women (three groups of 15 each) were selected for and completed the study. All the women were in good physical condition and had been taking oral contraceptives for at least 3 months. The oral contraceptives were used for contraceptive purposes only and not for regulation of the menstrual cycle. Subjects with a current type I hypersensitivity disorder (urticaria, eczema, hay fever, or asthma) were excluded from participation. Written informed consent was obtained from all volunteers before the start of the study.

Study Design

This study was an open-label, randomized, parallel-design, multiple-dose study designed to assess the dose-proportionality and pharmacokinetic characteristics of ganirelix after repeated SC administration. In addition, the pharmacodynamic effect on endogenous hormone levels (LH, FSH, and E_2) was investigated. The study was approved by the ethics committee of the institution and was performed in accordance with the latest revision of the Declaration of Helsinki.

After screening for and inclusion in the study, the volunteers discontinued the daily intake of contraceptive pills exactly 1 week before the first drug injection. After this pill-free period, they were randomized to receive 0.125 mg, 0.25 mg, or 0.50 mg of ganirelix (Org 37462, Antagon/Orgalutran) SC once daily for 7 days. All injections were given in the upper leg and alternated daily between the left and right legs. The volunteers were hospitalized from treatment day 6 until treatment day 8 and visited the clinical research institution on an ambulatory basis for drug administration and blood sampling on the other study days.

Assessments

Blood samples for the assessment of serum ganirelix levels were collected before each injection (predose) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, and 96 hours after the injection on day 7. Immediately after collection, blood samples were processed to serum and stored at −20°C until analysis. In addition, all the samples taken before the injections on days 1–7 and the samples taken at 2, 4, 8, 12, 16, 24, 36, 48, 72, and 96 hours after the injection on day 7 were analyzed for serum LH, FSH, and E_2 concentrations. Further, the date on which spontaneous menses occurred after the last drug injection was assessed for each subject. For the safety evaluation, all reported adverse events were documented.

Assay

For measurement of serum ganirelix concentrations, a validated RIA without prior sample purification was used, as previously described by Nerenberg et al. (6). The lower limit of quantification was 0.02 ng/mL. Calibration curves in the range of 0.20–2.56 ng/mL were linear, with correlation coefficients of at least 0.99. The coefficient of variation derived from analysis of quality control samples ranged from 5.5%–8.8%.

Serum LH, FSH, and E_2 levels were analyzed with the use of time-resolved fluoroimmunoassays (Delfia; Wallac Oy, Turkü, Finland). The detection limits for serum LH, FSH, and E_2 were 0.6 IU/L, 1 IU/L, and 13.6 pg/mL, respectively. The coefficients of variation derived from analysis of quality control samples for LH, FSH, and E_2 ranged from 2.8%–5.9%, 2.9%–3.5%, and 2.8%–5.9%, respectively.

Analysis

Pharmacokinetics

All subjects were included in the analysis of pharmacokinetic parameters. From the predose concentrations on days 1–7 and the concentration measured at 24 hours after the last dose, the time to achieve steady state (t_{ss}) and the mean steady-state trough concentration ($C_{ss,min}$) were calculated for each subject. From the serum ganirelix concentrations on day 7, the following pharmacokinetic parameters were calculated for each subject:

1. C_{max} and t_{max}: the peak concentration and the time of its occurrence were determined from the measured serum drug level data.
2. k_{el} and $t_{1/2}$: from the individual log concentration versus time plots, it was determined by visual inspection from which time onward to the last time point, the concentration versus time (C-t) plot was approximately linear. Using log-linear regression on these terminal data points of the C-t curve, the slope (k_{el}) was estimated; the elimination half-life ($t_{1/2}$) was calculated as $^e\log 2/k_{el}$. Concentrations lower than the detection limit in the elimination phase were ignored.
3. AUC_{0-24}: the area under the curve (AUC) over one dosing interval (24 hours) at steady state was calculated with the use of the linear trapezoidal rule. In case of a value lower than the detection limit, a concentration of zero was assumed.

Dose-proportionality

To test whether the pharmacokinetic characteristics of ganirelix after multiple, rising doses are linear, a one-way analysis of variance with dose as a fixed effect was performed on the ^{e}log-transformations of the parameters $C_{ss,min}$, C_{max}, AUC_{0-24}, and t½ (dose-normalized when appropriate). For t_{max} and t_{ss}, the nonparametric Kruskal-Wallis test was performed.

Pharmacodynamics

All the subjects were included in the pharmacodynamic analysis. For each subject, the change from baseline levels during treatment was determined for LH, FSH, and E_2. In addition, nadir levels of serum hormones were assessed after the last ganirelix injection on day 7. Further, for 40 subjects, the day that spontaneous menses returned after the last ganirelix injection was documented. For different reasons, the day that spontaneous menses returned could not be documented during the study period for the other 5 subjects.

RESULTS

Volunteers

Forty-five women with a mean age of 22.1 years participated in the study. They ranged from 50–81 kg in weight and from 156–184 cm in height, and had an average body mass index of 22.6 kg/m^2. There was no difference in demographic characteristics between the three treatment groups (data not shown).

Pharmacokinetics

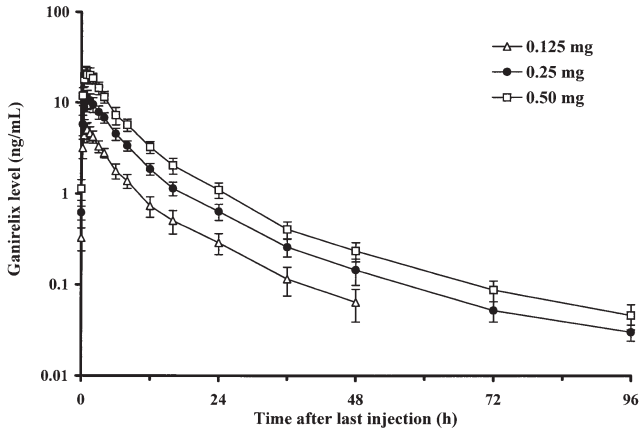
Steady-state levels of ganirelix were reached between day 2 and day 3 of treatment (data not shown). For each dose group, the mean serum concentrations of ganirelix after the injection on day 7 are presented in Figure 1. The concentration versus time (C-t) profiles were highly similar and increased linearly with each dose increment. The main pharmacokinetic parameters that were calculated from the ganirelix concentration data are presented in Table 1.

Steady-state trough concentrations averaged 0.31 ng/mL, 0.63 ng/mL, and 1.09 ng/mL for the 0.125-mg, 0.25-mg, and 0.50-mg dose groups, respectively. After the last injection on day 7, mean peak levels (C_{max}) were observed after approximately 1 hour and were 5.2 ng/mL, 11.2 ng/mL, and 22.2 ng/mL for the 0.125-mg, 0.25-mg, and 0.50-mg dose groups, respectively. The corresponding mean area under the concentration versus time (C-t) curves over one dosing interval (AUC_{0-24}) were 33 ng · h/mL, 77.1 ng · h/mL, and 137.8 ng · h/mL, respectively. Average elimination half-lives of 13.7 hours, 16.2 hours, and 16.3 hours were calculated for the 0.125-mg, 0.25-mg, and 0.50-mg dose groups, respectively.

No statistically significant dose effect was found for the dose-normalized $C_{ss,min}$ and C_{max}, indicating dose-proportionality for these parameters. Further, dose independence for the parameters t_{max} and t_{ss} was proved. A general overview of the calculated AUC_{0-24} values for the three dose groups appeared to indicate dose-proportionality for this parameter as well. Analysis of the results by analysis of variance, however, revealed that the AUC_{0-24} of the 0.25-mg dose group was statistically significantly higher than that of the other two dose groups. With respect to the elimination half-life, a statistically significant difference was found between the lowest dose group and the other two dose groups.

FIGURE 1

Log-linear plot of mean (±SD) serum ganirelix concentrations at steady state after multiple-dose SC administration of 0.125 mg, 0.25 mg, and 0.50 mg of ganirelix (15 subjects per dose group).



Oberyé. Ganirelix (Antagon/Orgalutran). Fertil Steril 1999.

Pharmacodynamics

The median predose serum LH, FSH, and E_2 levels in each treatment group, assessed during ganirelix treatment,

TABLE 1

Pharmacokinetic parameters calculated from serum ganirelix concentrations measured after once-daily SC administration of 0.125 mg, 0.25 mg, or 0.50 mg of ganirelix during cycle days 8–14 (n = 15 per group).

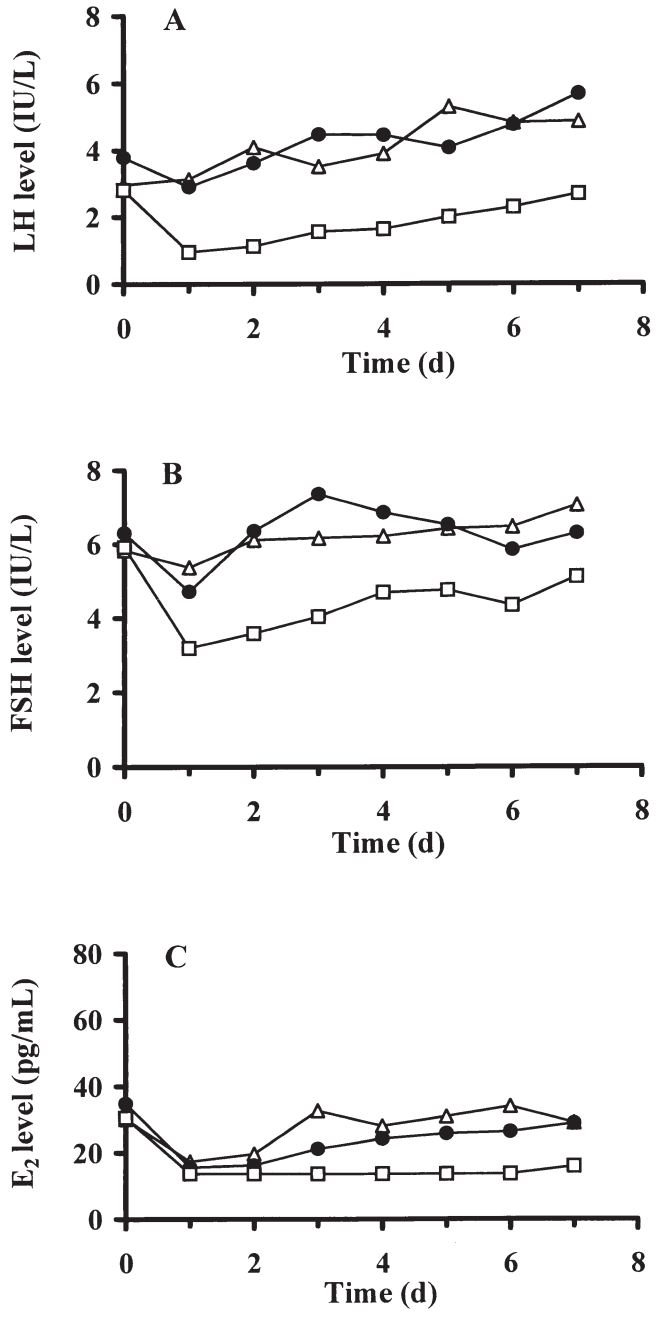
Pharmacokinetic parameter	Dose of ganirelix (mg)		
	0.125	0.25	0.50
$C_{ss,min}$ (ng/mL)	0.31 ± 0.09	0.63 ± 0.08	1.09 ± 0.25
C_{max} (ng/mL)	5.23 ± 0.80	11.16 ± 2.41	22.15 ± 3.43
t_{max} (h)	1.04 ± 0.47	1.14 ± 0.23	1.12 ± 0.40
AUC_{0-24} (ng · h/mL)	32.96 ± 4.20	77.13 ± 9.75	137.83 ± 17.02
t½ (h)	13.70 ± 3.43	16.23 ± 1.64	16.34 ± 1.02

Note: Values are means ± SD.

Oberyé. Ganirelix (Antagon/Orgalutran). Fertil Steril 1999.

FIGURE 2

Median predose serum hormone concentrations during multiple-dose SC administration of 0.125 mg (△), 0.25 mg (●), and 0.50 mg (□) of ganirelix (15 subjects per dose group). (**A**), LH concentrations. (**B**), FSH concentrations. (**C**), E_2 concentrations.



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are depicted in Figure 2. A decrease in predose serum hormone levels occurred after the first injection and was followed by a gradual recovery during the rest of the treatment period. For serum LH levels, this initial decrease after the first injection was less obvious for the 0.125-mg and 0.25-mg dose groups, whereas for serum FSH levels, a clear dose dependency in the suppressive effect after the first injection was observed. For serum E_2 levels, a comparable initial decrease was observed in all three dose groups.

Despite the fact that predose serum hormone levels gradually increased after the initial decrease that followed the first injection, they remained lower than or the same as levels seen in the early follicular phase. Serum E_2 levels remained extremely low or undetectable during the entire treatment period only in the 0.50-mg dose group. Overall, the suppressive effect on serum hormone levels was more pronounced in the 0.50-mg dose group than in the 0.25-mg and 0.125-mg dose groups; the last two groups demonstrated a comparable effect.

In Figure 3, the effect on pituitary hormone and E_2 levels after the last injection on day 7 and the recovery of serum hormone levels after the cessation of treatment is shown. Nadir LH levels were reached 4 hours after ganirelix injection in all three dose groups, whereas nadir FSH levels occurred later, namely 12 hours, 16 hours, and 16 hours after the last ganirelix injection in the 0.125-mg, 0.25-mg, and 0.50-mg dose groups, respectively (all median values).

In the 0.25-mg dose group, the decrease was calculated to be 74% and 32% for LH and FSH, respectively, compared with day 7 predose values. Levels of E_2 in the two lowest dose groups showed a relatively small decrease within the first 24 hours after the last injection. In the 0.25-mg dose group, a 25% decrease at 16 hours after injection was observed. In the highest dose group, no further decrease in E_2 levels was seen during the period of frequent sampling after the last injection because the levels already were close to the limit of detection.

After the cessation of treatment, the suppressive effect of ganirelix was rapidly reversed in all three dose groups. Pituitary hormone and E_2 levels returned to predose values within 24 hours after the last injection and increased even further in the 24 hours thereafter.

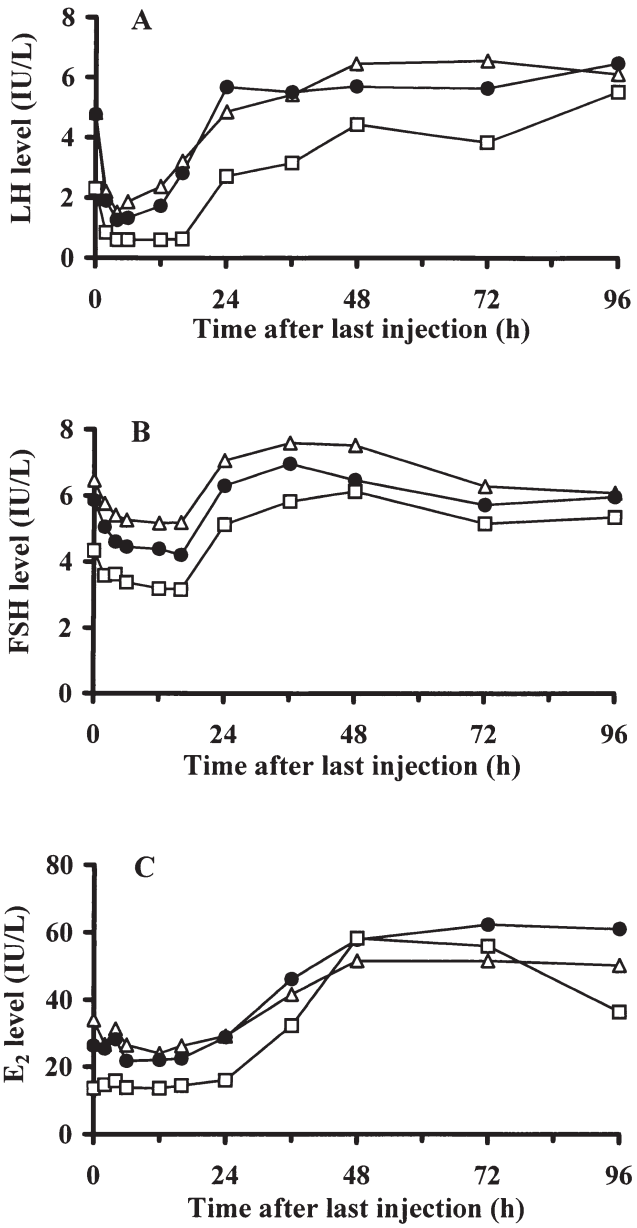
Rapid recovery of pituitary suppression also was illustrated by the data on the return of menses in the three treatment groups. Menses returned in a mean (±SD) of 21.6 ± 6.9 days, 22.9 ± 5.2 days, and 23.9 ± 6.2 days after the last ganirelix injection in the 0.125-mg, 0.25-mg, and 0.50-mg dose groups, respectively.

Safety

All the ganirelix treatment regimens were well tolerated. Most reported adverse events were of a mild intensity. No differences in the incidence of adverse events was detected among the three dose groups. The most frequently reported side effects included headache (32 subjects), injection site reactions (20 subjects), and fatigue (11 subjects).

FIGURE 3

Median serum hormone concentrations during one dosing interval and recovery after multiple-dose SC administration of 0.125 mg (△), 0.25 mg (●), and 0.50 mg (□) of ganirelix (15 subjects per dose group). (**A**), LH concentrations. (**B**), FSH concentrations. (**C**), E_2 concentrations.



Oberyé. Ganirelix (Antagon/Orgalutran). Fertil Steril 1999.

DISCUSSION

The primary aim of this study was to demonstrate dose-proportional pharmacokinetics within a dose range that included the selected therapeutic dose (0.25 mg) of ganirelix. To rule out the possible influence of the menstrual cycle phase on ganirelix pharmacokinetics and to allow treatment in cohorts, the subjects' menstrual cycles were synchronized with the use of oral contraceptives. Ganirelix treatment was started 1 week after the discontinuation of oral contraceptive therapy, when serum hormone levels were similar to those of the early follicular phase.

After multiple dosing, C_{max} and $C_{ss,min}$ showed a clear linear increase with each dose increment, indicating dose-proportionality for these parameters. A similar trend was observed for the AUC, but statistical analysis revealed that the dose-normalized AUC_{0-24} of the 0.25-mg dose group was significantly higher than that of the 0.125-mg and 0.50-mg dose groups. However, no consistent dose-dependent relation was present, because the AUC_{0-24} rose with the first dose increment and decreased again with the next dose increment. Therefore, we conclude that the statistically significant difference in AUC_{0-24} values does not have clinical implications and probably was caused by the low coefficient of variation (12%–13%).

The dose-proportionality of ganirelix pharmacokinetics within the dose range studied in the current trial is in agreement with the results of the ganirelix dose-finding study (1), in which doses of 0.625–2 mg were tested. Steady-state trough levels ($C_{ss,min}$) measured in the dose-finding trial were highly comparable to those measured in the present study and revealed dose-proportionality over the entire dose range.

The average elimination half-life after 7 days of SC treatment ranged from 13.7 hours in the 0.125-mg dose group to 16.2 hours in the 0.25-mg dose group and 16.3 hours in the 0.50-mg dose group. The half-life of the lowest dose group was comparable to the reported elimination half-life of approximately 13 hours in the single-dose study (5). The fact that the half-life in the lowest dose group was statistically significantly shorter than that in the higher dose groups may be related to several factors. First, a longer interval can be used for estimation of the half-life in higher dose groups because drug concentrations drop below the detection limit at a later time. Second, no distinct log-linear terminal phase was present in the higher dose groups because ganirelix concentrations kept deflecting in time. Third, the statistically significant difference could be due to the relatively low variability. However, it should be noted that the observed difference in average half-lives is minimal (14 hours vs. 16 hours) and of limited clinical relevance.

In contrast, for cetrorelix, average half-lives of 5–10 hours, calculated after single-dose SC administration of 0.25–1 mg, increased to 20–80 hours after multiple administration of the same doses (7). Such an extension might affect the duration of action and the reversibility of treatment.

Because all the women studied were using oral contraceptives until 7 days before the start of the study, their median baseline hormone levels were similar to those of the

early follicular phase (i.e., relatively low) just before the first ganirelix injection. Still, a decrease in LH, FSH, and E_2 levels was noted at 24 hours after the first injection. This initial decrease in predose levels gradually reversed during the rest of the treatment period in the two lowest dose groups, but suppression remained evident for the highest dose group. This gradual "escape" from pituitary suppression also has been reported with cetrorelix (7) and detirelix (8). The inability of the compounds to suppress serum hormone levels during the entire treatment period might be caused by the absence of the negative E_2 feedback (9) or by an increased GnRH pulse frequency during that particular phase of the cycle (10).

However, the results obtained during the interval of frequent sampling after the last injection show that serum hormone levels are suppressed temporarily but profoundly during the 24-hour period after each injection. For the 0.25-mg dose group, a nadir for LH of −74% at 4 hours after dosing and a nadir for FSH of −32% at 16 hours after dosing was calculated. This finding is in agreement with results reported for other GnRH antagonists, all of which suppressed LH levels to a larger extent than FSH levels (7, 11). In contrast, GnRH agonists have a different mechanism of action (pituitary desensitization and receptor down-regulation) and provide constant suppression of LH and FSH during treatment.

For cetrorelix, a comparable level of suppression has been reported (7). At 6 hours after the administration of a single dose of 0.25 mg, serum LH and FSH levels were maximally decreased by 75% and 23%, respectively, and at 12 hours, serum E_2 levels were maximally decreased by 35%. The fact that maximal suppression occurred at different times after the administration of cetrorelix and ganirelix probably is related to differences in study design.

Overall, the pharmacodynamic effects in the 0.125-mg dose group were similar to those in the 0.25-mg dose group, whereas serum hormone levels were suppressed more profoundly in the 0.50-mg dose group. On the other hand, in the ganirelix dose-finding study, a clear dose-dependent suppression of LH over a dose range of 0.625–2 mg was observed (1). The fact that dose-dependent suppression is less evident in the present study cannot be explained by a different systemic exposure to ganirelix because pharmacokinetics were dose-proportional over the studied dose range. Therefore, it is more likely that dose dependency could not be demonstrated because of the relatively small number of subjects evaluated in this study (15 subjects vs. 60 subjects in the dose-finding study). Further, the different physiologic conditions of the subjects could have had an effect; the women studied in the present trial started ganirelix treatment 1 week after discontinuing oral contraceptive therapy, whereas the women in the dose-finding study were undergoing a controlled ovarian stimulation protocol.

Concordant with the rapid elimination of ganirelix from the circulation, serum LH, FSH, and E_2 levels returned to baseline levels within 48 hours after the last ganirelix injection. This rapid recovery also was reflected by the early return of spontaneous menses. Menses returned an average of 3 weeks after the last injection; this means that the normal cycle length of 28 days was lengthened by the treatment period of 7 days.

A comparable delay in menses was reported by Nelson et al. (3) in a study in which they administered 1-mg and 2-mg doses of ganirelix for 8 days during approximately the same phase of the menstrual cycle. On the basis of changes in serum LH and progesterone levels measured after the treatment period, they hypothesized that follicular development was arrested during ganirelix treatment. However, other studies have demonstrated that the administration of high doses (approximately 10 mg) of GnRH antagonists may cause follicular atresia or luteolysis, depending on the phase of the menstrual cycle (12, 13). In view of the rapid return of menses and the very low doses of GnRH antagonist used in the current study, follicular growth might not have been arrested but the LH surge merely delayed.

Overall, we conclude from the findings in this study that the multiple-dose pharmacokinetics of ganirelix are dose-proportional within the dose range studied. Further, ganirelix induces a profound suppression of pituitary hormone secretion that is rapidly reversed when treatment is discontinued.

Acknowledgments: The authors thank the involved members of the staff of Pharma Bio-Research International B.V., Zuidlaren, the Netherlands, for their assistance during the conduct and evaluation of the trial.

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Clinical profiling of recombinant follicle stimulating hormone (rFSH; Puregon): relationship between serum FSH and efficacy

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Single-dose and multiple-rising dose studies of recombinant follicle stimulating hormone (rFSH) in hypogonadotrophic male and female volunteers demonstrated that the rate of FSH absorption after i.m. injection is higher in men than in women. In the absence of endogenous FSH, a correlation between serum FSH and body weight became apparent. The elimination half-life of rFSH was not different between the sexes and was comparable with urinary FSH. However, the in-vitro bio:immuno ratio of serum FSH was significantly higher after the administration of rFSH than after urinary FSH. When rFSH was administered daily with a fixed dose, steady state levels were reached within 3–5 days. Serum FSH concentrations increased in a dose-dependent manner when the daily dose was increased weekly over 3 weeks from 75 to 225 IU. In hypogonadotrophic women, rFSH induced normal follicular growth whereas oestrogen synthesis was impaired. In women pituitary suppressed by a high-dose oral contraceptive, the daily administration of 150 IU rFSH for 1 week induced more and larger antral follicles than the same regimen with urinary FSH, whereas the serum immunoactive FSH concentrations measured 24 h after each dosing were similar. It is concluded that even though equal or lower serum immunoactive FSH concentrations were obtained following the administration of rFSH compared with urinary FSH, circulating bioactive FSH concentrations were higher. Therefore, the conventional idea that serum immunoreactive FSH correlates positively with the magnitude of the ovarian response should be reconsidered.

Key words: FSH/pharmacodynamics/pharmacokinetics/Puregon/rFSH

Introduction

Follicle stimulating hormone (FSH) preparations are being used in the treatment of anovulatory infertility and in ovarian stimulation regimens prior to assisted reproduction technologies. With the advent of pure recombinant FSH (rFSH) it is likely that urinary preparations will be replaced by rFSH preparations. For that purpose, clinicians should gain knowledge of the main pharmacokinetic and pharmacodynamic properties of any new hormone preparation developed. The efficacy and safety of two rFSH preparations, i.e. Puregon (Organon, Oss, The Netherlands) and Gonal-F (Serono, Geneva, Switzerland), in patients undergoing ovarian stimulation have been reported since 1992 when the first pregnancies were established (Devroey *et al.*, 1992a; Germond *et al.*, 1992; Loumaye *et al.*, 1995; Out *et al.*, 1995).

This article reviews all relevant pharmacokinetic and dynamic data of Puregon which were mainly obtained in male and female volunteers.

Pharmacokinetic study models

Historical overview

The first human exposure studies of rFSH go back to early 1991, when single and multiple-rising doses were administered to gonadotrophin-deficient but otherwise healthy female and male volunteers (Schoot *et al.*, 1992, 1994; Matikainen *et al.*, 1993; Shoham *et al.*, 1993; Mannaerts *et al.*, 1993, 1996). These phase I studies were performed in

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four specialist reproductive endocrinology and infertility units. Apart from safety and pharmacokinetic data on rFSH, these studies provided for the first time information on the pharmacodynamics of FSH in the absence of luteinizing hormone (LH). With respect to the latter, it was found that FSH alone can induce normal follicular growth whereas FSH-induced oestrogen synthesis is impaired because of a lack of endogenous LH (Schoot *et al.*, 1992, 1994; Shoham *et al.*, 1993). Furthermore, it has been shown that the bioavailability of FSH after the administration of exogenous FSH is influenced by many factors, including gender, body weight and route of administration. Interestingly, circulating immunoreactive (I) FSH did not reflect circulating bioactive (B) FSH, in that B:I ratios of serum FSH after the administration of rFSH and urinary FSH appeared to be significantly different.

In late 1991, a phase II efficacy study was initiated in women undergoing in-vitro fertilization and embryo transfer to evaluate whether rFSH therapy could be combined with various gonadotrophin-releasing hormone (GnRH) agonist treatment regimens, inducing different degrees of pituitary suppression (Devroey *et al.*, 1994). The objective was to evaluate whether the amount of residual endogenous LH would be sufficient to support FSH-induced steroidogenesis and related reproductive functions. One of the first patients in this study was reported as having the first ongoing pregnancy (Devroey *et al.*, 1992a) and singleton term birth (Devroey *et al.*, 1992b) after ovarian stimulation with rFSH (Devroey *et al.*, 1993). Overall, the efficacy data indicated that rFSH stimulates multiple follicular development with corresponding rises in serum inhibin (LH-independent) and oestradiol (LH-dependent) concentrations. The rise in oestradiol concentration indicates that in women with normal menstrual cycles the amount of remaining endogenous LH after profound pituitary suppression (1–2 IU/l) is still sufficient to support rFSH-induced oestrogen biosynthesis (Devroey *et al.*, 1994). The outcome of this pilot efficacy study justified the further development of rFSH by means of several randomized, group-comparative, phase III studies. In parallel, dose proportionality and absolute bioavailability studies were initiated.

Interpretation of FSH activity as determined by various assays

For the detection, quantification and characterization of rFSH, three different types of assay were frequently applied, i.e. an in-vivo bioassay, an in-vitro bioassay and an immunoassay. The principles, quality controls, methodology and applications of these gonadotrophin assays have been described previously (Mannaerts *et al.*, 1987, 1991; Mason *et al.*, 1993). In-vivo bioassays are standard pharmacopoeia tests for the quantification of therapeutic preparations containing FSH and/or LH activity. Determining factors in these assays are gonadotrophin half-life and in-vivo metabolism. For the estimation of in-vivo bioactive FSH, a method developed by Steelman and Pohley (1953) was applied which measures the augmentation of ovarian weight in immature rats after 3 days of treatment with a FSH-containing preparation in the presence of a standard amount of human chorionic gonadotrophin (HCG). The suitability of this assay for pure FSH preparations, however, should be reconsidered, especially since clinical studies with rFSH have indicated that its predictive value might be limited (Out *et al.*, 1995).

Furthermore, because of their low sensitivity, in-vivo bioassays are unable to detect physiological amounts of gonadotrophins in body fluid samples. For the estimation of serum bioactive FSH, only in-vitro FSH bioassays are suitable which are based on the induction of FSH-specific processes in isolated target cells, i.e. granulosa or Sertoli cells. Obviously, such bioassays measure only the intrinsic bioactivity at the target level and do not take into account the elimination half-life and in-vivo metabolism. Comparative in-vitro studies for rFSH were performed in the rat Sertoli cell aromatase bioassay (Mannaerts *et al.*, 1991) and in the human granulosa cell bioassay (Mason *et al.*, 1993), and serum bioactive FSH after rFSH administration was established by means of the rat granulosa cell bioassay (Matikainen *et al.*, 1993). Recently the human FSH receptor has been cloned (Tilly *et al.*, 1992) and expressed in Chinese hamster ovary cells; it provides a continuous source for a homologous bioassay system and is thought to be more predictable for the actual efficacy.

To quantify the amount of circulating FSH, an immunoassay is most frequently applied. Such an assay is so-called structure specific because of the interaction of specific monoclonal antibodies with one or more antigenic epitopes. In clinical studies with rFSH, a time-resolved immunofluorometric sandwich assay (Delfia®) was applied which detects only intact human FSH molecules, does not crossreact with human thyroid-stimulating hormone, LH or HCG and which has a high sensitivity (detection limit 0.05 IU/l in terms of international standard 78/549). Because the antibodies in this assay, as in most immunoassays, recognize specific protein epitopes, this assay is not able to detect carbohydrate differences or alterations during clearance. Prior to the application of this assay it was confirmed that the assay recognizes the various rFSH isohormone fractions equally well because their specific immunoreactivities (specific activity per mg protein) are similar (de Leeuw *et al.*, 1996).

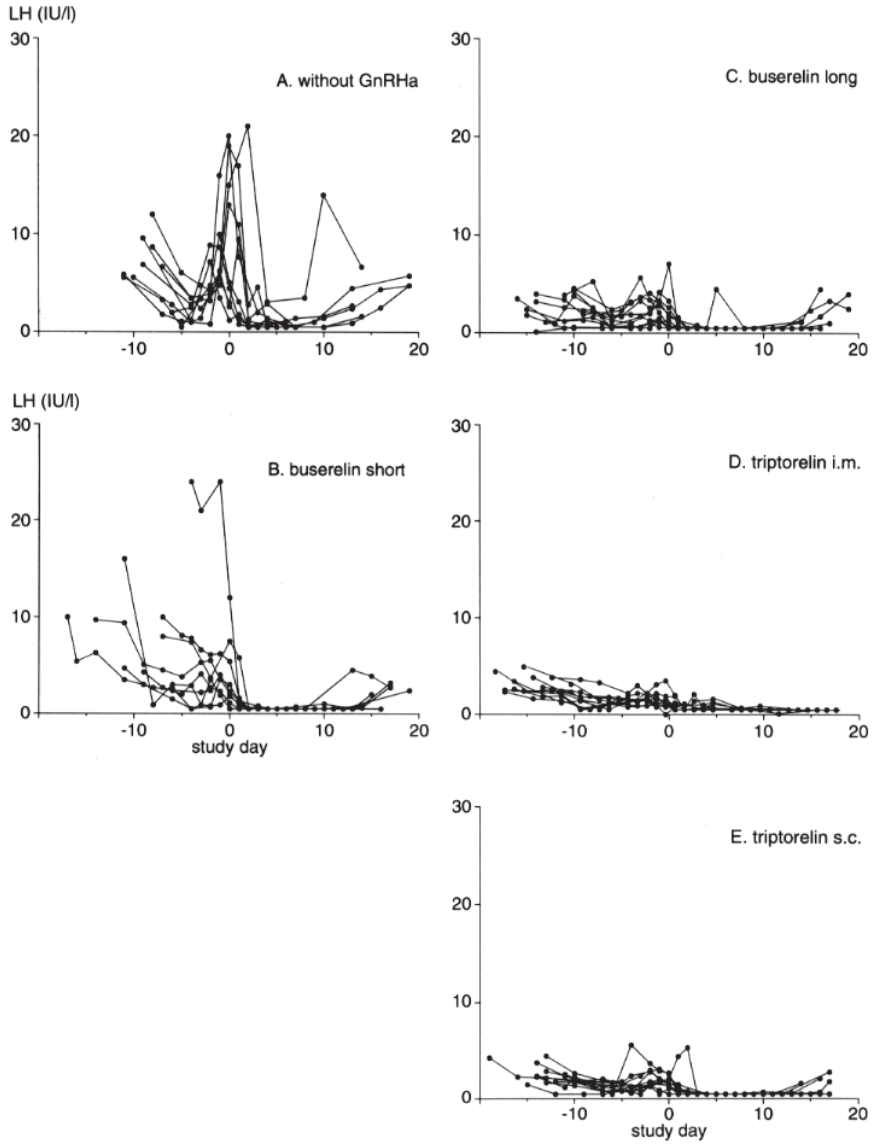

Figure 1. Individual plots of endogenous immunoreactive luteinizing hormone (LH) concentration measured in in-vitro fertilization patients during ovarian stimulation with recombinant follicle stimulating hormone (rFSH) only (**A**) or with rFSH in combination with buserelin intranasal spray (4× 150 µg) in a short (**B**) or long protocol (**C**) or with rFSH in combination with a long protocol of triptorelin, by either a single i.m. dose of 3.75 mg (**D**) or a daily s.c. dose of 200 µg (**E**). Day 0 is the day of human chorionic gonadotrophin administration.

In the calibration of samples, the outcome of the above-mentioned assays is never the same since different properties are measured and often different references are applied because various international standards have been defined for immuno- and bioassays. These preparations are of a urinary or pituitary origin and differ in their purity and isohormone profile. For the calibration of rFSH, a first international standard of rFSH will be established within the coming year.

Models to study the pharmacokinetic properties of exogenous FSH

A proper evaluation of serum FSH concentrations after the administration of exogenous FSH requires that interference by endogenous gonadotrophins is prevented as much as possible. The pulsatile pattern of endogenous FSH is known to vary in frequency and amplitude, especially during the normal menstrual cycle when the more acidic forms of FSH

in the follicular and luteal phases shift to more basic forms at the time of ovulation (Wide and Backos, 1993). In addition, a high FSH baseline may jeopardize a proper pharmacokinetic analysis. Therefore, pharmacokinetic studies of rFSH were performed in either gonadotrophin-deficient or pituitary-suppressed volunteers. In gonadotrophin-deficient volunteers, baseline concentrations of FSH and LH were extremely low (mostly <0.5 IU/l). These subjects with hypogonadotrophic hypogonadism included cases of hypophysectomy, isolated gonadotrophin deficiency and panhypopituitarism. Because these types of patient are very rare, further pharmacokinetic studies of rFSH were performed in healthy young women with induced pituitary suppression. The degree of pituitary suppression induced by GnRH agonists can vary widely and depends on the compound of choice, and the route and frequency of administration. As an example, Figure 1 shows individual endogenous LH concentrations measured in women stimulated with rFSH and different GnRH regimens for ovarian stimulation.

Obviously, triptorelin (Decapeptyl®) administered once as a depot formulation, or daily via the s.c. route, seems to cause a profound suppression. In principle, compounds such as triptorelin or goserelin (Zoladex®) can be used for pituitary down-regulation in pharmacokinetic models (Le Cotonnec *et al.*, 1994a,b), but in a three-way crossover study with rFSH it was revealed that maintenance doses of 1.8 mg triptorelin administered i.m. 2, 4 and 6 weeks after the first i.m. injection of 3.75 mg triptorelin may induce considerable flare-ups of FSH and/or LH, indicating incomplete down-regulation (unpublished data). For this reason, pharmacokinetic studies of rFSH were performed in women using the high-dose oral contraceptive Lyndiol® (2.5 mg lynestrenol and 0.05 mg ethinyl oestradiol) which decreases endogenous FSH (see Figure 3) and LH concentrations to at least the same low concentrations as encountered in subjects with hypogonadotrophic hypogonadism (Out *et al.*, 1996).

Table I. Summary table of the outcome of two single-dose and two multiple-dose pharmacokinetic studies with Puregon and comparisons with a preparation of urinary follicle stimulating hormone (FSH; Metrodin)

Subjects	Frequency, dose[a], drug	Route	No. of subjects	C_{max} (IU/l)	t_{max} (h)	AUC (IUh/l)	$t_{½}$ (h)
Gonadotrophin deficient						0–∞	
	Single dose	i.m.	7 men	7.4 ± 2.8	14 ± 8	452 ± 183	32 ± 12
	300 IU rFSH		8 women	4.3 ± 1.7	27 ± 5	339 ± 105	44 ± 14
	Single dose	i.m.	4 men	11.6 ± 1.7	9 ± 2	764 ± 190	43 ± 10
	300 IU urinary FSH		5 women	7.2 ± 2.3	21 ± 11	547 ±127	38 ± 9
	sequential						
Gonadotrophin deficient							
	Multiple-rising dose,	i.m.	9 men	–	–	–	48 ± 5
	75, 150 and 225 IU/day,	i.m.	7 women	–	–	–	39 ± 8
	each dose for 7 days						
Pituitary suppressed by Lyndiol						0–312	
	Single dose,	i.v.	13 women	43.0 ± 3.9	NA	588 ± 203	–
	300 IU Puregon,	i.m.	13 women	6.9 ± 2.9	18 ± 10	446 ± 136	–
	three-way crossover	s.c.	13 women	5.4 ± 0.7	17 ± 8	456 ± 141	–
Pituitary suppressed by Lyndiol							
	Daily dose for 7 days,					0–24	
	75 IU rFSH	i.m.	9 women	4.7 ± 1.5	8 ± 4	97 ± 22	27 ± 8
	150 IU rFSH		7 women	9.5 ± 2.6	10 ± 6	204 ± 49	30 ± 6
	225 IU rFSH		8 women	11.3 ± 1.8	11 ± 8	242 ± 44	29 ± 7
	or						
	150 IU urinary FSH,	i.m.	8 women	10.7 ± 2.0	4.8 ± 3.0	231 ± 45	30 ± 4
	parallel design						

AUC = area under the curve; C_{max} = peak FSH concentration; NA = not applicable; $t_{½}$ = calculated elimination half-life; t_{max} = time interval to peak serum FSH concentration.
[a]Dose in terms of in-vivo bioactive follicle stimulating hormone.

Pharmacokinetic properties: study outcome

The main pharmacokinetic parameters derived from serum immunoreactive FSH concentrations measured in two single-dose and two multiple-dose studies of rFSH are presented in Table I. In one single-dose study and in one multiple-dose study, a comparison was made with urinary FSH (Metrodin; Serono).

Single-dose pharmacokinetics

In gonadotrophin-deficient volunteers, who received a single i.m. injection of 300 IU rFSH in the buttock (Mannaerts *et al.*, 1993), the rate of FSH absorption was lower in women than in men, with lower peak FSH concentrations (C_{max}), a longer time interval to peak serum FSH concentration (t_{max}) and a smaller area under the serum concentration versus time curve (AUC), with the AUC tending to be smaller in women compared with men. These differences are thought to be related to the amount of gluteal s.c. fat, being greater in women than in men. Consequently, even though intended as a deep i.m. injection, women may receive part of the drug in their s.c. adipose layer, leading to slower absorption (see also below). Further studies are needed to determine this issue.

Increases in serum FSH measured after the single administration of rFSH or urinary FSH are presented in Figure 2. The calculated elimination half-life ($t_{½}$) of rFSH was 30–40 h and not significantly different between the sexes or between treatments. Interestingly, in both men and women a negative relationship between body weight and serum FSH concentration was revealed (r = 0.9 and 0.8 respectively). This correlation might be disturbed in normogonadotrophic subjects because of fluctuating, relatively high amounts of endogenous FSH. Nevertheless, various studies support the hypothesis that body weight is a major determining factor on the dose and duration of gonadotrophin stimulation in assisted reproduction. Further research should indicate whether the adjustment of FSH doses in relation to body weight can reduce ovarian response variability. When serum immunoreactive FSH concentrations after the administration of rFSH and urinary FSH were compared, no difference was noted for the time interval to reach peak FSH concentration; however, in both sexes it appeared that C_{max} was significantly ($P < 0.05$) lower after rFSH than after urinary FSH injection. Accordingly, the AUC was significantly ($P < 0.05$) lower after rFSH than after urinary FSH administration. Further research showed that the lower concentrations of immunoreactive FSH after rFSH administration are not predictive for its bioactivity at the target cell level.

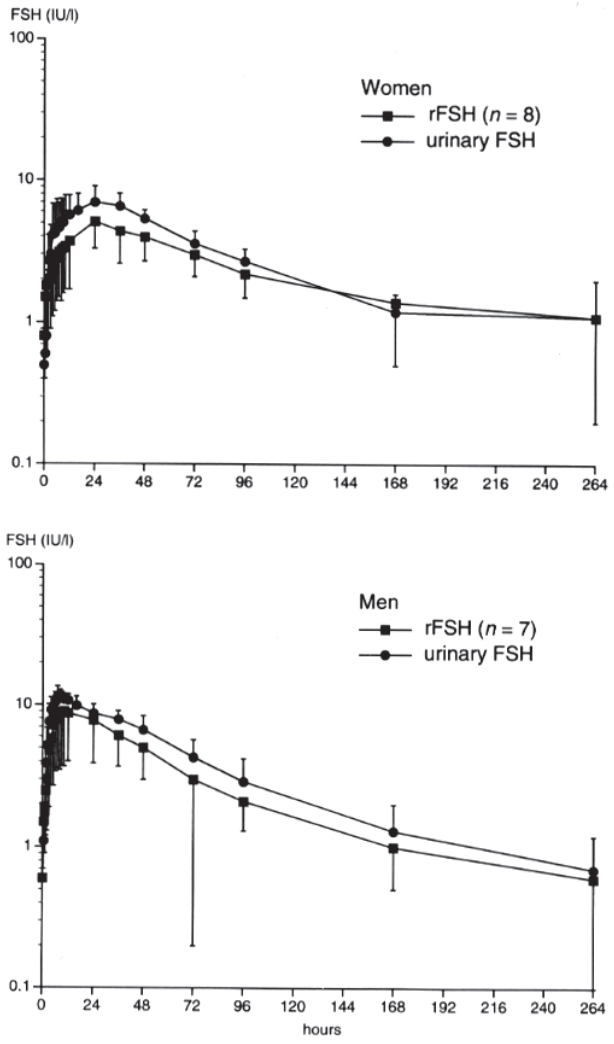

Figure 2. Mean curves of serum immunoreactive follicle stimulating hormone (FSH) after a single dose of 300 IU recombinant and urinary FSH in gonadotrophin-deficient women (upper panel) and men (lower panel).

Because of its high purity (>99%), rFSH is suitable for both i.m. and s.c. administration. The latter route is more suitable for self-administration, and its application will reduce time and the cost of medical staff. To examine whether the availability of rFSH after i.m. or s.c. injection is similar, an absolute bioavailability study of rFSH was undertaken in healthy women pituitary suppressed by the daily intake of Lyndiol. In this randomized, three-way crossover study, women received a single dose of 300 IU rFSH via the i.v., i.m. or s.c. route. Serum FSH concentrations measured after i.m. and s.c. injection are presented in Figure 3. The absorption of s.c. administered rFSH tended

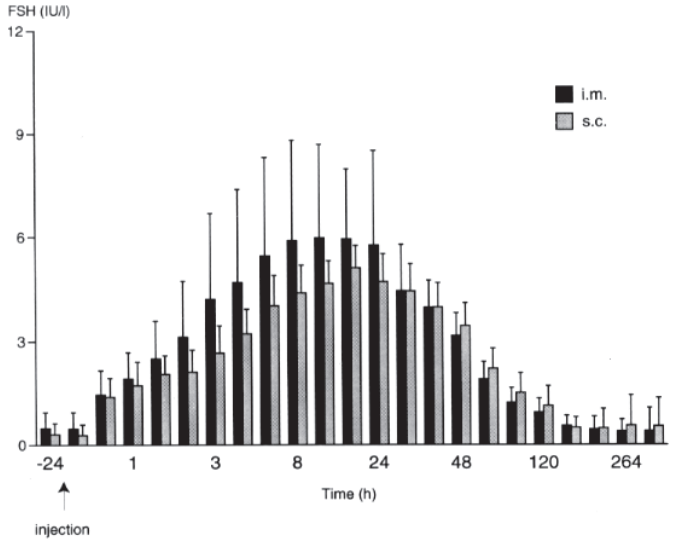

Figure 3. Mean (±SD) of serum immunoreactive follicle stimulating hormone (FSH) measured in 15 women pituitary suppressed by Lyndiol after the injection of a single dose of 300 IU recombinant FSH via the i.m. route (in the upper quadrant of the buttock) or via the s.c. route (under the skin of the abdominal wall).

to be slower, resulting in lower serum FSH concentrations during the absorption phase and in higher concentrations thereafter. Nevertheless, the extent of absorption (AUC) appeared to be equivalent and the bioavailability of the i.m. and s.c. routes in comparison with the i.v. route was almost equal, i.e. 76 and 78% respectively. Interestingly, the inter-subject variability for C_{max} after i.m. injection was much higher than after s.c. injection (coefficients of variation = 42 and 13% respectively), most probably because of the different amounts of adipose tissue and/or blood supply at the i.m. injection site.

Multiple-dose pharmacokinetics

Two multiple-dose studies of rFSH were performed, i.e. one study in gonadotrophin-deficient male and female volunteers and the other study in female volunteers pituitary suppressed by Lyndiol. In the first study, the safety of rFSH was the main objective, but in addition information on the pharmacokinetics and dynamics of rFSH was gathered. The second study focused mainly on the pharmacokinetic and pharmacodynamic properties of rFSH in comparison with those of urinary FSH (see also Table I).

During the multiple-rising dose study of rFSH in gonadotrophin-deficient subjects, the dose was increased at weekly intervals: the first 7 days, 75 IU/day; the subsequent 7 days, 150 IU/day; and the last 7 days, 225 IU/day. Serum FSH concentrations increased in a dose-dependent manner, and steady state levels were reached after 3–5 days of treatment (Mannaerts *et al.*, 1996). As a typical example, the individual serum immunoreactive FSH concentration measured in three gonadotrophin-deficient men are presented in Figure 4.

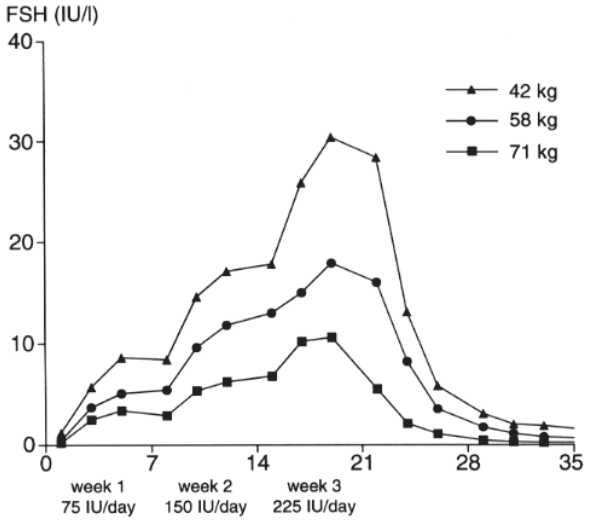

Figure 4. Individual graphs of serum immunoreactive follicle stimulating hormone (FSH) measured in three gonadotrophin-deficient men treated with single daily i.m. doses of recombinant FSH for 3 weeks. Doses were increased at weekly intervals from 75 to 225 IU/day.

An analysis of individual steady state levels and the body weight of all male volunteers indicated a negative correlation ($r = –0.6$) and supports the previously revealed negative correlation between C_{max} and body weight ($r = –0.9$) after single-dose administration. Accordingly, one subject weighing only 42 kg had relatively high increments of serum FSH (see also Figure 4). The pharmacokinetic evaluation in gonadotrophin-deficient women was hampered by the fact that four out of seven volunteers had to discontinue rFSH medication because of an increased risk of ovarian hyperstimulation (Schoot *et al.*, 1994). Median serum FSH steady state concentrations measured in gonadotrophin-deficient men and women prior to the first rFSH injection and 24 h after the completion of each treatment week are presented in Table II. Although the number of observations is small, especially in women, this study indicates, like the single-dose study, that the increase in serum FSH concentration is higher in men than in women, at least after deep i.m. injection in the buttock.

Table II. Median (range) serum immunoreactive follicle stimulating hormone (FSH) measured in gonadotrophin-deficient men and women during daily i.m. treatment for 21 days with multiple-rising doses of recombinant FSH (rFSH), i.e. the first 7 days 75 IU/day, the next 7 days 150 IU/day and the last 7 days 225 IU/day

Day	Gonadotrophin-deficient men		Gonadotrophin-deficient women	
	n	FSH (IU/l)	*n*	FSH (IU/l)
1	9	0.5 (<0.05–1.9)	7	0.3 (<0.05–1.2)
8	9	4.3 (2.0–8.4)	7	3.0 (1.8–4.9)
15	9	8.4 (4.9–17.8)	5	7.3 (4.2–8.5)
22	8	13.7 (5.6–28.4)	3	8.8 (8.3–11.7)

In accordance with the protocol, four out of seven female volunteers were discontinued during rFSH treatment because at least one follicle >14 mm in diameter was observed by ultrasonography.

Table III. Study design of a comparative, randomized, multiple-dose study in healthy women pituitary suppressed by Lyndiol

Study day	Treatment group			
	1	2	3	4
1	First day of menstrual bleeding			
8	Start daily Lyndiol intake			
29–35	rFSH, 75 IU/day	rFSH, 150 IU/day	rFSH, 225 IU/day	urinary FSH, 150 IU/day
42	Last Lyndiol intake			

The design of a group-comparative, randomized, multiple-dose study of rFSH in pituitary-suppressed healthy women of reproductive age is given in Table III. The pharmacokinetic outcome of this study is included in Table I. Steady state was reached after four doses of FSH for all groups. The dose-normalized C_{min} just before the last injection was comparable between groups, indicating dose proportionality in the 75–225 IU dose range. Immunoreactive serum FSH concentrations measured 24 h after each injection of 150 IU rFSH and 150 IU urinary FSH were similar.

Pharmacokinetic versus pharmacodynamic properties

As explained above, immunoassays only indicate the amount of intact FSH molecules present in the circulation and do not reflect the extent to which these molecules bind to the FSH receptor and are able to trigger target cells. In comparative pharmacokinetic studies of rFSH, in which equal doses of in-vivo bioactive FSH were administered, the amount of immunoreactive FSH after a single dose of rFSH was significantly lower than after a comparable dose of urinary FSH. Whether differences in serum immunoreactive FSH concentrations are predictive for the magnitude of ovarian response depends on the bioactivity of circulating FSH glycoforms. The latter can only be measured by an in-vitro bioassay, which was also applied in the single-dose study of rFSH in gonadotrophin-deficient volunteers. For this purpose, serum samples taken prior to recombinant or urinary FSH injection, and taken 6, 24 and 72 h thereafter, were analysed in both the immunofluorometric assay and the in-vitro granulosa cell bioassay (Matikainen *et al.*, 1993). The calculated ratio of the outcome of these assays, called the B:I ratio, is presented in Table IV.

In all subjects, endogenous bioactive FSH measured in pretreatment samples was under the detection limit of the bioassay (~3.7 IU/l). Individual B FSH concentrations measured at 6, 24 and 72 h after rFSH or urinary FSH administration were undetectable at all time points in one woman (body weight 85.8 kg) who was excluded from further evaluation. In all subjects treated with rFSH, B FSH was measurable for at least 72 h; during this period, no significant changes in the B:I ratio occurred. In addition, there was no significant difference in the B:I ratio between men and women. The B FSH concentrations after rFSH treatment tended to be higher than those after urinary FSH treatment, whereas the I FSH concentration showed an opposite tendency. As a result, in all post-treatment samples the B:I ratio was significantly higher after rFSH treatment than after urinary FSH treatment. This obviously

clinically relevant difference will have to be confirmed in similar studies comprising larger numbers of patients. Whether the higher serum B:I ratio of rFSH results in a greater ovarian response was established in the comparative multiple-dose study of rFSH in women pituitary suppressed by Lyndiol (Table III). Ovarian response was monitored daily by ultrasonography. A daily dose of 75 IU rFSH, given for 7 days, appeared to be too low to induce significant follicular growth (diameter of at least 10 mm) in any of the nine volunteers. In contrast, follicular growth was induced in all other subjects treated with 150 or 225 IU rFSH or 150 IU urinary FSH. The total number and size of follicles induced by 150 IU rFSH and 150 IU urinary FSH are depicted in Figure 5.

The difference in number and size distribution indicates the higher efficacy of rFSH in terms of inducing multiple follicular growth. This observation is in agreement with the higher B:I ratio of rFSH. Because rFSH contains more relatively basic isoforms and less relatively acidic isoforms than urinary FSH (de Leeuw *et al.*, 1996), and because basic isoforms are known to have a higher receptor affinity and intrinsic bioactivity than acidic isoforms, these findings may indicate that the relatively short-living basic FSH isoforms play an important role in the induction of (multiple) follicular growth, whereas the longer-living acidic FSH isoforms provide a certain maintenance of FSH above the required threshold. In comparison with other preparations, rFSH might trigger the ovary to a greater extent, especially during the first hours after injection.

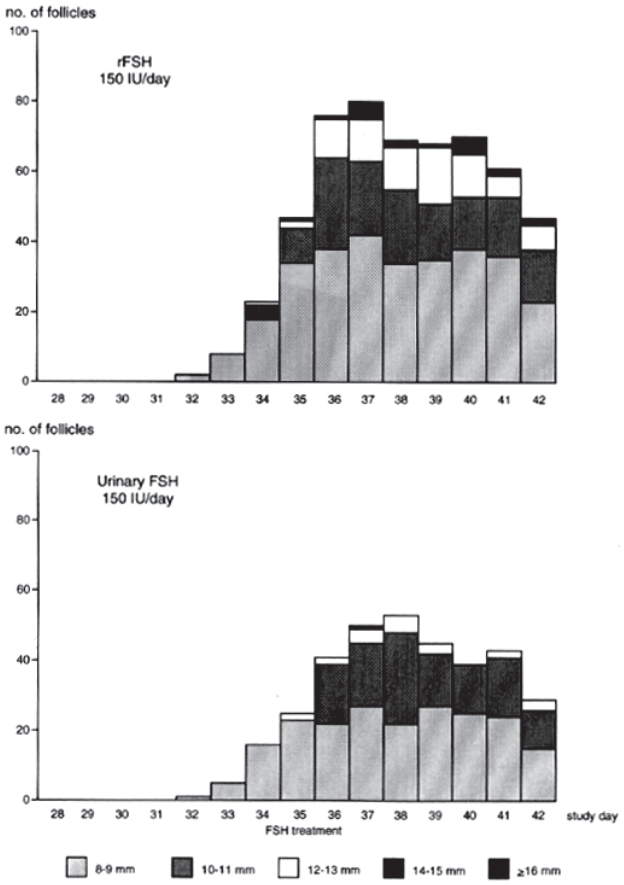

Figure 5. Total number and size of follicles in pituitary-suppressed women during and after daily i.m. treatment with 150 IU recombinant follicle stimulating hormone (FSH; upper panel) and 150 IU urinary FSH (lower panel).

Table IV. Bio:immuno ratios of serum follicle stimulating hormone (FSH) in gonadotrophin-deficient subjects measured prior to injection and 6, 24 and 72 h after the injection of 300 IU recombinant FSH (rFSH) and 300 IU urinary FSH

Time	Gonadotrophin-deficient men		Gonadotrophin-deficient women	
(h)	rFSH	Urinary FSH	rFSH	Urinary FSH
	(*n* = 7)	(*n* = 4)	(*n* = 7)	(*n* = 4)
0	–	–	–	–
6	4.1[a]	2.4	3.4[a]	1.8
	(2.2–5.5)	(1.4–3.5)	(ND–14.5)	(0.5–4.7)
24	4.4[a]	2.5	3.7[a]	1.6
	(3.1–5.6)	(ND–2.7)	(ND–7.7)	(0.7–2.7)
72	5.5[a]	1.1	4.6[a]	0.9
	(ND–21.0)	(ND–6.4)	(ND–9.7)	(ND–3.8)

ND = not detectable.
[a]Significantly ($P < 0.05$) higher for rFSH.

The ovarian response is modulated by various endocrine and paracrine factors like LH, GnRH, inhibin, activin, insulin and insulin-like growth factors. The role of LH during the induction of follicular growth was established in gonadotrophin-deficient women treated with multiple-rising doses of rFSH (Schoot *et al.*, 1994), which is devoid of any LH activity. The study design and steady state concentrations of serum FSH in these women have been described above. Interestingly, in hypogonadotrophic women, rFSH induced the normal growth of (multiple) ovarian follicles up to pre-ovulatory sizes (>15 mm), whereas only minor increases in serum oestradiol concentrations were observed because of the low availability of androgens. These data suggest a differential regulation of mitogenic and steroidogenic granulosa cell activity by FSH. Moreover, oestrogens appear to be less mandatory for human follicle development than previously believed. In accordance with the impaired oestrogen synthesis during ovarian stimulation in gonadotrophin-deficient women, women pituitary

suppressed by Lyndiol also had normal follicular growth without or with only minor rises in serum oestradiol concentration.

Conclusions

In pharmacokinetic studies, the amount of serum immunoreactive FSH is not only determined by the dose of exogenous FSH, but also by the body weight, gender and route of administration. After the administration of equal doses of in-vivo bioactive FSH, the amount of serum immunoreactive FSH might be significantly different between FSH preparations because of differences in their isohormone profiles. This may also explain why even though serum immunoreactive FSH concentrations are lower or equal, the actual bioactivity of this circulating FSH can be higher. Therefore, the conventional idea that serum immunoreactive FSH correlates positively with the magnitude of ovarian response should be reconsidered. It is suggested that relatively basic isohormones, because of their higher intrinsic bioactivity, play an important role in the induction of multiple follicular growth.

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3.3 Discussion

Phase I studies of follitropin-β, ganirelix and corifollitropin alfa in male and female volunteers indicated that all three drugs were well-tolerated and no antibodies were detected in any of the exposed volunteers following single or repeated drug administration [Mannaerts et al 1993; Mannaerts et al 1996a; Bouloux et al 2001]. Anti-drug antibody assays were initially based on a radioimmunoprecipitation assay applying ^{125}I-drug as a tracer. However, the ability to detect and measure specific anti-drug-antibodies was (and still is) complicated in the absence of appropriate positive controls and also because any drug remaining in the circulation could interfere in these assays [Mire-Sluis et al 2004]. In view of the small number of exposed subjects, the absence of anti-drug-antibodies during Phase I trials is no more than a first safety indicator, as a relatively small risk for drug-related hypersensitivity or immunogenicity can only be estimated after (repeated) exposure of hundreds of patients to the therapeutic dose in large phase III safety trials (see Chapter 6).

Regardless of the high purity of follitropin-β (> 10,000 IU/mg), the very first studies of follitropin-β in gonadotropin-deficient volunteers still applied the intramuscular route of administration [Mannaerts et al 1993; Mannaerts et al 1996a]. Following injection in the buttock, the rate of follitropin-β absorption was slower and lower in women than in men, probably due to the amount of gluteal subcutaneous fat being greater in women than in men. This hypothesis is supported by the finding that follitropin-β has a slower absorption with subcutaneous rather than with intramuscular injection, even though both routes have the same absolute bioavailability (76-78%) in comparison to the intravenous route [Mannaerts et al 1996b].

Probably due to their gonadotropin deficient status, for the first time an inverse relationship between body weight and serum FSH levels was documented following a single dose of follitropin-β administration. This association was also confirmed in the multiple-dose study of follitropin-β in gonadotropin-deficient male and female volunteers [Mannaerts et al 1996b]. Such an inverse relationship, although more pronounced, was also documented later for hCG [Chan et al 2003] and for corifollitropin alfa [Corifollitropin alfa Dose-finding group, 2008]. This phenomenon is explained by the fact that gonadotropins distribute mainly within the extra-cellular fluid space and the volume of distribution increases with body weight. However, adipose tissue does not markedly increase the volume distribution, due to its low content of extra-cellular water, explaining why serum gonadotropin levels may correlate better with body weight than with Body Mass Index (BMI) [Ledger et al 2011].

The pharmacokinetics of follitropin-β were described as dose-dependent, reaching a steady state within 4 to 5 days following daily administration, with peak levels 10-12 hrs (tmax) after drug administration and an elimination half-life of 30 to 40 hrs [Mannaerts et al 1996b]. Whether the exposure to follitropin-β measured by immunoassay was also predictive

for the ovarian response was examined by estimating the *in vitro* FSH bioactivity of serum samples collected over time following a single intramuscular injection of either follitropin-β or urinary FSH [Matikainen et al 1994]. Clearly the bioactivity:immunoreactivity (B:I) ratio was higher following follitropin-β injection than following urinary FSH injection due to a higher bioactivity and a lower immunoactivity, indicating a higher bioactivity of circulating FSH glycoforms. This higher serum B:I ratio of follitropin-β was confirmed in a subsequent comparative multi-dose study of follitropin-β in pituitary-suppressed volunteers, who developed more follicles, and follicles up to a larger size, following one week of treatment with daily 150 IU follitropin-β than with 150 IU urinary FSH [Mannaerts et al 1996b].

The pharmacokinetics of corifollitropin alfa were described in comparison to follitropin-β and differences included a 3-4 times slower absorption to serum peak levels (tmax) as well as a two-fold longer elimination half-life [Fauser et al 2009]. The pharmacokinetic profile of corfollitropin alfa implies that the highest FSH activity is reached 2 days after injection followed by decreasing FSH activity until day 8 of stimulation. Exposure to corifollitropin alfa should take into account a subject's body weight mainly because corifollitropin alfa is distributed within the extra-cellular fluid space, thus the volume of distribution of corifollitropin alfa typically increases with body weight. Short subjects with low body weight typically have less extra-cellular fluid than taller subjects with a correspondingly higher body weight. In contrast, for obese subjects, the impact of body weight on the extra-cellular volume is less apparent and adipose tissue does not markedly increase the volume distribution of corifollitropin alfa. Accordingly, the relationship between drug exposure and body weight tends to plateau for subjects weighing >90 kg [Ledger et al 2011; Figure 2].

In contrast to follitropin-β and corifollitropin alfa, the pharmacokinetics of the decapeptide ganirelix can be easily studied in healthy volunteers without interference due to endogenous hormones. Following a single subcutaneous dose of 0.25 mg ganirelix in healthy female volunteers of reproductive age, ganirelix was rapidly absorbed, reaching peak levels 1 to 2 hours (tmax) following injection. In comparison to the intravenous route, ganirelix has a high absolute bioavailability (>90%) after subcutaneous administration and a relatively short half-life of about 13 hrs regardless the route of administration [Oberyé et al 1999a]. Peak levels were reached 1-2 hrs following drug administration. Accordingly a subsequent multiple-dose study in the same type of volunteers indicated that exposure increased in a dose-proportional manner, with an elimination half-life of 16 hrs and a steady state within 2 to 3 days following daily 0.25 mg ganirelix administration [Oberyé et al 1999b].

The pharmacodynamic effect induced by fertility drugs in volunteers during phase I trials may be evaluated to estimate the therapeutic dose range for phase II dose-finding studies. Phase I studies usually start with single rising dose studies to establish the maximum tolerated dose. No or limited impact can be expected following a single dose of a short-acting

compound like follitropin-β [Mannaerts et al 1993]. In contrast, the ovarian response of pituitary-suppressed volunteers following a single dose of long-acting corifollitropin alfa may be assumed to be close to the anticipated ovarian response in IVF patients [Duijkers et al 2002]. Studying the pharmacodynamic properties of short-acting compounds in volunteers may require multiple rising dose studies. Accordingly, follitropin-β was tested with a daily fixed dose of 75 IU, 150 IU and 225 IU given for 7 days [Mannaerts et al 1996b]. If the therapeutic dose has already been selected following phase II studies, rising dose studies may still be performed, mainly to examine whether exposure and response are dose-proportional [Oberyé et al 1999b].

Chapter 4

Phase II Dose-finding Trials and Dose Selection

Chapter 4 Phase II Dose-finding Trials and Dose Selection

4.1 Introduction

The purpose of phase II studies

The primary objective of a phase II clinical trial is to determine whether a new drug in its final formulation has sufficient biological activity for the target indication to warrant more extensive development. Phase II studies should reveal the minimum effective dose(s) and/or the final treatment regimen to be tested on a large scale during phase III studies. As such, this stage in drug development is most critical as mistakes in trial design or misjudgment of trial data may lead to discontinuation of drug development at a late stage.

Extensive dose-finding studies are not always needed. For example, preclinical research on follitropin-β showed that its bioactivity was indistinguishable from urinary FSH and that the elimination half-life was not significantly different following a single dose administration in Beagle dogs or in gonadotrophin-deficient volunteers [De Leeuw et al 1996; Mannaerts et al 1996b]. Moreover, follitropin-β was (and still is) calibrated in the same *in vivo* bioassay as the urinary FSH/hMG preparations which were routinely applied, usually in a long GnRH agonist protocol. However, in contrast to urinary FSH/hMG preparations, follitropin-β lacked LH activity and therefore a phase II feasibility trial using different GnRH agonist regimens [Devroey et al 1994] inducing different degrees of pituitary suppression was deemed essential before moving forward into phase III trials.

Dose-finding study design

The classical approach of dose-finding for a new drug is to perform a randomized dose-finding study including at least 3 different dosages to demonstrate which dosage is too low and which dosage is too high for the anticipated indication. Ideally the study is blinded, at least for the applied dosages and a reference group is included, either for comparison or to ensure the validity of the study data. Whether a classical dose-finding study is needed depends on the specific drug under investigation and the information available from previous preclinical and clinical research.

The GnRH antagonist ganirelix was tested in relatively high dosages during phase I trials as Syntex intended to develop ganirelix for the treatment of endometriosis and hormone-dependent cancers. However, the development of ganirelix for the prevention of premature LH surges during ovarian stimulation required the selection of a relatively low dose of ganirelix that would prevent premature LH surges but would not result in a too profound LH suppression which was known to compromise clinical outcome. Therefore, a double-blind, randomized dose-finding study including 6 different dosages of ganirelix between 0.0625 mg

and 2 mg was performed.[Ganirelix dose-finding study group, 1998] and the study was supervised by an external independent advisory committee.

Selection of the optimal dose of corifollitropin alfa was more complex as it involved 2 components, namely dose and interval, as the optimal dose had to support multiple follicular growth for the first 7 days of ovarian stimulation. Based on the phase I study in pituitary-suppressed female volunteers [Duijkers et al 2002], a dose of at least 120 µg was thought to be required for this purpose. A phase IIa feasibility study was designed in which 120, 180 and 240 µg corifollitropin alfa were tested in groups of 24-25 patients each [Devroey et al 2004]. The study included a reference group of daily 150 IU rFSH, not for statistical comparison but as a control group to ensure the validity of the trial results. The outcome of this pilot study showed a relatively flat dose-response relationship in which the 3 test doses of corifollitropin alfa resulted in a similar number of oocytes (mean range, 11.0 –12.0) indicating that the maximum of the dose response curve was reached. Thus the phase IIb dose-finding study [Corifollitropin Alfa Dose-Finding Study Group, 2008] included 60, 120 and 180 µg corifollitropin alfa with 75 to 79 patients per group and one study reference group of daily 150 IU rFSH. The design of the phase II dose-response trial was based on the maximal probability of demonstrating a dose-relationship, wherein the number of oocytes was correlated with the corifollitropin alfa dose. The dose-finding study applied a fixed start of ganirelix on stimulation day 5, as there were a considerable number of subjects with early LH rises prior to the start of the GnRH antagonist on stimulation day 6 in the feasibility study [Devroey et al 2004].

Modeling & simulation and dose-selection

Modeling and simulation (M&S) is a valuable tool to integrate knowledge throughout the various stages of drug development. Models of pharmacokinetic-pharmacodynamic relationships may be used to predict clinical outcome, to optimize the design of trials and to select the most optimal dose(s) for further drug development. The design of the dose-finding trial of corifollitropin alfa was informed and optimized using M&S and the recommended doses for phase III development were also based on M&S, implying that selected doses may differ from doses actually tested during phase II development [Zandvliet et al 2011].

Based on the results of the phase II dose-response trial, a sophisticated data analysis was carried out to determine the precise recommended dose of corifollitropin alfa for phase III development. M&S was also applied to investigate the impact of various doses of corifollitropin alfa and patient characteristics on clinical outcome. The model framework that was used for the phase II dose-response trial design, also served as a basis for dose selection. It included the pharmacokinetics of corifollitropin alfa, inhibin-B levels, the initial follicular response at stimulation day 8, and the number of oocytes retrieved.

The value of serum inhibin-B as a biomarker for sufficient or insufficient FSH activity to support follicular development was demonstrated in the phase II dose-finding study in which several subjects, treated with the too low dose of 60 µg corifollitropin alfa, showed an early inhibin-B decrease, whereas follicular development still continued. A typical example of insufficient stimulation, reflected by an inhibin-B gap and delayed arrest of follicular growth, ultimately resulted in cycle cancellation [Corifollitropin Alfa Dose-Finding Study Group, 2008]. The pharmacokinetic data of the dose-finding study also supported the strong and inverse relationship between exposure and body weight. Thus the final dose selection of corifollitropin alfa included the outcome of the dose-finding trial in combination with robust modeling and simulation aiming at sufficient exposure to sustain multiple follicular development for an entire week taking into account the impact of body weight on exposure [De Greef et al 2010; Ledger et al 2011].

4.2 Results

- Devroey P, Mannaerts BMJL, Smitz J, Coelingh Bennink H, Van Steirteghem A. Clinical outcome of a pilot efficacy study on recombinant human FSH (Org 32489) combined with various GnRH agonist regimens. *Hum Reprod* 1994; 9: 1064-1069.

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Clinical outcome of a pilot efficacy study on recombinant human follicle-stimulating hormone (Org 32489) combined with various gonadotrophin-releasing hormone agonist regimens

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In total, 50 couples participated in a pilot study evaluating the efficacy of various regimens of gonadotrophin-releasing hormone agonist (GnRHa) in association with recombinant human follicle-stimulating hormone (recFSH) in women undergoing in-vitro fertilization (IVF) and embryo transfer. The women were treated with recFSH alone (group I), or with recFSH in conjunction with pituitary desensitization using a buserelin intranasal spray, 4×150 μg per day, in a short protocol (group II) or in a long protocol (group III), or using triptorelin in a long protocol, giving a single dose of 3.75 mg i.m. (group IV) or daily s.c. injections of 200 μg (group V). In all women, treatment with recFSH resulted in multiple follicular growth and rises of serum inhibin and oestradiol. The latter indicates that the amount of remaining luteinizing hormone (LH) was sufficient to support FSH-induced oestrogen biosynthesis. On the day of human chorionic gonadotrophin (HCG) administration, endogenous LH was most profoundly suppressed in subjects treated with triptorelin. The median number of ampoules and treatment days required in the various treatment groups varied from 21 to 36 ampoules and from 7 to 14 days, respectively. The median number of oocytes per group ranged from 9 to 11 and all cumulus–corona–oocyte complexes, with the exception of two, were classified as mature. The median fertilization and cleavage rates ranged between the treatment groups from 40 to 73% and from 73 to 100%, respectively. Fertilization failure of retrieved oocytes occurred in six couples with andrological or unexplained infertility. One patient had no transfer because of insufficient embryo quality. Finally, 43 couples had an embryo transfer and the median number of embryos replaced in each group was three per transfer. Clinical pregnancies were established in 10 women, two of whom had a miscarriage, resulting in eight ongoing pregnancies (18.6% per transfer) and the birth of nine healthy children. It is concluded from the current study that recFSH treatment is effective and safe for patients and their offspring.

Key words: GnRH agonist/recombinant FSH/ovarian stimulation

Introduction

The expression of human follicle-stimulating hormone (FSH) in Chinese hamster ovary cells transfected with both subunit genes has resulted in the synthesis of intact recombinant human FSH (recFSH) (Keene *et al.*, 1989; Van Wezenbeek *et al.*, 1990). In comparison with natural FSH preparations, purified (>99%) recFSH (Org 32489, Puregon®) has a very high specific bioactivity (~10 000 IU/mg protein) and lacks intrinsic luteinizing hormone (LH) activity (Mannaerts *et al.*,, 1991). Phase I studies of recFSH in gonadotrophin-deficient male and female volunteers revealed that recFSH is safe and well tolerated, and that the elimination half-life of recFSH is comparable to that of urinary FSH (Mannaerts *et al.*, 1993). In gonadotrophin-deficient women, daily administration of recFSH induced multiple follicular growth up to the pre-ovulatory stage, whereas oestrogen and androgen concentrations in serum and follicular fluid remained very low as compared to those measurable in normally cycling women (Schoot *et al.*, 1992; Shoham *et al.*, 1993). These data indicate that in the absence of LH activity, production of androgens in the theca cell is diminished and insufficient aromatase substrate becomes available for oestrogen conversion in the granulosa cell. Although the significance of oestrogens for follicle development can be questioned, it remains beyond dispute that oestrogens are mandatory for other reproductive processes, e.g. inducing the mid-cycle LH surge, endometrial proliferation and cervical mucus production.

Current FSH/human menopausal gonadotrophin (HMG) therapy for the induction of controlled superovulation is frequently combined with gonadotrophin-releasing hormone agonist (GnRHa) treatment to prevent premature luteinization. After an initial release of LH and FSH, continuous use of GnRHa induces a reversible state of hypogonadotrophic hypogonadism. The suppressive potency of GnRHa is related to their structure–receptor interaction, elimination half-life, dosage and route of administration (Barron *et al.*, 1982; Brogden *et al.*, 1989). Most frequently, short-acting GnRHa is given daily by subcutaneous injection or nasal spray, whereas long-acting agonists can be administered intramuscularly or subcutaneously as a single injection (Loumaye, 1990).

This pilot efficacy study in women undergoing in-vitro fertilization (IVF) and embryo transfer was undertaken to evaluate whether recFSH therapy can be combined with GnRHa treatment, i.e. whether the amount of remaining endogenous LH activity due to pituitary desensitization is sufficient to support recFSH-induced multiple follicular growth, steroidogenesis and related reproductive processes. Therefore various GnRHa/recFSH regimens, providing various degrees of pituitary

suppression, were applied: treatment with recFSH only, or treatment in conjunction with pituitary desensitization using a buserelin intranasal spray in a short or long protocol, or using triptorelin in a long protocol by two different dosages and routes.

Materials and methods

Patients

In total 50 out of 54 women of infertile couples completed this single-centre study up to oocyte retrieval, which was the study end-point. Two women became spontaneously pregnant before hormonal treatment was started; in one woman (group I) progesterone concentration was too high before starting treatment; one woman (group I) was discontinued because of a premature LH surge. The overall mean (± SD) age, body weight and height of patients who completed the study were 30.4 (± 3.3) years, 58.6 (± 7.7) kg and 164.8 (± 6.4) cm, respectively. All women had normal ovulatory cycles, proven ovarian response to clomiphene citrate (Clomid; Merell Dow, Switzerland) and/or HMG (Humegon, Oss, The Netherlands) and were scheduled for IVF and embryo transfer. The study protocol was approved by the institutional ethical committee and written informed consent was obtained from all patients.

Study design

This study was an open, non-randomized pilot study in which subjects were treated for one treatment cycle only. After screening the patients were numbered consecutively and allocated to five different treatment groups. Group I (n = 9) was treated with recFSH only; groups II (n = 9) and III (n = 11) were treated with recFSH in conjunction with buserelin intranasal spray (Suprecur; Hoechst, Frankfurt, Germany), 4×150 μg per day, in a short protocol (group II) or long protocol (group III); groups IV (n = 11) and V (n = 10) were treated with recFSH in combination with triptorelin (Decapeptyl; Ferring, Malmö, Sweden) in a long protocol either by administering one single dose of 3.75 mg i.m. (group IV) or 200 μg daily s.c. (group V). GnRHa treatment was started on the first day of the menstrual cycle. Patients allocated to group I and II started recFSH treatment 2 days later (cycle day 3). In groups III, IV and V, recFSH treatment was started after ~14 days of GnRHa pre-treatment when pituitary down-regulation was established (see Results). RecFSH (75 IU/ampoule, Puregon®, Org 32489; Organon International, Oss, The Netherlands) was administered once daily i.m. in the buttock. The daily dose of recFSH was fixed for at least the first 3 treatment days to one (group II), two (groups III, IV and V) or three (group I) ampoules. Thereafter, the daily dose was adjusted per patient based on the outcome of ultrasound and/or hormone measurements. In groups II–V ovulation was induced with 10 000 IU of human chorionic gonadotrophin (HCG) (Pregnyl; Organon, Oss, The Netherlands) when at least three follicles of ≥17 mm were detectable or earlier when serum oestradiol exceeded 300 pg/ml per follicle of ≥12 mm. In group I, HCG was administered when endogenous LH showed significant rises, indicating follicular luteinization and ovulation. Oocyte retrieval was performed by ultrasound-guided vaginal puncture and classification of cumulus–corona–oocyte complexes and embryos was performed as previously described (Staessen *et al.*, 1989). At transfer a maximum of three embryos was replaced into the uterus and supernumerary embryos were cryopreserved. All subjects received luteal phase support by means of intravaginally administered micronized natural progesterone (600 mg/day; Utrogestan, Piette, Belgium) (Smitz *et al.*, 1992). Clinical pregnancies were defined as gestations with embryonic sacs, as identified by ultrasound; ongoing pregnancies were confirmed when positive fetal heart activity was detected 12–16 weeks after embryo transfer.

Ultrasonography and (anti-)hormone assays

During each treatment cycle, transvaginal ultrasonography was performed at regular intervals to measure growth of individual follicles (≥12 mm). To monitor hormonal responses, morning blood samples were analysed for FSH, oestradiol, LH and progesterone by means of commercial assays as previously described by Smitz *et al.* (1988a). Inhibin concentrations on the first recFSH treatment day and on the day of administration of HCG were measured in 25 of the 50 patients by means of a commercially available enzyme immunoassay from Medgenix (Fleurus, Belgium). The antibodies of this assay cross-react with free α-subunits and with large precursor molecules (>33 kDa). The sensitivity of this assay was 0.6 U/ml and the intra-assay and inter-assay coefficients of variation were <10%. The induction of anti-FSH antibodies after recFSH treatment was judged by means of interaction with ^{125}I-labelled recFSH in a sensitive radioimmunoprecipitation assay as previously described by Mannaerts *et al.* (1993).

Statistical analysis

All statistical tests were two-tailed and carried out at the 5% level of significance. The comparison of groups was carried out using a non-parametric approach, i.e. the Kruskal–Wallis test was performed to detect significant differences in the five treatment groups and the Mann–Whitney test was performed for the two-by-two comparisons in cases of a global significant difference in the Kruskal–Wallis test.

Results

Patient characteristics

In this open study, a comparison of age, weight and height did not reveal any significant differences between patients of the five treatment groups (Table I). Causes of infertility were tubal (n = 21), andrological (n = 11), unexplained (n = 11) and endometriosis (n = 7). In total, 27 women suffered from primary infertility and 23 women from secondary infertility.

Baseline characteristics

The median number of days required for pituitary down-regulation was 14 days in groups III, IV and V and ranged between 12 and 37 days of GnRHa treatment. Median baseline concentrations (and ranges) of FSH, LH, oestradiol and progesterone are presented in Table II and represent values measured on the first recFSH treatment day (groups I, III, IV and V) or values at the start of buserelin administration in a short protocol (group II). As expected, endogenous FSH and LH

Table I. Clinical characteristics of patients

	Treatment group[a]				
	I	II	III	IV	V
Number of patients	9	9	11	11	10
Age (years)	30.2 (±3.2)	27.7 (±2.1)	31.5 (±2.9)	30.9 (±3.9)	31.4 (±2.7)
Weight (kg)	54.7 (±6.3)	59.4 (±6.7)	59.5 (±8.7)	59.5 (±7.0)	59.3 (±9.4)
Height (cm)	164 (± 7)	165 (± 4)	164 (± 8)	164 (± 6)	167 (± 8)
Cause of infertility					
tubal	5	3	5	3	5
andrological	—	2	5	4	—
endometriosis	1	1	—	1	4
unexplained	3	3	1	3	1
Parity					
primary infertility	4	6	6	7	4
secondary infertility	5	3	5	4	6

[a]Treatment in groups I, II, III, IV and V was respectively recFSH alone; recFSH + buserelin intranasal spray, short protocol; recFSH + buserelin intranasal spray, long protocol; recFSH + triptorelin, long protocol, single dose; and recFSH + triptorelin, long protocol, daily dose.

concentrations measured at menstrual cycle days 3 and 1 in groups I and II respectively were higher than those measured after 2 weeks of desensitization. After down-regulation with buserelin or triptorelin, median concentrations of serum FSH and LH were comparable and ranged between 3 and 4 IU/l and between 2.4 and 3.2 IU/l respectively. With respect to baseline values of oestradiol and progesterone, no differences were noted between the five treatment groups.

Treatment dosage and duration

In Table III the total number of recFSH ampoules, the number of treatment days required to induce multiple follicular growth and the calculated daily dose per group are presented. The median numbers of ampoules used in patients treated with recFSH combined with a long protocol of buserelin or triptorelin in groups III, IV and V were comparable, i.e. 36, 35 and 32 ampoules, respectively, though a large inter-individual variability was noted within each treatment group. The total amount of recFSH required in patients treated without GnRHa or with buserelin in a short protocol was considerably lower, i.e. 21 and 22 ampoules, respectively. For group I (without GnRHa) this result was explained by the fact that five out of nine patients showed rises of LH or progesterone during recFSH treatment and received HCG (10 000 IU) after only 5–7 treatment days. As a result, the median number of treatment days was only 7 days in group I, whereas this period was 12–14 days in the other groups. The calculated median daily dose was three ampoules in group I, two ampoules in group II and 2.5–2.7 ampoules in groups III, IV and V.

Ovarian stimulation

In all women, treatment with recFSH resulted in follicular growth. The total median number of follicles of ≥12 mm measured 8–72 h before HCG administration ranged from 7 (group III) up to 11 (group V). The median number of large pre-ovulatory follicles of ≥17 mm was 5.5 in group V, three in groups II and III, two in group IV and only one in group I.

Median values and ranges of hormones measured on the day of HCG administration are presented in Table IV. Serum FSH concentrations were highest in group I and lowest in group II with median daily doses of three and two ampoules of recFSH respectively (see above). Comparable concentrations of FSH were

Table II. Median (range) values of hormones measured at the first day of recFSH (group I, III, IV, V) or 2 days prior to the start of recFSH treatment (group II) at the start of buserelin treatment

	Treatment group[a]				
	I	II	III	IV	V
FSH (IU/l)	9 (6–14)	6 (3–9)	4 (2–10)	3 (1–11)	4 (<1–7)
LH (IU/l)	6.7 (5.6–12.0)	4.9 (2.4–7.3)	3.2 (0.1–4.6)	2.8 (2.3–4.9)	2.4 (1.5–4.5)
Oestradiol (pg/ml)	39 (17–59)	33 (21–38)	33 (14–65)	27 (<15–34)	24 (<20–266)
Progesterone (ng/ml)	0.5 (<0.1–1.5)	0.4 (0.1–0.7)	0.2 (<0.1–0.8)	0.3 (<0.1–0.4)	0.1 (<0.1–1.0)

[a]See Table I.
FSH = follicle-stimulating hormone; LH = luteinizing hormone.

Table III. Median (ranges) values of total number of recFSH ampoules, treatment days and recFSH ampoules per treatment day

	Treatment group[a]				
	I	II	III	IV	V
Ampoules	21 (15–27)	22 (7–50)	36 (20–57)	35 (24–81)	32 (20–49)
Treatment (days)	7 (5–9)	12 (7–17)	14 (9–16)	14 (12–18)	13 (10–18)
Ampoules/ day	3.0 (2.3–3.0)	2.0 (1.0–2.9)	2.7 (2.0–3.6)	2.6 (2.0–4.8)	2.5 (2.0–3.5)

[a]See Table I.

Table IV. Median (range) values of hormones measured in blood samples taken on the morning of the day of HCG administration

	Treatment group[a]				
	I	II	III	IV	V
FSH (IU/l)	21 (14–26)	13 (4–17)	15 (4–24)	17 (9–27)	17 (8–30)
LH (IU/l)	5.1 (1.2–20.0)	2.3 (<0.5–12.0)	1.3 (<0.5–7.1)	1.2 (0.8–3.5)	1.6 (<0.5–2.7)
Oestradiol (pg/ml)	1101 (684–2467)	1899 (948–2640)	1773 (711–2958)	1768 (781–2952)	1531 (893–3350)
Progesterone (ng/ml)	0.5 (0.2–1.6)	0.3 (0.2–1.3)	0.6 (<0.1–1.5)	0.5 (0.2–1.3)	0.9 (0.3–4.5)

[a]See Table I.

measured in groups III–V treated with a GnRHa in a long protocol. Median and maximal concentrations of circulating immunoactive LH reflected the degree of pituitary suppression due to various regimens applied, being most profound in subjects treated with triptorelin (Table IV).

No differences ($P = 0.6$) were noted between groups with respect to serum oestradiol concentrations. Serum progesterone concentrations were comparable between groups I–IV and higher in group V, which is in accordance with the larger number of pre-ovulatory follicles of ≥17 mm noted in this group.

Inhibin concentrations, assessed in 25 out of 50 patients prior to the first recFSH injection and on the day of HCG injection, were increased in all cases. The overall median value of serum inhibin was 11.3 (2.3–23.1) IU/l after recFSH treatment versus <0.6 (<0.6–1.8) IU/l prior to the first recFSH injection.

Oocyte retrieval, embryo transfer and cycle outcome

The total number of oocytes retrieved and fertilization and cleavage rates are given in Table V. The median number of oocytes retrieved ranged between 7 and 11 and was not significantly different between the treatment groups. All cumulus–corona–oocyte complexes were classified as mature, with the exception of two embryos in groups III and IV, respectively. The median fertilization and cleavage rates ranged between the treatment groups from 40 to 73% and from 73 to 100%, respectively. Attempts to fertilize oocytes failed in six couples with andrological ($n = 3$) and unexplained ($n = 3$) infertility. One patient had no transfer because of insufficient embryo quality. In total, 43 couples had an embryo transfer and the mean number of embryos replaced per group ranged between 2.3 and 2.8.

In group I no pregnancy was established, although one subject had a positive initial HCG test. In groups II–V clinical pregnancies were established in 10 women, two of whom had a miscarriage, resulting in eight ongoing pregnancies (18.6% per transfer) including one twin pregnancy (Table VI).

Pregnancy outcome

The ongoing pregnancies achieved were uneventful and nine healthy children, weighing (mean ± SD) 3087 ± 381 g, were born at a gestational age of 36 (twin) to 40 weeks. Extensive paediatric examination of the newborns revealed only minor malformations in the set of twins, i.e. one baby was diagnosed with mild hypospadias and the other with a torticollis. The latter disappeared within 10 weeks of kinesitherapy.

Table V. Median (range) number of oocytes recovered and fertilization and cleavage rates (%)

	Treatment group[a]				
	I	II	III	IV	V
Oocytes/retrieval	7 (3–23)	9 (6–13)	11 (2–18)	10 (4–20)	11 (6–19)
Fertilization rate	64 (0–100)	64 (0–100)	73 (43–100)	40 (0–87)	62 (26–89)
Cleavage rate	73 (50–100)	90 (57–100)	78 (33–100)	85 (71–100)	100 (40–100)

[a]See Table I.

Table VI. Treatment cycle outcome

	Treatment group[a]				
	I	II	III	IV	V
Transfers	7	7	11	8	10
Miscarriage			2		
Single vital pregnancy		2	2	2	1
Multiple vital pregnancy		1			

[a]See Table I.

Safety aspects

Daily i.m. injection of recFSH was well tolerated and was without induction of pain or skin redness. One woman who became pregnant after triptorelin/recFSH treatment (group IV) was hospitalized twice with the diagnosis of ovarian hyperstimulation, grade II. No other drug-related adverse experiences occurred. Anti-FSH antibody formation was not noted in any of the patients.

Discussion

This is the first efficacy study of recFSH (Org 32489) in healthy women undergoing IVF embryo transfer (Devroey *et al.*, 1993). The efficacy data show that recFSH stimulates normal multiple follicular development, as demonstrated by the number of pre-ovulatory follicles, rises of serum inhibin and oestradiol and the number of mature oocytes recovered. The increases of serum oestradiol indicate that the amount of remaining endogenous LH, even after profound pituitary suppression with triptorelin, is still sufficient to support FSH-induced oestrogen biosynthesis. Thus, in IVF patients with normal regular cycles the amount of LH required (threshold concentration) is extremely low. Furthermore, the study data demonstrate the successful establishment of pregnancies regardless of the GnRHa regimen applied, and the overall ongoing pregnancy rate was comparable to that previously reported after stimulation with gonadotrophins of urinary origin (Smitz *et al.*, 1988b, 1992). The case of one of the first patients in this study, allocated to group II, who was successfully treated with only nine ampoules of recFSH (675 IU) was reported previously as the first ongoing pregnancy and singleton term birth after ovarian stimulation with recFSH (Devroey *et al.*, 1992a,b). Another pregnancy obtained with GONAL-F® was published (Germond *et al.*, 1992).

The current pilot study did not intend to compare the success rates of different GnRHa/recFSH regimens. The number of observations was far too small and the inter-subject variability too large for this purpose. Comparison of ovarian responsivity due to treatment with recFSH alone, or with recFSH combined with GnRHa in a short protocol, with that induced by recFSH and GnRHa in a long protocol is complicated due to different baseline conditions of hormones and follicles at the start of recFSH therapy. Moreover, in the current study the starting dose of recFSH during the first 3 treatment days was fixed to three ampoules in patients treated with recFSH only, one ampoule (75 IU) in patients treated in the short protocol and two ampoules in those treated in a long protocol of GnRHa. Such treatment differences are likely to influence the initial selection of small antral follicles (Baird, 1987). Therefore, only the ovarian responses of subjects treated with buserelin (intranasal, group III) or triptorelin (i.m. or s.c., groups IV and V) in a long protocol are comparable, but the clinical outcome of these three treatment groups was very similar. The relatively low recFSH dose and short treatment period required in patients treated with a short protocol of buserelin, in which three out of nine patients became pregnant, illustrates the very favourable cost-efficacy ratio of this regimen.

In addition to actions on the anterior pituitary gland, GnRH and its agonists may exert direct effects on the ovary via highly specific GnRH receptors (Latouche *et al.*, 1989). It has been described previously that GnRHa may suppress FSH-induced cellular differentation and steroidogenesis by impairing the expression of LH receptors and induction of aromatase activity (Parinaud *et al.*, 1988; Guerrero *et al.*, 1993; Testart *et al.*, 1993). Such direct inhibitory effects might also contribute in a longer treatment period and/or higher dose of (rec)FSH/HMG required to induce ovulation when combined with GnRHa administration in a long protocol.

In summary, the successful treatment of IVF patients with recFSH in conjunction with various methods of pituitary desensitization is promising, since in the current study GnRHa/recFSH therapy appeared to be effective and safe for patients and their offspring. However, further clinical studies on recFSH treatment combined with pituitary desensitization and in comparison to urinary FSH/HMG will be required to prove the long-term efficacy and safety of this new biosynthetic hormone preparation.

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A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon®)

The ganirelix dose-finding study group*

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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 (rFSH; Puregon®) to establish the minimal effective dose preventing premature luteinizing hormone (LH) surges during ovarian stimulation. For ovarian stimulation, rFSH 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 gonadotrophin (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 rFSH 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 oestradiol fell with increasing Org 37462 dose. During Org 37462 treatment, serum LH concentrations ⩾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 ⩾11, ⩾15 and ⩾17 mm were similar in the six dose groups, whereas serum oestradiol 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 rFSH 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 zero (0.0625%) up to a maximum of 3.7% (0.5 mg group). 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.

Keywords: GnRH antagonist ganirelix/IVF/ovarian stimulation/recombinant FSH/LH surge prevention

Introduction

Gonadotrophin-releasing hormone (GnRH) analogues are used for a variety of disorders in which reversible suppression of the pituitary–gonadal axis is desired (Conn and Crowley, 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 gonadotrophins (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 gonadotrophins 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;

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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 gonadotrophins (HMG) were reported (Diedrich *et al*., 1994; Olivennes *et al*., 1994, 1995; 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 ⩾10 IU/l during Org 37462 treatment, the clinical outcome and the overall tolerance.

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 (rFSH). One patient who started rFSH 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 rFSH. 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 rFSH (Puregon®, NV Organon).

A schematic description of the applied treatment regimen is given in Figure 1. rFSH 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 rFSH, spontaneous pregnancy was excluded. During the first five treatment days, the daily dose of rFSH was fixed at 150 IU. After 5 days of rFSH treatment, Org 37462 treatment was begun with daily s.c. administration, the rFSH 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 gonadotrophin (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.

Assessments

During the first 5 days of only rFSH treatment, blood samples for hormone analysis were collected once daily in the morning just before rFSH 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, oestradiol 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 by 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 rFSH were reported to have follicular growth arrest and falling serum oestradiol 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 rFSH treatment but were switched during Org 37462 treatment to HMG, one patient stopped Org 37462 treatment 1 day before stopping rFSH treatment, and one patient donated her oocytes.

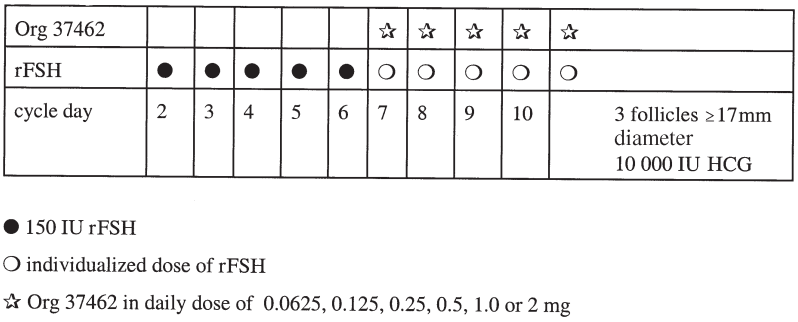

Figure 1. Schematic description of the treatment regimen using recombinant follicle stimulating hormone (rFSH) for ovarian stimulation and Org 37462 for the prevention of premature luteinizing hormone (LH) surges. HCG = human chorionic gonadotrophin.

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 and 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.

Results

Patient characteristics

The six treatment groups were comparable in demographic and infertility 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 infertility 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.

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 I. 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 rFSH to HMG because of insufficient ovarian response. All five women finally had embryo transfer, and one became pregnant.

Table I. Numbers of subjects per Org 37462 treatment group included in the efficacy analysis

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

HMG, human menopausal gonadotrophin; LH, luteinizing hormone; rFSH, recombinant follicle stimulating hormone.

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.

Serum hormone concentrations before Org 37462 treatment

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

A fixed daily dose of 150 IU rFSH for 5 days increased median serum FSH concentrations by 1.2 IU/L. Serum oestradiol 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 (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

Table II. Median and 5th and 95th percentiles (in parentheses) of serum hormone concentrations at the start of recombinant follicle stimulating hormone (rFSH) treatment (stimulation day 1) and before the first Org 37462 injection (stimulation day 6)

	FSH (IU/l)	LH (IU/l)	Androstenedione (ng/ml)	Oestradiol (pg/ml)	Progesterone (ng/ml)
Day 1 of rFSH	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 rFSH	7.7 (5.8–11.1)	1.8 (0.66–6.4)	2.0 (1.1–3.5)	301.0 (84.2–777)	0.4 (<0.25–0.85)

FSH = follicle stimulating hormone; LH = luteinizing hormone.

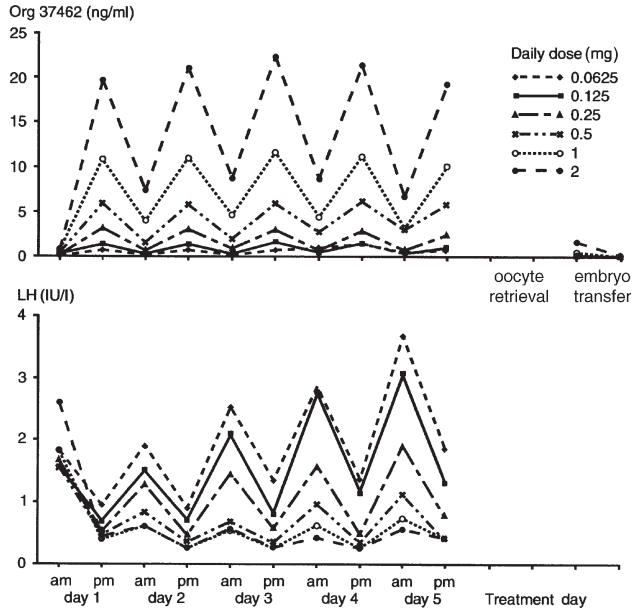

Figure 2. Serum Org 37462 concentrations (upper panel) and serum immunoreactive luteinizing hormone (LH) concentrations (lower panel) measured just before each Org 37462 injection in the morning (a.m.) and measured approximately 8 h later in the afternoon (p.m.) for patients with at least 5 days of Org 37462 treatment.

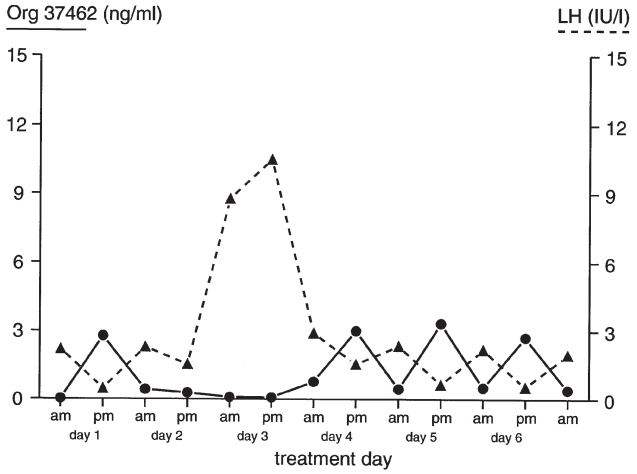

Figure 3. Typical example of a patient with an immediate luteinizing hormone (LH) rise due to non-compliance toward daily treatment with 0.25 mg Org 37462.

37462 administration, and 0.7, 1.5, 3.0, 6.1, 11.4 and 23.0 ng/ml about 8 h 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 oestradiol concentration after 5 days of once-daily 150 IU rFSH 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 oestradiol during the first five stimulation days were relatively high, ranging from 700 to 1860 pg/ml on the first day 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 3. The remaining 12 patients with an LH rise showed daily drug compliance during Org 37462 treatment (Figure 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).

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.

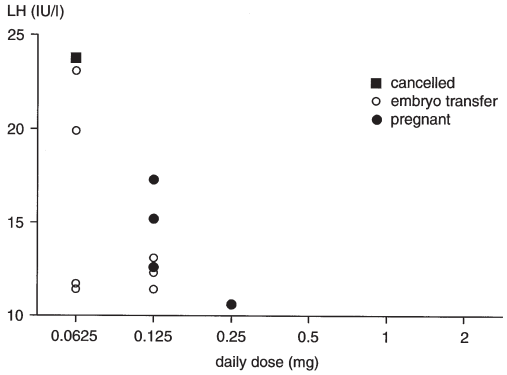

Figure 4. Scatter plot of individual luteinizing hormone (LH) values ⩾10 IU/l measured during Org 37462 treatment.

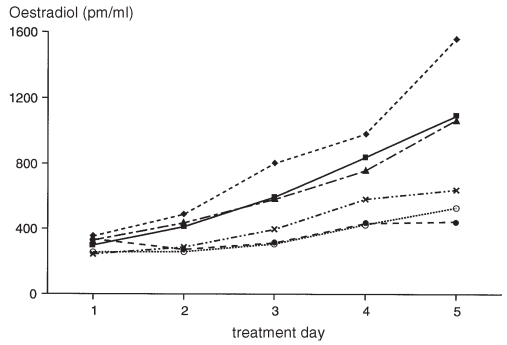

Figure 5. Serum oestradiol concentrations measured once daily just before each Org 37462 injection for patients with at least 5 days of Org 37462 treatment. See Figure 2 for key.

Total dose of rFSH and duration of Org 37462 treatment

Little difference was noted between the dose groups with respect to the total amount of rFSH 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, 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 rFSH treatment onwards, increases in serum oestradiol concentrations fell in relation to the increasing doses of Org 37462. In addition, in the 2 mg dose group, a slight decline in serum oestradiol was seen after the first Org 37462 administration (Figure 5).

Median values of hormone concentrations measured on the day of HCG administration are shown in Table III. 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 II). In parallel to endogenous LH, rises in serum oestradiol also decreased largely with increasing Org 37462 doses. Serum oestradiol 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 oestradiol 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 37462 treatment and on the day of HCG

On day 6 of rFSH 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.

Treatment outcome

The clinical outcome for the main parameters is given in Table IV. 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 IV), but the dose selection procedure indicated both 0.125 and 0.25 mg as eligible doses. In total, 13 miscarriages were reported, but

Table III. 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

	Daily dose of Org 37462 (mg)					
	0.0625	0.125	0.25	0.50	1	2
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)
Androstenedione (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)
Oestradiol (pg/ml)	1475 (645–3720)	1130 (462–3780)	1160 (384–3910)	823 (279–2720)	703 (284–2340)	430 (166–884)
Progesterone (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)

FSH = follicle stimulating hormone; LH = luteinizing hormone.

Table IV. Clinical outcome in the six different dose groups for all patients who started Org 37462 treatment (per attempt) unless otherwise indicated

	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 (2/54)	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[a]/27)	25.0 (15/60)	37.1 (23/62)	13.0 (7/54)	15.3 (9/59)	0 (0/23)

[a]Including one subject who was lost to follow-up after assessment of a vital pregnancy.
Values are mean ± SD, unless otherwise indicated.

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 oestradiol 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 6).

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 oestradiol 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).

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 1 h after Org 37462 injection was 20.5% and 1.2%, respectively. At this time point, skin redness was most

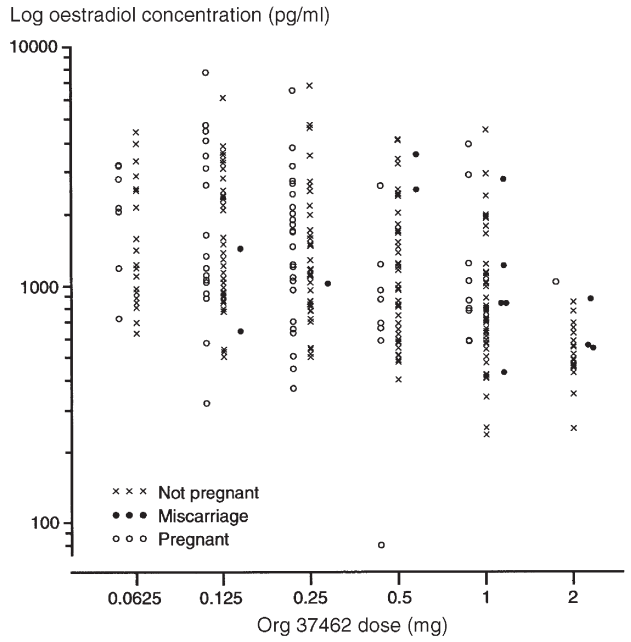

Figure 6. Scatter plot of individual serum oestradiol concentrations on the day of human chorionic gonadotrophin (HCG) of women who became pregnant and those who had a miscarriage or menses.

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.

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 rFSH, 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 (≥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 oestradiol 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 oestradiol 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 ≤1 IU/l, which is much lower than in patients undergoing controlled ovarian stimulation with rFSH 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 rFSH to HMG. Whether this reversal was related to the exogenous LH activity and/or increasing oestrogen concentrations remains unclear, but if so, this finding would be in contrast to those of previous studies in gonadotrophin-deficient women, demonstrating that despite minimal oestrogen increases, rFSH may induce normal follicle growth up to pre-ovulatory stages (Couzinet *et al*., 1988; Schoot *et al.*, 1992, 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 oestradiol 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 oestradiol rises were less apparent, as patients were treated daily with HMG containing LH activity (Albano *et al*., 1996, 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 oestradiol 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 rFSH (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 oestradiol 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 rFSH 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 rFSH used in a long protocol of the GnRH agonist buserelin (Out *et al.*, 1996). 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 gonadotrophins, 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 rFSH resulted in lower peak serum oestradiol concentrations than previously reported for rFSH 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 oestradiol 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.

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 and 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-hoc 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.

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A randomized dose–response trial of a single injection of corifollitropin alfa to sustain multifollicular growth during controlled ovarian stimulation†

The Corifollitropin Alfa Dose-finding Study Group[1]

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BACKGROUND: This study primarily investigated the dose–response relationship of corifollitropin alfa to initiate multifollicular development for the first 7 days of controlled ovarian stimulation (COS). METHODS: Women aged 20–39 years undergoing COS for *in vitro* fertilization or intracytoplasmic sperm injection were randomized to a single dose of corifollitropin alfa 60, 120 or 180 μg, or daily injections of 150 IU recombinant follicle-stimulating hormone (rFSH). Patients treated with corifollitropin alfa started fixed daily treatment with 150 IU rFSH on stimulation Day 8. Patients received a GnRH antagonist (ganirelix 0.25 mg/day) from stimulation Day 5 until the day of human chorionic gonadotrophin. RESULTS: Pharmacokinetics of corifollitropin alfa were dose-proportional. The main reason for not having embryo transfer was insufficient ovarian response in 30.8, 2.6, 3.8 and 7.4% of patients in the corifollitropin alfa 60, 120, 180 μg and rFSH groups, respectively. On Day 8, the mean (standard deviation) number of follicles ≥11 mm was 6.8 (4.4), 10.1 (6.1) and 12.8 (7.5), respectively. The number of cumulus–oocyte complexes retrieved showed a clear dose–response relationship ($P < 0.0001$), being 5.2 (5.5), 10.3 (6.3) and 12.5 (8.0) in the three dose groups, respectively. CONCLUSIONS: A single injection of corifollitropin alfa induces dose-related increase in multifollicular development and in the number of retrieved oocytes. The optimal dose for a 1-week interval is higher than 60 μg and lower than 180 μg and will be selected based on modelling and simulation taking into account insufficient stimulation as well as overstimulation. Clinical Trials gov: NCT00598208.

Keywords: corifollitropin alfa; rFSH; controlled ovarian stimulation; IVF; sustained follicle stimulant

Introduction

Current treatment regimens for controlled ovarian stimulation (COS) in women undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) usually require daily self-injection of follicle-stimulating hormone (FSH). Although advances in methods of FSH administration (e.g. self-injection with pen devices) have helped to lessen the burden for patients, the need for daily FSH injections is still present. New gonadotrophin preparations with longer half-lives are being developed that may lower the frequency of FSH administration (Fares *et al*., 1992; Duijkers *et al*., 2002; Klein *et al*., 2003; Perlman *et al*., 2003). When combined with the advantages of gonadotrophin-releasing hormone (GnRH) antagonist regimens (Hohmann *et al*., 2003; Kolibianakis *et al*., 2006; Tarlatzis *et al*., 2006; Heijnen *et al*., 2007), the development of new clinical protocols that can provide similar success rates with fewer injections may help reduce the treatment burden to further enhance the acceptability for patients undergoing COS.

Corifollitropin alfa (Org 36286, NV Organon, a part of Schering-Plough Corporation, Oss, the Netherlands), a sustained follicle stimulant, is a novel recombinant fertility hormone which consists of the α-subunit of human FSH and a hybrid subunit consisting of the carboxy terminal peptide (CTP) of the β-subunit of human chorionic gonadotrophin (hCG) coupled with the FSH β-subunit. This molecular structure includes O-linked carbohydrate chains at the CTP, which results in an increased half-life *in vivo* compared with recombinant FSH (rFSH) (Fares *et al*., 1992). Previous studies in hypogonadotrophic hypogonadal men, pituitary-suppressed healthy women and women with anovulatory infertility have indicated that the terminal half-life of corifollitropin alfa is approximately twice that of rFSH (Bouloux *et al*., 2001; Duijkers *et al*., 2002; Balen *et al*., 2004). These studies have also shown that administration of corifollitropin alfa is safe and well-tolerated and does not result in antibody formation (Bouloux *et al*., 2001; Duijkers *et al*., 2002; Balen *et al*., 2004).

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In a previous smaller study of corifollitropin alfa in women scheduled for IVF or ICSI, single doses of 120, 180 or 240 μg induced and maintained multiple follicular growth for 1 week in a GnRH antagonist protocol (Devroey *et al*., 2004). However, the outcome of this first trial showed that corifollitropin alfa was effective in the dose range of 120–240 μg, but that the dosages tested were too high to demonstrate a significant dose–response relationship. In addition, the amount of additional rFSH needed to reach the stage of hCG administration was similar in each of the corifollitropin alfa dose groups. The current study in women undergoing COS for IVF or ICSI was designed to investigate the dose–response relationship with respect to the number of oocytes retrieved following a single injection of corifollitropin alfa over a lower dose range (60–180 μg). The outcome of this investigation allowed subsequent modelling and simulation to identify the optimal dose of corifollitropin alfa in a 1-week regimen. A reference group of subjects treated with daily fixed 150 IU rFSH was used only as a positive control for a standard (mild) GnRH antagonist treatment regimen.

Materials and Methods

This was a multicentre, open-label, randomized study with three doses of corifollitropin alfa (60, 120 and 180 μg) in a COS regimen for IVF or ICSI. The primary objective of this study was to investigate the dose–response relationship of corifollitropin alfa. A treatment arm with rFSH was included for reference only. The study was approved by the Health Authority and Independent Medical Ethics Committee for each centre and conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guidelines and Good Clinical Practice. Written informed consent was provided by all patients.

Patients

Patients were women aged 20–39 years with a normal menstrual cycle (24–35 days) and a body mass index of 17–31 kg/m^2 with an indication for COS before IVF or ICSI. Women with a history of ovarian hyperstimulation syndrome (OHSS), polycystic ovary syndrome (PCOS) or any endocrine abnormality were excluded. Other exclusion criteria included a previous poor response to FSH or human menopausal gonadotrophin (hMG), more than three unsuccessful COS cycles since last ongoing pregnancy, fewer than two ovaries, abnormal hormone levels during Days 2–7 of the menstrual cycle, use of hormonal preparations within 1 month before treatment or previous use of corifollitropin alfa.

Study design

Patients were randomized after evaluation of screening data to confirm eligibility. Randomization to one of the four treatment groups (1:1:1:1 ratio) was stratified by age (<32 or ≥32 years) and by centre using a central remote allocation procedure using a fixed block size of four and a minimization algorithm combined with randomly permutated blocks. Patients randomized to corifollitropin alfa started ovarian stimulation on Day 2 or 3 of their menstrual cycle with a single subcutaneous (SC) dose of 60, 120 or 180 μg. This was followed 1 week later (treatment Day 8) by a fixed daily dose of SC rFSH 150 IU (follitropin beta, Follistim® AQ/Puregon®, NV Organon) up to the day of hCG administration. Patients in the reference group received a fixed daily dose of SC rFSH 150 IU from cycle Days 2 or 3 up to the day of hCG. FSH administration on the day of hCG was optional for all patients. To prevent premature luteinizing hormone (LH) surges, patients were treated with a fixed GnRH antagonist regimen of SC ganirelix 0.25 mg (ganirelix acetate injection, Orgalutran®, NV Organon) from stimulation Day 5 up to and including the day of hCG. Maximum total treatment duration was 19 days. To induce final oocyte maturation, hCG 10 000 IU (Pregnyl®, NV Organon) were to be given when three follicles ≥17 mm were observed by ultrasound scan (USS). Approximately 30–36 h thereafter, oocyte retrieval followed by IVF or ICSI was performed. At embryo transfer, which took place 2–5 days after oocyte retrieval, up to three embryos could be transferred. All patients received daily progesterone for luteal phase support for at least 2 weeks or until menses. Outcomes achieved with spare, frozen embryos were reported during the first year after treatment.

Assessments

Before the start of ovarian stimulation, pregnancy was excluded by means of an hCG test. A blood sample was obtained for hormone assessments, and USS was performed. Patients returned to the clinic for USS and blood sampling on Days 3 and 5, and then daily up to and including the day of hCG. Additional blood samples to assess hormone levels were collected on the day of oocyte retrieval, on the day of embryo transfer and 2 weeks after embryo transfer.

Validated immunoassays (Devroey *et al*., 2004) were performed at a central laboratory to measure serum levels of FSH, LH, estradiol (E_2), progesterone (NV Organon, Waltrop, Germany), inhibin-B, corifollitropin alfa and antibodies against corifollitropin alfa.

Statistics

The sample size was based on the primary objective to investigate the dose–response relationship for the number of cumulus–oocyte complexes retrieved (primary endpoint). Assuming a linear dose–response model with log-dose as explanatory variable, with a positive slope of 3.3 (implying a difference of four oocytes between the lowest and highest corifollitropin alfa doses) and a standard deviation (SD) of seven oocytes, a sample size of 80 patients per group was sufficient to detect a positive dose–response with a power of 90% at the 0.05 significance level.

The number of cumulus–oocyte complexes retrieved was defined as the primary outcome parameter for this trial and analyzed accordingly. The number of cumulus–oocyte complexes retrieved was analyzed per started cycle (number set to zero for patients without oocyte retrieval) and per oocyte retrieval. The dose–response was investigated using the log-dose effect in an analysis of covariance (ANCOVA) model, with log-dose as covariate and centre as factor. The treatment effect on the number of oocytes was also evaluated using an analysis of variance (ANOVA) model with centre and treatment as factors. Confidence intervals of the differences between each corifollitropin alfa dose group and the rFSH reference group were calculated using this model.

In addition, the number of follicles and E_2 levels on Day 8 were analyzed using the ANCOVA model, with log-dose as covariate and centre as factor.

Other variables that were evaluated included dose of rFSH required, number and size of follicles, serum hormone levels, fertilization rate, number and quality of embryos obtained and transferred, and pregnancy rates. Descriptive summary statistics were calculated for these parameters. Formal statistical comparisons between the corifollitropin alfa and rFSH treatment groups were not planned or performed for these other parameters.

All efficacy analyses were based on the intent-to-treat (ITT) population, which included all randomized patients who received corifollitropin alfa or at least one dose of rFSH. One patient

randomized to the 60 μg group was mistakenly treated with rFSH instead. This patient is included within the corifollitropin alfa 60 μg group for ITT analyses.

The pharmacokinetics of corifollitropin alfa were assessed using a one-compartment model with first-order absorption, first-order elimination and body weight as covariate and a non-linear mixed-effects model program (NONMEM) was used for the analysis. Population (mean) and individual parameter estimates were determined.

Results

A total of 325 patients were randomized at 17 centres across Europe (Fig. 1), 315 of whom received treatment. Non-treated patients dropped out because of spontaneous pregnancy or personal reasons. Treatment groups had comparable demographic and fertility characteristics at baseline (Table I). Mean age (SD) of the patients in this study was 32.1 (3.7) years, mean body weight (SD) was 64.5 (9.1) kg and mean body mass index (SD) was 23.0 (3.0) kg/m^2. Mean serum hormone levels were similar in all treatment groups at screening.

Thirty-nine patients who started ovarian stimulation with corifollitropin alfa or rFSH did not receive hCG, most of whom ($n = 23$) were in the corifollitropin alfa 60 μg group (Table I). Overall, 34 patients in the 60 μg group (44%), seven in the 120 μg group (9%), nine in the 180 μg group (11%) and 15 in the rFSH group (19%) were discontinued

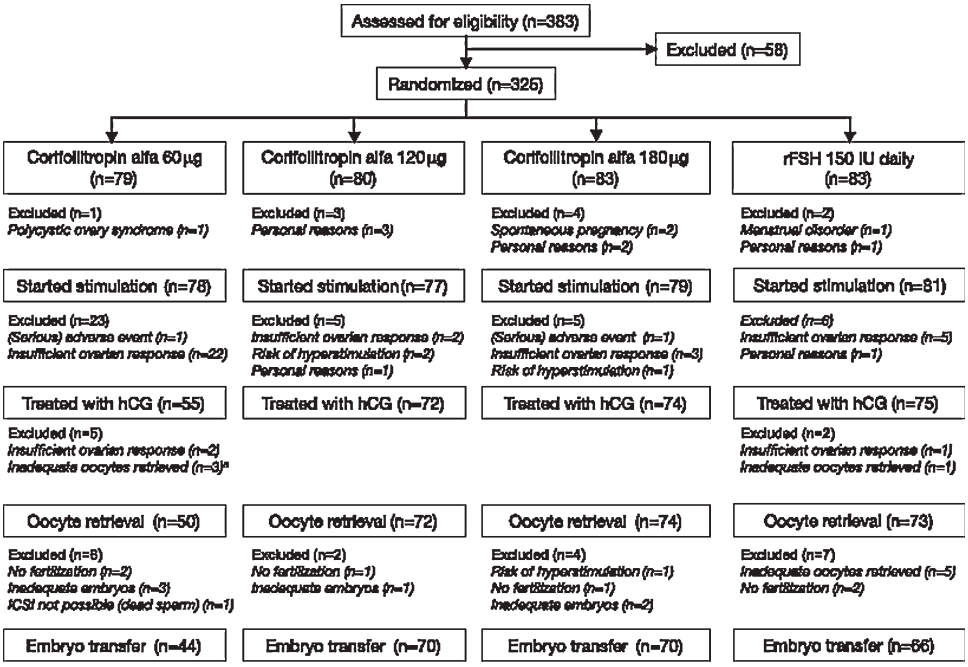

Figure 1: CONSORT diagram showing the flow of participants through each stage of this randomized controlled trial. hCG, human chorionic gonadotrophin; ICSI, intracytoplasmic sperm injection; rFSH, recombinant follicle-stimulating hormone. [a]Inadequate oocytes or embryos = none, too few or poor quality.

Table I. Baseline characteristics and disposition of patients by assigned treatment group (intent-to-treat population).

	Corifollitropin alfa			rFSH
	60 μg ($n = 78$)	120 μg ($n = 77$)	180 μg ($n = 79$)	150 IU daily ($n = 81$)
Age (years), mean (SD)	32.0 (3.5)	32.0 (4.1)	32.4 (3.5)	32.1 (3.8)
Body weight (kg), mean (SD)	63.8 (9.0)	64.8 (8.8)	64.0 (9.3)	65.2 (9.3)
Body mass index (kg/m^2), mean (SD)	22.7 (3.2)	23.5 (3.2)	22.7 (2.6)	23.1 (3.0)
Primary infertility, *n* (%)	40 (51.3)	46 (59.7)	31 (39.2)	42 (51.9)
Cause of infertility, *n* (%):				
Male factor	28 (35.9)	27 (35.1)	32 (40.5)	38 (46.9)
Endometriosis	3 (3.8)	8 (10.4)	6 (7.6)	6 (7.4)
Tubal	17 (21.8)	13 (16.9)	14 (17.7)	10 (12.3)
Other	1 (1.3)	3 (3.9)	0	1 (1.2)
Unknown	22 (28.2)	18 (23.4)	21 (26.6)	21 (25.9)
Multiple causes	7 (9.0)	8 (10.4)	6 (7.6)	5 (6.2)
Duration of infertility (years), mean (SD)	3.2 (1.8)	3.3 (1.9)	3.1 (2.5)	3.1 (2.2)
Started stimulation, *n* (%)	78[a] (100)	77 (100)	79 (100)	81 (100)
hCG, *n* (%)	55 (70.5)	72 (93.5)	74 (93.7)	75 (92.6)
Oocyte retrieval, *n* (%)	50 (64.1)	72 (93.5)	74 (93.7)	73 (90.1)
Embryo transfer, *n* (%)	44 (56.4)	70 (90.9)	70 (88.6)	66 (81.5)

[a]One patient randomized to corifollitropin alfa 60 μg was mistakenly treated with rFSH 150 IU daily.

Table II. Derived pharmacokinetic parameters [mean (SD)].

	Corifollitropin alfa		
	60 μg ($n = 75$)	120 μg ($n = 75$)	180 μg ($n = 76$)
$t_{\frac{1}{2}}$ (h)	65.7 (5.0)	65.3 (5.1)	66.0 (5.8)
t_{max} (h)	41.9 (6.1)	41.2 (6.0)	44.1 (7.2)
C_{max} (ng/ml)	1.90 (0.69)	3.71 (1.2)	5.53 (1.7)
dn-C_{max} ([ng/ml]/μg)	0.0317 (0.0114)	0.0309 (0.0101)	0.0307 (0.0096)
$AUC_{o\text{-}\infty}$ (ng.h/ml)	275 (86)	534 (156)	827 (233)
dn-$AUC_{o\text{-}\infty}$ ([ng.h/ml]/μg)	4.58 (1.4)	4.45 (1.3)	4.59 (1.3)
	Body weight		
	50 kg	65 kg	80 kg
$t_{\frac{1}{2}}$ (h)	63.8	66.6	68.2
C_{max} (ng/ml)	5.12	3.51	2.67
$AUC_{o\text{-}\infty}$ (ng.h/ml)	735	519	402

$AUC_{o\text{-}\infty}$, area under the curve from time zero to infinity; C_{max}, maximum serum concentration; dn, dose-normalized; $t_{1/2}$, terminal half-life; t_{max}, time to maximum serum concentration. Data based on all randomized patients treated with corifollitropin alfa with ≥1 serum concentration and documented dosing and sampling time.

(i.e. cycle cancelled) before embryo transfer. The main reason for cycle cancellation was insufficient ovarian response; occurring in 30.8, 2.6, 3.8 and 7.4% of patients in the corifollitropin alfa 60, 120, 180 μg and rFSH groups, respectively.

Controlled ovarian stimulation

Median duration of stimulation in the corifollitropin groups was 10–11 days. The total amount of rFSH required from Day 8 to reach the criteria of hCG administration was 600 IU (4 days), 450 IU (3 days) and 300 IU (2 days) in the 60, 120 and 180 μg groups, respectively. In the rFSH reference group, the median duration of stimulation was 10 days and the total amount of rFSH administered was 1350 IU.

Pharmacokinetics

Pharmacokinetic parameters for corifollitropin alfa are summarized in Table II. Maximum serum concentration (C_{max}) and area under the curve from time zero to infinity ($AUC_{0\text{-}\infty}$) of corifollitropin alfa were dose-proportional within the range 60–180 μg, whereas terminal half-life (mean 65–66 h) was independent of dose. Actual serum levels of corifollitropin alfa are plotted together with population-predicted curves for a patient with a body weight of 65 kg in Fig. 2. Corifollitropin alfa exposure showed an inverse relationship with body weight, which was found to be a significant covariate of clearance and volume of distribution. Using population-predicted parameters, C_{max} and $AUC_{0\text{-}\infty}$ were shown to be almost twice as high in a 50 kg body weight patient compared with a patient with a body weight of 80 kg, as depicted in the lower panel of Table II.

Follicular dynamics

From start of COS to treatment Day 6, the number and size of follicles were comparable between groups. However, from Day 6 onwards, the number of follicles ≥11 mm was higher in the corifollitropin alfa 120 and 180 μg groups compared with the 60 μg group. On Day 8, before administration of rFSH, the mean (SD) number of follicles ≥11 mm showed a dose-related increase ($P < 0.001$) and was 6.8 (4.4), 10.1 (6.1) and 12.8 (7.5) in the 60, 120 and 180 μg groups, respectively. On the day of hCG administration, this dose-related response was still apparent, and the mean (SD) number of follicles was, respectively, 11.4 (5.3), 13.5 (6.5) and 16.4 (7.2) (Fig. 3).

Serum hormones during the follicular and luteal phases

Median serum levels of LH, E_2 and inhibin-B for all patients who received hCG are shown in Fig. 4. After an initial decline in all groups, serum levels of LH increased from Day 3 to 5 in the corifollitropin alfa groups while continuing to decline in the rFSH group until Day 6. After the start of ganirelix treatment on stimulation Day 5, LH levels decreased in the

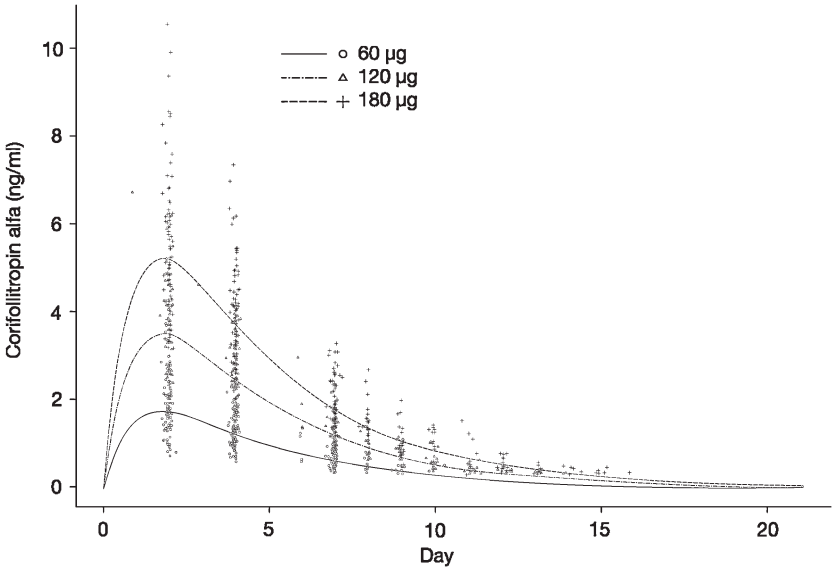

Figure 2: Population-predicted corifollitropin alfa versus time curves modelled on actual corifollitropin alfa concentrations assuming a body weight of 65 kg.

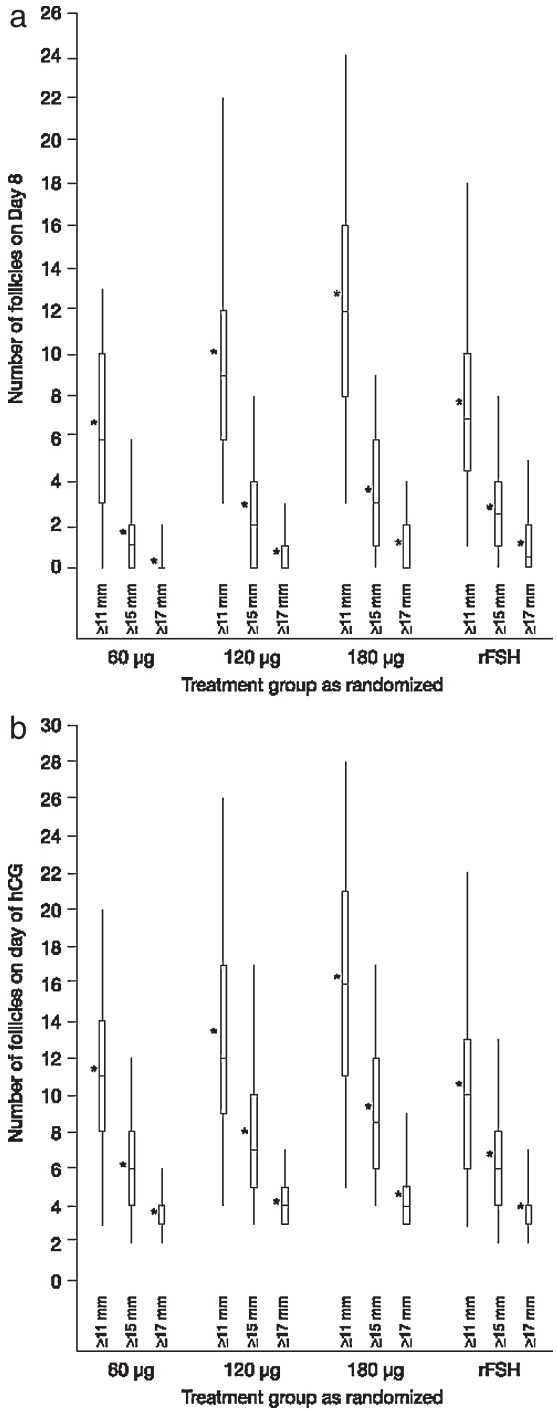

Figure 3: Mean number of follicles ≥11, ≥15 and ≥17 mm (**a**) on Day 8 (before administration of rFSH) and (**b**) on day of hCG administration (restricted to subjects who received hCG).
The box represents the P25, Median and P75. Asterisk represents the mean and whiskers represent the P5 and P95.

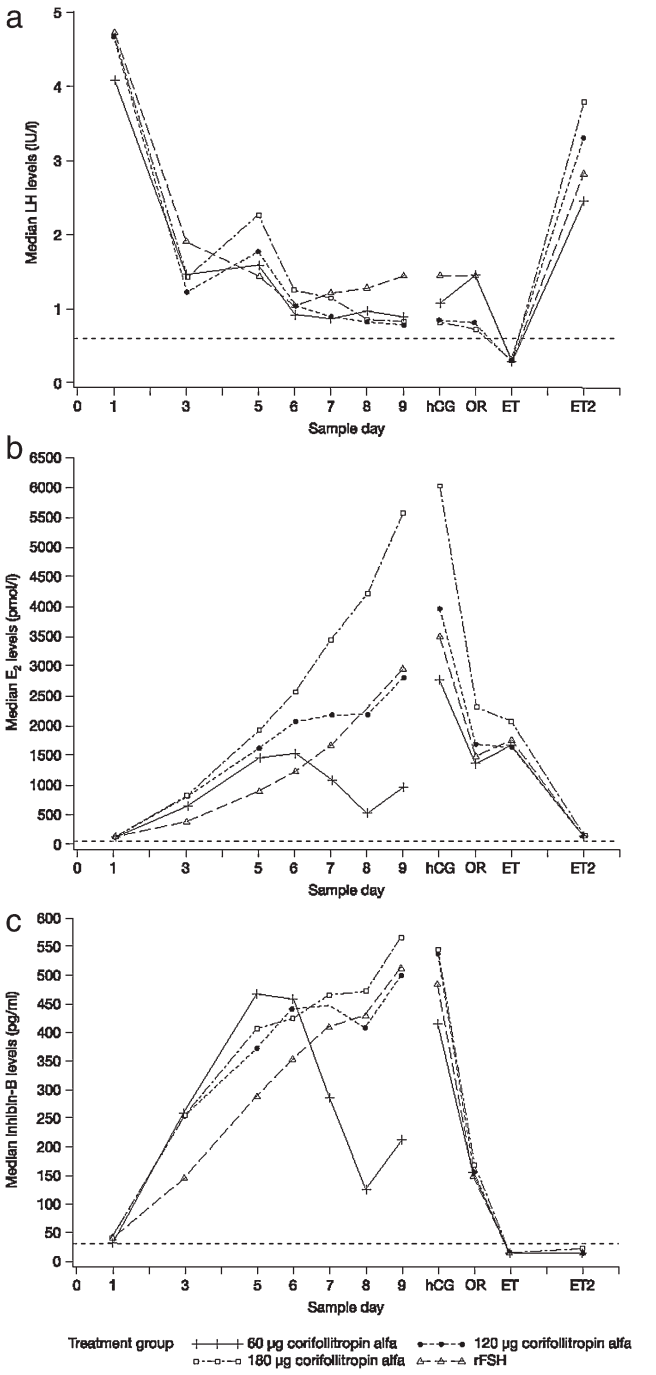

Figure 4: Median serum levels of (**a**) LH, (**b**) E_2 and (**c**) inhibin-B restricted to subjects with hCG administration.
ET, embryo transfer; ET2, 2 weeks after embryo transfer; OR, oocyte retrieval.

corifollitropin alfa groups until Day 9, whereas there was a slight increase in the rFSH group from Day 6 to 9. Median serum LH levels on the day of hCG were 1.1, 0.8 and 0.8 IU/l in the corifollitropin alfa 60, 120 and 180 µg groups, respectively, and 1.5 IU/l in the rFSH group.

On stimulation Day 5, premature LH rises (≥10 IU/l) were experienced by 12 patients before starting ganirelix (corifollitropin alfa 60 µg, $n = 1$; 120 µg, $n = 3$ and 180 µg, $n = 8$), 11 of whom also had concomitant rises in serum progesterone levels (≥3.18 nmol/l). Ten of these patients had a subsequent embryo transfer, of whom four became pregnant (one miscarried). LH rises were also observed in five patients after starting ganirelix (corifollitropin alfa 60 µg, $n = 3$; rFSH, $n = 2$). Three of these patients underwent embryo transfer but none became pregnant.

Median serum E_2 levels increased more rapidly in the corifollitropin alfa groups compared with the rFSH group during the first 6 days of stimulation. Between Day 6 and 8,

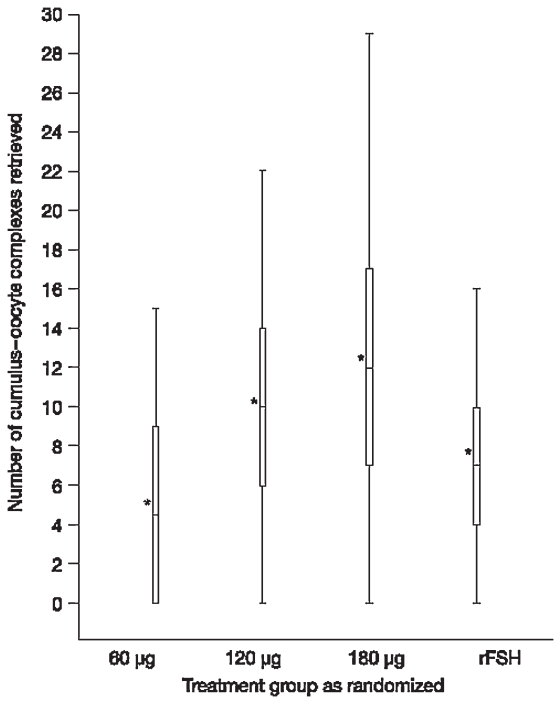

Figure 5: Number of cumulus–oocyte complexes retrieved per started cycle.
The box represents the P25, Median and P75. Asterisk represents the mean and whiskers present the P5 and P95.

levels declined in the 60 μg group, reached a plateau in the 120 μg group and continued to increase in the 180 μg group. On Day 8, serum E_2 levels showed a dose-related increase ($P < 0.001$). After the start of daily rFSH on Day 8, serum E_2 levels increased in all corifollitropin alfa groups. On day of hCG, serum E_2 levels were similar in the 60, 120 μg and rFSH groups, but ~1.5-fold higher in the 180 μg group.

Median serum inhibin-B levels followed a similar pattern as serum E_2 levels, with an initial rise in all groups. From Day 6 onwards, serum inhibin-B levels continued to increase in the 180 μg group and daily rFSH group, whereas inhibin-B reached a plateau from Day 6 to 8 in the 120 μg group and decreased in the 60 μg group.

Clinical outcomes

The number of cumulus–oocyte complexes retrieved after treatment with corifollitropin alfa showed a clear dose–response relationship ($P_{slope} < 0.0001$) (Fig. 5). The mean (SD) number of oocytes retrieved per started cycle was 5.2 (5.5), 10.3 (6.3) and 12.5 (8.0) in the corifollitropin alfa 60, 120 and 180 μg dose groups, respectively (Table III). Statistical testing indicated that all differences between the corifollitropin alfa groups reached significance [120 versus 60 μg: $P < 0.0001$, 95% confidence interval (CI) (3.07, 7.15); 180 versus 60 μg: $P < 0.0001$, 95% CI (5.37, 9.42) and 180 versus 120 μg: $P = 0.028$, 95% CI (0.25, 4.32)]. In the rFSH group, the mean number of oocytes (SD) was 7.7 (6.3), which was statistically significantly higher than in the 60 μg group [$P = 0.009$, 95% CI (0.69, 4.72)], but statistically significantly lower than in the 120 μg [$P = 0.020$, 95% CI (−4.43, −0.39)] and 180 μg [$P < 0.0001$, 95% CI (−6.71, −2.69)] dose groups, when tested using the ANOVA model.

Other clinical outcome parameters are summarized in Table III. The mean number of good-quality (Grade I and II) embryos obtained was higher in the 120 and 180 μg groups.

Table III. Clinical outcomes (intent-to-treat population).

	Corifollitropin alfa			rFSH
	60 μg ($n = 78$)	120 μg ($n = 77$)	180 μg ($n = 79$)	150 IU daily ($n = 81$)
Number of cumulus–oocyte complexes retrieved, mean (SD) per started cycle	5.2 (5.5)	10.3 (6.3)[a]	12.5 (8.0)[b]	7.7 (6.3)[c]
Fertilization procedure:				
IVF only	31 (63%)	40 (55%)	41 (55%)	38 (52%)
ICSI only	18 (36%)	30 (42%)	31 (42%)	31 (43%)
IVF+ICSI		2 (3%)	2 (3%)	4 (5%)
Number of metaphase II oocytes, mean (SD) for patients with oocyte retrieval and ICSI only	7.7 (5.5)	10.1 (6.0)	11.6 (6.6)	5.9 (3.0)
Fertilization rate[d], mean (SD)	60.5 (27.1)	65.2 (23.9)	59.8 (22.7)	61.7 (27.9)
Number of embryos obtained, mean (SD):				
Total[d]	4.9 (3.3)	7.1 (4.1)	8.2 (6.5)	5.1 (4.2)
Good-quality (Grade I and II)[d]	2.2 (2.0)	3.5 (2.7)	3.5 (3.4)	2.4 (2.3)
Good-quality transferred[e]	1.3 (0.7)	1.4 (0.7)	1.3 (0.7)	1.2 (0.7)
Implantation rate[e], mean (SD)	20.5 (34.6)	19.8 (38.3)	17.1 (37.0)	18.2 (35.8)
Vital pregnancy[f]:				
Per started cycle	12 (15%)	14 (18%)	12 (15%)	14 (17%)
Per transfer	12 (27%)	14 (20%)	12 (17%)	14 (21%)
Ongoing pregnancy[g]:				
Per started cycle	12 (15%)	12 (16%)	11 (14%)	11 (14%)
Per transfer	12 (27%)	12 (17%)	11 (16%)	11 (17%)
Ongoing pregnancy including 1 year cryocycles[h]	14 (18%)	21 (27%)	19 (24%)	16 (20%)
Number of cryocycles (patients, cryocycles)	8, 11	18, 25	23, 34	16, 22

[a]120 versus 60 μg: $P < 0.0001$, 95% CI [3.07, 7.15]; [b]180 versus 60 μg: $P < 0.0001$, 95% CI [5.37, 9.42] and 180 versus 120 μg: $P = 0.028$, 95% CI [0.25, 4.32]; [c]rFSH versus 60 μg: $P = 0.009$, 95% CI [0.69, 4.72], rFSH versus 120 μg: $P = 0.020$, 95% CI [−4.43, −0.39]), rFSH versus 180 μg: $P < 0.0001$, 95% CI [−6.71, −2.69]; [d]Restricted to patients with IVF or ICSI; [e]Restricted to patients with embryo transfer; [f]Intra-uterine pregnancy with ≥1 vital foetus confirmed by ultrasound scan; [g]≥1 vital foetus with heart activity confirmed by ultrasound scan after 12 weeks of gestation; [h]Ongoing pregnancy including fresh and cryo-replacement cycles.

The incidence of single embryo transfer of one good-quality embryo across the groups ranged from 37 to 53% and implantation rate ranged from 17 to 21%. In total, 46 ongoing pregnancies were achieved (corifollitropin alfa 60 μg, $n = 12$; 120 μg, $n = 12$; 180 μg, $n = 11$; rFSH, $n = 11$) and the percentage of ongoing pregnancies per transfer ranged from 16 to 27%. Ongoing pregnancies included nine sets of twins, one in the corifollitropin alfa 60 μg group, five in the 120 μg group, two in the 180 μg group and one in the rFSH group. Cumulative pregnancy rate, including fresh and cryopreserved embryos, was slightly higher in the corifollitropin alfa 120 and 180 μg groups.

Safety

In total, 13 subjects experienced 16 serious adverse events, including ectopic pregnancy, salmonellosis, mild pelvic pain, ovarian cyst, appendicitis, peritonitis and hyperemesis, all of which were considered by the investigator and study sponsor as unlikely to be related to study treatment. Six reports of OHSS were classed as serious adverse events, two in each of the corifollitropin alfa 120 and 180 μg groups and two in the rFSH group, all of which were considered possibly or probably related to study treatment. They included two severe cases (Grade III), one in the 120 μg group and one in the rFSH group, three moderate cases (Grade II) and one mild case (Grade I). Other serious adverse events considered at least possibly related to study treatment were a high-risk triple pregnancy in the 180 μg group and a report of severe pelvic pain in the rFSH group. Treatment with corifollitropin alfa did not induce hypersensitivity reactions, or antibodies against corifollitropin alfa.

Discussion

This study shows that a single injection of a new recombinant fertility hormone, corifollitropin alfa, can induce and sustain multifollicular growth for an entire week in women undergoing COS for IVF or ICSI. A single dose of corifollitropin alfa resulted in a dose-dependent, statistically significant increase in the number of cumulus–oocyte complexes retrieved over the dose range of 60–180 μg. This is consistent with the only previous smaller study of corifollitropin alfa in IVF patients, in which higher doses of 120–240 μg were tested (Devroey *et al*., 2004). In the current study, per started cycle, fewer cumulus–oocyte complexes were retrieved with 60 μg corifollitropin alfa compared with fixed 150 IU rFSH. The high cycle cancellation rate in the 60 μg group before hCG administration (30% and before embryo transfer 44%) indicates that this dose is too low to support the first 7 days of COS.

Cancellation rates with the higher corifollitropin alfa doses (9 and 11% with 120 and 180 μg, respectively) are consistent with those generally observed in COS protocols. However, the cancellation rate with daily rFSH was comparatively high (7% before hCG; 19% before embryo transfer), mainly due to insufficient ovarian response. This can be explained by the protocolized 150 IU regimen of rFSH, which implied that the dose could not be increased during stimulation if it appeared to be too low for a specific patient. The protocolized dosing regimen may also account for the relatively low number of oocytes retrieved in the rFSH group.

The open-label design of this study was deemed appropriate from a methodological perspective in view of the relatively objective and robust primary endpoint, number of oocytes retrieved, and within strict protocolized boundaries defining stimulation start-, dosing- and stop-criteria. Further studies are required to compare clinical outcomes with corifollitropin alfa and daily rFSH regimens.

Other clinical outcomes were generally comparable between the corifollitropin alfa groups. The overall pregnancy rate in the treatment cycles was relatively low, but comparable between all groups, including the daily rFSH reference group. Further analyses were hampered due to the small sample size, especially when looking at subpopulations (e.g. number of embryos transferred, day of embryo transfer, variation in luteal phase support), and did not reveal a clear explanation for this finding. The total number of embryos and number of good-quality embryos were higher with corifollitropin alfa 120 and 180 μg, possibly reflecting the higher oocyte yield in these groups. This may explain the slightly higher cumulative pregnancy rates in these groups, since more good-quality embryos may have been available for cryopreservation. Reliable estimation of the ongoing pregnancy rate following treatment with the corifollitropin alfa compared with rFSH will come from an adequately powered clinical trial using a similar patient population. The primary endpoint of this currently ongoing phase III trial is ongoing pregnancy.

Patients treated with corifollitropin alfa started daily rFSH treatment on Day 8 in order to reach the criteria for hCG administration. This was achieved with a median of just three injections of rFSH in the corifollitropin alfa 120 μg group and two injections in the 180 μg group. Although the 120 μg group required more daily rFSH than the 180 μg group, median duration of ovarian stimulation was the same (10 days) in both groups because the final dose of rFSH could have been given either on the day of hCG or the day before. This observation confirms previous findings, in which, on average, three daily injections of rFSH were required after a single injection of corifollitropin alfa (Devroey *et al*., 2004).

The occurrence of early increases in serum LH levels before the start of GnRH antagonist treatment was low but LH levels were assessed only once at stimulation Day 5. As described previously in patients treated with different starting doses of rFSH in a GnRH antagonist protocol (Out *et al*., 2004), the incidence of early LH rises was dose-dependent. Most of these patients (8/12) were treated with corifollitropin alfa 180 μg and all but one also had a rise in serum progesterone, indicating premature luteinization. The incidence of premature LH rise reported here is lower than that seen in the previous corifollitropin alfa IVF study, in which flexible initiation of GnRH antagonist administration (given when first follicle $\geq$14 mm) was associated with premature LH rises in 17.6% of corifollitropin alfa-treated patients and 8.3% of rFSH-treated patients (Devroey *et al*., 2004). However, the incidence is similar to that observed in previous studies using both fixed and flexible GnRH antagonist protocols in patients treated with daily rFSH 150 IU (The European Orgalutran Study Group *et al*., 2000; European and Middle East Orgalutran Study Group, 2001).

As also reported in the previous corifollitropin alfa IVF study, the pregnancy rate among patients with premature luteinization did not appear to be reduced, though the numbers included were small (four out of 12 patients).

The pharmacokinetics of corifollitropin alfa showed dose-proportionality over the range 60–180 μg. Terminal half-life was similar in all dose groups (65–66 h) and is consistent with results of previous studies in pituitary-suppressed female volunteers and IVF patients (Duijkers *et al*., 2002; Devroey *et al*., 2004). Body weight had a significant impact on exposure to corifollitropin alfa, expressed as C_{max} and $AUC_{0-\infty}$. A comparable effect of body weight on rFSH exposure during daily rFSH treatment has previously been shown (Mannaerts *et al*., 1996). Results of this study suggest that the exposure of subjects with different body weights should be taken into account in final dose selection of corifollitropin alfa for phase III studies.

The optimal dose of corifollitropin alfa is mainly determined by the predefined time interval (1 week) that a single administration needs to support multiple follicular growth. Clearly, cancellation due to insufficient circulating FSH should be prevented as well as overstimulation. Reflections of insufficient bioactive FSH are not only actual cancellations due to insufficient ovarian response, but also declining levels of serum inhibin-B during stimulation. Accordingly, the 60 μg dose is too low for the subset of subjects included in this trial. Based on the outcome of this trial and subsequent modelling and simulation, taking into account the body weight (exposure) of subjects, the optimal dose of corifollitropin alfa could be estimated. The outcome of simulations (not included in this manuscript) may also predict the number of oocytes that will be retrieved.

Treatment with a single injection of corifollitropin alfa was safe and well tolerated. The number of cases of OHSS was low and comparable with that reported for current rFSH regimens. Corifollitropin alfa was also shown to be non-immunogenic, with no antibody formation or hypersensitivity reaction being observed.

In conclusion, a single dose of corifollitropin alfa can sustain multifollicular growth for an entire week. This trial demonstrates that the optimal dose of corifollitropin alfa in a 1-week regimen is higher than 60 μg and lower than 180 μg. Treatment with corifollitropin alfa potentially offers the convenience of more injection-free days for patients undergoing COS for IVF and ICSI.

The Corifollitropin Alfa Dose-finding Study Group

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REVIEW

Corifollitropin alfa doses based on body weight: clinical overview of drug exposure and ovarian response

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Abstract Corifollitropin alfa is a new recombinant gonadotrophin with a different pharmacokinetic profile but similar pharmacodynamic properties to conventional recombinant FSH. A single dose of corifollitropin alfa sustains multiple follicular development during the first 7 days of ovarian stimulation. This review is based on results of phase II and III trials testing the selected dose of 150 μg corifollitropin alfa in subjects >60 kg and 100 μg in subjects ≤60 kg. Exposure to corifollitropin alfa is inversely related to body weight. The selected doses of 100 and 150 μg in subjects weighing ≤60 and >60 kg, respectively, provide, on average, equal drug exposure producing similar ovarian responses in terms of the number of growing follicles, serum oestradiol, inhibin B and number of oocytes retrieved. Clinicians treating IVF patients with corifollitropin alfa should alter their treatment paradigm as a lower or higher dose than recommended according to body weight does not affect the ovarian response, which depends mainly on the ovarian reserve. After decades of daily dosing with FSH preparations, corifollitropin alfa allows a simpler IVF treatment regime with fewer injections. Successful use of corifollitropin alfa requires assessment of patient suitability and dosing before the start of stimulation.

KEYWORDS: body weight, corifollitropin alfa, GnRH antagonist, IVF, ovarian response, pharmacokinetic/pharmacodynamic profile

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Introduction

Corifollitropin alfa is a new recombinant gonadotrophin with similar pharmacodynamic properties to conventional recombinant FSH (rFSH) in patients undergoing ovarian stimulation prior to IVF or intracytoplasmic sperm injection (ICSI) but with distinct pharmacokinetic characteristics conferring clinically relevant practical advantages (Fauser et al., 2010). A single injection of corifollitropin alfa is able to initiate and sustain multiple follicular developments during the first 7 days of ovarian stimulation prior to IVF or ICSI, thereby requiring fewer drug interventions.

Corifollitropin alfa is a recombinant fusion molecule composed of an α-subunit identical to that in human FSH and a β-subunit, which is a hybrid composed of the FSH β-subunit and carboxyterminal peptide of human chorionic gonadotrophin (HCG) β-subunit (Fares et al., 1992). Like rFSH, corifollitropin alfa only interacts with the FSH receptor and lacks LH activity (Fauser et al., 2009; LaPolt et al., 1992; Loutradis et al., 2010). The compound has been extensively tested in animal models and its profile in terms of absorption, distribution, metabolism and excretion is highly comparable to that of other gonadotrophins, especially FSH (LaPolt et al., 1992; Verbost et al., 2011). Corifollitropin alfa is stable in serum, readily distributes to the ovaries and is excreted renally. The metabolic fate consists of kidney clearance and the urinary excretion of the intact protein in parallel to kidney catabolism (Van Schanke et al., 2010).

Unlike rFSH, the dose of corifollitropin alfa cannot be converted into international units and is expressed in micrograms. Historically, the dose of FSH preparations was established using the Steelman Pohley assay that measured ovarian weight augmentation in immature rats receiving repeated injections of a standard dose of HCG and various doses of the FSH test preparation (Steelman and Pohley, 1953). However, the relationship between the dose of corifollitropin alfa and the increase of ovarian weight is different from that observed with daily FSH preparations. Thus the calculation of the in-vivo bioactivity of corifollitropin alfa, in terms of the current international FSH standard, does not provide a reliable guide to the best therapeutic dose in humans. For highly purified recombinant gonadotrophins, there are now more precise in-vitro bioassays combined with physico-chemical tests to predict the in-vivo biopotency (Driebergen and Baer, 2003; Mulders et al., 1997).

Exposure to corifollitropin alfa can be measured by either a specific immunoassay of corifollitropin alfa or of human FSH. Serum concentrations of corifollitropin alfa are assessed in a specific radioimmunoassay and expressed in ng/ml corifollitropin alfa as described by Bouloux et al. (2001). This assay does not cross-react with other gonadotrophins including FSH or HCG. After a single s.c. injection of corifollitropin alfa, the time to serum peak concentrations is approximately 44 h and its elimination half-life is approximately 69 h (Corifollitropin Alfa Dose-finding Group, 2008; Devroey et al., 2004; Duijkers et al., 2002), compared with approximately 34 h for conventional rFSH (Voortman et al., 1999). Due to this pharmacokinetic profile, a single dose of corifollitropin alfa can result in circulating concentrations that remain above the threshold of FSH activity to support multifollicular growth during an entire week. Whereas the pharmacokinetic profile of corifollitropin alfa is most suitable to induce and sustain multiple follicular development, it is less suitable to induce monofollicular growth and ovulation, as even low doses of corifollitropin alfa easily result in multiple follicular growth due to the relatively high corifollitropin alfa peak concentrations 2 days after injection (Balen et al., 2004).

The introduction of corifollitropin alfa requires clinicians supervising IVF/ICSI programmes to alter their treatment paradigm. In contrast to the use of daily FSH preparations, dose adjustments with corifollitropin alfa are neither possible nor required during the first 7 days of ovarian stimulation, as corifollitropin alfa remains pharmacologically active over this period. This overview explains the relationships between body weight, drug exposure and ovarian response to corifollitropin alfa using information derived from two large, randomized trials, Engage (Devroey et al., 2009) and Ensure (Corifollitropin Alfa Ensure Study Group, 2010). It compares the pharmacokinetics and pharmacodynamics of two doses of corifollitropin alfa and explores the rationale for dosing based on body weight and the reasons why corifollitropin alfa does not bring about a dose-dependent ovarian response as seen with daily rFSH.

Corifollitropin alfa treatment regimen

Corifollitropin alfa is administered as a single s.c. injection, preferably in the abdominal wall, during the early follicular phase of the menstrual cycle (cycle days 2 or 3 (stimulation day 1)). To prevent premature LH surges, a gonadotrophin-releasing hormone (GnRH) antagonist is started on stimulation days 5 or 6 depending on ovarian response (i.e. number and size of growing follicles and/or circulating oestradiol concentration) and continued daily until the day of triggering final oocyte maturation. Some patients may reach the criteria for triggering final oocyte maturation after the first 7 days of stimulation (early responders) and sustained FSH activity beyond this week would not be desirable. In other patients, treatment may continue with a daily dose of rFSH which may depend on the ovarian response of the patient. In normal responder patients a daily dose of 150 IU was shown to be sufficient to finish the treatment cycle (Corifollitropin Alfa Dose-finding Group, 2008) and administration of rFSH on the day of HCG is not required. If the patient at stimulation day 8 appears to have a relatively high ovarian response, like with daily rFSH treatment, patients may be coasted for up to 3 days (Devroey et al., 2009). Using a GnRH antagonist protocol, it is advisable to trigger final oocyte maturation in a timely manner, i.e. as soon as three follicles $\geq$17 mm are reached or on the day after this point is reached (Kolibianakis et al., 2005).

Immunoreactivity measured by FSH immunoassay (Delfia) after a single s.c. injection of corifollitropin alfa is shown in **Figure 1**. Due to cross-reactivity of most monoclonal antibodies directed against human FSH, corifollitropin alfa may also be detected with a commercial FSH immunoassay. In clinical practice, fertility specialists may wish to monitor immunoreactivity to verify drug compliance or drug expo-

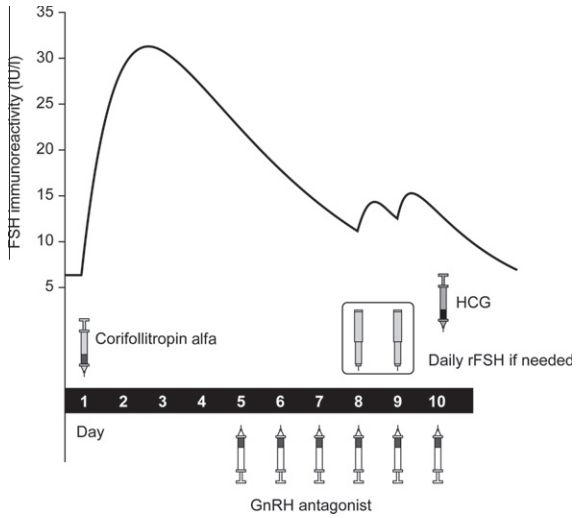

Figure 1 Schematic representation of the therapeutic interventions in the corifollitropin alfa treatment regimen in relation to immunoreactivity measured by FSH immunoassay. GnRH = gonadotrophin-releasing hormone; HCG = human chorionic gonadotrophin; rFSH = recombinant FSH.

sure throughout the treatment period. Although there may be a good correlation between serum concentrations measured by a specific corifollitropin alfa immunoassay and by a commercial FSH immunoassay (Devroey et al., 2004), the latter assay cannot be considered as an absolute quantitative measure as, instead of a corifollitropin alfa standard, a FSH standard is applied.

The efficacy and safety of the corifollitropin alfa treatment regimen was extensively evaluated in three phase III trials in which more than 1700 patients were exposed to either 100 or 150 μg corifollitropin alfa. Two out of these three trials were large, randomized, double-blind, double-dummy trials of corifollitropin alfa in women aged up to 36 years. In the Engage trial, patients with a body weight of >60 kg were treated with 150 μg corifollitropin alfa (n = 756) in the corifollitropin alfa arm, and in the Ensure trial, women with a body weight of ≤60 kg were treated with 100 μg corifollitropin alfa (n = 268) in the corifollitropin alfa arm (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009). Inclusion/exclusion criteria were identical in both trials, apart from patient weight, and populations were comparable including mean age, infertility duration, basal antral follicle count (AFC) and basal serum hormone concentrations (**Table 1**). Basal AFC was required to be ≤20 to exclude patients with polycystic ovaries (Balen et al., 2003) and (potential) high responder patients.

Impact of body weight on corifollitropin alfa exposure

Corifollitropin alfa is distributed within the extracellular fluid space, which increases with body weight. Consequently, the serum concentration of corifollitropin alfa is inversely related to body weight as initially demonstrated in the phase II dose–response study (Corifollitropin Alfa Dose-finding Group, 2008) and as confirmed in the phase III Engage and Ensure trials (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009; Fauser et al., 2010) (**Figure 2**). However, as shown in **Figure 2**, the impact of body weight on dose-normalized drug exposure is reduced as body weight increases, with a shallow slope in patients weighing ≥80 kg. These subjects are generally overweight and have a larger percentage of fat tissue compared with patients below this weight. Adipose tissue has a relatively low content of extracellular water, i.e. approximately 0.11 l/kg in adipose tissue versus 0.21 l/kg overall in non-obese subjects (Waki et al., 1991). Moreover, low perfusion may result in limited distribution of corifollitropin alfa to extracellular water in adipose tissue (Jonsson and

Table 1 Characteristics of patients in the Engage and Ensure phase III trials.

	Engage (150 μg corifollitropin alfa, women >60 kg)	*Ensure (100 μg corifollitropin alfa, women ≤60 kg)*
No. of patients (ITT group)	756	268
Age (years)	31.5 ± 3.3	30.9 ± 3.2
Body weight (kg)	68.8 ± 7.6	54.1 ± 4.2
BMI (kg/m^2)	24.8 ± 2.8	20.5 ± 1.5
FSH (IU/l, stimulation day 1)	6.7 ± 2.1	6.6 ± 1.8
AFC (stimulation day 1)	12.3 ± 4.6	11.1 ± 4.4
Cause of infertility (%)		
Male factor	51.3	47.4
Tubal factor	26.2	26.1
Endometriosis	14.4	11.9
Other	32.5	30.2
Duration of infertility (years)	3.3 ± 2.4	3.2 ± 2.2
First IVF cycle (%)	75.3	55.2

Values are mean ± SD unless otherwise indicated. AFC = antral follicle count (<11 mm); BMI = body mass index; ITT = intention-to-treat. Adapted from Corifollitropin alfa Alfa Ensure Study Group (2010) and Devroey et al. (2009).

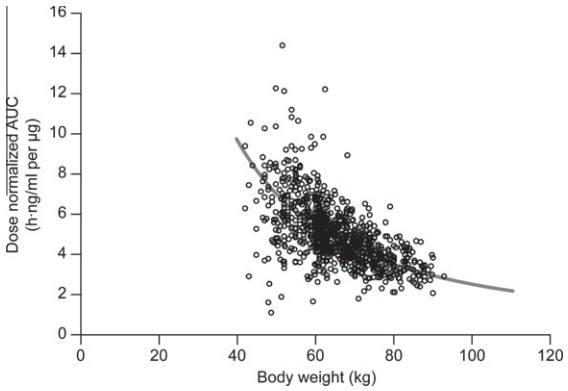

Figure 2 Relationship between dose-normalized drug exposure to corifollitropin alfa (exposure to corifollitropin alfa normalized to the administered dose, dose normalized area under the curve (AUC)) and patient body weight.

Johanson, 2002). Hence, adipose tissue does not markedly increase the volume distribution of corifollitropin alfa and body weight is a stronger determinant of drug exposure than body mass index (BMI). This may be explained by using a typical example of two subjects with a BMI of 23 kg/m^2, one 1.54 m (5.05 feet) tall weighing 55 kg, and the other 1.80 m (5.9 feet) tall weighing 75 kg. The volume of extracellular fluid is larger in the taller, heavier individual than in the shorter subject with lower body weight, whereas their BMI is the same. Treatment of each subject with the same dose of corifollitropin alfa would result in lower corifollitropin alfa concentrations in the subject weighing 75 kg than in the subject weighing 55 kg.

Rationale for dose selection

Dose selection of corifollitropin alfa was based on a phase II dose—response study in combination with a robust modelling and simulation study aiming at a 1-week interval that the compound had to sustain follicular development (de Greef et al., 2010). Taking into account the impact of body weight on exposure, this approach allowed the selection of the minimum effective corifollitropin alfa doses for phase III trials, even though the doses were not tested in phase II trials.

In a phase II, dose-finding study, patients weighing 50—90 kg were randomized to a single dose of 60, 120 or 180 µg corifollitropin alfa for the first 7 days of ovarian stimulation, or seven daily injections of 150 IU rFSH using a GnRH antagonist protocol (Corifollitropin Alfa Dose-finding Group, 2008). If needed, the treatment cycle was continued with daily 150 IU rFSH from stimulation day 8 onwards. This study showed that the optimal dose of corifollitropin alfa to sustain multiple follicular development during the first week of stimulation was greater than 60 µg and lower than 180 µg. The 60-µg dose appeared to be too low to retain serum concentrations of corifollitropin alfa above the threshold to support follicular development during the whole first week of stimulation and consequently cycles were cancelled in approximately one-third of subjects due to too low ovarian response. Cycle cancellation due to this reason was preceded by a decrease of serum inhibin B which appeared to be a sensitive marker of follicular maturation. On day 8, the mean number of follicles ≥11 mm in the corifollitropin alfa dose groups 60, 120 and 180 µg was 6.8, 10.1 and 12.8, respectively and the number of cumulus—oocyte—complexes retrieved showed a clear dose—response relationship. Together, these findings indicated that 60 µg corifollitropin alfa did not sustain ovarian stimulation for 10 weeks and resulted in underexposure to FSH activity in many individuals.

For a sustained ovarian response during a period of 7 days, serum concentrations of corifollitropin alfa should remain above a therapeutic threshold. Little or no follicular stimulation occurs below a corifollitropin alfa concentration of 1.10 ng/ml, whereas adequate multiple follicular development occurs above that threshold (de Greef et al., 2010; Macklon et al., 2006). For sustained drug exposure above the therapeutic threshold throughout the stimulation period of 7 days, a higher dose was needed in subjects weighing >60 kg while a lower dose was sufficient in subjects weighing ≤60 kg. Hence, the risk of cycle cancellation was minimized by the development of two doses of corifollitropin alfa, which would result in adequate follicular development while preventing overdosing in both body weight groups.

A pharmacokinetic—pharmacodynamic model was developed to describe the time profile of corifollitropin alfa concentrations, with multiple parameters reflecting ovarian response included in the model framework (de Greef et al., 2010; Zandvliet et al., 2011). Using this model, various doses of corifollitropin alfa were evaluated to ensure optimal drug exposure in all patients. The modelling results demonstrated that a dose of 100 µg would result in a sustained increase of inhibin B concentration, which reflected a sustained ovarian response, in 90% of the patients weighing 60 kg. This motivated the selection of the body weight cut-off of 60 kg. Simulations (1000 clinical trials in subjects ≤60 kg and 1000 trials in subjects >60 kg) were then performed to evaluate the cancellation rate for various dose concentrations. Treatment with a corifollitropin alfa dose of 100 µg for women weighing ≤60 kg was predicted to result in a minimal cancellation rate, whereas patients weighing >60 kg would require a higher dose of 150 µg corifollitropin alfa to prevent an increase in the cancellation rate.

Prediction models indicated that these corifollitropin alfa doses should result in similar drug exposure for patients in the indicated body weight categories. Therefore, the predicted mean number of oocytes per started stimulation cycle also approached a maximum in the simulation and was similar in the ≤60 kg and >60 kg weight groups (12.1 and 13.2, respectively) (de Greef et al., 2010) (**Figure 3**A and B). Treatment with a lower than recommended dose in each body weight group resulted in a significantly increased risk of cycle cancellation according to the simulated data. Conversely, the number of oocytes per started cycle reached a plateau at higher than recommended doses, and further increase in corifollitropin alfa dose beyond the recommended doses did not result in an increase in oocyte yield.

The recommended doses for each weight group were then tested in separate prospective clinical trials and were found to be adequate (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009).

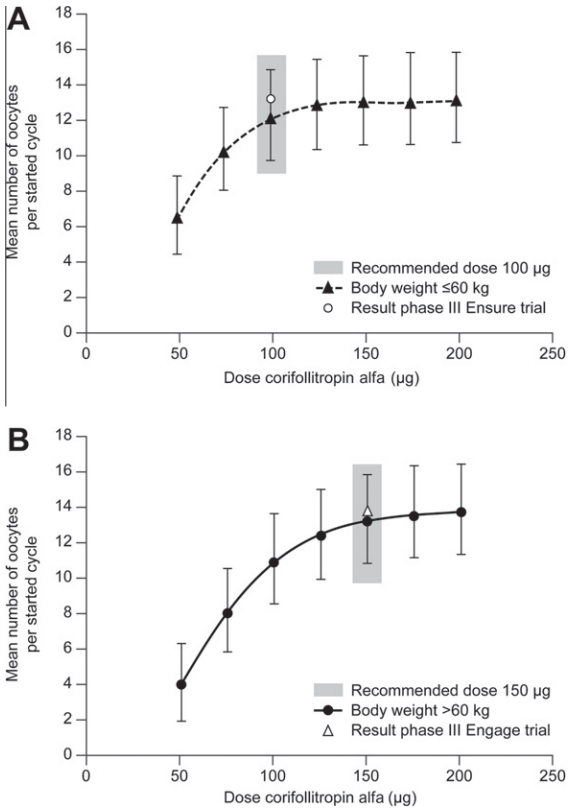

Figure 3 Relationship between mean number of oocytes per started cycle and dose of corifollitropin alfa: (A) 100 µg for subjects ≤60 kg and (B) 150 µg for subjects >60 kg. Adapted from de Greef et al. (2010).

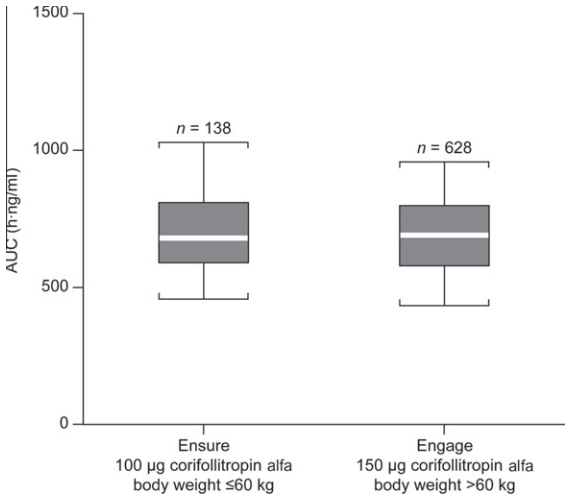

Figure 4 Exposure to corifollitropin alfa (AUC) in Caucasian subjects. Horizontal white marks represent median values, boxes represent interquartile ranges and whiskers represent 5—95th percentiles. Pharmacokinetic parameters obtained by population pharmacokinetic analysis.

Comparative stimulation characteristics in randomized controlled trials

The results of the Engage (150 µg corifollitropin alfa in subjects weighing >60 kg) and Ensure (100 µg corifollitropin alfa in subjects weighing ≤60 kg) trials confirmed that the recommended doses for the respective body weight groups are suitable for safe and effective treatment in ovarian stimulation (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009).

In these trials, the designated doses of corifollitropin alfa resulted in similar drug exposure. **Figure 4** illustrates that the median area under the curve (AUC) values in Caucasian subjects for the recommended corifollitropin alfa doses in their respective body weight groups were virtually identical in the two trials.

As expected, the pharmacodynamic profiles of corifollitropin alfa in the two body weight groups from Engage and Ensure were comparable, as shown in **Table 2** (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009; Fauser et al., 2010). Comparison of response characteristics revealed no difference in the number of days of stimulation in subjects treated with 150 or 100 µg corifollitropin alfa. The median duration of stimulation with corifollitropin alfa was 9 days in both trials. Thus, after a single injection of the designated corifollitropin alfa dose, subjects required on average 2 days of additional stimulation with rFSH to reach the criterion for triggering final oocyte maturation.

In the Engage trial, in subjects weighing >60 kg, the amount of daily rFSH applied to complete the stimulation was higher as most subjects received daily 200 IU (total 400 IU from day 8 onwards) whereas in the Ensure trial most subjects received daily 150 IU to finish the cycle (total 300 IU from day 8 onwards) (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009). However, the results of these studies and of the dose-finding trial indicated that in normal responders, 150 IU daily rFSH is sufficient to complete the stimulation until the criteria for giving HCG are reached, irrespective of body weight (Corifollitropin Alfa Dose-finding Group, 2008; de Greef et al., 2010).

There was no large difference between the number of growing follicles, rise in serum oestradiol and serum inhibin B concentrations, or the number of oocytes obtained between subjects weighing ≤60 kg or >60 kg after treatment with the designated doses of corifollitropin alfa (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009; Fauser et al., 2010). The number of follicles (≥11 mm) on stimulation day 8 and on the day of HCG administration and the number of oocytes per started cycle were marginally higher in Engage; however, the slightly higher ovarian response in the Engage trial may reflect that more subjects undergoing a first IVF cycle with a slightly higher AFC were enrolled (**Table 1**).

Serum concentrations of oestradiol, inhibin B, LH, FSH and progesterone for all subjects who received HCG exhibited similar patterns in both trials. Serum oestradiol increased from stimulation day 1 to day 8 and peaked on the day of HCG and then rapidly declined. In subjects weighing ≤60 kg or >60 kg, treatment with corifollitropin alfa resulted in a continuous rise of inhibin B during the first 5 days of stimulation, and then remained at the same concentration at stimulation day 8. After the day of HCG, serum inhibin B concentrations rapidly declined in both trials in those receiving corifollitropin alfa.

Table 2 Stimulation characteristics, ovarian response and hormones assessed in patients treated with 150 and 100 μg corifollitropin alfa according to body weight.

	Engage (150 μg corifollitropin alfa, women >60 kg)	*Ensure (100 μg corifollitropin alfa, women ≤60 kg)*
No. of patients (ITT group)	756	268
Duration of stimulation (days)	9 (6–18)	9 (6–15)
Total rFSH from day 8 onwards (IU)	400 (0–2000)	300 (0–1550)
Stimulation day 8		
Follicles ≥11 mm	12.8 ± 6.7	11.8 ± 6.1
Oestradiol (pmol/l)	2919 (151–31,122)	2899 (282–20,442)
Inhibin B (pg/ml)	457 (21–4000)	619 (56–4000)
rFSH (IU/l)	11.6 (4.2–24.4)	10.1 (5.0–18.1)
LH (IU/l)	1.0 (0.3–28.4)	0.8 (0.3–9.9)
Progesterone (nmol/l)	1.8 (0.6–38.5)	1.4 (0.6–3.3)
Day of HCG		
Follicles ≥11 mm	16.0 ± 7.0	14.9 ± 6.6
Oestradiol (pmol/l)	4661 (793–32,516)	4441 (690–20,442)
Inhibin B (pg/ml)	499 (5–1660)	729 (56–4000)
rFSH (IU/l)	12.3 (0.1–23.0)	11.5 (5.0–19.9)
LH (IU/l)	1.0 (0.3–28.4)	0.9 (0.3–9.9)
Progesterone (nmol/l)	2.8 (0.6–38.5)	2.1 (0.6–8.4)
Oocytes retrieved per started cycle	13.7 ± 8.2	13.3 ± 7.3

Values are mean ± SD or median (range). Hormone concentrations restricted to patients who received HCG.
HCG=human chorionic gonadotrophin; ITT = intention-to-treat; rFSH = recombinant FSH.
Adapted from Corifollitropin alfa Alfa Ensure Study Corifollitropin alfa Alfa Ensure Study (2010) and Devroey et al. (2009).

It is noteworthy that the number of oocytes obtained per started cycle was similar after treatment with 150 and 100 μg doses of corifollitropin alfa in the respective body weight categories (13.7 and 13.3, respectively) (**Table 2**). Moreover, the clinical data presented in the Engage and Ensure trials closely approximated the predictions of oocyte numbers per started cycle based on modelling and simulation (de Greef et al., 2010).

In the Engage trial, very limited data were available for subjects weighing >90 kg. However, body weight has only a minor impact on drug exposure in this subgroup as described previously (**Figure 2**). Hence, a dose of 150 μg corifollitropin alfa seems sufficient to initiate and sustain ovarian response in subjects weighing >90 kg.

In summary, the pharmacodynamic characteristics of a single s.c injection of 150 μg corifollitropin alfa in women weighing more than 60 kg are virtually identical to those observed after a single injection of 100 μg corifollitropin alfa in women weighing up to 60 kg. Treatment with the recommended corifollitropin alfa dose in the respective weight categories results in similar hormone patterns and number of follicles as well as an equal number of oocytes retrieved per started cycle. These data confirm that the two dosages in the recommended body weight groups provide similar drug exposure and, therefore, induce the same degree of ovarian response.

Predictors of ovarian response

When applying corifollitropin alfa for controlled ovarian stimulation, prediction of ovarian response may be helpful to decide on a patient's eligibility. Characteristics such as age, basal FSH concentration, AFC and/or anti-Müllerian hormone (AMH) are commonly used to identify potential poor or high responders (Broekmans et al., 2006; Broer et al., 2009). Treatment of (potential) high responders with corifollitropin alfa should be prevented since treatment recruits a slightly larger cohort of follicles than daily rFSH. Accordingly, patients with a basal AFC >20 (Broekmans et al., 2010) were excluded in clinical trials and for these patients treatment with corifollitropin alfa is not indicated.

The pooled data of the Engage and Ensure trial were applied to evaluate potential predictors of ovarian response for the selected doses of corifollitropin alfa. This analysis showed that the mean number of oocytes retrieved was not dependent on drug exposure or body weight for subjects treated with the correct dose of 100 or 150 μg corifollitropin alfa based on their body weight (**Figure 5**A–C). The mean number of oocytes retrieved per attempt was stable across quartiles of area under curve (AUC) or peak serum concentration (C_{max}) or body weight groups. As expected, the ovarian response induced by corifollitropin alfa depended mainly on clinically established predictors of ovarian response such as baseline FSH, AFC and age (**Figure 5**D–F). There was a general trend toward a higher ovarian response with an increasing AFC and the mean number of oocytes per attempt decreased with increasing baseline FSH and age.

In conclusion, a similar mean number of oocytes were retrieved in the Engage and Ensure trials after treatment with 150 and 100 μg corifollitropin alfa, respectively. Ovar-

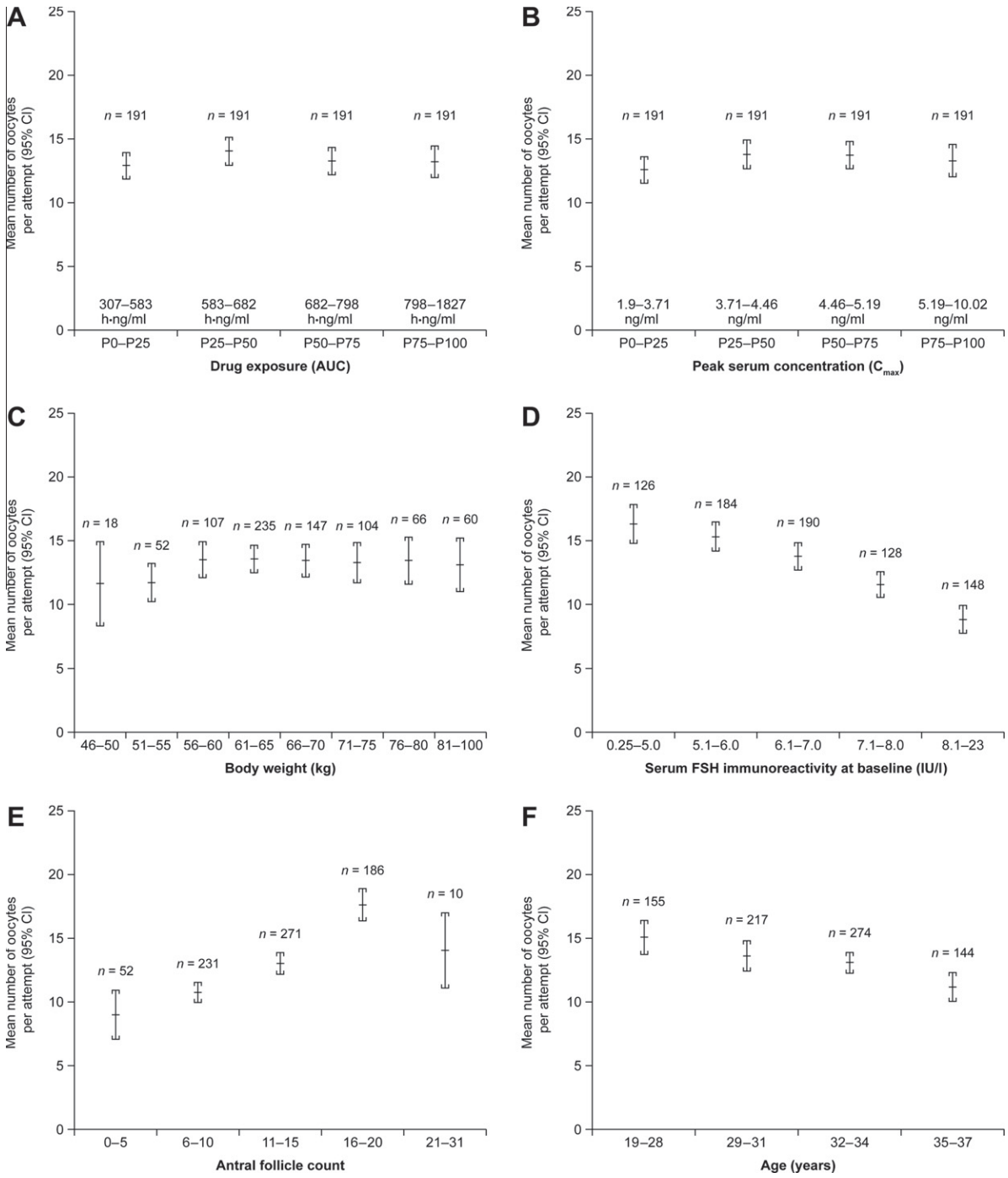

Figure 5 Relationship between number of oocytes and potential predictors of ovarian response in Caucasian subjects after treatment with recommended dose of corifollitropin alfa: number of oocytes versus (A) AUC; (B) C_{max}; (C) body weight; (D) basal FSH; (E) AFC; and (F) age. AFC = antral follicle count; AUC = area under curve; CI = confidence intervals; C_{max} = peak serum concentration; P = percentile.

ian response was not affected by body weight or drug exposure within the parameters set for either trial. In contrast, the ovarian reserve of the patient was a strong determinant of the ovarian response. This could not be compensated for by administration of a higher or lower dose of corifollitropin alfa. The following section provides further explanation and typical examples.

Dose recommendations

Individuals eligible for treatment with corifollitropin alfa are potential normal or low responders with an AFC $\leq$20. Patients should be treated with the appropriate dose of corifollitropin alfa based on their body weight (100 μg for subjects weighing $\leq$60 kg and 150 μg for subjects weighing

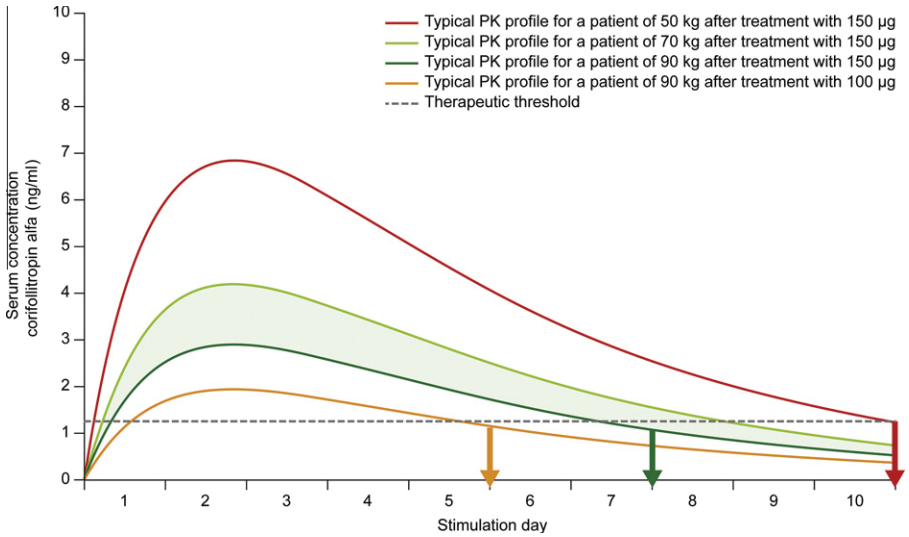

Figure 6 Time profiles of the corifollitropin alfa serum concentration after administration of a recommended (green), too low (orange) or too high (red) dose of corifollitropin alfa. The arrows indicate the time point when follicular stimulation is no longer sustained by a single s.c. dose of corifollitropin alfa. PK = pharmacokinetic.

>60 kg). The ovarian response will be determined by the ovarian reserve of the individual and not by the dose of corifollitropin alfa used. A lower dose does not result in a mild ovarian response and a higher dose does not result in an increased number of oocytes.

The consequences of misprescribing of corifollitropin alfa are illustrated in **Figure 6**. The exposure created in a subject weighing ≤60 kg overdosed with corifollitropin alfa 150 µg (red line) will not improve follicle recruitment (as shown in **Figure 3**A), but ovarian stimulation will be sustained for more than 1 week, whereas some subjects will reach the criteria for HCG injection after only 7 days. Thus, too high a corifollitropin alfa dose may result in too high FSH activity beyond stimulation day 8, but will not result in an improved ovarian response. Even in poor responders, 100 µg corifollitropin alfa is sufficient in subjects weighing ≤60 kg and 150 µg corifollitropin alfa is adequate in those with body weight >60 kg.

As previously stated, little or no follicular stimulation occurs below a corifollitropin alfa concentration of 1.10 ng/ml, whereas adequate multiple follicular development occurs above that threshold (de Greef et al., 2010). Underdosing by administering 100 µg corifollitropin alfa in a subject weighing >60 kg (yellow line; Figure 6) exceeds the therapeutic threshold for only a limited period of time. This dose will recruit a nearly maximum number of follicles (as shown in **Figure 3**B), but ovarian stimulation is not sustained after 5 days, which may result in a higher risk of cycle cancellation due to insufficient drug exposure on stimulation days 6 and 7. Even in high responder subjects, a lower dose may result in cycle cancellation and will not achieve milder ovarian stimulation.

The recommended single s.c. dose of 150 µg corifollitropin alfa in a subject weighing >60 kg (light green line) sustains follicular stimulation for at least a week. Even in women weighing >90 kg, a single 150-µg dose (dark green line; Figure 6) is thought to be adequate to initiate and sustain ovarian response for 7 days since body weight does not have a major impact on drug exposure in this subgroup (as shown in **Figure 2**).

Overall, the recommended dose of corifollitropin alfa (i.e. 100 µg for subjects weighing ≤60 kg and 150 µg for subjects weighing >60 kg) is safe and effective in potential normal or low responder individuals with an AFC ≤20. Women should not be treated with a lower or higher dose of corifollitropin alfa than recommended for their body weight in an attempt to adjust their ovarian response as this strategy may result in an increased risk of cycle cancellation or in too high FSH activity beyond stimulation day 8 (Table 3).

Table 3 Summary of dose recommendations of corifollitropin alfa in relation to body weight.

Dose	*Women ≤60 kg*	*Women >60 kg*
100 µg	Optimal sustained multiple follicular development for 7 days	Not indicated Results in too low corifollitropin alfa exposure and therefore increases the risk of cycle cancellation Will *not* result in milder stimulation
150 µg	Not indicated Results in too high corifollitropin alfa exposure beyond stimulation day 8 Will *not* result in a higher ovarian response	Optimal sustained multiple follicular development for 7 days

Conclusions

Two dosages of corifollitropin alfa have been developed resulting in similar drug exposure and a similar ovarian response across the complete range of body weight in subjects undergoing ovarian stimulation prior to IVF or ICSI. These two weight-based regimens are derived from pharmacokinetic/pharmacodynamic studies with extensive simulation and modelling, and their efficacy and safety have been confirmed in large prospective clinical trials.

As with daily rFSH treatment, prediction of the ovarian response due to corifollitropin alfa treatment includes factors such as age, AFC and serum AMH. Patients should be treated with a corifollitropin alfa dose based on their body weight (100 μg for subjects weighing ≤60 kg and 150 μg for subjects weighing >60 kg) which is a major determinant of exposure. The ovarian response cannot be reduced by decreasing the dose of corifollitropin alfa, therefore only (potential) normal or low responders with an AFC ≤20 should be treated with corifollitropin alfa.

A single injection of one of the two designated doses of corifollitropin alfa provides an effective alternative to the first seven conventional daily injections of gonadotrophins for IVF patients, resulting in less drug interventions and a simpler treatment regimen.

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4.3 Discussion

The phase II study of follitropin-β [Devroey et al 1994] was not a dose-finding study but a pilot study to demonstrate the feasibility of applying follitropin-β in different GnRH agonist protocols. A dose-finding study was not deemed necessary as the therapeutic dose was calibrated by the *in vivo* bioassay, as for urinary FSH/hMG preparations, and titrated depending on the ovarian response of the patient. The main difference was the high purity of the follitropin-β preparation that allowed subcutaneous, self-administration. In the absence of comparative pharmacokinetics of the subcutaneous and intramuscular route, the first studies of follitropin-β applied the intramuscular route. The highlight of this first IVF trial was reached in 1992 when the first pregnancy and birth of a healthy baby following treatment with follitropin-β was reported in the Lancet [Devroey et al 1992a; Devroey et al 1992b].

The first established pregnancy following ganirelix treatment and fresh embryo transfer was reported during the dose-finding study [Itskovitz et al 1998]. The ganirelix dose-finding study is a typical example of a classical, double-blind, dose-finding study including 333 patients and 6 dosages of which the lowest and highest dose were terminated prematurely because of too low and too high pituitary suppression, respectively [Ganirelix dose-finding study group, 1998]. The 0.25 mg ganirelix dose was selected as the minimal effective daily dose to prevent premature LH surges and interestingly, this dose also yielded the highest implantation rate and ongoing pregnancy rate per started cycle. Importantly, the six dose groups were similar in terms of the number and quality of oocytes and embryos obtained but the implantation rates in the highest dose groups were compromised. Follow-up data of cryopreserved embryos obtained during the dose-finding study did indicate that the high dosages had not adversely affected the potential of embryos to establish pregnancy in freeze-thaw cycles [Kol et al 1999]. Since no pregnancies were established in the 2 mg group following fresh embryo transfer, the impact of daily treatment with 2 mg ganirelix on endometrial development was studied in oocyte donors in a phase IV study [Simon et al 2005].

The phase II dose-finding study of corifollitropin alfa demonstrated that for subjects weighing 50 to 90 kg the optimal dose to sustain multiple follicular development during the first 7 days of ovarian stimulation is higher than 60 µg and lower than 180 µg [Corifollitropin Alfa Dose-Finding Study Group, 2008]. Subsequent dose selection was based on modeling and simulation taking into account the fact that exposure to corifollitropin alfa is inversely related to body weight and that FSH activity had to be retained for an entire week above the threshold required to support multiple follicular development [De Greef et al, 2010]. The main factors that were taken into account in the model were the pharmacokinetics of corifollitropin alfa, inhibin-B levels, the initial follicular response at stimulation day 8, and the number of oocytes retrieved. Simulation indicated that selecting only one dose of corifollitropin alfa for all subjects within different body weight categories would either lead to overdosing subjects

with a lower body weight or under-dosing subjects with a higher body weight. Thus, in line with the requirements to develop the lowest effective dose for the broadest range of body weights, two doses were selected i.e., 150 µg for subjects weighing >60 kg and 100 µg for subjects ≤ 60 kg. The lower dose of 100 µg together with the 60 kg cut-off was selected because simulation predicted the same average exposure and the same ovarian response as the 150 µg dose in subjects weighing >60 kg. This was prospectively confirmed in comparative phase III trials, 107012 (Ensure) and 38819 (Engage), both in terms of exposure and ovarian response [Ledger et al 2011].

Chapter 5

Phase III Randomized, Controlled Trials, Efficacy

Chapter 5 Phase III Randomized, Controlled Trials, Efficacy

5.1 Introduction

Phase III comparative trials are designed with adequate statistical power to demonstrate, in large numbers of patients, that the efficacy (and safety) of one or more selected dosages of a new drug or treatment is non-inferior, equivalent or superior to the active comparator or placebo, although the latter is less common in fertility trials. Preferably, phase III studies are performed with the final drug formulation and presentation, which may vary between pharmaceutical parenterals. The reference of choice is often the established standard of care for the targeted indication. The design of a phase III trial requires many different considerations including the study population and the primary endpoint which may determine the final labeling of the drug once approved, including the wording of the indication, contra-indications and warnings. To prevent bias, phase III studies should always be randomized and blinded studies should be preferred over open-label or assessor-blind studies. If the intended comparison is between different presentations, double-blind double-dummy studies may be indicated. Phase III trials may be split into two phases i.e. IIIa and IIIb. Data from phase IIIa trials contribute to the initial regulatory licence application, whereas phase IIIb trials are performed while the regulatory submission is pending.

Study population

The inclusion and exclusion criteria for patients in each study protocol determine the final study population. For infertile couples, the characteristics of the female partner should be specified one by one including age, body weight, BMI, ovarian reserve (antral follicle count (AFC)), cycle length, endocrine abnormalities, cause of infertility, duration of infertility and history of infertility treatment and outcome. Depending on the primary end-point or drug under investigation, certain patients may be less suitable and should be excluded. For the corifollitropin alfa studies (potential) high responders with an AFC > 20 were consistently excluded from the phase III program. If pregnancy is the primary endpoint of interest, one may want to exclude any pathology that is known to interfere with the probability of pregnancy. Younger patients provide more oocytes and have higher pregnancy rates than older patients, but age may also affect the total number of patients required if pregnancy rate is to become the primary endpoint.

Study endpoints

Phase III studies are often powered to exclude or demonstrate a clinically relevant difference of one or more efficacy endpoints and are less frequently powered to exclude or demonstrate a significant difference in safety endpoints. Efficacy endpoints in comparative phase III trials depend on the desired therapeutic indication and guidelines of Health Authorities may direct

the preferred study design and primary endpoint [ICH Note for Guidance E9 (Statistical Principles for Clinical Trials, 1998); ICH Note for Guidance E10 (Choice of Control Group, 2001); CHMP guideline on the choice of the non-inferiority margin, 2005]. Typically, trials that should support the treatment of patients undergoing controlled ovarian stimulation prior to IVF or ICSI may differ with respect to their efficacy endpoint which could focus on cancellation rates, the duration of stimulation, the total amount of FSH required, the number of cumulus-oocyte complexes retrieved, the number of metaphase II oocytes, the fertilization rate, the number and quality of embryos, the vital or ongoing pregnancy rates, or (single) live birth rate [Arce et al 2005]. The choice of each parameter as the primary endpoint should be justified prior to the study, but also all other clinically relevant efficacy endpoints, usually indicated as (key) secondary endpoints, should be specified. If a phase III study is not large enough to demonstrate or exclude a clinically relevant difference of one of the secondary endpoints of interest, a combined analysis of two or more randomized controlled trials may be considered.

Study designs and reference groups

Follitropin-β was developed both for ovulation induction and ovarian stimulation prior to ART. With respect to the latter, follitropin-β was compared to urinary FSH in a long GnRH agonist protocol which was, at that time, the gold standard. The most frequently applied GnRH agonists were intranasal buserelin (West-Europe), subcutaneous triptorelin (depot or daily, South-Europe) and daily subcutaneous leuprolide acetate (USA). In the first and largest comparative, assessor-blinded trial including 981 patients treated with a long protocol of intranasal buserelin and follitropin-β or urinary FSH (ratio 2:1), the primary end-points were the number of oocytes and the ongoing pregnancy rates [Out et al 1995]. Power calculations were based on the efficacy data of 1000 subjects. Assuming an ongoing pregnancy rate of 15% per started cycle, a difference of 6 to 7% in ongoing pregnancy rate would be detected between the groups with a probability of 80%. Assuming a SD of 6, the study would reveal a difference of 1.2 oocytes between the groups as statistically significant Two smaller comparative trials of follitropin-β, each including 100 patients, were performed to evaluate the efficacy when using no GnRH analogue [Jansen et al 1998] and using a long protocol of subcutaneous triptorelin [Hedon et al 1995]. Clearly, those trials were far too small to detect a clinically meaningful difference between the groups in ongoing pregnancy rates.

The first comparative phase III efficacy and safety study of ganirelix [The European Orgalutran® study group et al, 2000] was designed as an open-label, non-inferiority trial to show that the clinical outcome of the ganirelix regimen was “no worse” than the comparator i.e. a long GnRH agonist protocol. This first and largest comparative phase III trial included about 700 patients who were treated in a ratio of 2:1 with ganirelix or a long protocol of intranasal buserelin. The primary endpoint was the number of cumulus-oocytes complexes

recovered and the lower limit of the treatment difference was set at -3 oocytes. In addition, for the ongoing pregnancy rate per started cycle, a predefined treatment difference of -5% was considered to be acceptable anticipating an ongoing pregnancy rate of about 22%. Following the first large study, two smaller randomized controlled studies, each including approximately 300 patients, were performed using a long protocol of leuprolide acetate and triptorelin as a reference [The North American Ganirelix Study Group, 2001; The European and Middle East Orgalutran Study Group, 2001].

Many years later, the first comparative phase III study of corifollitropin alfa was also designed as a non-inferiority trial but with a double-blind, double-dummy design [Devroey et al 2009a; Fauser et al 2010]. The trial included 1506 patients (age 18-36 years; body weight > 60 kg) treated with a single dose of 150 µg corifollitropin alfa or 200 IU follitropin-β (ratio 1:1). The primary efficacy parameter was the ongoing pregnancy rate and the pre-defined non-inferiority margin of the treatment difference was -8%. This implied that if the lower limit of the 95% confidence interval (CI) was above -8%, the corifollitropin alfa treatment was considered non-inferior to the reference treatment. This lower margin was chosen as it was associated with an observed maximum treatment difference of 4% between the treatment groups, which was thought to be acceptable. The trial also included the number of oocytes retrieved as a co-primary endpoint with a predefined equivalence margin of -3 and +5 oocytes. The clinical justification was that -3 oocytes would translate into one good quality embryo less for transfer and that +5 oocytes would significantly increase the risk of OHSS by increasing the predicted ovarian response from approximately 13 to 18 oocytes [Papanikolaou et al 2006]. In parallel to this very large non-inferiority phase III study, a smaller double-blind double-dummy study was performed with 100 µg corifollitropin alfa including patients with a body weight ≤ 60 kg in the same age group from 18-36 years [Corifollitropin alfa ENSURE study group, 2010]. This trial was designed to show equivalence between 100 µg corifollitropin alfa and 150 IU follitropin--β (ratio 2:1) in terms of the number of oocytes retrieved using a predefined equivalence margin of -3 and +5 oocytes. Modeling and simulation had predicted that the 100 µg and 150 µg in the two body weight categories would provide the same exposure and therefore the same ovarian response [Ledger et al 2011]. Following both trials, another large double-blind double-dummy trial was performed with 150 µg corifollitropin alfa to confirm non-inferior pregnancy rates versus 300 IU follitropin-β in patients aged 35-42 years [Boostanfar et al, 2012b].

In summary, during phase III trials, follitropin-β was compared to urinary FSH and developed in a long GnRH agonist protocol. Second, the GnRH antagonist ganirelix was compared to a long GnRH agonist protocol and follitropin-β was used for ovarian stimulation. Finally corifollitropin alfa was compared to follitropin-β in a GnRH antagonist protocol. The

primary endpoints for the largest, randomized, controlled, phase III trials were consistently the number of oocytes retrieved and/or ongoing or vital pregnancy rates per started cycle.

5.2 Results

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A prospective, randomized, assessor-blind, multicentre study comparing recombinant and urinary follicle stimulating hormone (Puregon versus Metrodin) in in-vitro fertilization

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Urinary follicle stimulating hormone (FSH) is being used for the treatment of human infertility. Recently, FSH manufactured by means of recombinant DNA technology with a much higher purity (>99%) has become available. A prospective, randomized, assessor-blind, multicentre (n = 18) study was conducted in infertile women undergoing in-vitro fertilization comparing recombinant FSH (Org 32489, Puregon®) and urinary FSH (Metrodin®). Eligible subjects were randomized (recombinant versus urinary FSH = 3:2) and pretreated with buserelin for pituitary suppression. FSH was given until three or more follicles with a diameter of at least 17 mm were seen. After oocyte retrieval, fertilization routines were applied according to local procedures. No more than three embryos were replaced. In all, 585 subjects received recombinant FSH and 396 urinary FSH. Significantly more oocytes were retrieved after recombinant FSH treatment (mean adjusted for centre 10.84 versus 8.95, $P < 0.0001$). Ongoing pregnancy rates per attempt and transfer in the recombinant FSH group were 22.17 and 25.97% respectively, and in the urinary FSH group, 18.22 and 22.02% respectively (not significant). Ongoing pregnancy rates including pregnancies resulting from frozen–thawed embryo cycles were 25.7% for recombinant and 20.4% for urinary FSH (P = 0.05). Compared to urinary FSH, the total dose of FSH was significantly lower with recombinant FSH (2138 versus 2385 IU, $P < 0.0001$) in a significantly shorter treatment period (10.7 versus 11.3 days, $P < 0.0001$). No clinically relevant differences between recombinant and urinary FSH were seen with respect to safety variables. It is concluded that recombinant FSH (Puregon) is more effective than urinary FSH in inducing multifollicular development and achieving an ongoing pregnancy.

Key words: IVF/ovarian stimulation/Puregon/recombinant FSH

Introduction

For >30 years, human menopausal gonadotrophins (HMG) have been applied in the treatment of human infertility. Clinical applications include ovulation induction in clomiphene-resistant anovulatory women and ovarian stimulation in assisted reproduction techniques, e.g. in-vitro fertilization (IVF) (Breckwoldt and Zahradnik, 1991). Most HMG preparations contain either equal amounts of follicle stimulating hormone (FSH) and luteinizing hormone (LH) activity (FSH/LH ratio = 1) or mainly FSH activity with minor amounts of LH activity (urofollitrophin, FSH/LH ratio ≥60). The production of these hormones depends on the collection of huge amounts of urine. The use of urine sources implies limited product consistency and purity (1–5%).

Recently, FSH has been manufactured by means of recombinant DNA technology using a Chinese hamster ovary (CHO) cell line transfected with the genes encoding human FSH (Van Wezenbeek *et al.*, 1990). The final product (Org 32489, Puregon®) is purified up to 99% purity, does not contain any LH activity and is very similar to natural FSH (Hård *et al.*, 1990), although small differences in oligosaccharide moieties and isohormone composition are present.

Clinical experiences with recombinant FSH indicate the potential of the compound to induce follicular growth, and pregnancy can be achieved (Devroey *et al.*, 1994). In this paper, a multicentre trial is described evaluating the efficacy and safety of recombinant FSH to achieve ovarian stimulation in infertile women undergoing IVF, in comparison with urinary FSH.

Materials and methods

Patients

Between March 1992 and August 1993, infertile female subjects were recruited at 18 different IVF centres throughout Europe (see Acknowledgements section). The aim was to include 1000 patients. Inclusion criteria were as follows: patients had to be 18–39 years of age at the time of screening; have a cause of infertility which was potentially solvable by IVF; a maximum of three previous IVF or other assisted reproduction attempts in which oocytes were collected at least once; normal ovulatory cycles with a mean length of between 24 and 35 days and an intra-individual variation of plus or minus 3 days (but never outside the 24–35 days range); good physical and mental health; and a body weight 80–130% of the ideal body weight (adapted from the Metropolitan Life Insurance Company Tables).

Exclusion criteria were: infertility caused by endocrine abnormalities such as hyperprolactinaemia, polycystic ovary syndrome, and absence of ovarian function; male infertility as defined by $<10\times10^6$ spermatozoa/ml and/or <40% normal morphology and/or <40% normal motility; any ovarian and/or abdominal abnormality that would interfere with adequate ultrasound investigation; hypertension (sitting diastolic blood pressure >90 mm Hg and/or systolic blood

pressure >150 mm Hg); chronic cardiovascular, hepatic, renal, or pulmonary disease; a history of (within 12 months) or current abuse of alcohol or drugs; administration of non-registered investigational drugs within 3 months prior to screening. When all criteria were met, the subject was considered to be eligible. The study was approved by the Ethics Committee of each local hospital. All subjects gave written informed consent. This investigation was performed according to the Declaration of Helsinki and the European Community note on Good Clinical Practice for trials on medicinal products in the European Community (CPMP Working Party on Efficacy of Medicinal Products, 1990).

Study design

This was a randomized, assessor-blind, prospective, multicentre study comparing recombinant human FSH (Org 32489, Puregon®, NV Organon, Oss, The Netherlands, batch numbers CP 091134 and 091077) and urinary FSH (urofollitropin, Metrodin®, Ares-Serono, Switzerland, batch numbers CP 092139, 093057, 092047, 091163). The objective of the study was to assess the efficacy and safety of recombinant FSH in relation to urinary FSH for the induction of ovarian stimulation in infertile pituitary-suppressed subjects undergoing IVF. Eligible subjects were randomized by receiving a subject number from a randomization list corresponding with patient boxes in which the medication was kept. The randomization procedure included a ratio between recombinant and urinary FSH of 3:2. All centres followed an identical clinical protocol and used standardized case report forms.

Pituitary down-regulation started on the first day of the menstruation by means of intranasal buserelin (Suprecur®, Hoechst, Germany). The initial dose was 4×150 μg daily. When suppression was not achieved (serum oestradiol >200 pmol/l) after 14 days, the dose was doubled (4×300 μg daily). The buserelin intake was sustained throughout the FSH treatment. The FSH dose for the first 4 days was 150 or 225 IU (two or three ampoules i.m.). Afterwards, the dose was adjusted according to follicular development as assessed by ultrasound scanning. Since, for technical reasons, recombinant FSH was supplied in vials and urinary FSH in ampoules, a double-blind design was not feasible. Instead, an assessor-blind design was chosen in which preparation and administration of the medication was done by a study co-ordinator who took no part in any decision concerning the FSH dose during treatment. When at least three follicles ≥17 mm were present, 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl®, NV Organon, The Netherlands) was given i.m. to induce ovulation. Oocyte retrieval, fertilization procedures and embryo transfer were done according to the local standards. A maximum of three embryos was transferred. Luteal support was given during at least 2 weeks and included minimally three injections of 1500 IU HCG or at least 50 mg of progesterone daily i.m. or 400 mg progesterone daily intravaginally.

End-points

The primary outcome variables were the number of oocytes retrieved, and ongoing pregnancy rate per attempt and transfer as assessed by ultrasound scanning at least 12 weeks following embryo transfer.

Secondary variables included number of follicles ≥15 mm and ≥17 mm on the day of HCG administration, length of FSH treatment, total dose, serum concentrations of FSH and oestradiol on the day of administering HCG, number of mature oocytes recovered, number of high quality embryos, implantation rate, clinical pregnancy rates per attempt and transfer. Implantation rate was defined as the number of vital fetuses as assessed by ultrasound at least 12 weeks after embryo transfer, divided by the number of embryos transferred for each subject. The definition of a clinical pregnancy included miscarriages with or without proof of a vital fetus.

Fertilization and cleavage rates are not reported due to the heterogeneity in IVF routines across the centres.

The main safety parameters were the incidence of ovarian hyperstimulation syndrome (OHSS) and the development of anti-FSH antibodies and anti-CHO cell-derived protein antibodies. Also, common laboratory parameters were compared before and after treatment. These parameters included routine blood biochemistry as sodium, potassium, chloride, bicarbonate, phosphorus, calcium, glucose, urea, creatinine, alkaline phosphatase, alanine amino transferase, aspartase amino transferase, lactic dehydrogenase, total bilirubin, total protein, albumin; haematology parameters included haemoglobin, haematocrit, erythrocytes, leukocytes plus differentiation; urinalysis included quantitative estimation of pH and qualitative estimations of protein, acetone, glucose, and haemoglobin.

Assessments

At screening, the medical history was obtained and a physical examination was performed. Routine blood biochemistry, haematology and urinalysis were done and the following endocrinological parameters were measured: serum oestradiol, FSH, LH, progesterone, testosterone, prolactin, and dehydroepiandrosterone sulphate. An ultrasound scan was done to exclude ovarian abnormalities. Sperm analysis of the partner took place and was repeated at the time of fertilization.

Serum oestradiol concentrations were measured to ensure optimal pituitary suppression prior to the first FSH injection. Serum FSH, LH, oestradiol and progesterone were measured on the first day of FSH treatment and on the day of HCG administration. In between, assessments of serum oestradiol and LH were done on a regular basis. Frequent ultrasound scans were made to monitor follicular growth.

Spare serum samples for the determination of anti-FSH and anti-CHO-cell derived protein antibodies were taken before and after treatment. Routine blood biochemistry, haematology and urinalysis were repeated as soon as possible after FSH treatment had ended.

Classification of oocytes as either mature or immature and embryos as type 1, 2, 3, or 4 was done according to previously published criteria (Staessen *et al.*, 1989). Type 1 and 2 were considered to be high quality embryos.

Assays

Antibody assay

Blood samples processed to serum taken before and after FSH treatment were sent to NV Organon, The Netherlands for central determination of anti-FSH and anti-CHO cell-derived protein antibodies.

Anti-FSH antibodies. The presence of specific antibodies against human FSH was assessed by a semi-quantitative radioimmunoassay in duplicate. In short, ^{125}I-labelled recombinant FSH was allowed to react with antibodies present in the sample. The immune-complexes formed were subsequently precipitated with polyethylene glycol (PEG 8000). After removal of the supernatant, bound radioactivity in the pellet was quantified. A calibration curve with human anti-FSH antibodies that would allow quantitative determination of the serum anti-FSH antibody concentration could not be established, since there is no representative standard human anti-FSH antibody preparation available. Therefore, all values were expressed as percentage of the total amount of tracer added in the assay and were corrected for the non-specific binding. Clinically relevant antibody titres were defined as those yielding a binding percentage of >25%.

Anti-CHO cell-derived protein antibodies. The occurrence of antibodies against proteins from the CHO cell line was assessed by a

semi-quantitative enzyme-immunoassay. In short, CHO cell-derived proteins were coated to the wall of 96-well microtitre plates. Antibodies in the sample were allowed to bind to the solid-phase-coated CHO cell-derived proteins, where they were then detected with a horseradish peroxidase coupled second antibody [goat antihuman immunoglobulin (IgG)]. The end product of the enzyme reaction was quantified spectrophotometrically at 450 nm, corrected for the optical density at 690 nm. Each analytical run included a series of six concentrations in human serum of the IgG fraction of a rabbit polyclonal antiserum against CHO cell-derived proteins as positive control. The CHO cell-derived proteins used to obtain this antiserum and the CHO cell-derived proteins applied in the assay were purified from the culture supernatant of a mock-transfected CHO cell line.

Other assays

Sperm analysis and measurement of blood biochemistry, haematology, urinalysis and endocrinological parameters were done at the local hospital according to local standards. Follicular size was measured with local ultrasound equipment and a vaginal probe.

Sample size

Power calculations were performed in order to assess the magnitude of treatment effects capable of detection in this large study, and were based on efficacy data of 1000 subjects, assuming that at least 850 subjects had an oocyte retrieval and embryo transfer (Dupont and Plummer, 1990). When testing at the customary 5% significance level (two-sided), and assuming an SD of 6, a difference of 1.2 oocytes in the two treatment groups would have been detected statistically with a probability of 80%. With respect to dichotomous variables such as pregnancy, by assuming a pregnancy rate of 15% per attempt and 18% per transfer for one treatment group, a value per attempt (and transfer) as small as 9% (11%) or as large as 22% (26%) for the second group would have been detected statistically with an 80% probability, using a two-sided χ^2 test, again at the 5% significance level. Therefore, the size of the study ensured that fairly modest treatment effects would have been detected with a high probability.

Statistical analysis

For ordinal data a general parametric approach (Whitehead and Whitehead, 1991) of combining individual centre results was applied and used for communication of results and eventual analysis. For binary data (pregnancy outcome) the Mantel–Haenszel test statistic extended for multiple centres was used. In both cases the combination of centre results was expressed as means adjusted for centre and approximate confidence intervals (CI) were calculated based on the normal distribution.

All analyses were done on an intent-to-treat basis, including all subjects who received at least one ampoule of FSH. The main advantages of this rule were that more patients were available for final analysis of efficacy and that it more closely reflected how physicians evaluate a therapeutic agent in the clinical setting, outside an experimental control.

Results

Patients

A total of 1027 subjects (recombinant FSH: n = 615, urinary FSH: n = 412) was randomized, 1007 (recombinant FSH: n = 602, urinary FSH: n = 405) started buserelin pretreatment and 981 (recombinant FSH: n = 585, urinary FSH: n = 396) started FSH treatment. The number of subjects treated with FSH per centre was 10–146 (mean 54.5).

Both treatment groups were comparable in demographic and infertility characteristics (Table I). The main cause of infertility was tubal disease (64.4 and 64.1% for recombinant versus urinary FSH respectively). The mean duration of infertility for recombinant and urinary FSH was 6.3 and 6.1 years respectively.

Table I. Demographic and infertility characteristics

Characteristic	Recombinant FSH (n = 585)	Urinary FSH (n = 396)
Mean age (years)	32.2	32.3
Mean weight (kg)	61.3	61.2
Mean height (cm)	164.4	164.3
Number (%) of subjects with cause of infertility		
Tubal disease	377 (64.4)	254 (64.1)
Endometriosis	45 (7.7)	30 (7.6)
Tubal disease + endometriosis	23 (3.9)	15 (3.8)
Unknown	117 (20.0)	79 (19.9)
Other	23 (3.9)	18 (4.5)
Mean duration of infertility (years)	6.3	6.1
Number (%) of subjects with primary infertility	259 (44.3)	174 (43.9)
Number (%) of subjects with secondary infertility	326 (55.7)	222 (56.1)

Primary efficacy parameters

The results of the main efficacy parameters are given in Table II. In the recombinant FSH group, a mean number (adjusted for centre) of 10.84 oocytes was recovered, compared to 8.95 in the urinary FSH group. The difference of 1.89 was highly significant (P <0.0001; 95% CI 1.2–2.6). The mean number of oocytes recovered across the centres ranged from 7.2 to 16.4 for recombinant FSH and from 4.0 to 12.8 for urinary FSH, the differences varying from 0.62 to 5.50 oocytes. In all centres, more oocytes were retrieved after recombinant FSH treatment (Figure 1).

Ongoing pregnancy rates per attempt and transfer and adjusted for centre were 22.17 and 25.97% respectively for the recombinant FSH group, and 18.22 and 22.02% respectively, in the urinary FSH group. Until August 1994, 117 and 73 subjects in the recombinant and urinary FSH group respectively, subsequently underwent a natural cycle during which frozen–thawed embryos were replaced, resulting in 17 ongoing pregnancies in the recombinant FSH group and five in the urinary FSH group. A second 'frozen embryo' cycle was done in 26 and 15 women, which resulted in seven additional pregnancies: five in the recombinant and two in the urinary FSH group. Eight women had a third frozen embryo cycle and two subjects a fourth, which did not result in ongoing pregnancies. The mean number of embryos transferred in the frozen embryo cycles was 2.1 for both groups. In total, 22 additional pregnancies were obtained in the recombinant FSH group, and seven in the urinary FSH group, resulting in cumulative ongoing pregnancy rates (adjusted for centre) of 25.7 and 20.4% in favour of recombinant FSH (P = 0.05).

Secondary efficacy parameters

Results of the secondary parameters are given in Table III. On the day of HCG, significantly more follicles ≥15 mm were seen in the recombinant FSH group (n = 7.49, mean adjusted

Table II. Results on main parameters

Parameter	Mean adjusted for centre		Recombinant minus urinary FSH		
	Recombinant FSH	Urinary FSH	Difference	SE	95% CI
No. of oocytes recovered	10.84	8.95	1.89	0.37	1.2–2.6 ($P < 0.0001$)
Ongoing pregnancy rate (%) per attempt	22.17	18.22	3.95	2.59	−1.1–9.0 NS
Ongoing pregnancy rate (%) per transfer	25.97	22.02	3.95	3.01	−1.9–9.8 NS

CI = confidence interval.
NS = not significant.

for centre), compared to the urinary FSH group (n = 6.67, P = 0.0002, 95% CI of difference: 0.4–1.2). Figure 2 demonstrates that development of large (≥15 mm) follicles began to diverge between the groups after 5–6 days of FSH treatment. This higher number of follicles was associated with a significantly increased maximum serum oestradiol in the recombinant FSH group (mean adjusted for centre 6084 versus 5179 pmol/l, P <0.0001). On the day of HCG, FSH concentrations were significantly higher in the urinary FSH group (12.1 versus 11.5 IU/l, P = 0.03). A significantly lower total dose of recombinant FSH (mean adjusted for centre 2138 versus 2385 IU, P <0.0001) were needed in an also significantly shorter treatment period (10.7 versus 11.3 days, P <0.0001) compared to the urinary FSH group. There was no linear relationship between the number of ampoules administered and number of oocytes collected.

Mean LH concentrations after down-regulation before start of the FSH treatment were 1.6 and 1.7 IU/l, and on the day of HCG administration 1.2 and 1.3 IU/l in the recombinant and urinary FSH groups respectively.

After oocyte retrieval, more mature oocytes (difference 1.8, P <0.0001; 95% CI 1.1–2.4) were recovered and more high quality embryos (difference 0.5, P = 0.003; 95% CI 0.2–0.8) were obtained in the recombinant FSH group.

No significant differences between recombinant and urinary FSH were seen in the number of follicles ≥17 mm on the day of HCG, the implantation rate and the clinical pregnancy rates per attempt and transfer (see Table III).

The mean number of oocytes with two pronuclei was 6.8 and 5.6 in the recombinant and urinary FSH groups respectively, as assessed 12–18 h after incubation with semen. Oocytes with three or more pronuclei were seen in 208 subjects (38.2%) in the recombinant FSH group, compared to 117 (32.4%) in the urinary FSH group. A mean number of 2.58 and 1.81 embryos were frozen in the recombinant and urinary FSH groups respectively.

Cycle cancellations

In all, 152 patients started FSH treatment but did not have an embryo transfer (recombinant FSH: n = 85, 14.5%; urinary FSH: n = 67, 16.9%; not significantly different). Low ovarian response was reported in 27 subjects in the recombinant FSH group (4.6%) and in 30 in the urinary FSH group (7.6%). The risk of OHSS was the reason for cancellation in 12 recombinant

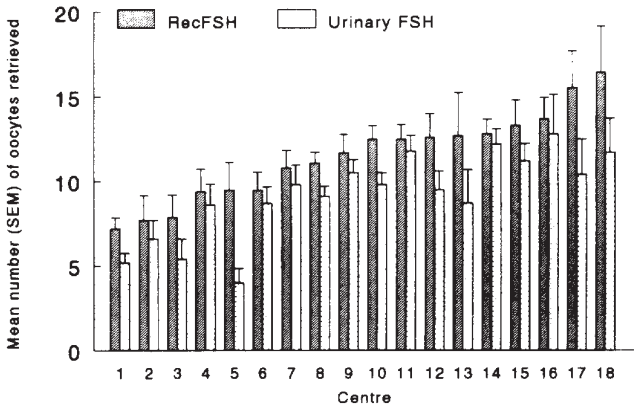

Figure 1. Mean (+ SEM) number of oocytes retrieved per centre. FSH = follicle stimulating hormone. Rec = recombinant.

FSH-treated subjects (2.1%) and six urinary FSH-treated subjects (1.5%). Unsuccessful fertilization was the reason for premature discontinuation in 29 subjects (5.0%) in the recombinant FSH group, compared to 16 (4.0%) in the urinary FSH group.

Safety

OHSS leading to hospitalization was seen in 19 out of 585 recombinant FSH-treated subjects (3.2%) compared with eight out of 396 urinary FSH-treated subjects (2.0%, not significantly different). In 545 recombinant FSH-treated and 353 urinary FSH-treated subjects spare serum samples could be assessed for the presence of anti-FSH and anti-CHO cell-derived protein antibodies. No significant rises of serum antibody concentrations were found. Clinically relevant changes from base line of routine blood biochemistry, haematology and urinalysis were not detected.

Discussion

To our knowledge, this is the largest prospective, randomized clinical trial ever performed in IVF. In total, 585 subjects received recombinant FSH (Puregon®) and 396 urinary FSH (Metrodin®) for ovarian stimulation. The aim of stimulation was to increase the number of oocytes for assisted reproduction. Therefore, the number of oocytes retrieved was chosen as

Table III. Results on secondary parameters

Parameter	Mean adjusted for centre		Recombinant minus urinary FSH		
	Recombinant FSH	Urinary FSH	Difference	SE	95% CI
No. of follicles ≥15 mm on day of HCG	7.49	6.67	0.81	0.22	0.4–1.2 (P = 0.0002)
No. of follicles ≥ 17 mm on day of HCG	4.61	4.38	0.23	0.14	−0.0–0.5 NS
Maximum serum oestradiol (pmol/l)	6084	5179	905	210	494–1317 (P < 0.0001)
Serum FSH on day of HCG (IU/l)	11.5	12.1	−0.6	0.26	−1.1 to −0.1 (P = 0.03)
Total no. of ampoules used	28.5	31.8	−3.3	0.62	−4.5 to −2.1 (P < 0.0001)
Treatment length (days)	10.7	11.3	−0.6	0.13	−0.9 to −0.3 (P < 0.0001)
No. of mature oocytes recovered	8.55	6.76	1.79	0.33	1.1–2.4 (P < 0.0001)
No. of high quality embryos	3.11	2.61	0.50	0.17	0.2–0.8 (P = 0.003)
Implantation rate (%)	0.11	0.09	0.01	0.02	−0.02–0.05 NS
Clinical pregnancy rate (%) per attempt	29.29	25.30	3.99	2.88	−1.6–9.6 NS
Clinical pregnancy rate (%) per transfer	34.30	30.46	3.84	3.31	−2.6–10.3 NS

CI = confidence interval.
NS = not significant.
HCG = human chorionic gonadotrophin.
FSH = follicle stimulating hormone.

one of the main efficacy parameters in this study. Ovarian stimulation was continued until there was evidence of adequate multiple follicular development (at least three follicles ≥17 mm in diameter). The fact that the treatment groups did not differ significantly with respect to the number of follicles ≥17 mm on the day of HCG indicated that the stimulation procedures were carried out in a similar way. However, a significantly higher number of oocytes was retrieved in the recombinant FSH group. Despite the large well-known differences between centres in overall number of oocytes retrieved, this finding was consistently in favour of recombinant FSH throughout all centres (Figure 1). Accordingly, a larger cohort of follicles was recruited in these subjects, as illustrated by the significantly higher number of follicles ≥15 mm in the recombinant FSH group seen on the last ultrasound before oocyte retrieval. The significantly higher maximum serum oestradiol concentration in the recombinant FSH group is most likely a reflection of this larger number of follicles. Interestingly, overall concentrations of immunoreactive FSH on the day of HCG were slightly but significantly lower in the recombinant FSH group, even though different types of FSH assays were applied which increased the overall variability. This might be related to the significantly lower amount of recombinant FSH administered and the significantly shorter treatment period in this group. It also illustrates that FSH concentrations based on immunoassay measurements have only limited value in assessing the true potency, since they only reflect the number of circulating FSH molecules but not their actual biological activity.

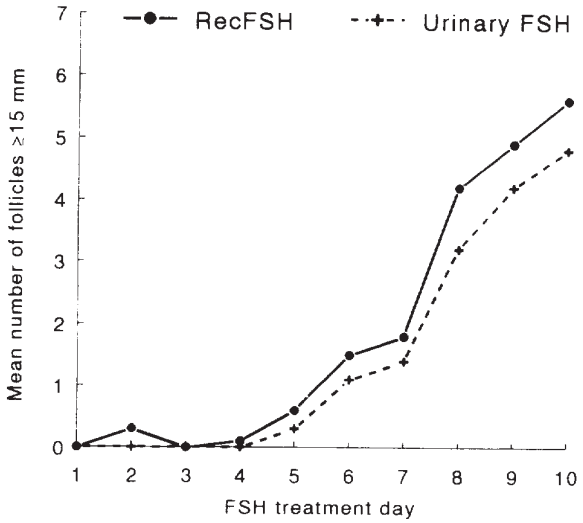

Figure 2. Mean number of follicles ≥15 mm related to the follicle stimulating hormone (FSH) treatment day. Rec = recombinant.

According to pharmacopeial requirements (Council of Europe, 1986), the FSH activity of a batch is calibrated in the in-vivo Steelman–Pohley rat assay, against an International Standard Preparation (Steelman and Pohley, 1953). This rat model is apparently not valid to predict clinical activity in the human, given the differences we found using nominally equal preparations with the same declared content, namely 75 IU in-vivo bioactivity per ampoule.

Possible factors which might explain the higher potency of recombinant FSH compared to urinary FSH include subtle differences at the level of the oligosaccharide moieties of the molecules, differences in isohormone composition (Matikainen *et al.*, 1994), or the proteinaceous contaminants in the urinary product inhibiting FSH action and the pharmaceutical formulation. Further research is needed to elucidate the influence of these factors on the clinical efficacy of gonadotrophin preparations. Not all recombinant FSH products give identical results with IVF, or are superior to urinary FSH (recombinant human FSH study group, 1995). In fact, in that study oestradiol levels at the day of HCG administration were significantly lower in the recombinant FSH group. This surely suggests that differences between various recombinant FSH preparations exist.

With respect to the other main efficacy parameter, ongoing pregnancy rate, no statistically significant difference between the recombinant and urinary FSH groups was found. This is to be expected since the significant treatment differences in favour of recombinant FSH, such as the higher number of oocytes retrieved and the larger number of high quality embryos, are basically nullified since both groups 'restarted' treatment at an equal position at the moment of transfer of a fixed maximum number of embryos. In both treatment groups a mean of 2.4 embryos were replaced. However, differences in ongoing pregnancy rates including 'frozen embryo' cycles reached statistical significance in favour of recombinant FSH ($P = 0.05$). This might be due to the availability of better quality embryos after recombinant FSH treatment, next to the obvious reason that merely the presence of more embryos ultimately will lead to more pregnancies.

The most important side-effect of gonadotrophin treatment in ovarian stimulation is the occurrence of OHSS. This possibly life-threatening condition is characterized in its most serious forms by ascites, haemoconcentration, coagulation and electrolyte disorders and extreme ovarian enlargement (Rizk and Smitz, 1992). Despite the higher number of follicles recruited and the increased serum oestradiol concentration on the day of HCG administration, which are both risk factors for the development of the syndrome, its incidence was not statistically significantly higher after recombinant FSH treatment. However, the incidence of severe hyperstimulation requiring hospitalization is so low that the power of the study would be insufficient to detect a significant difference of 2%. Therefore, given the higher potency of recombinant FSH, careful monitoring to prevent the occurrence of this syndrome is essential.

Recombinant and natural FSH differ slightly at the level of carbohydrate moieties (Hård *et al.*, 1990) and potentially, minute amounts of host cell-originating contaminations might be present in the recombinant preparation. To investigate the immunogenic characteristics, and to rule out any risk, antibody development against FSH and CHO cell-derived proteins was assayed. Antibody formation was not seen in any of the recombinant FSH-treated patients.

In conclusion, this study has demonstrated that recombinant FSH (Puregon®) is more efficacious than urinary FSH (Metrodin®) as assessed by the number of oocytes retrieved. Pregnancy rates including frozen embryo cycles were significantly higher after recombinant FSH treatment. Recombinant and urinary FSH are equally safe.

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Treatment with the gonadotrophin-releasing hormone antagonist ganirelix in women undergoing ovarian stimulation with recombinant follicle stimulating hormone is effective, safe and convenient: results of a controlled, randomized, multicentre trial

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A multicentre, open-label, randomized study of the gonadotrophin-releasing hormone (GnRH) antagonist ganirelix (Orgalutran®/Antagon™) was performed in women undergoing ovarian stimulation with recombinant FSH (rFSH: Puregon®). The study was designed as a non-inferiority study using a long protocol of buserelin (intranasal) and rFSH 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 infertility 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 rFSH 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 ≥10 IU/l) was 2.8% versus 1.3% during rFSH stimulation in the buserelin group. On the day of triggering ovulation by human chorionic gonadotrophin (HCG), the mean number of follicles ≥11 mm diameter was 10.7 and 11.8, and the median serum oestradiol 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.

Key words: buserelin/ganirelix/GnRH agonist/GnRH antagonist/ICSI/IVF/ovarian stimulation/recombinant FSH

Introduction

Ganirelix is the active ingredient of Orgalutran® and Antagon™, a gonadotrophin-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 (Oberyé *et al.*, 1999a).

In current practice, GnRH agonists are used to suppress endogenous gonadotrophins during ovarian stimulation (Loumaye, 1990). However, agonists initially stimulate the release of gonadotrophins (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 gonadotrophins 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 (Oberyé *et al.*, 1999b). Moreover, after

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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 gonadotrophins, 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 oestradiol concentrations, the use of GnRH agonist instead of human chorionic gonadotrophin (HCG) is thought to prevent the clinical manifestation of OHSS (Itskovitz *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, 1999). 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 (Ganirelix dose-finding study group, 1998; Itskovitz-Eldor *et al.*, 1998). In this study, patients were treated with a fixed dose of 150 IU rFSH 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; Oberyé *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 rFSH for IVF or intracytoplasmic sperm injection (ICSI).

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 and MacRae, 1999), stratifying for centre age, for primary or secondary infertility, and for IVF or ICSI.

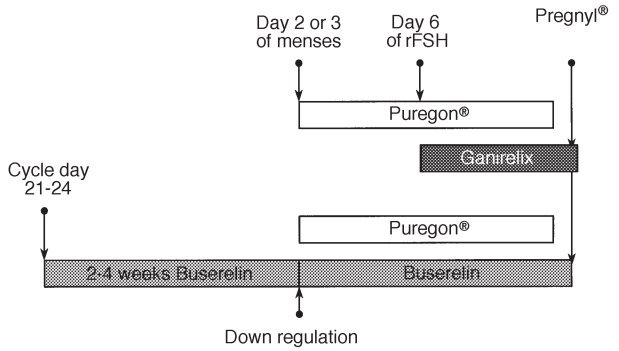

Figure 1. Schematic of the treatment regimen with ganirelix (upper part of diagram) and with a long protocol of intranasal buserelin (lower part) in patients undergoing ovarian stimulation with recombinant FSH (rFSH; Puregon®).

A diagrammatic representation of the applied treatment regimens is shown in Figure 1. Injections of rFSH (Puregon®; NV Organon) and ganirelix were given in the morning. In the ganirelix group, treatment with rFSH was started in patients on day 2 or 3 of the menstrual cycle by a once-daily s.c. injection. After 5 days of rFSH 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 oestradiol 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 was discontinued.

In both treatment groups, ovarian stimulation was started with a fixed daily dose of 150 IU rFSH for the first five treatment days. From day 6 onwards, the dose of rFSH was adapted depending on the ovarian response as monitored via ultrasonography. On the day of HCG, rFSH was not administered.

HCG (10 000 IU, Pregnyl®; NV Organon) was administered when at least three follicles of $\geqslant$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 rFSH, 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 rFSH, a blood sample

was taken for hormone assessment and ultrasonography (USS) was performed. From rFSH 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, oestradiol 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 rFSH 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 and 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.

Table I. Demographics and infertility characteristics

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 infertility (years)[a]	4.5 ± 2.7	4.4 ± 2.7
Main causes of infertility (%)		
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

[a]Values are mean ± SD.

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 rFSH treatment when patients were not yet exposed to ganirelix.

Results

Patient characteristics

The two treatment groups were similar with respect to age, body height, weight and BMI (see Table I). 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 infertility 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.

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 rFSH or GnRH analogue treatment. The number of patients per treatment stage is presented in Table II.

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

Table II. Number of patients per treatment stage

	Ganirelix	Buserelin
No. of subjects randomized to group	463	238
Treated with buserelin	–	237 (100)
Treated with rFSH	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)

[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 gonadotrophin.

insemination instead of oocyte retrieval. In the buserelin group, seven patients (3.0%) were discontinued before starting rFSH due to insufficient down-regulation. In the ganirelix group, two patients (0.4%) were discontinued because of premature luteinization.

Duration of GnRH analogue treatment and total dose of rFSH

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 rFSH treatment was 9 (6–18) and 10 (6–19) days respectively. The total amount of rFSH administered was in total 1500 (900–5400) 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 oestradiol 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 cut-down 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.

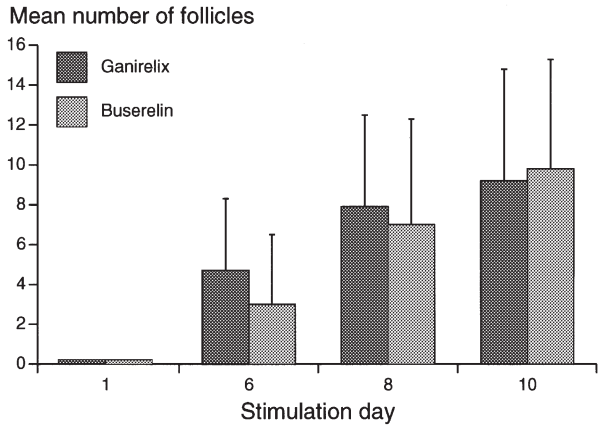

Figure 2. Number of follicles of ⩾11 mm diameter on stimulation days 1, 6, 8 and 10 of rFSH for patients with at least 9 days of stimulation. Values are mean ± SD.

Table III. 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

	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)
Oestradiol (pg/ml)	1190 (373–3105)	1700 (527–4070)
Progesterone (ng/ml)	0.7 (0.4–1.6)	0.7 (0.4–1.6)
Oestradiol/follicle (pg/ml)	111	144

[a]Includes one patient who received HCG because of premature luteinization.

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. The number and size of follicles measured at the day of HCG administration are summarized in Table III.

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 rFSH 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). 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

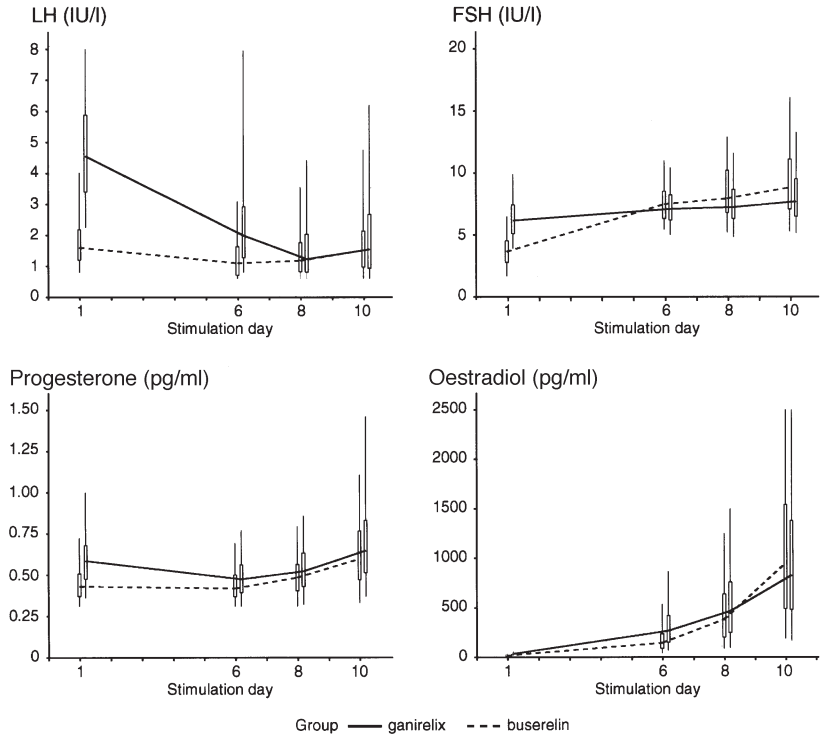

Figure 3. Serum hormone concentrations on stimulation days 1, 6, 8 and 10 of rFSH for patients with at least 9 days of stimulation. The boxes indicate the 75% and 25% percentiles, the vertical lines indicate the 95% and 5% percentiles, and median values are connected.

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 III), as also reflected by the comparable mean number of follicles ⩾17 mm at the day of HCG administration (4.9 versus 5.2).

Serum hormone concentrations

Median serum FSH, LH, oestradiol 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 3. At the start of rFSH 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 oestradiol concentrations in the ganirelix group. Median serum oestradiol 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 oestradiol concentrations followed the pattern of follicular growth in that on day 8 the oestradiol concentrations were only 128 pg/ml higher in the ganirelix group than in the buserelin group, whereas on the day of HCG (see Table III) the opposite was observed, with serum oestradiol concentration being about 500 pg/ml lower in the ganirelix group than in the buserelin group (1190 versus 1700 pg/ml). Finally, serum oestradiol content per follicle (⩾11 mm) was lower in the ganirelix group than in the buserelin group.

Individual serum LH values measured on day 6 and measured on the day of HCG were plotted in Figure 4. 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 4) or between serum oestradiol 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 IV. 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.

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.

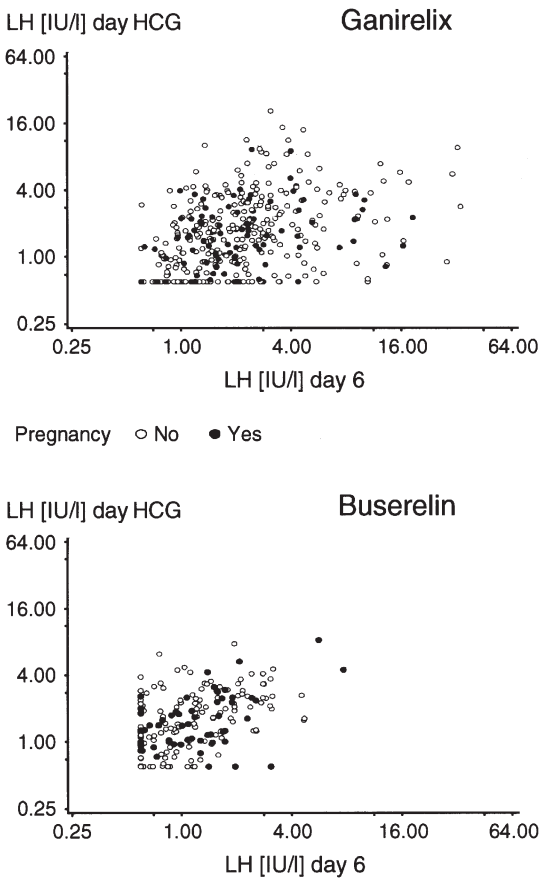

Figure 4. Concentrations of serum LH in individual patients on day 6 of stimulation versus serum LH on the day of HCG. Filled points represent values of patients who became pregnant.

Table IV. 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

	Ganirelix	Buserelin
Cumulus–oocyte complexes/attempt	n = 463	n = 238
	8.7 ± 5.6	9.7 ± 6.2
Number of oocytes before IVF	n = 234	n = 123
	9.6 ± 5.7	10.3 ± 5.7
Number of oocytes before ICSI	n = 196	n = 95
	8.6 ± 4.8	10.3 ± 5.9
Cumulus–oocyte complexes/retrieval	n = 440	n = 221
	9.1 ± 5.4	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

ICSI = intracytoplasmic sperm injection.

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 V. 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.

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

	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

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 VI).

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

Table VI. Outcome of 53 first freeze–thaw cycles

	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. of 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

[a]Data are missing for one cycle.

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.

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.

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 $\geqslant$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 (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 oestradiol concentrations during the first days of stimulation. When follicular growth is progressing and oestradiol 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 oestradiol 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 $\geqslant$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 rFSH 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 rFSH treatment was one day shorter and the amount of rFSH required was lower in the ganirelix group. Since the number of subjects without any follicles $\geqslant$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.*, 1988).

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 oestradiol, which is explained by the different endocrine status of the patient at the start of stimulation. In view of this different follicular pattern, rFSH 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 oestradiol. The smaller cohort of follicles and the lower oestradiol 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 nor cetrorelix exert any significant action on ovarian steroidogenesis (Ortmann *et al*., 1999; Verbost *et al*., 1999), 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 (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.*, 1995). 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 gonadotrophin (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 gonadotrophin 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 oestradiol concentration before HCG administration, and a higher production of oestradiol per oocyte recovered (Cramer *et al*., 1999). In the ganirelix group, the opposite was shown in that before HCG administration oestradiol concentrations were lower, and also the oestradiol production per follicle was lower in the ganirelix group than in the buserelin group. Although in the current study neither serum LH nor oestradiol 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.

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human reproduction

ORIGINAL ARTICLE *Infertility*

A double-blind, non-inferiority RCT comparing corifollitropin alfa and recombinant FSH during the first seven days of ovarian stimulation using a GnRH antagonist protocol

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BACKGROUND: Corifollitropin alfa, a fusion protein lacking LH activity, has a longer elimination half-life and extended time to peak levels than recombinant FSH (rFSH). A single injection of corifollitropin alfa may replace seven daily gonadotrophin injections during the first week of ovarian stimulation.

METHODS: In this large, double-blind, randomized, non-inferiority trial the ongoing pregnancy rates were assessed after one injection of 150 μg corifollitropin alfa during the first week of stimulation and compared with daily injections of 200 IU rFSH using a standard GnRH antagonist protocol.

RESULTS: The study population comprised 1506 treated patients with mean age of 31.5 years and body weight of 68.6 kg. Ongoing pregnancy rates of 38.9% for the corifollitropin alfa group and 38.1% for rFSH were achieved, with an estimated non-significant difference of 0.9% [95% confidence interval (CI): −3.9; 5.7] in favor of corifollitropin alfa. Stratified analyses of pregnancy rates confirmed robustness of this primary outcome by showing similar results regardless of IVF or ICSI, or number of embryos transferred. A slightly higher follicular response with corifollitropin alfa resulted in a higher number of cumulus–oocyte-complexes compared with rFSH [estimated difference 1.2 (95% CI: 0.5; 1.9)], whereas median duration of stimulation was equal (9 days) and incidence of (moderate/severe) ovarian hyperstimulation syndrome was the same (4.1 and 2.7%, respectively $P = 0.15$).

CONCLUSION: Corifollitropin alfa is a novel and effective treatment option for potential normal responder patients undergoing ovarian stimulation with GnRH antagonist co-treatment for IVF resulting in a high ongoing pregnancy rate, equal to that achieved with daily rFSH.
The trial was registered under ClinicalTrials.gov identifier NTC00696800.

Key words: corifollitropin alfa / sustained follicle stimulant / FSH / ovarian stimulation / IVF

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Introduction

Corifollitropin alfa is the first hybrid molecule with sustained follicle-stimulating activity. Corifollitropin alfa is a recombinant fusion protein composed of FSH and the carboxy terminal peptide (CTP) of the hCG β-subunit (Fares *et al*., 1992). Like recombinant FSH (rFSH), corifollitropin alfa interacts only with the FSH-receptor and lacks LH activity (Lapolt *et al*., 1992; Fauser *et al*., 2009). However, corifollitropin alfa has an approximately 2-fold longer elimination half-life ($t_{1/2}$) and an almost 4-fold extended time-interval (t_{max}) to peak serum levels (C_{max}) (Duijkers *et al*., 2002). Due to this pharmacokinetic profile, corifollitropin alfa can function as a sustained follicle stimulant with a similar pharmacodynamic profile as rFSH, but with the ability to initiate and sustain multiple follicular growth for an entire week. Consequently, a single s.c. injection of the recommended dose of corifollitropin alfa can replace the first seven injections of any daily FSH preparation in an ovarian stimulation treatment cycle prior to IVF.

The need for simplified treatment approaches which will lessen the treatment burden of IVF is self-evident. The IVF treatment process itself is increasingly recognized as contributing to the physical, psychological and emotional burden on infertility patients (Boivin and Takefman, 1996; Cousineau and Domar, 2007). Infertile patients experience high levels of distress and their level of anxiety and depression is equivalent to that experienced by women with cancer or heart disease (Domar *et al*., 1993). A number of IVF studies in which treatment costs were reimbursed have nevertheless reported drop-out rates well above 50% before completing their covered number of cycles (Land *et al*., 1997; Olivius *et al*., 2002; Schröder *et al*., 2004), largely due to the psychological impact of treatment (Olivius *et al*., 2004; Rajhkowa *et al*., 2006). Thus, the primary reason for treatment discontinuation is not based on physician recommendation, but is because patients are too distressed to continue (Hammarberg *et al*., 2001).

In GnRH antagonist co-treatment stimulation protocols, the duration of stimulation is reduced compared with GnRH agonist protocols and less FSH is used to reach the same criteria for administering hCG (Tarlatzis *et al*., 2006). Interestingly, a prospective cohort study comparing a GnRH antagonist protocol with a conventional long GnRH agonist protocol demonstrated a significantly reduced drop-out rate in the antagonist group, indicating that the impact of the treatment strategy is an important factor determining the risk of drop-out (Verberg *et al*., 2008). Clearly, simple treatment regimens that lessen the burden of IVF improve the overall patient experience, and encourage lower drop-out rates (Olivennes 2003, Heijnen *et al*., 2004, Pennings and Ombelet, 2007). Last but not least, fewer injections to be given may improve drug compliance and/or prevent errors during drug administration.

Developing corifollitropin alfa in a short GnRH antagonist protocol may add to the further reduction of the treatment intensity experienced by patients undergoing ovarian stimulation for IVF. Following the dose-finding trial (The Corifollitropin alfa Dose-finding Study Group, 2008) and subsequent pharmacokinetic/pharmacodynamic modeling (De Greef *et al*., 2007) it was concluded that the recommended dose of corifollitropin alfa was 100 μg for subjects with body weight of ≤60 kg and 150 μg for subjects with body weight of >60 kg. Based on simulations, these two dosages of corifollitropin alfa will provide the same exposure and the same degree of ovarian response in the recommended body weight groups. After a single injection of corifollitropin alfa on menstrual cycle day 2 or 3, treatment may be continued with a daily dose of rFSH from stimulation day 8 onwards if needed. Patients who reach the criteria of triggering final oocyte maturation prior to day 8 of stimulation do not require any daily FSH to be administered.

First and foremost the question that needs to be addressed is whether the new corifollitropin alfa regimen results in the same success rates as a daily FSH regimen with GnRH antagonist co-treatment. To this end, the aim of the ENGAGE trial was to investigate whether the ongoing pregnancy rates of the new corifollitropin alfa regimen were comparable to a daily rFSH regimen in patients undergoing ovarian stimulation prior to IVF. The sample size required to sufficiently power this trial for ongoing pregnancy rate as a primary end-point renders this the largest double-blind randomized trial in the field of assisted reproductive technology (ART) to date.

Materials and Methods

The ENGAGE trial was a multi-center, randomized, double-blind double-dummy, non-inferiority clinical trial involving 14 centers in North America (13 centers in USA and one in Canada) and 20 centers in Europe (three in Spain and The UK; two in Belgium, Czech Republic, Finland, France, Norway, and Sweden; one in Denmark and The Netherlands) and conducted between June 2006 and January 2008.

The study was approved by the Independent Medical Ethics Committee or Institutional Review Board for each center as well as by the responsible Health Authority and was conducted in accordance with the Declaration of Helsinki, International Conference on Harmonization guidelines for Good Clinical Practice, and local regulatory requirements. An Independent Data Safety Monitoring Board was appointed to monitor the safety of subjects participating in the trial, and written informed consent was provided by all patients.

Study population

Women aged 18–36 years with a body weight of more than 60 kg up to and including 90 kg, a BMI of 18–32 kg/m^2, a menstrual cycle length of 24–35 days, access to ejaculatory sperm and an indication for controlled ovarian stimulation (COS) before IVF or ICSI were eligible to enroll in the study. Patients who had a (history of) an endocrine abnormality, an abnormal outcome of blood biochemistry or hematology, an abnormal cervical smear, a chronic disease, relevant ovarian-, tubal- or uterine-pathology that could interfere with the COS treatment (e.g. endometrioma >0 mm or fibroids ≥5 cm), embryo implantation or pregnancy were not to be included in the trial. Patients who had a history of ovarian hyperresponse (more than 30 follicles ≥11 mm) or ovarian hyperstimulation syndrome (OHSS), polycystic ovary syndrome (PCOS) or a basal antral follicle count (AFC) of more than 20 on ultrasound (<11 mm, both ovaries combined) were excluded from participation. Other exclusion criteria included a previously low ovarian response to FSH or hMG treatment (i.e. cycle cancelled due to insufficient ovarian response or less than four oocytes obtained), an FSH or LH over 12 IU/L in the early follicular phase, more than three consecutive unsuccessful IVF cycles since the last ongoing pregnancy, a history of recurrent miscarriage (three or more), or currently smoking more than five cigarettes per day.

Study design

The trial was designed as a randomized, double-blind, double-dummy, active-controlled, non-inferiority trial to compare the efficacy of a single injection of corifollitropin alfa during the first week of stimulation with

7 daily injections of rFSH for inducing and sustaining multifollicular growth during COS. Randomization to one of the two treatment arms (1:1 ratio) was done per center and stratified by age (<32 and≥32 years) by central remote allocation using randomly permutated blocks with an undisclosed fixed block size of four.

Stimulation regimen and ART procedures

All patients were to start their treatment cycle on menstrual cycle day 2 or 3 as depicted in Fig. 1. Per protocol all injections were to be administered in the morning. Patients started stimulation with a single s.c. injection of 150 μg (0.5 mL) corifollitropin alfa (NV Organon, The Netherlands) or matching placebo. Injections could be done by the patient herself, her partner or the medical staff. To conceal treatment allocation all patients also started daily s.c. injection of 200 IU rFSH (follitropin beta, Puregon®/Follistim® AQ Cartridge, NV Organon, The Netherlands) or matching placebo on the same day (Stimulation Day 1) using the Puregon®/Follistim Pen®. Daily active or placebo ('dummy') rFSH injections were continued through the first 7 days of stimulation. The chosen reference dose of 200 IU rFSH daily was considered the optimal choice for the included patient population weighing over 60 kg in a global trial combining European and North American sites. This dose was fixed for the first 5 days of stimulation. This is in line with the suggestion by Arce *et al*. (2005) who recommended a fixed starting dose for at least 5–7 days in all efficacy trials as only after such period the impact of the administered FSH dose can be adequately evaluated. Moreover, this is considered appropriate because daily FSH only reaches steady state after 3–5 days of dosing (Mannaerts *et al*., 1993). A reduction of the rFSH dose was allowed from stimulation day 6 onward in case of too high an ovarian response, at the discretion of the investigator. When no follicle ≥11 mm was visible on ultrasound scan (USS) before injection on stimulation day 8 the cycle was to be cancelled due to insufficient ovarian response. From stimulation day 8 onwards, treatment in both groups was continued with a daily s.c. dose of (active) rFSH up to and including the day of hCG administration. The maximum rFSH dose to continue treatment after the first 7 days was 200 IU but the dose could be reduced when desired. For normal responders, the recommended daily dose of rFSH was 150 IU. Whenever deemed required by the investigator, rFSH administration could be withheld for a maximum of 3 days (coasting) up to and including the day of hCG administration. In case there was a too high ovarian response as per the investigator's opinion, the cycle could be cancelled at any time. However, in case of a risk for OHSS, defined as more than 30 follicles of ≥11 mm on USS, hCG was always to be withheld and the treatment cycle was to be cancelled per protocol. The maximum total duration of stimulation was 19 days.

To prevent premature LH surges the GnRH antagonist ganirelix (0.25 mg, Orgalutran®/ganirelix acetate injection, NV Organon, The Netherlands) was administered once daily s.c. starting on stimulation day 5 up to and including the day of hCG. Urinary hCG (10 000 IU) was administered to induce final oocyte maturation as soon as at least three follicles of ≥17 mm were observed by USS. Investigators were allowed to delay hCG administration for 1 day when preferred for practical reasons. In case there was a too high ovarian response in the opinion of the investigator, a lower dose (5000 IU hCG) could be used. About 34–36 h thereafter, oocyte retrieval followed by standard IVF or ICSI was to be performed. Embryo quality was evaluated for all available embryos on day 3 of culture by the local embryologist using a protocol-defined guideline based on the following parameters: number of blastomeres, degree of fragmentation, blastomere size uniformity and presence or absence of multinucleation. Embryos graded as grade 1 (6–10 cells, no fragmentation and equal blastomere size) or grade 2 (allowing up to 20% fragmentation) were qualified as good quality embryos. The quality of embryos continued in culture after day 3 was reassessed on the day of transfer or freezing using grading criteria appropriate for the stage of embryo culture. At embryo transfer, 3 or 5 days after oocyte retrieval, one or two embryos were to be transferred. The decision on the day of transfer and number of embryos to be transferred was made by the investigator. To support implantation and early pregnancy, luteal phase support with progesterone (at least 600 mg/day vaginally or at least 50 mg/day i.m., to be prescribed locally) was started on the day of oocyte retrieval and continued for at least 6 weeks, or either up to menses or up to a negative pregnancy test performed at least 14 days after embryo transfer.

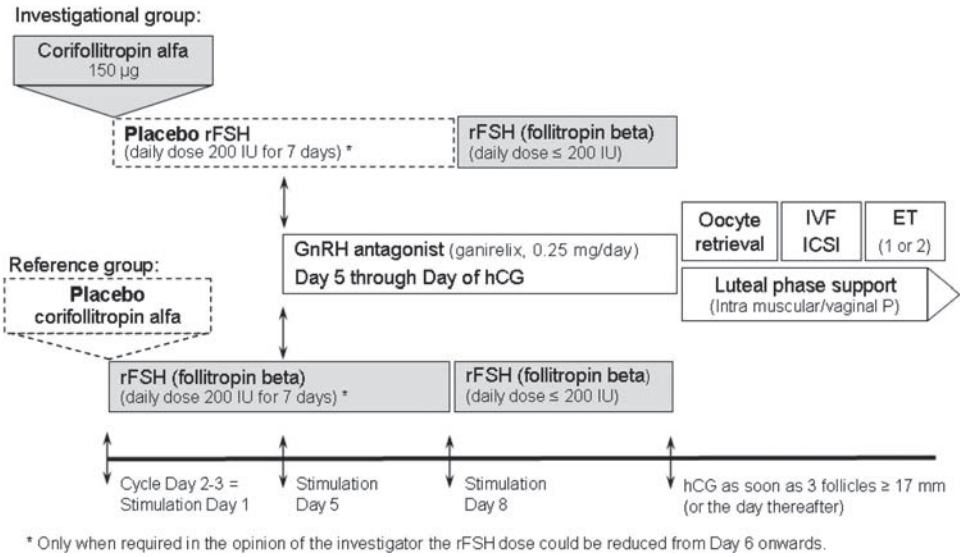

Figure 1 Graphical illustration of the treatment regimens applied in this trial. Upper panel depicts investigational group (corifollitropin alfa), lower panel depicts reference group (rFSH).
rFSH: recombinant FSH, P: progesterone.

Assessments

Before the start of ovarian stimulation, pregnancy was excluded by means of an hCG test, a blood sample was obtained for hormone assessments, and USS was performed to measure and count visible follicles. Local tolerance parameters (pain, itching, swelling and redness) were assessed by the clinical staff 30 min after injection for both (placebo) corifollitropin alfa and (placebo) rFSH injection sites. Patients returned to the clinic for USS and blood sampling on stimulation days 5 and 8, and then daily up to and including the day of hCG administration for USS only. Additional blood samples were collected on the day of embryo transfer and 2 weeks after embryo transfer. Patients who left the study prior to embryo transfer were sampled for hormones and antibody assessments at the day of discontinuation and 2 weeks thereafter.

Validated immunoassays were performed at a central laboratory (Schering-Plough, Oss, The Netherlands and Waltrop, Germany) to measure serum levels of FSH, LH, estradiol (E_2), progesterone, inhibin-B and antibodies against corifollitropin alfa (Devroey *et al.*, 2004).

End-points

Ongoing pregnancy, defined as presence of at least one fetus with heart activity at least 10 weeks after embryo transfer as assessed by USS or Doppler, or confirmed by live birth, was the primary end-point for this trial. This can be considered as the best and closest estimate of the ultimate treatment success (delivery of a healthy baby) and excludes subjects with a miscarriage during the first 10 weeks of pregnancy (Arce *et al.*, 2005). In addition, the number of retrieved oocytes was considered as co-primary end-point in this trial being more proximately related to the pharmacological effect of the two treatment regimens which are compared in this trial. Other clinical outcome parameters evaluated included dose of rFSH required, duration of stimulation, number and size of follicles, serum hormone levels, fertilization rate, number and quality of embryos obtained and pregnancy rates. The ultimate live birth rate, the health of the offspring, and outcomes achieved with spare, frozen embryos will be reported separately when follow-up has been completed.

Occurrence of (serious) adverse events, including moderate and severe OHSS as per World Health Organization criteria (WHO, 1973), outcome of local tolerance and immune response assessments were evaluated as safety end-points.

Statistical analysis

The sample size needed for this trial was largely determined by the chosen pre-defined non-inferiority margin (i.e. smaller margin requires larger sample size to maintain the same power) but also depended on the anticipated ongoing pregnancy rate (i.e. higher pregnancy rate requires larger sample size) to be expected in a combined trial comprising sites in Europe, USA and Canada. For this global trial a non-inferiority margin of 8% was considered appropriate. This implies that if the lower bound of the 95% confidence interval (CI) for the estimated difference in ongoing pregnancy rates between treatment groups (corifollitropin alfa minus rFSH) was determined to be above −8%, corifollitropin alfa could be considered non-inferior to rFSH. Although such a difference would be relevant for an individual subject seeking to become pregnant after IVF treatment it should be considered in the context of existing differences in routine pregnancy rates between centers, countries and regions (Gleicher *et al.*, 2006, 2007). A sample size of at least 1380 subjects was calculated to be the minimum required to demonstrate non-inferiority with a power of 90%, using a −8% non-inferiority margin for the lower limit of the two-sided 95% CI, and assuming an ongoing pregnancy rate of 30%. Based on these data, a minimum of 700 subjects per group, in total at least 1400 subjects, were to be randomized which makes this the largest double-blind comparative randomized trial performed to date.

For analysis of the primary end-point, ongoing pregnancy, the treatment groups were formally compared with a generalized linear model for the ongoing pregnancy rate including covariates treatment group, age ($<$32 years, $\geq$32 years) and region (Europe, North America). The difference between the two treatment groups (corifollitropin alfa − rFSH) and its associated two-sided 95% likelihood-based CI was estimated and a predefined non-inferiority margin of 8% was applied. Additional explorative analyses were performed to investigate robustness of the primary end-point results. No adjustment for multiplicity to correct for repeated testing was performed.

The number of cumulus–oocyte-complexes retrieved was defined as co-primary end-point in this trial. As there is an optimal range of oocytes obtained in response to ovarian stimulation, below and above which the success rates of IVF are compromised (Van der Gaast *et al.*, 2006) equivalence testing was deemed appropriate. Therefore, equivalence margins for the difference in the number of oocytes retrieved were predefined to be −3 and +5 oocytes. If the new corifollitropin alfa regimen resulted in three or more oocytes less than the reference treatment, such difference was considered as clinically relevant because three oocytes usually result in one good quality embryo for transfer or freezing. Anticipating obtaining an average of 12–13 oocytes with the applied rFSH doses in the reference group, an excess of more than five oocytes would be undesirable as patients with more than 18 oocytes retrieved are described to be at increased risk of developing OHSS (Papanikolaou *et al.*, 2006; Verwoerd *et al.*, 2008). Hence, an upper margin of +5 oocytes is applied for the difference in the number of oocytes retrieved between the treatment groups. The treatment groups were formally compared using analysis of variance for the number of oocytes including covariates treatment group, age ($<$32 years versus $\geq$32 years) and center. The estimate of the difference between the two treatment groups and its associated two-sided 95% CI was given. In case the 95% CI of the difference exceeded −3 or +5 oocytes corifollitropin alfa treatment was not considered equivalent to the rFSH treatment.

Efficacy analyses are based on the intention-to-treat (ITT) population entailing all patients randomized and treated, analyzing them according to their allocated treatment (i.e. 'as randomized'). Both ITT and per protocol (PP) analyses were performed (see Supplementary Data), but as the results were very similar, only the ITT results are presented here. The main efficacy analyses were performed 'per started cycle' (i.e. including all subjects who started treatment regardless whether they discontinued) as this provides the most conservative efficacy estimates as it also accounts for any unintended interference of premature cycle cancellations. For safety end-point analyses, patients are grouped 'as treated' which implied that three patients (one randomized to corifollitropin alfa and two patients to rFSH) treated inadvertently with the wrong (comparator) medication type are analyzed according to the treatment they actually received. Statistical significance was set at $P < 0.05$.

Results

A total of 1696 patients signed informed consent for eligibility evaluation and participation in this trial (Fig. 2). Subsequently a total of 187 patients failed screening or dropped out due to personal reasons prior to treatment allocation. Eventually, 1509 patients were randomized to one of the two treatment groups of which 1506 patients actually started stimulation. The remaining three patients were discontinued prior to the start of treatment (one for personal reasons and two were found to violate entry criteria after randomization but before commencing treatment).

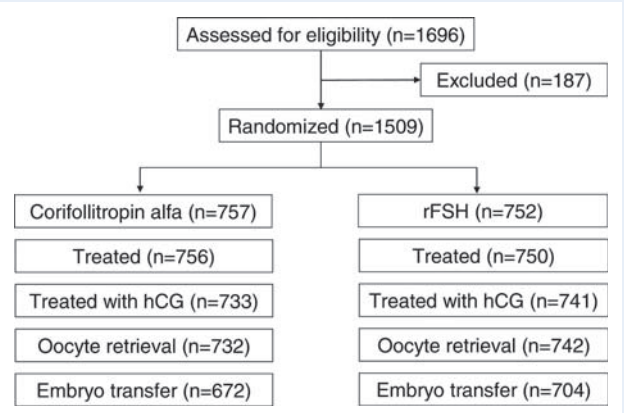

Figure 2 Flow chart showing the number of participants at each stage of the clinical trial.

Demographics of the ITT population as well as relevant fertility characteristics, USS findings and hormone profiles were comparable in the two groups (Table I). Mean age (SD) of the patients included in this trial was 31.5 (3.3) years, body weight was 68.6 (7.5) kg and BMI was 24.8 (2.7) kg/m^2. The average duration of infertility was 3.3 (2.3) years and for 74% of the patients this was their first treatment cycle. On stimulation day 1, no differences were observed between the treatment groups in the basal AFC or baseline serum hormone levels (Table I).

Primary end-point: ongoing pregnancy rate

Results of the primary end-point analyses are presented in Table II. High ongoing pregnancy rates of 38.9% for the corifollitropin alfa group and 38.1% for the rFSH group were obtained per started cycle. The estimated treatment difference, adjusted for age group (<32 years, ≥32 years) and region (Europe, North America), was +0.9% [95% CI: (−3.9; 5.7)] in favor of corifollitropin alfa.

Co-primary end-point: number of cumulus–oocyte-complexes retrieved

The number of cumulus–oocyte-complexes retrieved was defined as co-primary end-point in this trial. The mean (SD) number of oocytes retrieved in the corifollitropin alfa group was 13.7 (8.2) which was higher than the mean of 12.5 (6.7) obtained in the rFSH group (Table II). The estimated treatment difference was 1.2 oocytes in favor of corifollitropin alfa ($P = 0.001$), while the 95% CI was (0.5; 1.9). This indicates that the number of oocytes retrieved in the two treatment groups was at least equivalent based on the pre-defined equivalence range.

Other clinical outcome parameters

The median duration of stimulation was 9 days for both treatment groups, which implied patients treated with corifollitropin alfa needed on average 2 days of rFSH to complete their treatment cycle (Table III). Per protocol, the dose of (placebo) rFSH could be reduced (not increased) from stimulation day 6 onwards. Dose decreases on stimulation days 6 or 7 were recorded slightly more often in the corifollitropin alfa group (85/750, 11.3%) than in the rFSH group (62/741, 8.4%). Coasting by withholding rFSH for 2 or more days was applied in 1.6% [12/733, 95% CI: (0.7; 2.6)] of patients in the corifollitropin alfa group and in 2.2% [16/741, 95% CI: (1.0; 3.3)] of patients in the rFSH group. A fixed dose regimen from stimulation day 8 up to but not including the day of hCG was used in 72.1% (413/573) of the corifollitropin alfa group and 81.0% (430/531) of the rFSH group. After a single injection of corifollitropin alfa, 249 out of 756 patients (32.9%) reached the criteria for giving hCG before or on stimulation day 8: the ongoing pregnancy rate for this subgroup of patients was 44.0%.

Eventually, 97.0% of the patients in the corifollitropin alfa group and 98.8% of the rFSH group received hCG to induce final oocyte maturation. The vast majority received 10 000 IU hCG (76.9% (581/756) and 85.6% (642/750) of the patients in the corifollitropin alfa and rFSH group, respectively), while a dose of 5000 IU hCG was used

Table I Demographics, fertility characteristics and baseline (stimulation day 1) ultrasound scan (USS) and serum hormone levels per treatment group (intent-to-treat population)

	Corifollitropin alfa (n = 756)	rFSH (n = 750)
Demographics		
Age (years)	31.5 (3.3)	31.5 (3.2)
Body weight (kg)	68.8 (7.6)	68.4 (7.3)
BMI (kg/m^2)	24.8 (2.8)	24.8 (2.7)
Race, n (%)		
Asian	21 (2.8)	21 (2.8)
Black	33 (4.4)	28 (3.7)
Caucasian	643 (85.1)	650 (86.7)
Other	59 (7.8)	51 (6.8)
Fertility characteristics		
Primary infertility, n (%)	403 (53.3)	393 (52.4)
Duration of infertility (years)	3.3 (2.4)	3.2 (2.2)
Cause of infertility[a], n (%)		
Male factor	388 (51.3)	347 (46.3)
Tubal factor	198 (26.2)	191 (25.5)
Endometriosis	109 (14.4)	115 (15.3)
Other/unexplained	246 (32.5)	262 (34.9)
First IVF cycle, n (%)	569 (75.3)	552 (73.6)
Stimulation day 1		
Total ovarian volume (mL)[b]	13.2 (8.1)	13.2 (7.1)
Basal AFC (<11 mm)	12.3 (4.6)	12.4 (4.4)
FSH (IU/L)	6.7 (2.1)	6.6 (1.9)
LH (IU/L)	4.8 (2.0)	4.7(1.8)
E_2 (pmol/L)	126.1 (39.3)	124.8 (37.4)
Progesterone (nmol/L)	1.8 (1.3)	1.8 (1.4)

AFC: antral follicle count, E_2: estradiol, rFSH: recombinant FSH.
Numbers are mean (SD) unless otherwise indicated.
[a]A patient can have multiple causes of infertility.
[b]According to the formula for a polate ellipsoid: 0.523 × longitudinal × antero-posterior × transverse diameters as measured per transvaginal ultrasound.

Table II Primary end-points of the ENGAGE trial: ongoing pregnancy rate (assessed at least 10 weeks after embryo transfer) and the mean (SD) number of cumulus–oocyte-complexes retrieved (intent-to-treat population)

	Corifollitropin alfa ($n = 756$)	rFSH ($n = 750$)	Estimated difference[a)] [95% CI], *P*-value
Ongoing pregnancies (*n*)	294	286	
Per started cycle (%)	38.9%	38.1%	0.9 [−3.9; 5.7], $P = 0.71$
Per embryo transfer (%)	43.8%	40.6%	3.1 [−2.0; 8.2], $P = 0.24$
Cumulus–oocyte-complexes			
Per started cycle	13.7 (8.2)	12.5 (6.7)	1.2 [0.5; 1.9], $P = 0.001$
Per oocyte retrieval	14.1 (7.9)	12.7 (6.7)	1.6 [0.8; 2.3], $P < 0.001$

P-value corresponds to the test whether the treatment difference equals zero.
[a)]Estimated treatment difference (corifollitropin alfa − rFSH) adjusted for covariates.

Table III Clinical parameters from stimulation phase up to embryo transfer (intent-to-treat population)

	Corifollitropin alfa ($n = 756$), median (range)	rFSH ($n = 750$), median (range)
Stimulation characteristics[a)]		
Total dose of rFSH (IU)	400 (0–2000)	1800 (400–2800)
Total dose of rFSH from day 8 onwards (IU)	400 (0–2000)	400 (0–1400)
Total duration of stimulation (days)[b)]	9 (6–18)	9 (6–15)
Follicles, day of hCG[a)]		
≥11 mm	16.0 (7.0)	13.9 (6.1)
≥15 mm	9.6 (4.8)	8.7 (4.0)
≥17 mm	5.7 (3.2)	5.6 (2.9)
Serum parameters, day of hCG[a)]		
FSH (IU/L)	12.5 (3.3)	11.6 (2.8)
LH (IU/L)	1.4 (1.8)	1.9 (1.6)
E_2 (pmol/L)	5508.8 (3469.8)	5165.3 (2998.2)
Inhibin-B (pg/mL)	610.3 (492.3)	614.8 (435.6)
Progesterone (nmol/L)	3.0 (2.1)	3.2 (1.5)
Clinical outcome per started cycle		
Oocytes retrieved, ICSI only	13.8 (7.6)	12.1 (6.3)
Metaphase II oocytes (ICSI only), % of total	10.8 (6.5), 78.9 (18.9)	9.2 (5.1), 77.4 (18.1)
Fertilization rate (%)[c,d)]	66.0 (23.4)	67.6 (22.9)
Total number of embryos obtained (day 3)[c)]	8.3 (5.6)	7.4 (4.8)
Excellent (top) quality embryos (grade 1)[c)]	2.6 (3.4)	2.5 (3.4)
Good quality embryos (grade 1+2)[c)]	4.6 (4.3)	4.4 (3.9)
Single embryo transfer (%)[d)]	25.7	27.0
Embryos transferred[e)]	1.7 (0.4)	1.7 (0.4)
Embryos cryopreserved[f)]	4.3 (3.6)	3.9 (2.7)

Numbers are mean (SD) unless otherwise indicated.
[a)]Restricted to patients with hCG injection.
[b)]Number of days up to and including the day of hCG administration.
[c)]Restricted to patients with IVF and/or ICSI.
[d)]Defined as 100 times the number of mature oocytes (with two pronuclei) obtained divided by the number of oocytes used for fertilization.
[e)]Restricted to patients with embryo transfer.
[f)]Restricted to patients with cryopreserved embryos.

for 19.8% (150/756) and 13.1% (98/750) of the respective groups. Only the most frequent reasons for discontinuation *prior to hCG* are mentioned. In total, 23 (3.0%) of the patients in the corifollitropin alfa group and 7 (0.9%) of the patients in the rFSH group discontinued prior to hCG. In the rFSH group, 2 patients discontinued after the oocyte retrieval procedure, but it appeared that they had not received hCG. Taking this into account, totals add up correctly to the number of subjects treated with hCG (733 = 756−23, 741 = 750−7−2).

On the day of hCG, the mean (SD) number of follicles measuring ≥11 mm on USS was 16.0 (7.0) for the corifollitropin alfa group and 13.9 (6.1) for the rFSH reference group (Table III). When comparing serum hormones on the day of hCG administration comparable levels were observed in both treatment groups. Oocyte retrieval was performed in 96.8% of the patients in the corifollitropin alfa group and 98.9% in the rFSH group. A higher mean number of cumulus–oocyte-complexes was retrieved in the corifollitropin alfa group (Table II). Oocyte maturity was assessed for ICSI patients and also showed a higher number and percentage of mature oocytes (Table III) in the corifollitropin alfa group (mean 10.8, 78.9%) compared with the rFSH group (mean 9.2, 77.4%). A comparable fertilization rate was observed between the groups resulting in a mean (SD) of 8.3 (5.6) embryos obtained on day 3 of culture in the corifollitropin alfa group and 7.4 (4.8) embryos in the rFSH group for patients with IVF and/or ICSI. Embryo quality, as assessed by the local embryologist, was similar in both groups.

Ultimately, 672 patients (88.9%) in the corifollitropin alfa group and 704 patients (93.9%) in the rFSH group had embryo transfer in this trial. Between hCG administration and embryo transfer 39 out of 756 patients (5.2%) in the corifollitropin alfa group and 34 out of 750 (4.5%) patients in the rFSH group were discontinued as a result of too few or too low quality oocytes, lack of fertilization or poor embryo development. During this period 6/756 patients (0.8%) in the corifollitropin alfa group and none in the rFSH group were discontinued due to a too high ovarian response or risk of OHSS. Although no embryo transfer was performed, fertilized oocytes or embryos were cryopreserved for all these six patients. When embryo transfer was performed, single embryo transfer was carried out in slightly more than a quarter of all transfers (Table III). On average, 1.7 (0.4) embryos were transferred in both treatment groups. For 51.7 and 53.2% of the patients in the corifollitropin alfa and rFSH groups, a mean (SD) of 4.3 (3.6) and 3.9 (2.7) supernumerary embryos have been cryopreserved, respectively.

In the corifollitropin alfa group 29.2% (221/756), and in the rFSH group 30.3% (227/750), received only i.m. luteal phase progesterone support, whereas respectively 52.0% (393/756) and 54.9% (412/750) received only intravaginal progesterone support. Other subjects received a combination of routes.

Pregnancy rates (confirmed by positive hCG test) of 48.1 and 46.9% were achieved in the corifollitropin alfa and rFSH groups, respectively, followed by a comparable number of early pregnancy losses during the first 10 weeks after embryo transfer. Accordingly, similar ongoing pregnancy rates were observed in both groups (Table IV). There was no relevant difference in the ongoing pregnancy rates between subjects who received the hCG injection on the same day that three follicles ≥17 mm were observed (40.0 and 37.8% for corifollitropin alfa and rFSH, respectively) versus subjects who received hCG one day later (38.9 and 41.8% for corifollitropin alfa and rFSH, respectively).

The robustness of this primary efficacy outcome was explored in specific subsets of patients grouped according to the ART procedure-related factors, i.e. undergoing IVF or ICSI, having single or double embryo transfer and having embryo transfer on day 3 or day 5 (Fig. 3). The ongoing pregnancy rates in each subset were similar for corifollitropin alfa and rFSH and there was no significant interaction with the type of treatment factor (IVF or ICSI), nor for the number of embryos transferred (one or two). A borderline significant interaction with the type of treatment was only observed for the factor day of transfer ($P = 0.045$) as a result of higher pregnancy rates for day 3 transfers with corifollitropin alfa whereas, for day 5 transfers, the rFSH group showed marginally higher pregnancy rates (Fig. 3). The subset analyses together confirm the robustness of the observed difference in pregnancy rates, independent of ART procedure-related parameters.

Table IV Clinical efficacy outcomes per started cycle (intent-to-treat population)

	Corifollitropin alfa ($n = 756$)	rFSH ($n = 750$)	P-value[a]
Positive hCG test[b], *n* (%)	364 (48.1)	352 (46.9)	0.64
Clinical pregnancy[c], *n* (%)	322 (42.6)	308 (41.1)	0.57
Vital pregnancy[d], *n* (%)	302 (39.9)	293 (39.1)	0.75
Ongoing pregnancy[e], *n* (%)	294 (38.9)	286 (38.1)	0.71
Multiple pregnancy[f], *n* (%)	83 (28.2)	66 (23.1)	0.18
Early miscarriage[g], *n* (%)	27 (8.4)	21 (6.8)	0.55

[a]*P*-values are based on Fisher's exact test, except for ongoing pregnancy where the *P*-value is based on the likelihood ratio test corresponding to the generalized linear model with covariates treatment group, age class (<32 years, ≥32 years) and region (Europe, North America).
[b]Positive hCG test at least 14 days after embryo transfer or USS with at least one gestational sac.
[c]Clinical pregnancy: gestational sac on USS.
[d]Vital pregnancy: gestational sac + fetal heartbeat.
[e]Ongoing pregnancy: vital fetus at least 10 weeks after embryo transfer or live birth.
[f]Per ongoing pregnancy.
[g]Per clinical pregnancy.

Multiple pregnancy rates per ongoing pregnancy were 28.2% and 23.1% in the corifollitropin alfa group and the rFSH group, respectively (Table IV). Although this difference is not significant, it is in line with the slightly higher implantation rate defined as 100 times the maximum number of gestational sacs divided by the number of embryos transferred per subject for corifollitropin alfa [observed means (SD) were 36.2% (41.6%) versus 32.2% (40.1%)].

Safety

In total, 16 subjects (2.1%) in the corifollitropin alfa group discontinued due to a (serious) adverse event [SAE, two (0.3%) before and 14 (1.9%) following oocyte retrieval] as compared with three subjects (0.4%) in the rFSH group [two (0.3%) before and one (0.1%) after oocyte retrieval]. A total of 53 patients in the corifollitropin alfa-treated group (7.0%) and 47 patients in the rFSH-treated group (6.3%) developed OHSS in this trial. The incidences of (moderate/severe) OHSS were 4.1% [31/755, 95% CI: (2.6; 5.6)] and 2.7% [20/750, 95% CI: (1.5; 3.9)] for the corifollitropin alfa and rFSH group, respectively, which was not significant (Fisher exact $P = 0.15$).

An equal number of 37 SAEs was reported for the corifollitropin alfa and the rFSH-treated groups. Most frequently reported SAEs were OHSS [14 patients (1.9%) treated with corifollitropin alfa and 9 patients (1.2%] treated with rFSH) and (ruptured) ectopic pregnancy [7 patients (0.9%) and 9 patients (1.2%), respectively] while, respectively, the most frequently reported AEs were procedural pain (22.3

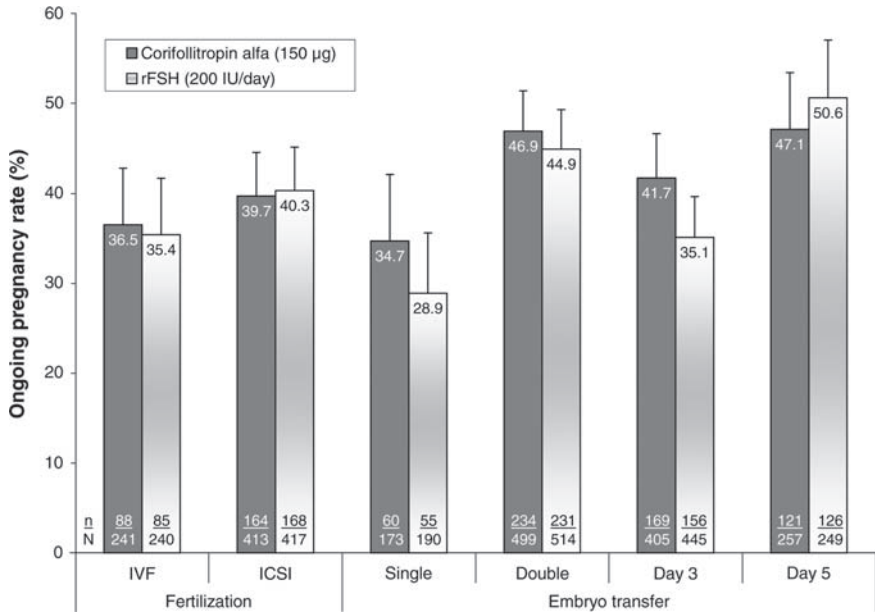

Figure 3 Ongoing pregnancy rates stratified for fertilization procedure, number of embryos transferred and day of transfer. Subset with double embryo transfer includes three patients with three embryos transferred.

and 20.1%), pregnancy-related events including (missed) abortion (13.8 and 11.2%), pelvic pain (12.1 and 12.3%), pelvic discomfort (11.5 and 11.6%) and headache (10.5 and 15.2%). There were no drug-related immune responses or moderate or severe local tolerance reactions observed in this trial.

Discussion

The current ENGAGE trial was a double-blind, double-dummy trial initiated to investigate whether the new corifollitropin alfa regimen provides similar success rates compared with the current care. The double-dummy approach guaranteed the blinding of medication during the trial and prevented any bias in terms of treatment decisions. Owing to the fact that the trial was powered to enable comparison of the ongoing pregnancy rates as the primary study end-point, this is the largest double-blind efficacy trial in IVF performed to date. The outcome of the trial provides compelling evidence of equal efficacy in terms of ongoing pregnancy rates, because the point estimates i.e. 38.9 and 38.1% for the ongoing pregnancy rates were very close for the two treatment groups and the 95% lower limit of the difference was only −3.9%. Subset analyses of subjects undergoing IVF or ICSI and of subjects who had single or double embryo transfer reveal consistently high ongoing pregnancy rates, similar between the treatment groups, which confirm the robustness of the primary end-point. Even though an equal number and quality of embryos were replaced in both treatment groups, the multiple pregnancy rate in the corifollitropin alfa group tended to be higher (+4.4% absolute risk increase) than in the reference group, which may be related to the slightly higher implantation rate. This increase, although not significant, cannot be ruled out and may prove to be clinically relevant, given the low power of the study to detect such a difference (*c.*22%). In this context, it should be emphasized that both treatment arms used a 'protocolized' fixed dose treatment regimen applying GnRH antagonist (ganirelix) co-treatment, which confirms the successful outcome of a patient-friendly short GnRH antagonist protocol in corifollitropin alfa, as well as rFSH, treatment cycles. In addition, one-third of the patients treated with a single injection of corifollitropin alfa reached the criteria for hCG injection prior to or on stimulation day 8, omitting the need for any additional FSH injections. The ongoing pregnancy rate of this subset of good responder patients was 44.0%, thus 5.9% higher than the overall group of subjects treated with corifollitropin alfa.

A hybrid molecule composed of human FSH and the CTP of hCG was first described by Boime and colleagues (Fares *et al.*, 1992). In subsequent clinical trials the new recombinant fertility hormone exhibited a slower absorption (and subsequent rise to peak levels) and an approximately 2-fold longer $t_{1/2}$ compared with rFSH (Bouloux *et al.*, 2001; Duijkers *et al.*, 2002). After corifollitropin alfa injection, peak levels of FSH activity are reached within 2 days whereas steady state levels with daily FSH are reached only after 4–5 days (Mannaerts *et al.*, 1996). These pharmacokinetic properties of corifollitropin alfa create an opportunity to further simplify ovarian stimulation protocols for IVF by omitting the need for daily gonadotrophin injections during the first week of stimulation (Fauser *et al.*, 2009). In the current trial, the difference in exposure during the first days of stimulation may have resulted in a slightly higher ovarian response in the corifollitropin alfa treated patients, as previous trials with rFSH have shown that the number of follicles recruited increases with the starting dose of rFSH given (Wikland *et al.*, 2001; Out *et al.*, 2004). In view of the equal pregnancy rates, the current trial does not suggest that the higher exposure to FSH immunoactivity during the first days of stimulation interferes with the endometrial receptivity.

In this trial hormonal pretreatment with oral contraceptives or supplementation with LH or hCG during the treatment phase was not allowed per protocol. The efficacy results in this large, global, multi-center trial suggest that the amount of endogenous LH during treatment with either corifollitropin alfa or daily rFSH in a standard GnRH antagonist protocol is sufficient to support high success rates in terms of ongoing pregnancies (Cedrin *et al*., 2004).

Because of concerns that the relatively high exposure to corifollitropin alfa during the first days of stimulation might initiate an early rise of LH (Devroey *et al*., 2004), all patients in this trial started treatment with ganirelix on stimulation day 5. This fixed start of GnRH antagonist co-treatment was considered advantageous from an efficacy perspective as this may result in higher pregnancy rates, as well as from a methodological point of view as this reduces variability in the applied treatment regimens (Kolibianakis *et al*., 2004; Al-Inany *et al*., 2005; Arce *et al*., 2005).

The corifollitropin alfa regimen used in this trial comprised a single dose of corifollitropin alfa followed by daily doses of rFSH (as needed up to a daily maximum of 200 IU). This corifollitropin regimen was compared with a fixed dose of 200 IU/day rFSH for the first 7 days, but with the option to decrease the daily FSH dose in cases when hyper-response was observed. Thus there was a difference in patient management options, with no option to adjust the dosing during the first week of stimulation in the corifollitropin arm. After corifollitropin alfa injection, serum FSH activity declines from stimulation day 3 (C_{max}) onwards, but further reduction of exposure during the first week of stimulation cannot be attained.

The validity of the claimed efficacy and safety data of this trial are limited to the study population only. Patients with known risk factors for a hyper-response, such as patients with a history of OHSS, with PCOS or with a high AFC (>20) were excluded from the current trial. In addition, patients with a history of low ovarian response in a previous IVF cycle were excluded. However, it should be noted that 74% of the patients included in this trial underwent their first IVF cycle for which the ovarian response is less predictable and will still include low- and high responder patients.

In this large cohort of (potential) normal responder patients, corifollitropin alfa has been shown to be well tolerated and non-immunogenic. In line with the higher ovarian response, the observed incidence of OHSS was higher in the corifollitropin alfa group, but the difference was not significant. Our study was not sufficiently powered to detect an underlying difference in the incidence of OHSS, and therefore its actual presence cannot be excluded. In line with the recruitment of slightly more follicles by corifollitropin alfa, dose reductions during stimulation were more frequently made in the corifollitropin alfa group than in the reference group. Clearly, a minority of subjects required a dose reduction on stimulation day 6 or 7, which may have affected the ovarian response in the rFSH group rather than in the corifollitropin alfa group in which only the placebo was reduced.

Appropriate clinical monitoring and lowering or withholding the daily rFSH dose to complete the cycle, as well as lowering or withholding the dose of hCG to trigger final oocyte maturation, or cryopreservation of all embryos obtained are options which can be considered as part of patient management to minimize the risk of OHSS (Delvigne and Rozenberg, 2002).

Owing to the double-blind design of the trial, requiring all patients to be treated with an equal number of injections, no comparative data could be collected on perceived patient convenience or preference for the corifollitropin alfa regimen. Since a single injection of corifollitropin alfa replaces the first seven daily injections of rFSH during ovarian stimulation, the intuitive advantages of such a simpler treatment regimen are obvious, but need to be confirmed in clinical practice. In addition to patient preference, future controlled trials may examine the efficacy of corifollitropin alfa in (potential) poor responding patients as well as the safety in (potential) high responders.

In conclusion, in this study we tested the efficacy (in terms of ongoing pregnancy rates) of substituting the first 7 daily doses of rFSH with a single injection of corifollitropin alfa in women undergoing ovarian stimulation for IVF/ICSI using rFSH and GnRH antagonists. Our data demonstrate that a single corifollitropin alfa injection results in an ongoing pregnancy rate which is equal to that of a daily rFSH regimen. Combined with appropriate patient selection and state-of-the-art clinical management during the stimulation phase of the treatment cycle, corifollitropin alfa potentially offers an attractive new treatment option for patients undergoing ovarian stimulation during ART.

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ARTICLE

Corifollitropin alfa for ovarian stimulation in IVF: a randomized trial in lower-body-weight women

The corifollitropin alfa Ensure study group [1]

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Abstract In this double-blind, double-dummy, randomized, equivalence trial (Ensure), 396 women weighing 60 kg or less who underwent controlled ovarian stimulation prior to IVF or intracytoplasmic sperm injection were randomized in a 2:1 ratio to a single dose of 100 μg corifollitropin alfa or daily 150 IU recombinant FSH (rFSH) for the first 7 days of stimulation in a gonadotrophin-releasing hormone antagonist protocol. The mean ± SD number of oocytes retrieved per started cycle was 13.3 ± 7.3 for corifollitropin alfa versus 10.6 ± 5.9 for rFSH. The estimated treatment difference of +2.5 oocytes (95% CI 1.2—3.9) in favour of corifollitropin alfa ($P < 0.001$) was well within the predefined equivalence margin. The median (range) duration of stimulation was 9 (6—15) days in both groups. In 32.8% of the patients, one injection of corifollitropin alfa was sufficient to reach the human chorionic gonadotrophin criterion. The incidence of moderate and severe ovarian hyperstimulation syndrome was 3.4% for corifollitropin alfa and 1.6% for rFSH. A dose of 100 μg corifollitropin alfa offers a simplified treatment option for potential normal responder patients with a lower body weight.

KEYWORDS: body weight, corifollitropin alfa, follicle stimulant, IVF, ovarian stimulation, sustained

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Introduction

Corifollitropin alfa is a novel recombinant hormone designed as a sustained follicle stimulant. A single dose administered in the early follicular phase of the menstrual cycle initiates and sustains multiple follicular development for 7 days (Corifollitropin Alfa Dose-Finding Study Group, 2008). Corifollitropin alfa contains the alfa-subunit of human FSH coupled to a hybrid subunit composed of the sequence of the β-subunit of human FSH and the carboxy-terminal peptide of the β-subunit of human chorionic gonadotrophin (HCG) (Fares et al., 1992; Fauser et al., 2009). The available preclinical and clinical data on corifollitropin alfa show that the compound has a prolonged half-life and a slower absorption to serum peak concentrations. Therefore, a single dose of corifollitropin alfa remains effective for a whole week in contrast to recombinant human FSH (rFSH), which is to be injected daily (Bouloux et al., 2001; Duijkers et al., 2002; Fares et al., 1992). The efficacy of corifollitropin alfa has initially been investigated in a small feasibility trial (Devroey et al., 2004), followed by a larger multicentre dose-finding trial in women undergoing ovarian stimulation for IVF or intracytoplasmic sperm injection (ICSI) (Corifollitropin Alfa Dose-Finding Study Group, 2008). The results of the dose-finding trial indicated a significant dose—response relationship with respect to the number of cumulus—oocyte—complexes retrieved.

The phase II data were combined with historical data in a modelling and simulation project that was initiated to predict the effects of a range of single doses of corifollitropin alfa followed by daily rFSH treatment for ovarian stimulation. By taking various variables (including age and body weight) into account, the modelling revealed that 100 μg is the most optimal corifollitropin alfa dose in the desired 1-week regimen for women with a body weight up to and including 60 kg and provides an exposure similar to the exposure provided by 150 μg in women weighing more than 60 kg. Equal exposure to those two dosages would also imply equal ovarian response (De Greef et al., 2007).

The anticipated therapeutic indication for corifollitropin alfa is ovarian stimulation for the development of multiple follicles and pregnancy in women participating in an assisted reproductive technology programme. Corifollitropin alfa has been developed in a gonadotrophin-releasing hormone (GnRH) antagonist protocol (Al-Inany et al., 2007; Hohmann et al., 2003; Tarlatzis et al., 2006). The application of a simplified GnRH antagonist protocol has been suggested as the preferred option for predicted normal responders (Devroey et al., 2009a). This treatment regimen may reduce the psychological distress and the drop-out rates of IVF patients (Heijnen et al., 2007; Verberg et al., 2008). It is anticipated that the replacement of the first seven injections of daily FSH by a single injection of corifollitropin alfa may further lower the injection burden of patients undergoing ovarian stimulation (Fauser et al., 2009).

In the Engage trial, more than 1500 patients were treated either with 150 μg corifollitropin alfa or 200 IU rFSH in a standardized GnRH antagonist protocol. The pregnancy rates (38.9% versus 38.1%) confirmed equal efficacy in terms of ongoing pregnancy rates (Devroey et al., 2009b).

The main objective of this comparative, double-blind trial (Ensure) in patients undergoing ovarian stimulation prior to IVF or ICSI was to assess the efficacy and safety of a lower dose of 100 μg corifollitropin alfa in patients weighing up to 60 kg, using a fixed daily dose of 150 IU rFSH as a reference.

Materials and methods

The Ensure trial was a multicentre, multinational, randomized, double-blind, double-dummy equivalence trial involving 14 centres in Europe (three in Austria, two each in Czech Republic, France, Spain, Poland and Sweden and one in Denmark) and five centres in Asia (three in Korea and two in Taiwan). The study was approved by the local health authorities and the independent medical ethics committee for each centre and conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guidelines and Good Clinical Practice. Written informed consent was provided by all patients.

Patients

Women aged 18—36 years, weighing 60 kg or less, a body mass index of 18—32 kg/m^2, a normal menstrual cycle length (24—35 days), access to ejaculatory spermatozoa and an indication for ovarian stimulation for IVF or ICSI were eligible to enrol in the study. The exclusion criteria were the same as those reported in the Engage trial (Devroey et al., 2009b); thus, patients with a history of ovarian hyperresponse to ovarian stimulation (more than 30 follicles ≥11 mm) or ovarian hyperstimulation syndrome (OHSS), polycystic ovary syndrome or more than 20 basal antral follicles on ultrasound (<11 mm, both ovaries combined) were excluded. Similarly, patients with a history of no or low ovarian response (i.e. cycle cancelled due to insufficient response or less than four oocytes obtained) or more than three unsuccessful ovarian stimulation cycles since the last established ongoing pregnancy were excluded.

Study design

The Ensure trial was designed as a multicentre, multinational, randomized, double-blind, double-dummy equivalence trial. After evaluation of screening data to confirm eligibility, patients were randomized just before the start of stimulation. Randomization to one of the two treatment groups in a 2:1 ratio (investigational group: reference group) was performed at each centre and stratified by age (<32 or ≥32 years) and planned fertilization procedure (IVF or ICSI) by central remote allocation using randomly permutated blocks with an undisclosed fixed block size of three.

The treatment regimen is depicted in **Figure 1**. All patients were to start ovarian stimulation on day 2 or 3 of their menstrual cycle with a single subcutaneous (s.c.) injection of 100 μg (0.5 ml) corifollitropin alfa (N.V. Organon, The Netherlands) or placebo injection. Injections could be given

by the patient herself, her partner or the medical staff. To conceal treatment allocation, all patients also started daily s.c. injection of rFSH (150 IU/day) or placebo on the same day (stimulation day 1) using the Puregon/Follistim Pen (N.V. Organon). Daily active or placebo rFSH injections were continued through the first 7 days of stimulation. The chosen reference dose of 150 IU rFSH daily was fixed for the first 5 days of stimulation, but could be reduced or increased up to a maximum of 200 IU from day 6 onwards. If no follicle ≥11 mm was visible on ultrasound scan (USS) before injection on day 8, the cycle was to be cancelled due to insufficient ovarian response. From day 8 onwards, treatment with (open-label) rFSH was continued in both the investigational and reference group and the dose could be reduced or increased from 150 IU based on the observed follicular response (maximum rFSH dose 200 IU/day) up to the day of HCG administration. The investigator was allowed to withhold rFSH administration for a maximum of 3 days (coasting) up to and including the day of HCG administration. If, in the opinion of the investigator, the ovarian response was too high, the investigator was allowed to cancel the cycle at any time. However, in case of a risk for OHSS, i.e. more than 30 follicles ≥11 mm on USS, HCG was to be withheld and the treatment cycle was to be cancelled per protocol. Starting on stimulation day 5, all patients were scheduled to receive 0.25 μg ganirelix acetate injection (Orgalutran; N.V. Organon) up to and including the day of HCG to prevent premature LH surges. To induce final oocyte maturation 10,000 IU HCG (Pregnyl; N.V. Organon), or 5000 IU HCG in case of a high ovarian response, was to be given when three follicles ≥17 mm in mean diameter were observed by transvaginal USS. Investigators were allowed to delay HCG administration for 1 day when preferred for practical reasons. All IVF or ICSI procedures after HCG administration were equal to those described by Devroey et al. (2009b) for the Engage trial. At embryo transfer, which took place 3 or 5 days after oocyte retrieval, a maximum of two embryos could be transferred. Daily progesterone (at least 600 mg/day vaginally or at least 50 mg/day intramuscularly, to be prescribed locally) for luteal phase support was to be administered for at least 6 weeks if pregnant or until menses.

Assessments

USS assessments were performed on stimulation days 1, 3, 5 and 8 and then daily up to and including the day of HCG. Serum FSH, LH, oestradiol, progesterone and inhibin B were analysed prior to injection on days 1, 3, 5 and 8, day of HCG, day of embryo transfer and at the visit 2 weeks after embryo transfer or at cycle discontinuation and 2—3 weeks after cycle discontinuation.

Local injection site tolerance was assessed by each centre's medical staff at 30 min after drug administration on stimulation day 1. Injection site reactions were scored none, mild, moderate or severe for four parameters (pain, itching, swelling and redness). Assessment of serum anti-corifollitropin alfa antibodies was performed by radioimmunoassay on day 1 prior to injection and 2 weeks after embryo transfer or at cycle discontinuation and 2—3 weeks after cycle discontinuation.

Validated immunoassays (Devroey et al., 2004) were performed at a central laboratory (MSD, Oss, The Netherlands and Waltrop, Germany) to measure serum concentrations of corifollitropin alfa, FSH, LH, oestradiol, progesterone, inhibin B and anti-corifollitropin alfa antibodies.

A pregnancy test (serum or urinary HCG) was performed at least 2 weeks after embryo transfer. In case of a pregnancy, USS was performed at 5—6 weeks and at ≥10 weeks after embryo transfer to establish the presence of an ongoing pregnancy.

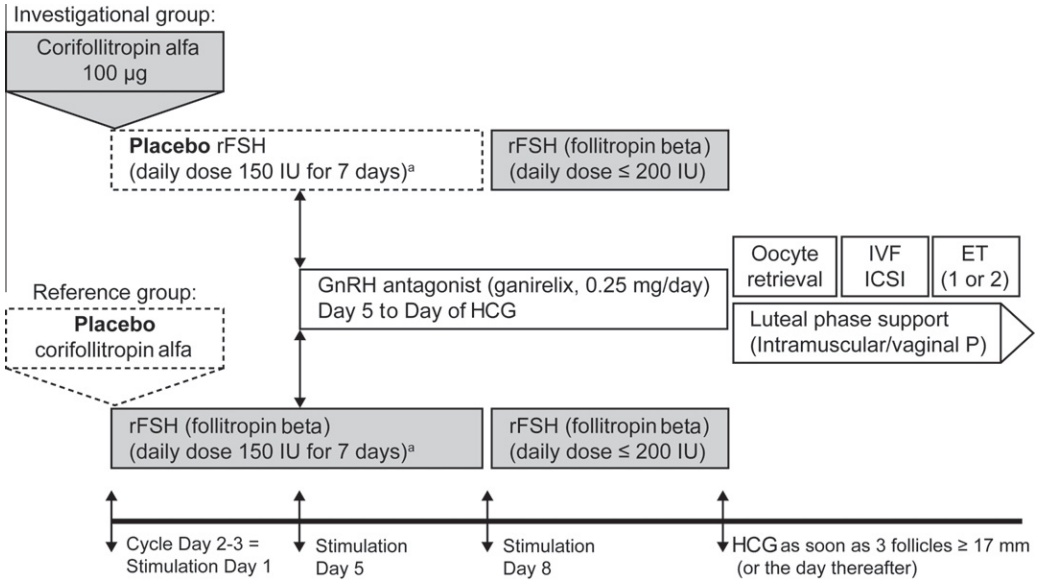

Figure 1 Graphical illustration of the treatment regimens applied in this double-blind, double-dummy trial. ET, embryo transfer; GnRH, gonadotrophin-releasing hormone; HCG, human chorionic gonadotrophin; ICSI, intracytoplasmic sperm injection; P, progesterone; rFSH, recombinant FSH. [a]Only when required, in the opinion of the investigator, the rFSH dose could be adjusted from day 6 onwards.

Study endpoints and statistics

The primary objective was to show that the corifollitropin alfa regimen, in terms of the number of cumulus–oocyte–complexes retrieved, was equivalent to the reference treatment (predefined equivalence range: −3 to +5 oocytes). The number of oocytes was chosen as the primary endpoint of the trial, as it reflects best the pharmacological effect of the new corifollitropin alfa regimen, which in comparison to daily FSH should not provide less than three oocytes (which usually result in one good-quality embryo) and not more than five oocytes as that could increase the risk of OHSS significantly. Randomizing patients in a 2:1 ratio (twice as many patients in the investigational group as in the reference group), a total of at least 330 patients (220 patients in the investigational group, 110 patients in the reference group) ensured 90% power of the trial, assuming a standard deviation of almost 8 for the number of oocytes retrieved. The treatment groups were formally compared with analysis of variance for the number of cumulus–oocyte–complexes, including covariates treatment group, age (<32 or ≥32 years), planned fertilization procedure (IVF or ICSI) and centre.

Descriptive statistics, including mean and standard deviation, were calculated for other endpoints, including dose of rFSH required from day 8 to the day of HCG administration, serum FSH, LH, oestradiol, inhibin B and progesterone concentrations, number and size distribution of follicles (≥11, ≥15 and ≥17 mm) during stimulation and on the day of HCG administration, number and quality of oocytes, fertilization rate (defined as 100 times the ratio of the number of fertilized two pronuclei (2PN) oocytes obtained and the number of oocytes used for fertilization), number and quality of embryos, implantation rate (defined as 100 times the maximum number of gestational sacs as assessed by any USS after embryo transfer divided by the number of embryos transferred (per subject), maximized to 100%), miscarriage rate and pregnancy rate. Patients who received corifollitropin alfa or rFSH but did not have embryo transfer were considered to be cancelled. The percentage of cancelled patients was compared between the treatment groups using Fisher's exact test.

Occurrence of (serious) adverse events, including moderate and severe OHSS as per the World Health Organization criteria (WHO, 1973), outcome of local tolerance and immune response assessments were evaluated as safety endpoints. The percentage of patients with moderate or severe OHSS was compared between the treatment groups using Fisher's exact test.

All efficacy analyses were based on the intent-to-treat (ITT) population, which included all randomized patients who received corifollitropin alfa or at least one dose of rFSH. Patients were grouped based on the treatment to which they had been randomized. One patient treated with rFSH was not randomized via the interactive voice response system and is therefore not part of the ITT population. Safety analyses were performed on the all-subjects-treated group, which comprised all the patients who received either corifollitropin alfa or rFSH, with patients grouped according to the active treatment that they actually received.

Results

Patient characteristics and disposition

A total of 396 patients were randomized (2:1 ratio) and treated: 268 patients with corifollitropin alfa and 128 patients with rFSH (**Table 1**). The two treatment groups were

Table 1 Patient disposition.

	100 μg corifollitropin alfa	*150 IU rFSH*
Randomized	268 (100)	128 (100)[a]
Started stimulation	268 (100)	128 (100)
Cancelled	2	1
	Insufficient ovarian response (investigator opinion) (*n* = 1)	Too high ovarian response (*n* = 1)
	Patient's decision (*n* = 1)	
Treated with HCG	266 (99.3)	127 (99.2)
Oocyte retrieval	266 (99.3)	127 (99.2)
Cancelled	20	7
	Risk of OHSS (*n* = 1)	Too high ovarian response (*n* = 1)
	Too high ovarian response (*n* = 5)	No/too few/bad quality oocytes retrieved (*n* = 2)
	No/too few/bad quality oocytes retrieved (*n* = 2)	No or abnormal fertilization (*n* = 2)
	No or abnormal fertilization (*n* = 4)	No/too few/bad quality embryos for transfer (*n* = 1)
	No/too few/bad quality embryos for transfer (*n* = 7)	No fertilization possible (*n* = 1)
	Suspicious pulmonary tuberculosis (*n* = 1)	
Embryo transfer	246 (91.8)	120 (94.5)

Values in brackets are percentages. HCG, human chorionic gonadotrophin; OHSS, ovarian hyperstimulation syndrome; rFSH, recombinant FSH.
[a]Excluding one patient who was not randomized via the interactive voice response system, but was treated.

comparable with respect to demographics, fertility characteristics, lifestyle characteristics, ultrasound findings and hormone profiles (**Table 2**). The mean age ± SD of the patients included in this trial was 31.0 ± 3.1 years and their mean body weight was 54.2 ± 4.2 kg. In total, 44.4% of all patients were Asian. Overall, the average duration of infertility was 3.2 ± 2.2 years, 61.9% of the patients presented with primary infertility and 56.8% of the patients had no previous IVF cycle. The most frequently reported cause of infertility was male factor (49.5%). On stimulation day 1, the number of basal antral follicles was comparable between the treatment groups and serum LH and FSH concentrations were normal for the early follicular phase.

Three patients who started ovarian stimulation did not receive HCG: two in the corifollitropin alfa group (0.7%) and one in the rFSH group (0.8%). Overall, in 22 (8.2%) patients in the corifollitropin alfa group and eight (6.3%) in the rFSH group, the cycle was cancelled (i.e. did not have embryo transfer). The cancellation rates in both treatment groups were low and not statistically significantly different between the groups (Fisher's exact test). The main reason for cycle cancellation was 'No/too few/bad quality embryos for transfer', occurring in 2.6% and 0.8% of patients of the corifollitropin alfa group and the rFSH group, respectively (**Table 1**).

Primary endpoint

The mean ± SD number of cumulus—oocyte—complexes retrieved per started cycle in the ITT group was 13.3 ± 7.3 in the corifollitropin alfa group versus 10.6 ± 5.9 in the reference group. The estimated treatment difference was +2.5 oocytes in favour of corifollitropin alfa ($P < 0.001$). With a 95% confidence interval of 1.2—3.9, the two treatment groups are considered equivalent based on the predefined equivalence range of (—3, +5) oocytes, despite any statistical significance of the difference.

Table 2 Demographics, fertility and lifestyle characteristics and baseline (stimulation day 1) ultrasound scan and serum hormone concentrations per treatment group (intent-to-treat population).

	100 μg corifollitropin alfa (n = 268)	*150 IU rFSH (n = 128)*
Demographics		
Age (years)	30.9 ± 3.2	31.1 ± 3.0
Body weight (kg)	54.1 ± 4.2	54.4 ± 4.2
Body mass index (kg/m^2)	20.5 ± 1.5	20.6 ± 1.6
Race		
Asian	120 (44.8)	56 (43.8)
Black	1 (0.4)	0 (0.0)
Caucasian	147 (54.9)	72 (56.3)
Fertility characteristics		
Primary infertility	60.8	64.1
Duration of infertility (years)	3.2 ± 2.2	3.3 ± 2.1
Cause of infertility[a]		
Male factor	127 (47.4)	69 (53.9)
Tubal factor	70 (26.1)	31 (24.2)
Endometriosis	32 (11.9)	11 (8.6)
Cervical mucus problems	3 (1.1)	1 (0.8)
Unexplained fertility	74 (27.6)	33 (25.8)
Other	7 (2.6)	2 (1.6)
No previous IVF cycles	55.2	60.2
Lifestyle characteristics		
Smoking (max. five/day)	20 (7.5)	10 (7.8)
Alcohol consumption	65 (24.3)	31 (24.2)
Stimulation day 1		
Total ovarian volume (ml)	10.7 ± 6.2	10.4 ± 5.4
Number of basal antral follicles (<11 mm)	11.1 ± 4.4	11.4 ± 4.3
FSH (IU/l)	6.5 (1—16)	6.6 (2—15)
LH (IU/l)	4.5 (<0.6—13)	4.1 (<0.6—15)

Values are mean ± SD, number (%), % or median (range). rFSH, recombinant FSH.
[a]A patient can have multiple causes of infertility.

Other endpoints

Pharmacokinetic evaluation of the serum corifollitropin alfa concentrations revealed an average elimination half-life ($t_{1/2}$) of 73.1 h. The time to reach the maximum serum concentration (t_{max}) was 46.2 h.

Figure 2 presents serum rFSH immunoreactivity during ovarian stimulation for corifollitropin alfa and daily rFSH. Upon starting stimulation with a single subcutaneous 100 μg corifollitropin alfa injection, median serum rFSH immunoreactivity showed a rapid increase at day 3 (median value: 26.3 IU/l) and a rapid decline thereafter to reach a median value of 10.1 IU/l at day 8. In the daily rFSH group, median serum FSH immunoreactivity increased until day 5 (10.2 IU/l) and then reached a plateau to a median value of 10.3 IU/l at day 8. From day 8 onwards, there were no relevant differences in median serum FSH immunoreactivity between the two treatment groups.

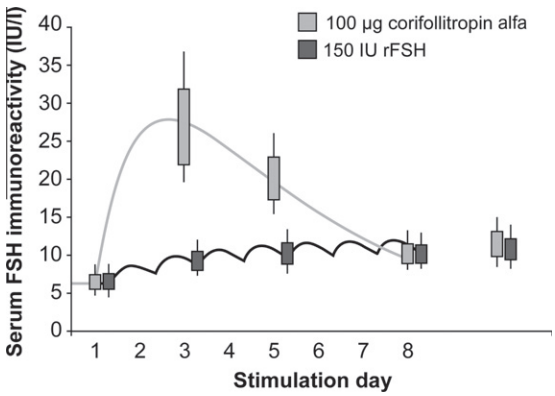

Figure 2 Serum recombinant FSH (rFSH) immunoreactivity during ovarian stimulation for the corifollitropin alfa regimen and the reference treatment with daily rFSH. The boxes represent interquartile ranges and the whiskers represent 10–90th centiles. The lines represent typical time profiles of FSH immunoreactivity for a subject with a body weight of 55 kg (simulated from available data).

The median duration of stimulation was 9 days in both treatment groups (**Table 3**); thus, after corifollitropin alfa injection on average only 2 days of rFSH were required until HCG administration. The median total amount of rFSH required from day 8 to HCG administration was 300 IU for the corifollitropin alfa group and 275 IU for the rFSH treatment group (**Table 3**). **Figure 3** presents the frequency distribution of the day when HCG criteria were met in each treatment group. One-third (32.8%) of the patients in the corifollitropin alfa group met the criterion for HCG injection before or on stimulation day 8. From stimulation day 8 onwards, a fixed dose of 150 rFSH per day was used in 56.1% of the corifollitropin alfa group and in 68.5% of the rFSH group. Per protocol, the dose of (placebo) rFSH could be reduced or increased from stimulation day 6 onwards. Dose decreases on stimulation days 6 and/or 7 were recorded more often in the corifollitropin alfa group (6.4%) than in the rFSH group (0.8%), while this was vice versa for dose increases (3.4% versus 6.4%, respectively). Most patients (all-subjects-treated group) received 10,000 IU HCG (80.2% and 89.1% of the patients in the corifollitropin alfa and rFSH group, respectively) and a dose of 5000 IU HCG was used for 19.0% and 10.1% of the respective groups.

In the corifollitropin alfa group, slightly more follicles (≥11 mm) were recruited during the first week of stimulation compared with the rFSH group (**Table 3**). On stimulation day 8, the mean ± SD number of follicles ≥11 mm was 11.8 ± 6.1 in the corifollitropin alfa group and 10.6 ± 5.3 in the rFSH group. On the day of HCG, the total number of follicles ≥11 mm had increased to 14.9 ± 6.6 follicles in

Table 3 Stimulation characteristics and follicle growth (intent-to-treat population).

	100 μg corifollitropin alfa (n = 268)	*150 IU rFSH (n = 128)*
Stimulation characteristics[a]		
Total duration of stimulation (days)	9 (6–15)	9 (6–15)
Total dose of rFSH (IU)	300 (0–1550)	1350 (825–2650)
Total dose of rFSH from day 8 onwards (IU)	300 (0–1550)	275 (0–1600)
Patients reaching HCG criterion on or before stimulation day 8 (%)	32.8	39.8
Follicles, stimulation day 8		
≥11 mm	11.8 ± 6.1	10.6 ± 5.3
≥15 mm	5.0 ± 4.6	5.1 ± 4.5
≥17 mm	2.0 ± 3.0	2.4 ± 3.1
Follicles on day of HCG[a]		
≥11 mm	14.9 ± 6.6	12.9 ± 5.8
≥15 mm	9.4 ± 4.9	8.5 ± 4.4
≥17 mm	5.3 ± 3.0	5.1 ± 3.0

Values are median (range), % or mean ± SD unless otherwise stated. HCG, human chorionic gonadotrophin; rFSH, recombinant FSH.
[a]Restricted to patients with human chorionic gonadotrophin injection.

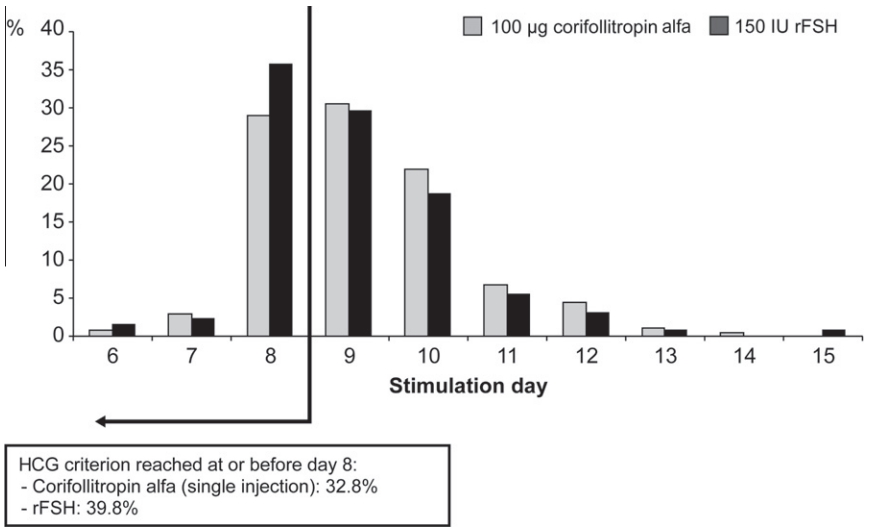

Figure 3 Graphical presentation of the percentage of patients per stimulation day reaching the human chorionic gonadotrophin (HCG) criterion (as soon as three follicles ≥17 mm) (intent-to-treat group). rFSH, recombinant FSH.

the corifollitropin alfa group and to 12.9 ± 5.8 follicles in the rFSH group.

Serum concentrations of oestradiol, inhibin B, LH and progesterone for all patients who received HCG are shown in **Figure 4A—D**. During the first 5 days of stimulation, serum oestradiol and inhibin B concentrations tended to increase more rapidly in the corifollitropin alfa group than in the rFSH group. From stimulation day 5 to stimulation day 8, serum oestradiol and inhibin B concentrations continued to rise in both treatment groups, but the increase was

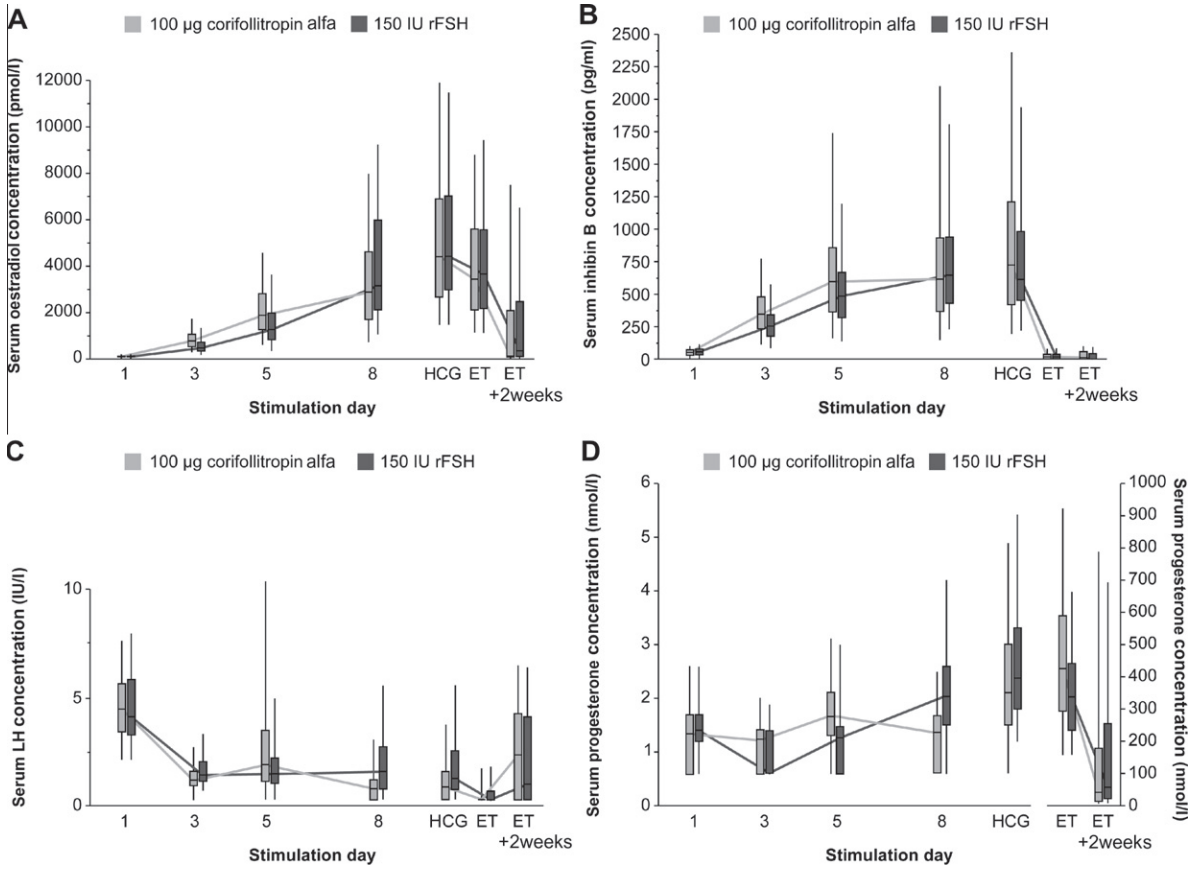

Figure 4 Serum concentrations of (**A**) oestradiol, (**B**) inhibin B, (**C**) LH and (**D**) progesterone per treatment group during and after stimulation restricted to patients with human chorionic gonadotrophin (HCG) injection (intent-to-treat group). The boxes indicate the 25—75% centiles, the whiskers indicate the 5—95% centiles and median values are connected. ET, embryo transfer; rFSH, recombinant FSH.

less pronounced in the corifollitropin alfa group than in the rFSH group.

Serum LH concentrations rapidly declined from stimulation day 1 to 3 in both treatment groups. On stimulation day 5 (prior to starting GnRH antagonist co-treatment) the incidence of premature LH rises (value $\geq$10 IU/l) was 5.2% (14 out of 268) of the patients in the corifollitropin alfa group and 3.9% (five out of 128) of the patients in the rFSH group. Overall, on stimulation day 5, a larger variation in LH concentrations was observed in the corifollitropin alfa group with a somewhat higher median LH value as compared with rFSH. Upon initiation of GnRH antagonist administration at day 5, LH concentrations decreased again in the corifollitropin alfa group, whereas the median LH values in the rFSH group did not change from day 3 until day 8. During GnRH antagonist treatment, none of the patients in the corifollitropin alfa group and two patients (1.6%) in the rFSH group experienced an LH rise.

Table 4 Clinical outcome per started cycle (intent-to-treat population).

	100 μg corifollitropin alfa (n = 268)	*150 IU rFSH (n = 128)*
Cumulus–oocyte–complexes retrieved (primary endpoint)	13.3 ± 7.3	10.6 ± 5.9
Fertilization procedure[a]		
IVF only	83 (31.4)	37 (29.8)
ICSI only	137 (51.9)	72 (58.1)
IVF + ICSI	44 (16.7)	15 (12.1)
Number and quality of oocytes (ICSI only)		
Number of oocytes	12.7 ± 6.8	9.9 ± 5.4
Metaphase II oocytes	10.7 ± 6.4	7.8 ± 4.8
Percentage of total	82.5	79.1
Fertilization outcome		
Fertilization rate[a,b]	67.6 ± 22.5	67.7 ± 25.4
2PN fertilized oocytes obtained[a]	7.8 ± 4.7	6.2 ± 3.9
2PN fertilized oocytes used for embryo development[a]	6.8 ± 3.6	5.8 ± 3.8
Number and quality of embryos		
Total number of embryos obtained (day 3)[a]	7.1 ± 4.2	6.1 ± 4.1
Good-quality embryos (grade 1 and 2)[a]	3.4 ± 3.0	3.0 ± 3.0
Percentage of total[a]	50.1	49.1
Single embryo transfer[c]	46 (18.7)	26 (21.7)
Embryos transferred[c]	1.8 ± 0.4	1.8 ± 0.4
Good-quality embryos transferred[c]	1.3 ± 0.8	1.3 ± 0.8
Embryos cryopreserved	2.0 ± 3.0	1.7 ± 2.6
Implantation rate[c]	23.4 ± 37.1	28.5 ± 38.6
Clinical outcome		
Biochemical pregnancy[d]	101 (37.7)	58 (45.3)
Clinical pregnancy[e]	78 (29.1)	48 (37.5)
Vital pregnancy[f]	69 (25.7)	45 (35.2)
Ongoing pregnancy[g]	68 (25.4)	44 (34.4)
Twin pregnancy[h]	19 (27.9)	10 (22.7)
Miscarriage[i]	10 (12.8)	4 (8.3)

Values are mean ± SD or number (%) unless otherwise stated.
[a] Restricted to patients with IVF and/or intracytoplasmic sperm injection (ICSI).
[b] Defined as 100 times the number of mature oocytes (with two pronuclei (2PN)) obtained divided by the number of oocytes used for fertilization.
[c] Restricted to patients with embryo transfer.
[d] Positive human chorionic gonadotrophin (HCG) test performed 2 weeks after embryo transfer.
[e] Clinical pregnancy: gestational sac on ultrasound scan.
[f] Vital pregnancy: gestational sac + fetal heartbeat.
[g] Ongoing pregnancy: vital fetus at least 10 weeks after embryo transfer or live birth.
[h] Per ongoing pregnancy.
[i] Per clinical pregnancy.

At the start of stimulation, serum progesterone concentrations were similar between the two treatment groups and remained low throughout ovarian stimulation in both groups (**Figure 4D**).

ICSI was the most frequently used fertilization procedure (51.9% in the corifollitropin alfa group and 58.1% in the rFSH group). In patients with ICSI only, the mean number of metaphase II oocytes as a percentage of the total number of oocytes was 82.5% in the corifollitropin alfa group and this was comparable to the mean percentage of 79.1% in the rFSH group (**Table 4**).

For patients with IVF and/or ICSI, the fertilization rate was similar between the two treatment groups (67.6% versus 67.7%). The mean number of fertilized 2PN oocytes obtained and used for embryo development was 7.8 and 6.8, respectively, for the corifollitropin alfa group and 6.2 and 5.8, respectively, for the rFSH group (**Table 4**). This difference between obtained and used is explained by the fact that for a total of 49 patients a subset of fertilized 2PN oocytes were reported to be lost or used for other purposes, of which more than 90% was cryopreserved.

The mean ± SD number of good-quality (Grade 1 and 2) embryos obtained at day 3 in patients with IVF and/or ICSI was 3.4 ± 3.0 and 3.0 ± 3.0 in the corifollitropin alfa and rFSH groups, respectively (**Table 4**). Although the majority of patients had two embryos transferred, the mean ± SD number of good-quality embryos transferred was 1.3 ± 0.8 in both groups. The mean ± SD number of embryos cryopreserved was 2.0 ± 3.0 and 1.7 ± 2.6, in the corifollitropin alfa and rFSH groups, respectively. The mean ± SD implantation rates for patients with embryo transfer were 23.4 ± 37.1% in the corifollitropin alfa group and 28.5 ± 38.6% in the rFSH group.

Per started cycle, the biochemical and clinical pregnancy rates were 37.7% and 29.1%, respectively, for the corifollitropin alfa group and 45.3% and 37.5%, respectively, for the rFSH group. The vital and ongoing pregnancy rates per started cycle were 25.7% and 25.4%, respectively, for the corifollitropin alfa group and 35.2% and 34.4%, respectively, for the rFSH group. Analysis of the ongoing pregnancy rate per started cycle showed that the *P*-value for the estimated difference in ongoing pregnancy rate between the corifollitropin alfa and rFSH treatment groups was not statistically significant at a 5% level. For both treatment groups, the majority of the ongoing pregnancies were singletons: 72.1% ($n = 49$) in the corifollitropin alfa group and 77.3% ($n = 34$) in the rFSH group. In total, 29 twin pregnancies were reported: 19 (27.9%) in the corifollitropin alfa group and 10 (22.7%) in the rFSH group. Eight patients (7.9%) in the corifollitropin alfa group and four patients (6.9%) in the rFSH group had an ectopic pregnancy per biochemical pregnancy. In total, 14 patients with a clinical pregnancy had a miscarriage: 10 (12.8%) in the corifollitropin alfa group and four (8.3%) in the rFSH group.

Safety

In total, 20 patients (7.5%) in the corifollitropin alfa group reported 22 serious adverse events and eight patients (6.3%) in the rFSH group reported nine serious adverse events. The most frequently reported serious adverse event was ectopic pregnancy, which occurred with the similar incidence of 3.0% and 3.1%, in the corifollitropin alfa and rFSH groups, respectively. The percentage of patients reporting adverse events was comparable between the two treatment groups: 55.2% in the corifollitropin alfa group and 53.5% in the rFSH group. None of the patients had discontinued the trial due to an adverse event or serious adverse event. The most frequently reported adverse events in the corifollitropin alfa and rFSH groups, respectively, were pelvic discomfort (10.1% and 14.7%), pelvic pain (10.4% and 10.9%), antepartum haemorrhage (6.0% and 12.4%) and headache (8.2% and 8.5%). A total of 18 patients in the corifollitropin alfa-treated group (6.7%) and six patients in the rFSH-treated group (4.7%) developed OHSS in this trial. The incidences of (moderate/severe) OHSS were 3.4% and 1.6% for the corifollitropin alfa and rFSH groups, respectively; the difference was not statistically significant at a 5% level (Fisher's exact test).

No drug-related hypersensitivity reactions were reported following corifollitropin alfa injection. With respect to local tolerance, none of the patients had any moderate or severe local reaction at the site of injection. In total, 267 patients treated with corifollitropin alfa and screened in the anti-corifollitropin alfa antibody assay were found negative, indicating that none of the patients had developed anti-corifollitropin alfa antibodies.

Discussion

The current trial was undertaken to confirm the efficacy and safety of a single subcutaneous injection of 100 μg corifollitropin alfa in patients weighing up to 60 kg using daily 150 IU rFSH as a reference. With respect to the primary endpoint, corifollitropin alfa provided significantly more oocytes (+2.5 oocytes, 95% confidence interval 1.2−3.9), but the estimated difference was well within the pre-set equivalence margin. Twice as many patients were randomized to the corifollitropin alfa group as compared with the rFSH reference group. This 2:1 randomization ratio was used to collect more (safety) information on the investigational product and did not introduce bias due to the double-blind, randomized design of the trial. rFSH (reference group) has already been on the market for several years and the safety and efficacy profile of this reference compound is well established (Kolibianakis et al. 2007).

Previously, the dose-finding trial of corifollitropin alfa indicated that body weight is a major determinant of exposure to corifollitropin alfa and treatment outcome (Corifollitropin Alfa Dose-Finding Study Group, 2008). In the current trial, a single dose of 100 μg corifollitropin alfa was sufficient to maintain multiple follicular development during the first week of stimulation in patients weighing ≤60 kg given the low cancellation rate in this group prior to the day of HCG.

The median duration of stimulation in the 100 μg corifollitropin alfa group was 9 days and was equal to the reference group using daily 150 IU rFSH in the same GnRH antagonist protocol. After a single injection of 100 μg corifollitropin alfa, patients required on average 2 days of additional stimulation with rFSH to reach the criterion for triggering final oocyte maturation. In 32.8% of the patients,

a single injection of corifollitropin alfa was sufficient to reach the HCG criterion, without the need for any additional rFSH.

The stimulation characteristics of 100 μg corifollitropin alfa in patients weighing at most 60 kg are identical to those observed after a single injection of 150 μg corifollitropin alfa in patients weighing more than 60 kg, as investigated in the large Engage trial (Devroey et al. 2009b). In the current double-blind trial, treatment with corifollitropin alfa resulted in an equal number of cumulus—oocyte—complexes retrieved per started cycle as in the Engage trial, i.e. 13.3 in the Ensure trial versus 13.7 in the Engage trial. These data confirm that the two dosages in the recommended body weight groups provide similar exposure and, therefore, induce the same degree of ovarian response. In comparison to the reference groups, the difference in number of oocytes retrieved is determined by the daily dose of rFSH, which was 150 IU in the current trial and 200 IU in the Engage trial. Accordingly, the estimated treatment difference in terms of the number of oocytes retrieved was 2.5 oocytes in the current trial versus 1.2 oocytes in the Engage trial. In terms of other clinical outcome parameters such as the maturity of oocytes retrieved, the fertilization rate and the number of good-quality embryos obtained, comparable results were obtained in both the Ensure and Engage trials.

In the Engage trial involving more than 1500 patients, results demonstrated an equal and high ongoing pregnancy rate in patients treated with corifollitropin alfa versus daily rFSH. In the current trial, ongoing pregnancy rate was a secondary endpoint and, in contrast to the Engage trial, this trial was not powered to assess non-inferiority in the ongoing pregnancy rates between the treatment groups. Observed ongoing pregnancy rates were 25.4% and 34.4% for the 100 μg corifollitropin alfa group and 150 IU rFSH group, respectively, and this difference was not statistically significant. Given the fact that other outcome parameters such as oocyte maturity, fertilization rate and number of good-quality embryos transferred were comparable to the reference group, the apparent difference in ongoing pregnancy rates in the current trial is considered a chance finding.

In this trial, the incidence of ectopic pregnancy in both treatment groups was twice as high as the reported incidence in IVF practice (Fernandez and Gervaise, 2004). Known risk factors for ectopic pregnancy include the method of embryo transfer, previous ectopic pregnancy, previous tubal surgery or pathology, previous spontaneous abortion and previous genital infections. In the current study, one patient had a history of three previous ectopic pregnancies whereas seven additional patients had a history of previous spontaneous abortion, endometriosis, tubal or unexplained infertility, which may have contributed to the relatively high incidences in this trial.

A single injection of 100 μg corifollitropin alfa had a safety profile comparable to daily doses of 150 IU rFSH in terms of the incidence and type of reported serious adverse events and adverse events. In line with the higher ovarian response, the incidence of moderate/severe OHSS tended to be higher after treatment with corifollitropin alfa than after treatment with rFSH, although the difference was not statistically significant.

Corifollitropin alfa was well tolerated at the site of injection. No drug-related hypersensitivity reactions or anti-corifollitropin alfa antibodies were reported following injection of 100 μg corifollitropin alfa. These findings are consistent with the outcome of previous phase I to III trials (Balen et al. 2004; Beckers et al. 2003; Bouloux et al. 2001; Corifollitropin Alfa Dose-Finding Study Group, 2008; Devroey et al. 2004, 2009b; Duijkers et al. 2002).

In conclusion, a lower dose of corifollitropin alfa (100 μg) offers a simplified treatment option for potential normal-responder patients with a lower body weight (at most 60 kg) undergoing controlled ovarian stimulation prior to IVF or ICSI. Compared with the reference group, treated with a fixed starting dose of 150 IU rFSH, the ovarian response is higher following corifollitropin alfa but well within the predefined equivalence margin whereas the duration of stimulation is equally short. One-third of the patients studied had complete multiple follicular development up to three follicles ≥17 mm based on a single injection of 100 μg corifollitropin alfa and did not need additional rFSH injections.

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5.3 Discussion

Phase III trials were designed to demonstrate how effective and safe new fertility drugs are in comparison to the "current care". In time, the latter shifted from urinary FSH to rFSH and from a long GnRH agonist protocol to a short GnRH antagonist protocol. Non-inferiority trials are often designed for new drugs or regimens that do not necessarily improve efficacy, but may favor safety or convenience of treatment. Non-inferiority trials are indicated to demonstrate that the test product is not less efficacious than the comparator by more than a pre-specified amount which is known as the non-inferiority margin. For an active controlled equivalence trial, both the upper and the lower equivalence margins are to be specified, while only the lower margin of the difference is needed for the active controlled non-inferiority trial.

For the convenience of the reader, the outcome of the primary endpoints for the 3 largest phase III trials of respectively follitropin-β, ganirelix and corifollitropin alfa is summarized in Table 2. Comparison of the ongoing pregnancy rates of these 3 trials indicates how much the average pregnancy rates have increased over time in Europe from 20% at the time of the follitropin-β studies, to 25% at the time of the ganirelix studies to 30% at the time of the corifollitropin alfa studies [Boostanfar et al 2012a].

The comparative study of follitropin-β [Out et al 1995] demonstrated that the latter was slightly more effective than urinary FSH (Metrodin) by providing a significantly higher number of oocytes retrieved. This was related to the relatively more basic FSH isoforms in follitropin-β [De Leeuw et al 1996]. The difference in the total number and size of follicles induced by a daily dose of 150 IU follitropin-β or by urinary FSH was previously also noted in pituitary-suppressed female volunteers [Mannaerts et al 1996b]. More oocytes were not associated with statistically significant higher ongoing pregnancy rates following fresh embryo transfer, but did lead to a 5.0% higher ongoing pregnancy rate following combined analysis of 3 randomized controlled trials and fresh embryo transfer, a treatment difference which further increased to 6.4% when the cryopreserved embryos were included in the analysis [Out et al 1997].

Studies comparing the GnRH antagonist ganirelix with long protocols of GnRH agonist have consistently indicated that the cohort of recruited follicles is smaller following GnRH antagonist treatment. The difference in the largest ganirelix trial [The European Orgalutran study group, Borm and Mannaerts, 2000] was -1.0 oocyte and the lower limit of the 95% CI was within the predefined non-inferiority margin of -3 oocytes (see Table I). The treatment difference for the ongoing pregnancy rate was -5.4% which was not statistically significant but the point estimate passed the predefined treatment difference (delta) of 5%. Following this first randomized controlled trial, many other prospective controlled studies and (systematic) reviews have demonstrated the advantages of GnRH antagonist treatment over GnRH agonists in assisted reproduction, including: (i) the immediate reversibility of drug effects; (ii) a requirement for less FSH; (iii) a shortened duration of stimulation; (iv) a

reduced incidence of ovarian hyperstimulation syndrome (OHSS); and (v) reduced patient burden [Tarlatzis et al 2006; Heijnen et al 2007; Devroey et al 2009b]. The latest Cochrane analysis [Al-Inany et al 2011] including 7511 patients comparing the antagonist to the long agonist protocols concludes that there is no evidence of a statistically significant difference in ongoing pregnancy (28 randomized controlled studies; Odds Ratio 0.87, 95% CI 0.77 to 1.00).

Table 2 Outcome primary end-points of largest phase III RCTs for follitropin-β, ganirelix and corifollitropin alfa, one treatment cycle, fresh embryo transfer

	Age yrs	Oocytes	Treatment difference (95% CI)	Ongoing PR	Treatment Difference (95% CI)
Follitropin-β vs urinary FSH, Start dose 150 or 225 IU Long protocol of buserelin (EU study) **1992-1993**	18-39	10.8 vs 9.0	1.9 (1.2-2.6)	22.2 vs 18.2	4.0 (-1.1-9.0)
Ganirelix vs long protocol buserelin Stimulation with follitropin-β, Start dose 150 IU (EU study) **1997-1998**	18-39	8.7 vs 9.7	-1.0 (-1.8-0.2)	20.3 vs 25.7	-5.4% (-11.9-1.0)
Corifollitropin alfa vs follitropin-β 150 μg vs 200 IU for first 7 days Ganirelix (EU and US study) **2006-2007**	18-36	13.7 vs 12.5	1.2 (0.5-1.9)	38.9 vs 38.1*	0.9 (-3.9-5.7)

* Ongoing pregnancy rates were 30% vs 45 % in Europe vs North America [Boostanfar et al 2012a]

Last but not least, the largest phase III comparative trial of corifollitropin alfa [Devroey et al 2009a] demonstrated that in a GnRH antagonist protocol, more follicles were recruited and thus more oocytes were retrieved following treatment with 150 μg corifollitropin alfa than following a daily dose of 200 IU follitropin-β treatment during the first 7 days of stimulation. The estimated treatment difference (95% CI) was 1.2 (0.5-1.9) oocytes and the 95% CI was within the predefined equivalence interval of -3 to 5 oocytes (see Table II). The treatment difference for the ongoing pregnancy rate was +0.9% with a lower 95% confidence limit of -3.9% thus well within the predefined non-inferiority margin of -8%. Including the cyropreserved embryos, the calculated ongoing pregnancy rate per started cycle was 47.2% in patients treated with corifollitropin alfa vs 44.9% in patients treated with follitropin-β [Boostanfar et al 2012a].

In conclusion, phase III studies should be sufficiently powered to exclude or detect a clinically meaningful difference of the primary endpoint between treatment groups. The number of oocytes per started cycle may be considered an appropriate pharmacological outcome parameter for FSH preparations, also reflecting the number of subjects with too low or too high ovarian response which is directly related to the risk for cycle cancellation or the risk for OHSS. The ongoing pregnancy rate per started cycle as a primary endpoint requires much larger comparative studies which should include the outcome of cryopreserved embryos to estimate the contribution made by surplus oocytes.

Chapter 6

Phase III Randomized, Controlled Trials, Safety

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6.1 Introduction

In general, safety data for drugs in development are evaluated based on the reported Adverse Experiences or Events (AEs) which may be considered Serious (SAEs) and/or drug-related by the investigator. Adverse events may include any unfavorable and unintended change in medical signs and symptoms observed during the study, regardless of whether it is considered related to the use of the study drug or not.

For all new fertility drugs and treatment regimens the incidence of OHSS should be documented in detail, distinguishing mild, moderate and severe cases with either early or late onset.

Parenteral (glyco)proteins in clinical research deserve particularly close monitoring of specific events such as any (immediate) allergic reaction or immune response following (repeated) drug administration. Monitoring for drug-induced antibodies is complex [White paper: Mire-Sluis et al 2004; Guidance for Industry Assay Development for Immunogenicity Testing of Therapeutic Proteins, FDA Center for Drug Evaluation and Research, 2009] and requires testing of pre- and post-treatment samples in consecutive assays including a screening assay, a confirmation assay and a neutralization assay.

Last but not least, documenting the health of infants born following treatment of infertile couples with new fertility drugs is paramount.

Ovarian Hyperstimulation Syndrome (OHSS)

Ovarian hyperstimulation syndrome (OHSS) is a potentially serious complication following ovulation induction or ovarian stimulation prior to IVF or ICSI [Delvigne and Rozenberg, 2002 and 2003]. Following multiple follicular development, the risk of developing OHSS is related to the degree of the ovarian response in combination with the exposure to exogenous or endogenous hCG, which triggers the signs and symptoms of OHSS. In the natural cycle following monofollicular growth and an endogenous LH surge, OHSS is very rare but has been described in patients with an FSH-receptor mutation causing crossreactivity of the receptor with hCG [Vasseur et al 2003].

OHSS is often graded into 3 categories as being either mild, moderate or severe. Mild OHSS occurs in the majority of OHSS cases of ovarian stimulation and does not require special treatment, in contrast to moderate or severe OHSS which are considered more clinically relevant. There are two main clinical forms of OHSS, namely, early OHSS and late OHSS [Papanikolaou et al 2006]. OHSS with early onset may result from a too high ovarian response to gonadotropins, whereas late OHSS is induced by endogenous hCG produced during early pregnancy. Early OHSS can be prevented by individualized treatment following the prediction of potential high responders [Nyboe Andersen et al 2011]. The risk of OHSS is

largely reduced by the application of a lower starting dose of rFSH/hMG and a GnRH antagonist protocol instead of a long GnRH agonist protocol [Kolibianakis et al 2006; Al Inany et al 2011].

Within each protocol, the risk of OHSS increases with the number of follicles, rather than with increasing serum estradiol levels. In a GnRH antagonist protocol, the best threshold to identify patients at risk of severe OHSS is the development of more than 18 to 19 follicles ≥ 11 mm on the day of hCG [Papanikolaou et al 2006; Mannaerts et al 2012]. For these patients preventive measures should be considered by switching from hCG to GnRH agonist to trigger final oocyte maturation [Devroey et al 2011].

Treatment of potential high responders with corifollitropin alfa is contra-indicated as they should be treated with low doses of daily FSH. Still, in normal responder patients treated with a ganirelix protocol, corifollitropin alfa recruits more follicles than daily 150 or 200 IU follitropin-β resulting in 1 to 2 extra oocytes [Devroey et al 2009a; Corifollitropin alfa Ensure Group, 2010]. To evaluate whether this higher ovarian response also implies a higher risk of OHSS, a pooled analysis was undertaken of individual patient data collected during the phase III studies of corifollitropin alfa [Tarlatzis et al, 2012].

Hypersensitivity & immunogenicity

Delivering pharmacological doses of a therapeutic (glyco)protein to humans may raise concerns of inducing a (general) hypersensitivity or anaphylactic reaction, with or without the formation of anti-drug antibodies. Whereas hypersensitivity or allergic reactions may be observed during clinical trials, an immune response may be induced without (acute) signs and symptoms and needs to be monitored following drug exposure. The likelihood of an immune response may depend on the size of the molecule, the purity and pharmaceutical formulation, and the route and frequency of administration [Schellekens, 2002]. The main factors contributing to immunogenicity are impurities and the presence of aggregates. In the majority of cases, induced antibodies have no biological or clinical effects, and if they do neutralize, the most common clinical effect is the loss of efficacy.

Two reviews [Koren et al 2002; Wadha et al 2003] were published concerning the immune response to therapeutic proteins in humans which reported the absence of antibody response to exogenous urinary and recombinant FSH. There are a few references that report on the presence of circulating immunoglobulins crossreacting with native FSH, however, it is not clear whether these antibodies lower endogenous FSH. Anti-FSH antibodies may be naturally occurring antibodies associated with peripheral FSH concentrations and produced in higher levels in infertile women [Haller et al 2007]. Case reports have also been published on the presence of (transient) anti-hCG antibodies in hypogonadotropic hypogonadal males with long-term hCG treatment and in infertile women with recurrent pregnancy loss, but to date no induced antibodies have been reported following treatment with recombinant hCG.

The final therapeutic product of corifollitropin alfa (purity > 99%, formulation similar to follitropin-β) is administered as a single subcutaneous injection of 100 µg or 150 µg per IVF cycle. The risk of this new therapeutic recombinant protein becoming immunogenic is considered low as it incorporates the expression of two human genes resulting in one β-subunit without additional linkage amino acids. Nevertheless, this needs to be confirmed in target populations. In a phase III trial of women undergoing several consecutive treatment cycles with corifollitropin alfa there were no related allergic reactions and/or induced antibodies [Norman et al 2011].

Pregnancy and infant follow-up

A general concern for new drugs or new ART procedures is the possible risk of perinatal complications or congenital birth defects. In general, new drugs or procedures are only accepted as safe following reassuring results from relatively large follow-up studies, often including pooled data from various intervention studies [Out et al 1999; Bonduelle et al 2002; Bonduelle et al 2010]. The currently available literature data do not indicate that treatment with gonadotropins or GnRH analogues would increase the risk of congenital malformations. Like follitropin-β, corifollitropin alfa only interacts with the FSH receptor and the receptor-binding and -activation by corifollitropin alfa is comparable to that by follitropin-β [Fauser et al 2009]. Therefore, it may be assumed that the risks associated with corifollitropin alfa treatment for patients and their offspring are comparable to those associated with other FSH preparations. The pregnancy and neonatal follow-up program of corifollitropin alfa was designed prospectively with predefined parameters including complications during pregnancy, mode of delivery, complications during delivery and neonatal outcome including congenital malformations [Bonduelle et al 2012].

6.2 Results

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ARTICLE

Comparative incidence of ovarian hyperstimulation syndrome following ovarian stimulation with corifollitropin alfa or recombinant FSH

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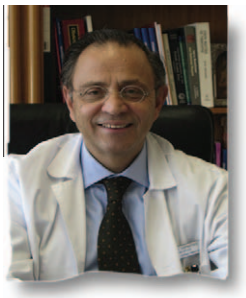

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Abstract Corifollitropin alfa is a novel recombinant gonadotrophin with sustained follicle-stimulating activity. A single injection can replace seven daily injections of recombinant follicle-stimulating hormone (rFSH) during the first week of ovarian stimulation. All cases of ovarian hyperstimulation syndrome (OHSS) with corifollitropin alfa intervention in a gonadotrophin-releasing hormone antagonist protocol have been assessed in three large trials: Engage, Ensure and Trust. Overall, 1705 patients received corifollitropin alfa and 5.6% experienced mild, moderate or severe OHSS. In the randomized controlled trials, Engage and Ensure, the pooled incidence of OHSS with corifollitropin alfa was 6.9% (71/1023 patients) compared with 6.0% (53/880 patients) in the rFSH group. Adjusted for trial, the odds ratio for OHSS was 1.18 (95% CI 0.81–1.71) indicating that the risk of OHSS for corifollitropin alfa was similar to that for rFSH. The incidence of mild, moderate and severe OHSS was 3.0%, 2.2% and 1.8%, respectively, with corifollitropin alfa, with 1.9% requiring hospitalization, and 3.5%, 1.3% and 1.3%, respectively, in the rFSH arms, with 0.9% requiring hospitalization. Despite a higher ovarian response with corifollitropin alfa compared with rFSH for the first 7 days of ovarian stimulation, the incidence of OHSS was similar.

KEYWORDS: corifollitropin alfa, gonadotrophin-releasing hormone antagonist, OHSS, ovarian stimulation, recombinant FSH, assisted reproductive technology

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is a potentially serious complication of ovulation induction and ovarian stimulation for assisted reproductive technology (Delvigne and Rozenberg, 2002, 2003). Two main clinical forms of OHSS, distinguished by time of onset, are described in the literature and appear to be distinct in their aetiology (Mathur et al., 2000). Early OHSS generally occurs within 10 days after human chorionic gonadotrophin (HCG) administration, is an acute effect of exogenous HCG administration and is correlated to the magnitude of the preovulatory ovarian response to stimulation (Lyons et al., 1994; Mathur et al., 2000). Late OHSS generally occurs more than 10 days after HCG administration, is linked to endogenous HCG production by an implanting embryo or HCG administration for luteal-phase support and is poorly correlated to the preovulatory ovarian response to stimulation (Mathur et al., 2000; Papanikolaou et al., 2006). Late OHSS is more likely to be severe than early OHSS (Mathur et al., 2000).

To date, it is difficult to estimate the risk for developing OHSS in the absence of known predisposing factors. However, patient characteristics associated with the ovarian reserve, including age, serum follicle-stimulating hormone (FSH) concentrations, antral follicle count (AFC), inhibin B concentrations and anti-Müllerian hormone concentrations, may be used to estimate the ovarian response prior to ovarian stimulation.

The risk of early-onset OHSS increases in high responders to ovarian stimulation, as measured by the number of ovarian follicles, serum oestradiol concentrations on the day of HCG administration and the number of oocytes retrieved (Mathur et al., 2000; Verwoerd et al., 2008). Patients with more than 18 follicles $\geq$11 mm are at increased risk of developing OHSS (Papanikolaou et al., 2006).

Gonadotrophin-releasing hormone (GnRH) antagonist protocols appear to be effective in reducing the development of OHSS (Kolibianakis et al., 2006). Comparative clinical trials of GnRH antagonist versus long GnRH agonist protocols have shown that long protocols are associated with the recruitment of more follicles and oocytes and eventually a higher incidence of OHSS (Kolibianakis et al., 2006).

Corifollitropin alfa is a novel recombinant gonadotrophin, a single dose of which is capable of initiating and sustaining multifollicular growth during the first 7 days of ovarian stimulation. The pharmacokinetic profile of corifollitropin alfa is characterized by a slow absorption, resulting in peak concentrations 2 days after injection with a steady decline afterwards. Its long elimination half-life accommodates a sufficiently high FSH threshold window to support ovarian stimulation over an entire week (Duijkers et al., 2002; Fauser et al., 2010). This sustained activity may be perceived to be a risk factor for overstimulation. Corifollitropin alfa has equivalent efficacy to recombinant FSH (rFSH) for achieving ongoing pregnancies in a GnRH antagonist protocol but offers a simplified treatment regimen with fewer injections (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009). It does promote a slightly higher ovarian response compared with daily rFSH, most likely due to the higher FSH activity during the first few days of stimulation (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009).

The current study assessed the incidence of OHSS with corifollitropin alfa intervention in a GnRH antagonist protocol for ovarian stimulation. Cases of OHSS from three phase-3 trials with corifollitropin alfa that were primarily designed, powered and conducted to assess ongoing pregnancy rates (Engage; Devroey et al., 2009), number of oocytes (Ensure; Corifollitropin Alfa Ensure Study Group, 2010) and immunogenicity (Trust; Norman et al., 2011) were captured. Because of the relative rarity of severe OHSS, a pooled analysis of the Engage and Ensure trials (two trials of similar design with rFSH as the comparator) was conducted to provide a reliable estimate of the incidence of OHSS.

Materials and methods

Phase-3 trials

The incidence, severity and time of onset of OHSS were assessed in three trials of corifollitropin alfa (Elonva; N.V. Organon) in a GnRH antagonist protocol in normogonadotrophic women with an indication for ovarian stimulation for IVF/intracytoplasmic sperm injection (ICSI). Details of these trials, Engage (Devroey et al., 2009), Ensure (Corifollitropin Alfa Ensure Study Group, 2010) and Trust (Norman et al., 2011), have been reported previously.

Engage and Ensure were double-blind, double-dummy randomized controlled trials that compared the efficacy of a single injection of corifollitropin alfa during the first 7 days of ovarian stimulation with daily injections of rFSH in a GnRH antagonist (ganirelix) protocol.

Trust was an open, uncontrolled trial that evaluated the safety and tolerability of repeated cycles (up to three per patient) with a single injection of corifollitropin alfa for the first 7 days of ovarian stimulation in a GnRH antagonist protocol. In the current analysis, data from the first cycle only were included.

Study population

In the Engage trial participants aged 18–36 years with a bodyweight of 61–90 kg and body mass index (BMI) 18–32 kg/m^2 received either corifollitropin alfa 150 μg (n = 755) or seven daily injections of rFSH 200 IU (Puregon/Follistim Pen; N.V. Organon) (n = 751). In the Ensure trial, participants aged 18–36 years with a body weight $\leq$60 kg and BMI 18–32 kg/m^2 received either corifollitropin alfa 100 μg (n = 268) or seven daily injections of rFSH 150 IU (n = 129). The different doses of corifollitropin alfa (100 μg for patients $\leq$60 kg and 150 μg for patients $>$60 kg) provide a similar exposure in these two different bodyweight groups (de Greef et al., 2010; Ledger et al., 2011). In the Trust trial, participants aged 18–39 years with a bodyweight of $>$60 kg and BMI of 18–29 kg/m^2 received corifollitropin alfa 150 μg (n = 682).

Patients with a history of ovarian hyper-response to ovarian stimulation (more than 30 follicles $\geq$11 mm), of OHSS or polycystic ovarian syndrome or with more than 20 basal antral follicles on ultrasound ($<$11 mm, both ovaries combined), i.e. all predisposing factors that confer an increased

risk of developing OHSS during ovarian stimulation (Delvigne and Rozenberg, 2002; Lee et al., 2008), were excluded. Patients with a history of low or no ovarian response or more than three unsuccessful ovarian stimulation cycles since the last established pregnancy were also excluded.

Study design

In these trials, corifollitropin alfa treatment (or daily rFSH for Engage and Ensure) started on menstrual cycle day 2 or 3 (stimulation day 1). From stimulation day 8 onwards, treatment was continued as needed with a daily subcutaneous dose of ≤200 IU of rFSH (Engage and Ensure) or ≤225 IU FSH/human menopausal gonadotrophin (Trust) up to and including (optional in Trust) the day of HCG administration. To prevent premature luteinizing hormone surges, the GnRH antagonist ganirelix (0.25 mg, Orgalutran/ganirelix acetate injection; N.V. Organon) was administered once daily subcutaneously, starting on stimulation day 5, up to and including the day of HCG injection. Urinary HCG 10,000 IU (or 5000 IU in the case of a high ovarian response; Engage, Ensure and Trust) or recombinant HCG 250 μg (Trust) was administered to induce final oocyte maturation as soon as three follicles ≥17 mm were observed by ultrasound scan or the next day. Oocyte retrieval was performed 34–36 h later, followed by either IVF or ICSI (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009; Norman et al., 2011).

The dose of rFSH could be reduced from day 6 onwards in case of too high an ovarian response in the Engage and Ensure trials and from day 8 as appropriate in the Trust trial. The investigator could reduce the dose of rFSH (dose tapering) or withhold rFSH administration for a maximum of 3 days (coasting) up to and including the day of HCG administration. In the case of too high an ovarian response, the cycle could be cancelled at any time. However, if there was a risk of OHSS, defined as >30 follicles ≥11 mm on ultrasound scan, HCG was withheld and the treatment cycle was cancelled. The maximum total duration of stimulation was 19 days.

Patients who were cancelled from the treatment cycle because of hyper-response were included in the analysis with 0 oocytes if they did not reach oocyte retrieval.

Assessments

Cases of OHSS were categorized as mild, moderate or severe according to World Health Organization guidelines (WHO, 1973), with a slight modification to the classification of mild OHSS to require the presence of abdominal discomfort, including abdominal pain: (i) mild OHSS (grade I): excessive steroid secretion and ovarian enlargement (5–7 cm), accompanied by abdominal discomfort, including abdominal pain; (ii) moderate OHSS (grade II): distinct ovarian cysts (ovary size 8–10 cm), accompanied by abdominal pain and tension, nausea, vomiting and diarrhoea; and (iii) severe OHSS (grade III): enlarged cystic ovaries (ovary size >10 cm), accompanied by ascites and occasionally hydrothorax; abdominal tension and pain may be severe; pronounced hydrothorax together with an abdominal cavity filled with cysts and fluid elevating the diaphragm may cause severe breathing difficulties; large quantities of fluid inside the cysts and in the peritoneal and pleural cavities cause haemoconcentration and increased blood viscosity; in rare cases, the syndrome may be further complicated by the occurrence of thromboembolic phenomena.

OHSS that occurred less than 10 days after oocyte retrieval was termed early-onset OHSS and OHSS that occurred 10 or more days after oocyte retrieval was termed late-onset OHSS.

Statistical analysis

Demographics and infertility characteristics were summarized per trial. Data were pooled for the two comparative randomized controlled trials (Engage and Ensure) and presented separately for the Trust trial, as there was no comparator rFSH arm in the latter and there were differences from the other two trials in the patient population.

The most important baseline and treatment characteristics were summarized for patients with OHSS versus without OHSS per treatment group for the randomized, controlled trials, pooled.

To examine whether the incidence of OHSS was possibly related to differences in serum FSH activity at day 8 of stimulation, the incidence of OHSS in the Engage and Ensure trials was evaluated both in the corifollitropin alfa and the rFSH arms for patients with relatively low, average or high serum FSH concentrations on day 8 (<P25, P25–75 and >P75).

From the two randomized trials, an odds ratio (OR) for OHSS (corifollitropin alfa versus rFSH) was derived and stratified by trial. Additionally, a logistic regression model was fitted, with covariate treatment group incorporated into the model and other covariates selected in a stepwise fashion. Covariates started within the stepwise selection were study, age, bodyweight, BMI, AFC, FSH at stimulation day 1 and 8, duration of stimulation, HCG received, number of follicles and serum oestradiol concentration (log transformed with base 10) at day of HCG and number of oocytes retrieved. Forward selection ($P \leq 0.05$ for entry) and backward elimination ($P > 0.05$ for removal) led to the same set of covariates.

In order to retain all 1903 subjects in the final model, missing covariate values for FSH (109 subjects) and log serum oestradiol (128 subjects) were replaced by their population medians. The number of oocytes was set to 0 for subjects who did not reach oocyte retrieval, thus there were no missing values for the number of oocytes. Note that for subjects who discontinued due to hyper-response the chance of developing OHSS is substantially reduced by withholding HCG and therefore it is also justified to include these patients with 0 oocytes in the model. The receiver operating characteristic (ROC) curve for the final model was plotted and the associated area under the curve (AUC) was calculated.

Results

Patient demographics in the three trials

Overall, 2585 patients were treated with either corifollitropin alfa ($n = 1705$) or rFSH ($n = 880$) in the three trials. The patient demographics and fertility characteristics per trial are summarized in **Table 1**.

Table 1 Overall patient demographics and fertility characteristics.

Variable	*Engage* (n = *1506*)	*Ensure* (n = *397*)	*Trust* (n = *682*)
Age (years)	31.5 ± 3.3	31.0 ± 3.1	32.9 ± 3.6
Bodyweight (kg)	68.6 ± 7.5	54.2 ± 4.2	67.0 ± 6.5
Body mass index (kg/m^2)	24.8 ± 2.7	20.6 ± 1.5	24.2 ± 2.4
Race			
Asian	42 (2.8)	177 (44.6)	9 (1.3)
Black	61 (4.1)	1 (0.3)	20 (2.9)
Caucasian	1293 (85.9)	219 (55.2)	640 (93.8)
Other	110 (7.3)	0	13 (1.9)
Fertility characteristics			
Primary infertility	796 (52.9)	246 (62.0)	392 (57.5)
Duration of infertility (years)	3.3 ± 2.3	3.2 ± 2.2	3.8 ± 3.0
Cause of infertility[a]			
Male factor	735 (48.8)	196 (49.4)	405 (59.4)
Tubal factor	389 (25.8)	101 (25.4)	165 (24.2)
Endometriosis	224 (14.9)	43 (10.8)	80 (11.7)
Cervical mucous problems	11 (0.7)	4 (1.0)	7 (1.0)
Unexplained infertility	413 (27.4)	108 (27.2)	131 (19.2)
Other	115 (7.6)	9 (2.3)	5 (0.7)
Stimulation day 1			
FSH (IU/l)	6.4 (4.2, 10.0)	6.5 (4.1, 9.8)	6.8 (4.4, 11.3)
Antral follicle count <11 mm	12.4 ± 4.5	11.2 ± 4.4	11.0 ± 4.9

All values are means ± SD, *n* (%) or median (5th and 95th percentiles). All patients treated.
[a]Subjects can have more than one cause of infertility, so percentages do not necessarily add up to 100%.

The study populations in the two randomized controlled trials (Engage and Ensure) were broadly comparable to those in the uncontrolled trial (Trust). However, participants in the Trust trial were slightly older (mean 32.9 years) than in the Engage (31.5 years) and Ensure trials (31.0 years), as the upper limit for inclusion in Trust was 39 years, compared with 36 years in the Engage and Ensure trials. In line with this age difference, the basal AFC was also slightly lower in the Trust trial (11.0 versus 12.4 for Engage and versus 11.2 for Ensure). The median FSH concentration at stimulation day 1 was also slightly higher in the Trust population.

In the randomized controlled trials, cycle cancellations due to too high an ovarian response or risk of OHSS occurred before HCG and oocyte retrieval for 12 subjects (0.6%; eight subjects [0.8%] in the corifollitropin alfa arm and four subjects [0.5%] in the rFSH arm) and for 12 subjects after oocyte retrieval (0.6%, 12 [1.2%] in the corifollitropin alfa arm and none [0.0%] in the rFSH arm). In the Trust trial, 12 subjects [1.8%] discontinued before HCG for this reason and four subjects (0.6%) after oocyte retrieval.

Characteristics of patients with and without OHSS (pooled data from Engage and Ensure)

The demographic and fertility characteristics of patients with OHSS (any grade) treated with either corifollitropin alfa or rFSH are compared with those without OHSS in **Table 2**, which shows the pooled data for the two randomized controlled trials. Patients with OHSS were younger ($P < 0.01$) and had a higher AFC ($P < 0.01$) and lower FSH concentrations ($P < 0.01$) at stimulation day 1 than patients without OHSS. On the day of HCG administration, patients with OHSS had more follicles ≥11 mm ($P < 0.01$) and higher serum oestradiol concentrations ($P < 0.01$) than those with no OHSS. The number of oocytes retrieved per started cycle was higher in patients with OHSS than in those without OHSS ($P < 0.01$). This holds regardless of whether subjects were treated with a single dose of corifollitropin alfa or daily rFSH for the first 7 days of ovarian stimulation.

Incidence of OHSS

In total, over the three trials, 5.6% (95/1705) of the patients treated with corifollitropin alfa experienced signs or symptoms of OHSS (mild, moderate or severe).

In the two randomized controlled trials (Engage and Ensure), the pooled overall incidence of OHSS in the corifollitropin alfa group was 6.9% (71/1023), compared with 6.0% (53/880) in the rFSH group. The (unadjusted) corifollitropin alfa to rFSH OR for OHSS was 1.16. Adjusted for trial, the OR was 1.18 (95% CI 0.81−1.71), indicating that the risk of OHSS for corifollitropin alfa was similar to that for rFSH. Considering the severity of OHSS encountered, the incidence of mild, moderate and severe OHSS in the corifollitropin alfa-treated patients was 3.0%, 2.2% and 1.8%, respectively, with 1.9% requiring hospitalization, and in the rFSH-treated patients 3.5%, 1.3% and 1.3%, respectively, with 0.9% requiring hospitalization (**Figure 1A**). The pooled data from the two randomized controlled trials on OHSS of all grades of severity showed that early onset OHSS developed in 4.6% of patients (47/1023) in the corifollitropin alfa group and 3.6% (32/880) in the rFSH group whilst late-onset OHSS developed in 2.3% of patients (24/1023) in the corifollitropin alfa group and 2.4% (21/880) in the rFSH group (**Figure 1A**).

Table 2 Characteristics of patients with mild, moderate or severe ovarian hyperstimulation syndrome (OHSS) and patients with no OHSS (pooled data from two randomized controlled trials, Engage and Ensure).

	OHSS		*Without OHSS*		*P-value OHSS versus without OHSS*
	Corifollitropin alfa (n = 71)	*rFSH (n = 53)*	*Corifollitropin alfa (n = 952)*	*rFSH (n = 827)*	
Age (years)	30.6 ± 3.1	30.3 ± 3.3	31.4 ± 3.3	31.5 ± 3.2	<0.01[a]
Bodyweight (kg)	64.0 ± 9.7	66.4 ± 6.8	65.0 ± 9.4	66.3 ± 8.7	NS
Body mass index (kg/m^2)	23.5 ± 3.1	24.2 ± 2.5	23.7 ± 3.1	24.2 ± 3.0	NS
AFC <11 mm at stimulation day 1	13.9 ± 4.5	13.9 ± 3.1	11.8 ± 4.5	12.1 ± 4.5	<0.01[a]
FSH at stimulation day 1 (IU/l)	5.8 (4.0, 8.5)	5.9 (4.4, 7.7)	6.5 (4.3, 10.3)	6.5 (4.1, 10.1)	<0.01[a]
FSH at stimulation day 8 (IU/l)	10.9 (6.6, 16.1)	10.7 (7.4, 14.3)	11.1 (7.3, 16.1)	11.3 (7.9, 15.9)	0.03[a]
Duration of stimulation (days)	9.4 ± 1.2	9.1 ± 1.3	9.4 ± 1.7	9.2 ± 1.3	NS
HCG administered	71 (100.0)	52 (98.1)	927 (97.4)	818 (98.9)	NS
Dose of HCG[c]					
10,000 IU	47 (66.2)	46 (88.5)	749 (80.8)	711 (86.9)	
5000 IU	23 (32.4)	6 (11.5)	177 (19.1)	106 (13.0)	
Other	1 (1.4)	0 (0.0)	1 (0.1)	1 (0.1)	
Follicles ≥11 mm on day of HCG	21.5 ± 8.5	17.6 ± 6.1	15.2 ± 6.6	13.5 ± 6.0	<0.01[a]
Serum oestradiol concentration on day of HCG (pmol/l)	7193 (2767, 17,212)	6606 (2463, 12,001)	4367 (1534, 11,083)	4404 (1677, 10,349)	<0.01[b]
Oocytes retrieved	21.4 ± 9.8	17.0 ± 6.9	13.0 ± 7.5	11.9 ± 6.5	<0.01[c]

All values are means ± SD, *n* (%) or median (5th and 95th percentiles).
AFC = antral follicle count; HCG = human chorionic gonadotrophin; rFSH = recombinant FSH.
[a]*P*-value adjusted for treatment group and trial.
[b]*P*-value per treatment group (*P*-values for corifollitropin alfa and rFSH groups were both <0.01).
[c]Restricted to subjects with HCG.

In the uncontrolled Trust trial including 682 slightly older patients undergoing their first corifollitropin alfa treatment cycle, the overall incidence of OHSS (mild, moderate and severe) was 3.5% (24/682). The incidence of mild, moderate and severe OHSS was 1.8%, 0.9% and 0.9%, respectively, with 1.2% hospitalizations. The incidence of early-onset OHSS was 2.6% (18/682), and that for late-onset OHSS was 0.9% (6/682; **Figure 1B**).

There was no difference in the incidence of OHSS for patients starting on cycle day 2 or 3 with corifollitropin alfa treatment. Combining the three phase-3 trials, patients treated with corifollitropin alfa starting on day 2 had an incidence of 3.1% (25/812) moderate/severe OHSS and patients starting on day 3 had an incidence of 3.2% (27/836) moderate and severe OHSS. The overall incidence of OHSS was 5.3% versus 6.0%. This analysis is based on the all-subjects-treated-group, restricted to subjects treated with HCG.

Incidence of OHSS by FSH concentration on day 8

The FSH percentile concentrations P25, P50 and P75 on stimulation day 8 in the corifollitropin alfa and rFSH arms were, respectively, 9.7, 11.6, 13.5 IU/l and 9.8, 11.4, 13.2 IU/l in the Engage trial, and 9.0, 10.1, 11.6 IU/l and 9.1, 10.3, 11.6 IU/l in the Ensure trial (**Table 3**).

The incidence of OHSS in the corifollitropin alfa and rFSH treatment groups according to serum FSH concentrations (percentiles <P25, P25–P75 and >P75) on stimulation day 8 in the Engage and Ensure trial are shown in **Table 3**. Patients with higher FSH concentrations on day 8 did not have a higher risk of OHSS.

Logistic regression model for OHSS

Results for the logistic regression model for OHSS are given in **Table 4** and the associated ROC curve is given in **Figure 2**. The AUC for the final model was 0.753. It appeared that higher FSH concentrations on day 1 were associated with a lower probability of OHSS, whereas higher serum oestradiol concentrations on the day of HCG and a higher number of oocytes retrieved were associated with a higher probability of OHSS. It should be noted that the OR for these continuous covariates should be interpreted per unit increase. For the number of oocytes, for example, the OHSS OR increases by a factor of 1.08 for every additional oocyte. The adjusted

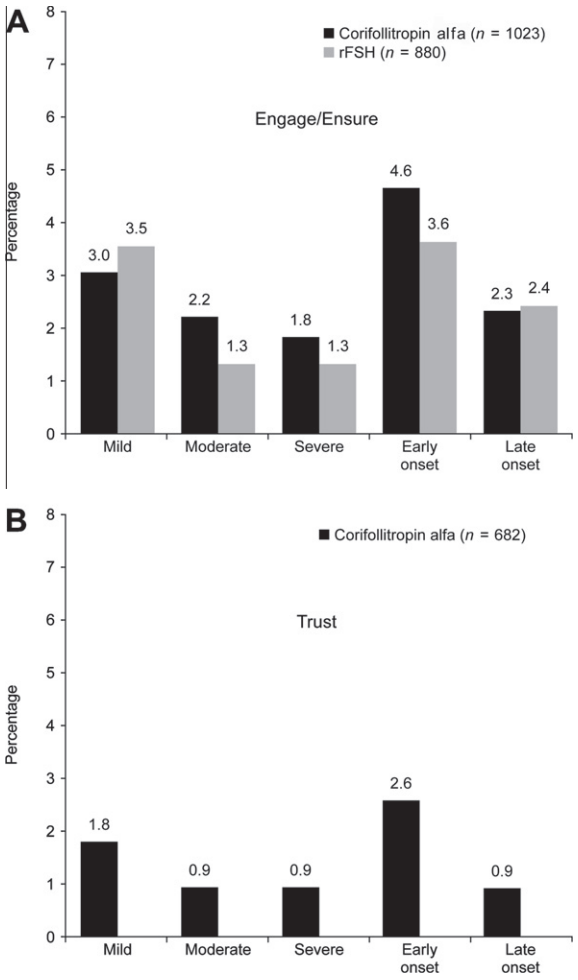

Figure 1 Ovarian hyperstimulation syndrome in patients treated with corifollitropin alfa or recombinant FSH (rFSH). Data from (A) Engage and Ensure (pooled) and (B) Trust.

corifollitropin alfa to rFSH OR was 0.99 (95% CI 0.67—1.45), again indicating that the OHSS risks for corifollitropin alfa and rFSH were similar. Other covariates, including age and AFC (which also had a univariate association with OHSS) did not contribute statistically to the multivariate model.

Another application of the logistic regression model is that the (absolute) risk of OHSS can be calculated for any given patient. For example, for a patient with median values for FSH on day 1 of 6.4 IU/l, serum oestradiol on the day of HCG of 4514 pmol/l and with 12 oocytes retrieved, the OHSS risk would be 4.5% if this patient were to be treated with corifollitropin alfa, versus 4.6% if they were to be treated with rFSH.

Discussion

The overall incidence of OHSS (5.6%), and the incidence per severity and per time of onset in all three phase-3 trials with corifollitropin alfa treatment for the first 7 days of ovarian stimulation to date (Engage, Ensure and Trust), are in line with those anticipated with daily rFSH treatment in this relatively young IVF population. Accordingly, the incidence of mild, moderate and severe OHSS (3.0%, 2.2% and 1.8%, respectively) in the current evaluation of patients (mean age 31 years) treated with corifollitropin alfa for ovarian stimulation in two large randomized controlled trials is lower than or within the range of previously reported incidences of OHSS for patients undergoing ovarian stimulation for IVF/ICSI (mild OHSS 8—33%, moderate 3—6% and severe OHSS 0.1—2%) (Delvigne and Rozenberg, 2002; Alper et al., 2009). The current analysis of incidence of OHSS with the same grading criteria in two large comparative randomized controlled trials is important, because the variety of grading systems for OHSS severity and the inclusion of different patient populations and treatment regimens has made it difficult to compare incidences of OHSS reported in the literature, and the wide incidence range for OHSS severity grades reported most likely reflects this (Brinsden et al., 1995; Golan and Weissman, 2009; Papanikolaou et al., 2006; WHO, 1973).

The current pooled analysis of data from two large randomized controlled trials indicates that the risk of OHSS following corifollitropin alfa treatment for ovarian stimulation tends to be higher than with rFSH treatment, with a difference of 1.4% in the incidence of moderate and/or severe OHSS and of 0.5% for severe OHSS. These differences, which are not statistically significant, are considered to be small and acceptable in view of the significant estimated difference of 2% (95% CI, 3% to 0%) in the incidence of severe OHSS reported in over 3000 patients from 15 randomized controlled trials treated with either the long GnRH agonist

Table 3 Incidence of OHSS according to FSH concentration percentiles on stimulation day 8: individual data from Engage and Ensure.

	Corifollitropin alfa			*rFSH*		
	<P25	*P25—P75*	*>P75*	*<P25*	*P25—P75*	*>P75*
Engage, *N*	174	357	175	170	347	163
FSH (IU/l)	P25 = 9.68	P50 = 11.60	P75 = 13.50	P25 = 9.83	P50 = 11.40	P75 = 13.20
Any OHSS (*n*, %)	16 (9.2)	20 (5.6)	12 (6.9)	13 (7.6)	23 (6.6)	4 (2.5)
Ensure, *N*	64	131	62	30	61	30
FSH (IU/l)	P25 = 9.02	P50 = 10.10	P75 = 11.60	P25 = 9.10	P50 = 10.30	P75 = 11.60
Any OHSS (*n*, %)	5 (7.8)	8 (6.1)	5 (8.1)	1 (3.3)	4 (6.6)	0 (0.0)
Randomized controlled trials combined, *N*	238	488	237	200	408	193
Any OHSS (*n*, %)	21 (8.8)	28 (5.7)	17 (7.2)	14 (7.0)	27 (6.6)	4 (2.1)

OHSS = ovarian hyperstimulation syndrome; rFSH = recombinant FSH.

Table 4 Logistic regression model for ovarian hyperstimulation syndrome (any grade).

Factor	*DF*	*Estimate*	*SE*	*OR*	*95% CI*	P-*value*
Intercept	1	−6.986	1.693			<0.0001
Corifollitropin alfa[a]	1	−0.013	0.197	0.99	0.67−1.45	0.9486
FSH day 1[b]	1	−0.182	0.071	0.83	0.73−0.96	0.0100
^{10}log oestradiol[c]	1	1.152	0.456	3.16	1.29−7.74	0.0115
No. of oocytes[d]	1	0.075	0.013	1.08	1.05−1.11	<0.0001

DF = degrees of freedom; OR = odds ratio; SE = standard error.
[a]Versus rFSH.[b]Per IU/l increase.[c]Per log unit increase.
[d]Per unit increase.

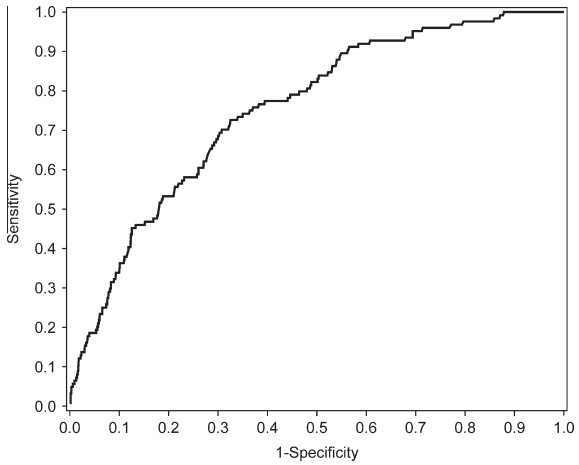

Figure 2 Receiver operating characteristic curve for logistic regression model. The area under the curve is 0.753, showing the sensitivity of the final logistic regression model.

protocol (average incidence 4.3% for severe OHSS) or with the GnRH antagonist protocol (average incidence 2.6%). By analogy to the difference between corifollitropin alfa and rFSH, the higher incidence of OHSS with the GnRH agonist may be related to the higher ovarian response compared with GnRH antagonist protocols (Kolibianakis et al., 2006). The difference with daily FSH is that following corifollitropin alfa administration, exposure largely reduces from stimulation day 3 to day 8 as in a step-down protocol which may be helpful in preventing overstimulation (Ledger et al., 2011).

There are several adaptations that can be made to individualize ovarian stimulation treatment protocols in order to reduce the likelihood of OHSS if the ovarian response during stimulation treatment cycles is too high (Humaidan et al., 2010b). Treatment with daily rFSH can be withheld for a maximum of 3 days (coasting), administration of HCG to trigger final oocyte maturation can be delayed, the dose of HCG can be reduced, all embryos may be frozen to prevent pregnancy, or worst case the cycle is cancelled as HCG is withheld. All these preventive measures were allowed in the current protocol and, whereas the cancellation prior to HCG was similar between the two treatment groups, it should be noted that fresh embryo transfer was more often omitted in the corifollitropin alfa group. Another effective measure which was not allowed in the current trial, but which prevents cycle cancellation, is the utilization of GnRH agonist (instead of HCG) to trigger final oocyte maturation (Devroey et al., 2011; Engmann et al., 2008; Griesinger et al., 2011). Subsequent cryopreservation of embryos would circumvent the luteal-phase insufficiency following triggering with GnRH agonists whereas additional luteal-phase support would allow transfer in the same treatment cycle with good clinical outcome (Humaidan et al., 2010a,2010b). The corifollitropin alfa treatment regimen has the flexibility to incorporate these modifications according to the patient's ovarian response.

In the current randomized controlled trials, the incidence of OHSS (mild, moderate and severe combined) was 6.9% and 6.0% in the corifollitropin alfa and rFSH treatment arms, respectively. The incidence of late-onset OHSS was similar in both treatment groups (2.3% and 2.4%) but there was a trend for a higher incidence of early-onset OHSS in the corifollitropin alfa group (4.6% compared with 3.6% in the rFSH group). Because early OHSS is related to the ovarian response (Lyons et al., 1994; Mathur et al., 2000), this may reflect the higher ovarian response observed with corifollitropin alfa treatment compared with rFSH (13.7 oocytes retrieved versus 12.5, respectively, in the Engage study and 13.3 versus 10.6, respectively, in the Ensure study) (Corifollitropin Alfa Ensure Study Group, 2010; Devroey et al., 2009). In the Trust trial too, early-onset OHSS was more prevalent (2.6%) than late-onset OHSS (0.9%), although both incidences were lower than in the Engage and Ensure trials.

The more frequent observation of early rather than late OHSS is in accordance with a prospective cohort study of 1801 patients undergoing ovarian stimulation with rFSH in a GnRH antagonist protocol for IVF/ICSI, which reported that moderate and severe early OHSS were more frequently observed (1.2% of patients) than late OHSS (0.9%; Papanikolaou et al., 2006).

In the Trust trial, the observed incidence of OHSS in patients receiving corifollitropin alfa was lower (moderate OHSS 0.9%, severe OHSS 0.9%) than in the corifollitropin alfa arms of the randomized controlled trials. It should be noted that the Trust trial was conducted more closely in line with current medical practice; only 33.6% of the patients received FSH/human menopausal gonadotrophin on the day of HCG for triggering final oocyte maturation (compared with approximately 65% in the randomized trials) and 23.3% of patients received 250 μg recombinant HCG (~6500 IU) instead of 10,000 IU urinary HCG. Furthermore, in patients

who required additional FSH injections from day 8 onwards, a lower dose than 200 IU was more frequently given than in Engage to complete ovarian stimulation (Norman et al., 2011). The patients enrolled in the Trust trial were also older (mean age 33 years) than in the Engage and Ensure trials (mean age 31 years) and had a lower mean basal AFC, both factors related to ovarian reserve, which impacts on the likelihood of OHSS development.

The lack of association between OHSS incidence and higher FSH concentrations on stimulation day 8 in the Engage and Ensure trials supports the premise that the risk of OHSS (for both corifollitropin alfa and rFSH treatment regimens) is more likely to be related to the ovarian reserve and subsequent ovarian response than to a higher FSH activity during the first week of corifollitropin alfa treatment.

In the current pooled analysis of data from the Engage and Ensure trials, patients with OHSS symptoms were younger than those who did not develop OHSS. This is in line with previous studies, which show a trend towards increased risk of OHSS in younger women (Delvigne and Rozenberg, 2002). The higher AFC in the OHSS group is also in line with previous publications, which indicate that increased AFC may be a predictor for hyper-response (Kwee et al., 2007; Verhagen et al., 2008).

Ovarian response characteristics of patients with OHSS compared with those without OHSS include an increased number of follicles $\geq$11 mm and higher serum oestradiol concentrations on the day of HCG, and higher number of oocytes retrieved (Humaidan et al., 2010b; Mathur et al., 2000; Verwoerd et al., 2008). These associations were observed for both the corifollitropin alfa and rFSH treatment groups. The characteristics of women with OHSS in the corifollitropin alfa treatment arms were indistinguishable from women with OHSS in the rFSH treatment arms.

Stepwise logistic regression supports these discussion points, since treatment group and FSH concentration on day 8 did not contribute to or appear in the final model, whilst FSH concentration on day 1 (which partly reflects the ovarian age) and serum oestradiol on the day of HCG did. The number of oocytes appeared to be more important than the number of follicles on day of HCG, but these are of course highly correlated. Whilst colinearity may be expected between serum oestradiol concentrations and the number of oocytes retrieved, both remained independent in the final model. On the other hand, the fact that AFC itself did not appear in the final model does not imply that AFC has no predictive value at all, only that it did not add much to other, possibly correlated, factors already included in the model.

OHSS is a potentially serious complication of ovarian stimulation for IVF/ICSI, but preventive measures can be taken to minimize this risk both following corifollitropin alfa or daily FSH treatment. These include careful monitoring of the ovarian reserve prior to stimulation and the ovarian response during stimulation, especially for patients who have not previously undergone IVF treatment and whose response to ovarian stimulation is unknown. Thus, ultrasonographic assessments of follicular development and determination of serum oestradiol concentrations prior to and during ovarian stimulation treatment should be performed. In the case of too high an ovarian response during stimulation treatment cycles, adjustments, as described earlier (Humaidan et al., 2010b), can be made within both corifollitropin alfa and rFSH treatment regimens to reduce the related risk of OHSS. Last but not least, it is not recommended to treat potential high responder patients with corifollitropin alfa or to apply corifollitropin alfa in a long GnRH agonist protocol, as a small uncontrolled study (Fatemi et al., 2010) suggests a higher ovarian response than in combination with a GnRH antagonist.

In conclusion, patients who developed OHSS had a higher ovarian reserve and higher ovarian response without any notable differences between the two treatment groups. Despite a higher ovarian response with a single injection of corifollitropin alfa compared with daily rFSH for the first 7 days of ovarian stimulation, the incidence of OHSS was not statistically different between the two treatment regimens. This study combines the first two controlled phase-3 trials but another large randomized controlled trial of corifollitropin alfa is underway (NCT01144416); thus, the conclusions of this current combined analysis need to be confirmed in the near future. Preferably this new meta-analysis should be performed by using individual patient data rather than by using summary statistics of published manuscripts.

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Appendix

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ORIGINAL ARTICLE *Reproductive endocrinology*

Repeated ovarian stimulation with corifollitropin alfa in patients in a GnRH antagonist protocol: no concern for immunogenicity

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BACKGROUND: One injection of corifollitropin alfa replaces the first seven daily FSH injections in controlled ovarian stimulation (COS) cycles. Repeated treatment with therapeutic proteins may cause immune responses or hypersensitivity reactions. We assessed the immunogenicity and safety of corifollitropin alfa treatment in up to three COS cycles.

METHODS: In this multicentre, phase III uncontrolled trial, patients (>60 kg) started treatment with one injection of 150 μg corifollitropin alfa on cycle Day 2 or 3 of menses and 0.25 mg ganielix on stimulation Day 5 or 6. Primary outcome measures were antibody formation against corifollitropin alfa (using highly sensitive radioimmunoprecipitation assay), hypersensitivity reactions, local tolerance and adverse events (AEs).

RESULTS: First, second and third COS cycles were started by 682, 375 and 198 patients, respectively. No clinically relevant immunogenicity or drug-related hypersensitivity was observed. For 192 patients undergoing their third cycle a post-treatment blood sample was negative in the anti-corifollitropin antibody assay, resulting in an upper limit of the one-sided 95% confidence interval (CI) of 1.5%. Most frequent AEs were procedural pain (17.7%, 95% CI: 14.9–20.8%), headache (9.1%, 95% CI: 7.0–11.5%) and pelvic pain (7.6%, 95% CI: 5.7–9.9%).

Cumulative ongoing pregnancy rate after three cycles, including frozen-thawed embryo transfer cycles and spontaneous pregnancies, was 61% (95% CI: 56–65%) after censoring for patients who discontinued.

CONCLUSIONS: Treatment with corifollitropin alfa can safely and effectively initiate and sustain ovarian stimulation during the first 7 days of COS in normal responder patients undergoing up to three treatment cycles, without concerns of immunogenicity.

The trial was registered under ClinicalTrials.gov identifier NCT00696878.

Key words: corifollitropin alfa / immunogenicity / drug safety / controlled ovarian stimulation / pregnancy rate

Introduction

Corifollitropin alfa is a recombinant fusion protein consisting of the α-subunit of human FSH and a hybrid β-subunit composed of the sequence of the β-subunit of human FSH and the carboxy-terminal peptide of the β-subunit of hCG. Compared with recombinant (r) FSH, corifollitropin alfa has a prolonged half-life and slower absorption to peak serum levels (Fares *et al.*, 1992; LaPolt *et al.*, 1992; Duijkers *et al.*, 2002; Fauser *et al.*, 2009). It has been demonstrated that corifollitropin alfa can initiate and sustain follicular growth for 1 week (Duijkers *et al.*, 2002), so that a single injection can replace the first seven daily injections of gonadotrophin in each ovarian stimulation treatment cycle prior to assisted reproduction. In view of the inverse relationship between exposure and body weight, based on the results of the phase II dose-finding study in combination with modelling and simulation, 100 and 150 μg corifollitropin alfa and a 60 kg body weight cut-off were selected to result in similar exposure and therefore similar ovarian response for all body weight groups (De Greef *et al.*, 2010).

[†] The list of Trust investigators available in Appendix.

Delivering pharmacological doses of a therapeutic (fusion) protein may raise concerns of inducing an immune response or hypersensitivity reaction (Schellekens, 2002). To monitor the potential immunogenicity of corifollitropin alfa, a testing strategy was designed in line with Mire-Sluis *et al.* (2004) to evaluate all patients exposed to corifollitropin alfa in up to three treatment cycles. The theoretical probability of corifollitropin alfa being immunogenic (Schellekens, 2002) in humans is estimated to be low based on the molecular structure, purity and formulation (Fauser *et al.*, 2009). Also, it is injected only once per treatment cycle with the majority of patients requiring no more than three to four treatment cycles. To date, up to four injections of 15 μg corifollitropin alfa in hypogonadotrophic, hypogonadal men (Bouloux *et al.*, 2001) or a single injection of 100 or 150 μg corifollitropin alfa in more than 1000 patients has not induced any hypersensitivity reaction or an immune response (Devroey *et al.*, 2009; The corifollitropin alfa Ensure study group, 2010).

The primary objective of the Trust trial was to assess the immunogenicity of repeated exposure to 150 μg corifollitropin alfa in a standard GnRH antagonist protocol in normal responder patients weighing >60 kg with a normal BMI (18–29 kg/m^2) and a regular menstrual cycle undergoing up to three cycles of controlled ovarian stimulation (COS) with corifollitropin alfa for IVF and/or ICSI. In addition, the overall safety and efficacy of the new corifollitropin alfa regimen used during sequential cycles was evaluated.

Materials and Methods

The Trust trial was a multicentre, open-label, uncontrolled clinical trial carried out in 30 centres in Australia, Europe (Denmark, France, Germany, Hungary, Italy, The Netherlands, Norway and Sweden) and South America (Argentina, Brazil and Chile) between September 2006 and May 2009.

The study was conducted in accordance with principles of good clinical practice and was approved by the appropriate institutional review boards and regulatory agencies. Written informed consent was provided by all subjects.

Study population

Patients aged 18–39 years with a body weight of >60 kg, a BMI of 18–29 kg/m^2, menstrual cycle length within 24–35 days range, access to ejaculatory sperm and an indication for COS for infertility using IVF or ICSI were eligible to enrol in the study.

Patients were excluded from the study if one or more of the following conditions were present: a history of, or any current (treated), endocrine abnormality; clinically relevant abnormal laboratory values or chronic disease; or relevant ovarian or tubal pathology that could interfere with ovarian stimulation. Patients were also excluded if they had a prior history of ovarian hyper-response or ovarian hyperstimulation syndrome (OHSS) (>30 follicles ≥11 mm), polycystic ovary syndrome or >20 basal antral follicles on ultrasound (<11 mm, both ovaries combined). Other exclusion criteria included a previously low ovarian response to FSH or hMG treatment (i.e. cycle cancelled due to inadequate ovarian response or three or less oocytes obtained), FSH or LH levels >12 IU/L in the early follicular phase, more than three unsuccessful IVF cycles since the last ongoing pregnancy or abnormal karyotype in the subject or her partner.

Study design

The trial was designed to assess the safety, including the local and general tolerance, of corifollitropin alfa in healthy female partners of infertile couples undergoing COS for IVF or ICSI. For each subject, the trial period covered one to three stimulated treatment cycles and no more than six frozen-thawed embryo transfer (FTET) cycles from the first two treatment cycles. The study design per cycle is summarized in Fig. 1.

All subjects started their COS cycle on menstrual cycle Day 2 or 3 (stimulation Day 1). Subjects started stimulation with a single s.c. injection of 150 μg (0.5 ml) corifollitropin alfa (Elonva®, N.V. Organon, The Netherlands). Injections were carried out by the patient herself (44.6%) or by a medically qualified person (55.4%). From stimulation Day 8 onwards, treatment was continued with a daily s.c. dose of FSH (follitropin alfa, follitropin beta or menotropins) until the day of hCG administration (FSH administration on the day of hCG administration was optional). The maximum FSH dose for continuing treatment was 225 IU but this dose could be reduced as appropriate. For normal responders, the recommended daily dose of FSH was 150 IU. The investigator was allowed

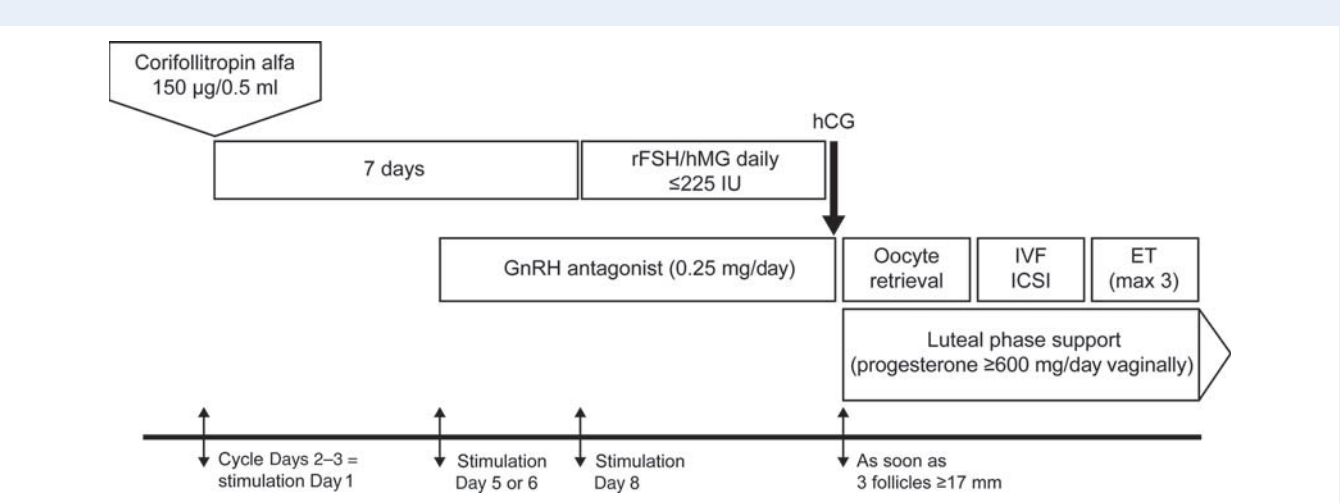

Figure 1 Treatment scheme during the first stimulation cycle. In case patients did not become pregnant, the same treatment was applied in the second and third stimulation cycle. Between two treatment cycles patients could have replacement of cryopreserved oocytes or embryos obtained in a previous treatment cycle. ET, embryo transfer; rFSH, recombinant FSH.

to withhold FSH administration for a maximum of 3 days (coasting) up to and including the day of hCG administration. If the ovarian response was too high in the opinion of the investigator, the investigator was allowed to cancel the cycle at any time. However, if there was a risk of OHSS, defined as >30 follicles ≥11 mm on transvaginal ultrasound, hCG was withheld and the treatment cycle was cancelled per protocol. The maximum total duration of stimulation was 19 days.

To prevent premature LH surges, a GnRH antagonist (0.25 mg; ganirelix or cetrorelix acetate) was administered once daily starting on stimulation Day 5 or 6 up to and including the day of hCG. Either urinary hCG (10 000 IU or 5000 IU in case of a high ovarian response) or rhCG (250 μg) was administered to induce final oocyte maturation as soon as three follicles ≥17 mm were observed by ultrasound. Investigators were allowed to delay hCG administration for 1 day when preferred for practical reasons.

Approximately 34–36 h after hCG administration, oocytes were retrieved and standard IVF or ICSI was performed. At embryo transfer, 3 or 5 days after oocyte retrieval, a maximum of three embryos were transferred. To support implantation and early pregnancy, progesterone (≥600 mg/day vaginally) was started on the day of oocyte retrieval and continued for 5–6 weeks or up to menses or a negative pregnancy test performed at least 14 days after embryo transfer. Cryopreservation of human embryos was, as per local embryo protection law, not allowed in Chile, Germany and Italy: in these countries super numerous two pronuclei oocytes instead of embryos were frozen.

Assessments

Before the start of COS, subjects underwent an hCG test to exclude pregnancy, a clinical examination and ultrasound scan to assess the number of antral follicles, and each subject gave a blood sample for laboratory assessments. On stimulation Day 1, 30 min after injection of corifollitropin alfa, subjects underwent a clinical examination during which medical staff assessed injection-site pain, itching, swelling and redness.

Subjects returned to the clinic on stimulation Day 5 or 6 (before the first administration of the GnRH antagonist) and Day 8 for assessment of the size and number of follicles and hormone concentrations, and then at least every other day up to and including the day of rhCG administration for assessment of the size and number of follicles.

Immunogenicity

Immunogenicity was determined by monitoring the development of potential anti-corifollitropin alfa antibodies. A pretreatment and post-treatment sample was obtained from each subject after each treatment cycle, 2 weeks after embryo transfer or 2–3 weeks after cycle discontinuation. To monitor the potential immunogenicity of corifollitropin alfa, a testing strategy was designed to monitor all patients after each exposure to the drug up to three treatment cycles. For that purpose, a highly sensitive anti-corifollitropin alfa antibody assay was designed and validated according to the white paper of Mire-Sluis *et al.* (2004). The screening assay was a validated, sensitive radioimmunoprecipitation assay able to detect any immune response, regardless of titre, affinity or class of immunoglobulins (sensitivity 1.37 ng antibody/ml serum). A patient-population-specific floating cut-off point was established in serum samples from over 300 IVF patients collected at various time points of the menstrual cycle. If a post-treatment sample was above the predefined cut-off point, pretreatment and post-treatment samples were compared to determine whether the response could be drug induced. Post-treatment samples, which had a statistically higher assay response than pretreatment samples as determined by a paired *t*-test and in which binding affinity was depletable with corifollitropin alfa, were reported and further evaluated to assess the titre and isotypes, specificity (cross-reactivity with rFSH, rLH or hCG) and their neutralizing potential in an *in vitro* bioassay.

Adverse events

Adverse events (AEs) and serious AEs (SAEs) were assessed whenever they occurred. AEs were defined as any unfavourable sign, symptom or disease that occurred during the study period. Moderate or severe local tolerance reactions up to 24 h after any injection of corifollitropin alfa, OHSS, ectopic pregnancy and miscarriage were always considered at least an AE. OHSS was graded as mild, moderate or severe, according to the OHSS guidelines (WHO Scientific Group, 1973). If the same patient was reported with more than one grade of OHSS, only the highest severity of OHSS was included.

SAEs were defined as AEs that were life-threatening, required (prolonged) hospitalization or resulted in persistent or significant disability or incapacity.

End-points

The primary outcome measures were antibody formation against corifollitropin alfa, hypersensitivity reactions, local tolerance and occurrence of AEs or SAEs, including the incidence of OHSS.

The secondary outcome measures were efficacy, determined for each COS cycle, including the number of cumulus–oocyte complexes (COC) retrieved, the number and quality of embryos obtained and transferred, the ongoing pregnancy rates and the cumulative pregnancy rate.

Statistical analysis

A sample size of 600 subjects was planned, with the anticipation that 50% of patients starting each cycle would start the subsequent cycle, thus giving 300 subjects starting Cycle 2 and 150 starting Cycle 3. If no immunogenicity to corifollitropin alfa was observed with 150 subjects undergoing three treatment cycles, this allowed for an upper limit of the one-sided 95% confidence interval (CI) of 2%, the target set for this study.

The cumulative ongoing pregnancy rate was calculated using the Kaplan–Meier approach (Kaplan and Meier, 1958) and included all ongoing pregnancies from the treatment regimen, including fresh cycles, frozen-thawed cycles and spontaneous pregnancies (Vail and Gardener, 2003). Patients who discontinued treatment without becoming pregnant after Cycles 1 or 2 or after FTET cycles were censored assuming that patients who did not return for a subsequent IVF cycle would have had the same chance of an ongoing pregnancy as patients who continued treatment.

Results

Patient characteristics and disposition

A total of 682 patients were included in the trial, 117 in Australia, 304 in Europe and 261 in South America. The mean (SD) age, body weight and BMI were 32.9 (3.6) years, 67.0 (6.5) kg and 24.2 (2.4) kg/m^2, respectively. The most frequently reported cause of infertility was male factor (59.4%) followed by tubal factor (24.2%) and unexplained infertility (19.2%). In total 392 subjects (57.5%) suffered from primary infertility and 290 subjects (42.5%) presented with secondary infertility. The mean (SD) duration of infertility was 3.8 (3.0) years.

Of the 682 patients who started their first COS treatment cycle with corifollitropin alfa, 375 patients continued with a second cycle and 198 patients started their third treatment cycle. Fig. 2 shows the numbers of patients in each cycle continuing treatment at each stage of the protocol.

The cancellation rate per cycle (i.e. patients who started treatment but did not have embryo transfer in that cycle) was 9.7, 9.3 and 10.1% for Cycles 1, 2 and 3, respectively. The overall reasons for cycle

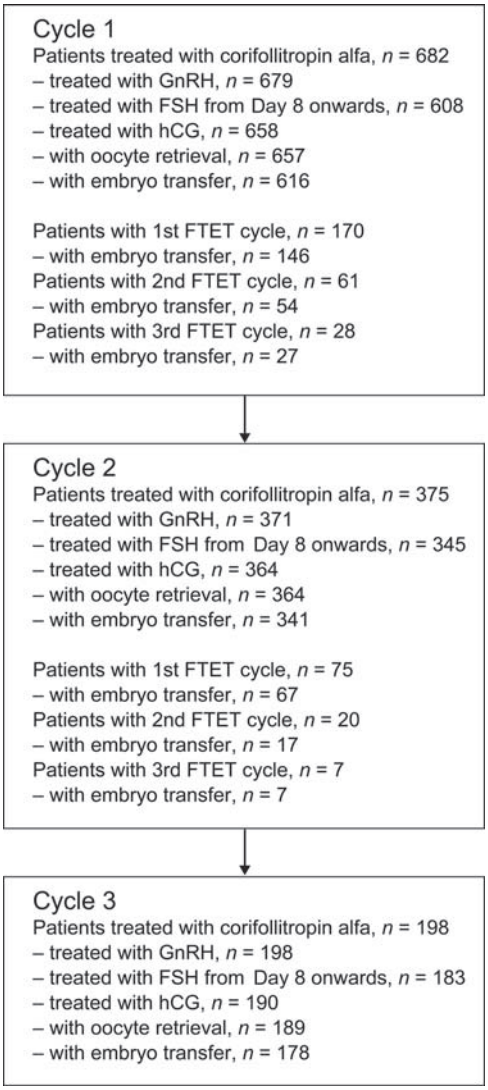

Figure 2 Flow of patients through the treatment cycles. FTET, frozen-thawed embryo transfer.

discontinuation are shown in Table I. Reasons for trial discontinuation (not undergoing embryo transfer in Cycle 3) were AEs or SAEs (1.2%, $n = 8$, pregnancy (including spontaneous pregnancy) after treatment Cycles 1 or 2 or after an FTET cycle (44.6%, $n = 304$), withdrawal of consent (12.8%, $n = 87$), termination of the trial (4.7%, $n = 32$), non-SAE events in Cycle 3 (2.6%, $n = 18$) and other reasons (8.1%, $n = 55$). Among the most frequent other reasons to discontinue the trial were too high an ovarian response in the previous treatment cycle(s) (2.9%, $n = 20$) and too low an ovarian response (1.2%, $n = 8$).

Safety end-points

Immunogenicity

Post-treatment serum samples for assessment in the anti-corifollitropin alfa antibody assay were available for 681 out of 682 (99.9%), 372 out of 375 (99.2%) and 192 out of 198 (97.0%) patients who underwent one, two and three COS cycles, respectively. All post-treatment samples were reported negative, with the exception of one post-treatment sample taken after Cycle 2. This sample appeared to have a statistically significant increased binding ($P < 0.05$) in the antibody assay that was depletable by corifollitropin alfa and rFSH, but not by rLH or hCG. However, the binding of the post-treatment sample was so low (titre 2) that isotyping was technically impossible. The post-treatment sample was without neutralizing activity, thus it did not interfere with the bioactivity of corifollitropin alfa or rFSH. For this subject no AEs were reported in either Cycle 1 or 2 and an additional blood sample taken 6 months after the previous sample tested negative. In view of the very low titre and the absence of neutralizing activity, of moderate or severe local tolerance reactions and of any AEs, the test result was judged as not clinically relevant. The subject, who discontinued from the trial after Cycle 2, became pregnant thereafter in a treatment cycle with daily FSH. The upper limit of the one-sided 95% CI for the incidence of immunogenicity for subjects with three treatment cycles was 1.5%.

Table I Patients discontinued from treatment cycles, i.e. those who received corifollitropin alfa treatment but did not undergo embryo transfer.

	Treatment cycle		
	Cycle 1 (*N* = 682)	Cycle 2 (*N* = 375)	Cycle 3 (*N* = 198)
Patients with cycle discontinuation, *n* (%)	66 (9.7)	35 (9.3)	20 (10.1)
AE or SAE, *n* (%)	6 (0.9)	3 (0.8)	2 (1.0)
Insufficient ovarian response, *n* (%)	8 (1.2)	5 (1.3)	7 (3.5)
Risk of OHSS, *n* (%)	7 (1.0)	1 (0.3)	0 (0)
Too high ovarian response[a], *n* (%)	9 (1.3)	6 (1.6)	1 (0.5)
Insufficient number and quality of oocytes retrieved, *n* (%)	6 (0.9)	5 (1.3)	1 (0.5)
No or abnormal fertilization, *n* (%)	18 (2.6)	9 (2.4)	3 (1.5)
Insufficient number and quality of embryos for transfer, *n* (%)	6 (0.9)	3 (0.8)	5 (2.5)
Other reasons, *n* (%)	6 (0.9)	3 (0.8)	1 (0.5)

AE, adverse event; OHSS, ovarian hyperstimulation syndrome; SAE, serious adverse event.
[a]In the view of the investigator.

Hypersensitivity

No drug-related hypersensitivity reactions, including skin rash, urticaria, hypotension, allergic asthma, chest tightness, bronchospasm, dyspnoea and wheezing, were reported following injection of corifollitropin alfa.

Local tolerance at the injection site

Local tolerance reactions were mild, mainly redness and occurred in 2.5, 4.3 and 2.5% of patients in Cycles 1, 2, and 3, respectively. There were no moderate or severe injection-site reactions (Table II).

Adverse events

In Cycles 1, 2 and 3, 46.8, 35.2 and 31.3% of patients, respectively, had at least one AE (Table II). Overall, including all treatment cycles, the most common reported AEs were procedural pain related to oocyte retrieval (17.7%, 95% CI: 14.9–20.8%), headache (9.1%, 95% CI: 7.0–11.5%) and pelvic pain (7.6%, 95% CI: 5.7–9.9%).The incidences of these AEs per treatment cycle are presented in Table II. In Cycles 1, 2 and 3, 11.1, 3.7 and 2.0% of AEs were considered to be related to treatment.

AEs of severe intensity were uncommon, occurring in 2.5, 1.3 and 0.5% of patients in Cycles 1, 2 and 3, respectively. A total of 63 SAEs were reported in 47 patients (6.9%) overall, and occurred in 3.4, 1.6 and 1.5% of patients, respectively according to COS treatment Cycles 1, 2 or 3. Among the reported SAEs were eight ectopic pregnancies, two ruptured ectopic pregnancies, one heterotopic pregnancy, three missed abortions, two spontaneous abortions and two imminent abortions. In total, 15 SAEs were considered to be related to trial medication, including all 10 occurrences of OHSS. In addition, one subject in Cycle 1 with OHSS experienced a recoverable pulmonary embolism.

Table II Incidence of subjects with at least one (serious) adverse event, with OHSS or with mild local tolerance reaction.

	Cycle 1 (*N* = 682)	Cycle 2 (*N* = 375)	Cycle 3 (*N* = 198)
Subjects with AEs, % (95% CI)	46.8 (43.0–50.6)	35.2 (30.4–40.3)	31.3 (24.9–38.3)
Procedural pain	14.2 (11.7–17.1)	11.2 (8.2–14.8)	10.1 (6.3–15.2)
Headache	5.6 (4.0–7.6)	5.3 (3.3–8.1)	5.6 (2.8–9.7)
Pelvic pain	4.8 (3.4–6.7)	3.7 (2.1–6.2)	2.5 (0.8–5.8)
Subjects with SAEs, % (95% CI)	3.4 (2.1–5.0)	1.6 (0.6–3.4)	1.5 (0.3–4.4)
OHSS, any grade, % (95% CI)	3.5 (2.3–5.2)	1.9 (0.8–3.8)	0
Mild	1.8	0.8	
Moderate	0.9	0.5	
Severe	0.9	0.5	
Local tolerance			
Mild, % (95% CI)	2.5 (1.5–4.0)	4.3 (2.5–6.8)	2.5 (0.8–5.8)

CI, confidence interval.

Incidence of OHSS

OHSS was reported in 24 patients (3.5%) in their first cycle of COS and in seven patients (1.9%) in the second cycle and did not occur during the third treatment cycle (Table II). One subject experienced OHSS in Cycle 1 and Cycle 2. It should be noted that 25 subjects discontinued the trial after the first ($n = 16$) or after the second ($n = 9$) cycle because of too high an ovarian response or signs or symptoms of OHSS. In total 15 cases of OHSS were considered mild, eight cases were considered moderate and eight cases were reported as severe OHSS. In the first cycle, eight patients were hospitalized, one with mild, one with moderate and six with severe OHSS. In the second cycle, two patients with OHSS were hospitalized, one with mild and one with severe OHSS. One subject who experienced severe OHSS in the second cycle received ambulant treatment in the fertility clinic and was not hospitalized.

Table III Amount of recombinant FSH/hMG and hCG administered and numbers of cumulus–oocyte complexes retrieved, embryos obtained and embryos transferred.

	Treatment cycle		
	Cycle 1 (*N* = 682)	Cycle 2 (*N* = 375)	Cycle 3 (*N* = 198)
Total dose of rFSH/hMG administered from Day 8 (IU)[a]	400 (0, 2100)	450 (0, 1950)	450 (0, 2250)
Total duration of stimulation (days)[a]	10 (7, 18)	10 (6, 19)	10 (7, 18)
Dose of hCG administered, *n* (%)			
None	24 (3.5)	11 (2.9)	8 (4.0)
5000 IU (urinary)	60 (8.8)	25 (6.7)	10 (5.1)
10 000 IU (urinary)	439 (64.4)	245 (65.3)	132 (66.7)
250 μg (recombinant)	159 (23.3)	94 (25.1)	48 (24.2)
Number of cumulus–oocyte complexes per started cycle[b]	11.9 (7.2)	11.5 (6.8)	11.3 (7.6)
Number of embryos obtained on Day 3[c]	6.4 (4.5)	6.5 (4.4)	6.6 (4.8)
Number of good quality embryos obtained on Day 3[c]	3.2 (3.1)	2.9 (2.8)	2.8 (2.7)
Number of embryos transferred[c]	1.9 (0.7)	2.1 (0.7)	2.2 (0.7)
Number of good quality embryos transferred[d]	1.4 (0.9)	1.5 (1.0)	1.6 (1.0)

[a]Median (min, max) and restricted to patients with hCG injection.
[b]Mean (SD); per started cycle.
[c]Restricted to subjects with IVF and/or ICSI.
[d]Restricted to subjects with embryo transfer.

Efficacy outcomes

In the first, second and third treatment cycles, corifollitropin alfa was self-administered by 39.9, 48.8 and 52.5% of the patients, respectively. The hCG criteria were reached on stimulation Day 8 or before Day 8 for 122 subjects (17.9%) in Cycle 1, for 60 subjects (16.0%) in Cycle 2 and for 36 subjects (18.2%) in Cycle 3. The median dose of rFSH/hMG was 150 IU per day in all three treatment cycles and the total median dose was 400, 450 and 450 IU in Cycles 1, 2 and 3, respectively. The majority of patients (>85%) finished their first, second or third cycle with rFSH and the minority (≤0.6%) used hMG. The duration of stimulation (10 days) was consistent from Cycles 1 to 3 (Table III).

In total 50.6, 57.1 and 62.6% of patients in Cycles 1, 2 and 3, respectively, started the GnRH antagonist on stimulation Day 5 and 47.4, 41.3 and 37.4% in Cycles 1, 2 and 3, respectively, started on Day 6. The majority of patients received ganirelix (79.6, 82.7 and 96% in Cycles 1, 2 and 3, respectively) and a minority cetrorelix (19.6, 15.5 and 3.5% in Cycles 1, 2 and 3, respectively). The amount of urinary or rhCG administered in each cycle to trigger final oocyte maturation is given in Table III. The number of COCs retrieved and embryos obtained and transferred were similar for the three treatment cycles (Table III).

Table IV For fresh cycles only: pregnancy rates per started cycle and implantation rates per embryo transfer.

	Treatment cycle		
	Cycle 1 (*N* = 682)	Cycle 2 (*N* = 375)	Cycle 3 (*N* = 198)
Biochemical pregnancy (%)[a]	31.4	28.5	27.8
Clinical pregnancy (%)[b]	26.2	23.5	24.2
Vital pregnancy (%)[c]	23.0	21.3	21.2
Ongoing pregnancy (%)[d]	22.7	20.5	20.7
Singletons	81.9	83.1	87.8
Twins	16.8	16.9	12.2
Triplets	1.3	1.3	0
Implantation rate, % (*n*[e])	21.2 (*n* = 616)	16.6 (*n* = 340)	16.3 (*n* = 178)

[a]Biochemical pregnancy: pregnancy proven by a biochemical pregnancy test or with ultrasound showing at least one gestational sac.
[b]Clinical pregnancy: presence of at least one gestational sac as assessed by ultrasound.
[c]Vital pregnancy: presence of at least one fetus with heart activity as assessed by ultrasound.
[d]Ongoing pregnancy: presence of at least one fetus with heart activity at least 10 weeks after embryo transfer or live birth.
[e]Patients with embryo transfer.

Pregnancy rates

Table IV shows the pregnancy rates for patients who started ovarian stimulation with corifollitropin alfa, which was consistent over Cycles 1, 2 and 3. The ongoing pregnancy rate per started cycle was 22.7% in the first cycle, 20.5% in the second cycle and 20.7% in the third cycle. However, the pregnancy rates varied widely among the 30 participating sites: the 10th and 90th percentiles for the ongoing pregnancy rate per site in Cycle 1 were 11.6 and 43.7%, respectively. Most ongoing pregnancies were singletons (81.9–87.8% in each cycle). Of the 46 multiple pregnancies, 28 occurred in Cycle 1, 13 in Cycle 2 and 5 in Cycle 3. In Cycle 1 there were 26 twin and 2 triple pregnancies; in Cycle 2 there were 12 twin pregnancies and 1 triple pregnancy; and in Cycle 3 there were 5 twin pregnancies. The miscarriage rate per clinical pregnancy was 12.8, 12.5 and 14.6% for Cycles 1, 2 and 3, respectively.

In Cycle 1, coasting was applied in 15 patients with hCG injection and 5 out of these 15 patients had an ongoing pregnancy (33.3%). In Cycle 2, coasting was applied for 9 patients with hCG injection and 1 patient became pregnant (11.1%). In Cycle 3, coasting was applied for 1 patient who did not become pregnant.

The cumulative ongoing pregnancy rate after three COS cycles including in-between FTET cycles and spontaneous pregnancies was

Table V Cumulative ongoing pregnancy rate: pregnancies after treatment, FTET cycles and spontaneous pregnancies.

	Ongoing pregnancies	Cumulative incidence	95% Confidence interval[a]
Cycle 1	155	0.23	0.20–0.26
Spontaneous pregnancies after cycle 1	11	0.25	0.22–0.28
FTET cycles between Cycle 1 and Cycle 2	37	0.31	0.27–0.35
Spontaneous pregnancies after the FTET cycles	4	0.32	0.28–0.35
Cycle 2	76[b]	0.45	0.42–0.50
Spontaneous pregnancies after Cycle 2	8	0.47	0.43–0.52
FTET cycles between Cycle 2 and Cycle 3	11	0.50	0.46–0.54
Spontaneous pregnancies after the FTET cycles	3	0.51	0.47–0.55
Cycle 3	40[c]	0.61	0.56–0.65

FTET, frozen-thawed embryo transfer.
[a]Limits of the 95% CI for the cumulative incidence rate.
[b]One woman registered ongoing pregnancy twice, once in a FTET cycle between treatment Cycles 1 and 2 and once in Cycle 2; she was not counted as pregnant in Cycle 2 since per protocol she discontinued the trial without being treated in another cycle.
[c]One woman registered pregnancy in Cycles 2 and 3; she was only counted as pregnant in Cycle 2 since per protocol she discontinued the trial after her pregnancy in Cycle 2.

51%; after censoring for patients who discontinued treatment, the rate was 61% (Table V).

Discussion

In the current trial, exposure to a single injection of corifollitropin alfa in up to three repeated COS cycles using a standard GnRH antagonist protocol in 682 patients weighing >60 kg with normal BMI and a regular menstrual cycle was safe and well-tolerated without concerns of immunogenicity. The antibody assay used was a highly sensitive assay in which a relatively small difference between binding affinity of the pretreatment and post-treatment sample could lead to a statistically significant increased binding to corifollitropin alfa. To assess whether an elevated assay response was indicative of the presence of corifollitropin alfa-specific antibodies or whether it concerned a non-specific interaction, an immunodepletion assay was also performed. The post-treatment sample of one patient in her second treatment cycle was depletable with corifollitropin alfa and rFSH. However, this sample was negative in the neutralizing activity assay, and thus the antibodies did not interfere with the FSH bioactivity of either gonadotrophin preparation.

There is in general limited information on immune responses to therapeutic gonadotrophins in women undergoing treatment for infertility. To date, two reviews have reported the absence of antibody response to exogenous urinary or rFSH (Koren *et al.*, 2002; Wadhwa *et al.*, 2003). The incidence of antibody formation against hCG has been reported in hypogonadotrophic hypogonadal males who received long-term treatment with urinary products resulting in loss of efficacy of the drug (reported incidence of antibody formation: 0–40%), but so far no antibodies have been reported in humans using rhCG (Moudgal *et al.*, 1997). The presence of circulating immunoglobulins cross-reacting with endogenous FSH has been reported (Meyer *et al.*, 1990; Gobert *et al.*, 2001; Haller *et al.*, 2005; Haller *et al.*, 2007); however it is not stated whether these antibodies lowered the endogenous FSH levels by neutralization. Anti-FSH antibodies may be naturally occurring antibodies associated with peripheral FSH concentrations and produced in higher levels in infertile women (Haller *et al.*, 2007). Case reports have also been published on the presence of (transient) anti-hCG antibodies in infertile women with recurrent pregnancy loss (Pala *et al.*, 1988; Amato *et al.*, 2002).

In addition to the lack of antibody immune responses against corifollitropin alfa in this trial, there were also no treatment-related hypersensitivity reactions; injection-site reactions were only mild in nature. Lack of immunogenicity with repeated ($n = 3$) cycles of corifollitropin alfa treatment is consistent with the previous study of Bouloux *et al.* (2001). This is also consistent with the findings of the Engage trial, in which there were no drug-related immune responses to corifollitropin alfa and no moderate or severe reactions at the injection site (Devroey *et al.*, 2009). In the current study, mild local tolerance reactions were observed in fewer than 5% of subjects in each cycle. This is similar to the reported 30 min local tolerance reactions in the Engage trial when reactions were also only mild, 6.1% in the corifollitropin alfa arm and 6.1% in the rFSH arm (Devroey *et al.*, 2009).

In the current trial, the overall incidence of OHSS in the first cycle was 3.5 versus 7.0% in the Engage trial (Devroey *et al.*, 2009) and the incidence of moderate or severe cases of OHSS was 1.8 versus 3.9% in the Engage trial. The lower incidence of OHSS in the trial reported here is likely to be related to the patient population included, who were slightly older (32.9 years in the current study versus 31.5 years in Engage) and who had a lower basal antral follicle count (10.9 in the current study and 12.3 in Engage) (Devroey *et al.*, 2009). In addition, the current trial was conducted more closely in line with current medical practice; only 43.5% of the patients received FSH/hMG on the day of hCG for triggering final oocyte maturation and 23.3% of patients received 250 μg rhCG (~6500 IU) instead of 10 000 IU urinary hCG. Furthermore, in patients who required additional FSH injections from Day 8 onwards, a lower dose than 200 IU was more frequently given to complete ovarian stimulation. The nature and incidence of reported SAEs other than OHSS (ectopic pregnancies, procedural pain, pelvic pain, pelvic discomfort and headache) were similar to those reported in both the corifollitropin alfa and rFSH arms of the Engage trial (Devroey *et al.*, 2009).

Concerning efficacy variables, the mean number of COCs retrieved per attempt was similar (11.9–11.3) across the three treatment cycles, and the mean number of embryos (6.4–6.6) and good-quality embryos (3.2–2.8) obtained was also consistent across the three treatment cycles. The ongoing pregnancy rate per started cycle was comparable between the three cycles which is in line with consistent pregnancy rates across cycles in patients receiving their first six cycles of IVF treatment (Malizia *et al.*, 2009). Since patients included were not allowed more than three IVF cycles prior to the trial, none of the patients had more than six treatment cycles.

The mean ongoing pregnancy rate in cycle 1 was lower than has been reported previously for patients undergoing a similar COS treatment regimen with corifollitropin alfa [22.7% in the current study compared with 38.9% in the Engage trial (Devroey *et al.*, 2009)]. However, there was a considerable range of pregnancy rates among the different participating sites with some sites providing high ongoing pregnancy rates of over 40%. In contrast to the Engage trial, the current trial did not include any IVF units within the USA, the latter often having higher success rates than the rest of the world (Baker *et al.*, 2010) but did include IVF clinics in countries such as Italy, Germany and Chile with restrictions in terms of embryo selection for transfer and cryopreservation of embryos/zygotes. This implied that in these clinics, maximally three zygotes were kept in culture to develop into embryos and that these embryos were transferred, regardless of their quality. In addition to different IVF units contributing to the current trial outcome, the patient population was slightly older than that in the Engage trial, with a lower ovarian reserve, as mentioned before. Nevertheless, half of the patients were pregnant after undergoing one, two or three treatment cycles without any unexpected AEs, with very few cases of OHSS and with an excellent local and general tolerance.

In conclusion, the results of this trial suggest that repeated treatment cycles with a single injection of 150 μg corifollitropin alfa can be safely and effectively applied in potential normal responder patients undergoing COS prior to IVF or ICSI, without concerns for immunogenicity.

Authors' roles

R.N., F.Z. and B.S. are investigators who contributed to the acquisition and interpretation of data in this manuscript to which they provided intellectual content. M.M. was responsible for the statistical analysis and J.E., E.H. and B.M. were responsible for the design of the study,

interpretation of data and drafting of this article. B.M. and R.N. wrote the manuscript.

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Conflict of interest

R.N. has served as chairperson on the Medical Advisory Board of Schering-Plough in Australia and Asia-Pacific and has received speaker honoraria from Schering-Plough. F.Z. declares no conflicts of interest. B.S. has served on the Advisory Board for Schering-Plough. J.E., E.H., M.M. and B.M. are employees of MSD.

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ORIGINAL ARTICLE *Reproductive endocrinology*

Prospective follow-up of 838 fetuses conceived after ovarian stimulation with corifollitropin alfa: comparative and overall neonatal outcome

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STUDY QUESTION: Is treatment with corifollitropin alfa, a new recombinant gonadotrophin with sustained follicle-stimulating activity, safe in terms of perinatal complications and birth defects in infants conceived following corifollitropin alfa treatment for contolled ovarian stimulation (COS)?

SUMMARY ANSWER: In terms of neonatal outcome and risk of malformations, treatment with a single dose of corifollitropin alfa during COS is as safe as treatment with daily recombinant FSH (rFSH).

WHAT IS KNOWN AND WHAT THIS PAPER ADDS: This is the first pooled analysis of individual safety data in terms of neonatal outcome and major and minor congenital malformations collected following intervention trials of corifollitropin alfa.

DESIGN: Pregnancy and follow-up studies were conducted prospectively and data were collected from all Phase II and III trials with corifollitropin alfa intervention, including two comparative randomized controlled trials (RCTs) in which patients received either a single dose of corifollitropin alfa or daily rFSH for the first 7 days of COS. Patients with ongoing pregnancies at 10 weeks after embryo transfer were followed up to labour and the health of the offspring was assessed up to 4–12 weeks after birth.

PARTICIPANTS AND SETTING: Following corifollitropin alfa treatment prior to IVF or ICSI, the health of 677 pregnant women, 838 fetuses and 806 live born infants was evaluated.

MAIN RESULTS AND THE ROLE OF CHANCE: Among 440 fetuses in the corifollitropin alfa arm and 381 fetuses in the rFSH arm of the two RCTs, there were 424 (96.4%) and 370 (98.7%) live births, respectively. Neonatal characteristics, the frequency of premature births and the incidence of infant adverse events were similar in both treatment arms. The overall incidence of any congenital malformations in live born infants was 16.3 and 17.0%, with major malformation rates of 4.0 and 5.4% in the corifollitropin alfa and rFSH groups, respectively [odds ratio (OR) for major malformations, 0.71; 95% confidence interval, 0.36–1.38]. From 838 fetuses assessed in all corifollitropin alfa intervention trials, there were 806 (96.2%) live births with a major malformation rate of 4.5% in live born infants.

BIAS, CONFOUNDING AND OTHER REASONS FOR CAUTION: Both RCTs had a double-blind and active-controlled design and the adjudication of congenital malformations was also performed in a blinded fashion. As the total number of major malformations was limited (37), the confidence interval around the OR was rather wide.

GENERALISABILITY TO OTHER POPULATIONS: The similarity of corifollitropin alfa and rFSH with respect to the incidence of congenital malformations was consistent across the RCTs and pregnancy type (singleton, multiple). This suggests that this similarity could hold in general. Overall incidences, however, may depend on the definitions of malformations and rules to adjudicate these events as major or minor.

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TRIAL REGISTRATION NUMBERS: NCT00703014, NCT00702624, NCT 00702195, NCT 00702195, NCT 00702988, NCT 00702520, NCT 00702338 and NCT 00702234.

Key words: assisted reproduction techniques / controlled ovarian stimulation / congenital malformations / corifollitropin alfa / GnRH antagonist

Introduction

The health of children born following assisted reproduction technology (ART) using any new procedures or compounds is paramount. Any concerns about the safety of these procedures/compounds may be allayed by evaluating the risk of perinatal complications or birth defects. Obviously, ovarian stimulation protocols for IVF/ICSI should not compromise the health of the mother during pregnancy or the infants born to these mothers (Ericson and Källén, 2001; Anthony *et al*., 2002; Bonduelle *et al*., 2002; Hansen *et al*., 2002).

To date, there have been no reports that specific drugs used in ART programmes increase these potential risks. However, it has been documented that IVF/ICSI children have an increased risk (relative risk of 1.3) of major malformations, especially cardiac malformations (Wen *et al*., 2010; Tararbit *et al*., 2011), when compared with the general population (Sutcliffe and Ludwig, 2007). The reason for this increased risk is not fully understood but may be related to the underlying cause of the infertility of the parents (Zhu *et al*., 2006).

Corifollitropin alfa is a novel recombinant gonadotrophin, used to induce multifollicular development prior to IVF or ICSI, with high specific affinity for the FSH receptor (Fauser *et al*., 2009). Its FSH receptor binding specificity and activation are comparable with that of recombinant FSH (rFSH) and it lacks intrinsic activity for the LH receptor and the thyroid-stimulating hormone receptor (Verbost *et al*., 2011). Therefore, it may be assumed that the risks associated with corifollitropin alfa treatment for women trying to achieve a pregnancy and their offspring are comparable with those associated with rFSH treatment, but this assumption is unproved.

Two large-scale, prospective, multinational, double-blind, randomized Phase III trials have confirmed the effectiveness of one injection of corifollitropin alfa for the first 7 days of controlled ovarian stimulation (COS) compared with daily rFSH injections in achieving good ongoing pregnancy rates in a GnRH antagonist protocol (Devroey *et al*., 2009; Corifollitropin Alfa Ensure Study Group, 2010).

Analyses of these two comparative randomized controlled trials (RCTs) may be considered most valid when comparing corifollitropin alfa with daily FSH because the same double-blind protocol design was followed and the same inclusion/exclusion criteria used with the exception of body weight. In the two recommended body weight categories, the two dosages were also shown to provide equal exposure and the same ovarian response (Ledger *et al*., 2011).

In addition, the current investigation is a follow-up of all fetuses and infants from two comparative RCTs with corifollitropin alfa versus rFSH, and all Phase II and III trials with corifollitropin alfa intervention.

Materials and Methods

Follow-up of Phase III RCTs

Two follow-up protocols (NCT00703014 and NCT00702624) collected the safety follow-up data of neonatal outcomes from two Phase III, double-blind, RCTs (see Table I), Engage (NCT00696800) and Ensure (NCT00702845) trials have been described previously (Devroey *et al*., 2009; Corifollitropin Alfa Ensure Study Group, 2010).

Table I Phase II and III trials included in the pregnancy and infant follow-up after corifollitropin alfa intervention.

Phase	Trial with follow-up	Pregnant women in follow-up (n = 677)	Fetuses in follow-up (n = 838)	Live born infants in follow-up (n = 806)
II	NCT 00702585, Balen *et al.* (2004)	2	2	2
	NCT 00702806, Devroey *et al.* (2004)	16	20	19
	NCT 00598208, The Corifollitropin Alfa Dose-finding Study Group (2008)	33	40	36
	NCT 00702351, Fatemi *et al.* (2010)	15	19	19
	NCT 00697255, Sterrenburg *et al.* (2009)	1	2	2
III	NCT 00696800, Engage trial, Devroey *et al.* (2009)	274	352	344
	NCT 00702845, Ensure trial, Corifollitropin alfa Ensure study group (2010)	68	88	80
	NCT 00696878, Trust trial, Norman *et al.* (2011)	268	315	304

Patients received a single injection of corifollitropin alfa (Elonva,® N.V. Organon, The Netherlands) in a dose of 100 or 150 μg during the first 7 days of COS. Patients were recruited using identical inclusion and exclusion criteria with the exception of body weight: patients in the Engage trial weighed ≥60 kg ($N = 1506$) and patients in the Ensure trial weighed ≤60 kg ($N = 396$). All patients were treated from stimulation Day 5 onward with a GnRH antagonist, and ovarian stimulation was initiated in the Engage trial with either 150 μg corifollitropin alfa or daily 200 IU rFSH (treatment ratio: 1:1) and in the Ensure trial with 100 μg corifollitropin alfa or 150 IU rFSH (treatment ratio: 2:1). Apart from these differences in dosages according to body weight, the treatment regimens were identical. All subjects with an ultrasonically confirmed ongoing pregnancy of at least 10 weeks after embryo transfer were eligible for enrolment in the pregnancy and neonatal follow-up study. The studies were approved by the local health authorities and the independent medical ethics committee of each study centre, and all patients had to provide informed consent to participate in this follow-up study. Pregnant patients only completed the trial if the examination of the newborns at 4–12 weeks' post-partum was completed.

Follow-up on other Phase II and III trials

Data on fetal and neonatal outcomes were also collected from eight international trials, five Phase II and three Phase III trials with corifollitropin alfa intervention (see Table I). Patients received corifollitropin alfa in doses ranging from 7.5 to 240 μg during the first 7 days of COS. The following other trials included were:

(i) NCT 00702585 (pregnancy and infant follow-up trial NCT 00702195) was a randomized, double-blind, placebo-controlled, comparative trial to investigate the optimal dose of a single administration of corifollitropin alfa (7.5, 15 and 30 μg) to induce monofollicular ovulation in women with World Health Organization (WHO) Group II anovulatory infertility (Balen *et al.*, 2004).

(ii) NCT 00702806 (pregnancy and infant follow-up trial NCT 00702195) was an open-label, prospective, randomized, comparative clinical trial to investigate the appropriate dose of a single injection of corifollitropin alfa (100, 180 and 240 μg) versus daily 150 IU of rFSH to initiate multiple follicular growth in a COS protocol for IVF or IVF/ICSI (Devroey *et al.*, 2004).

(iii) NCT 00598208 (pregnancy and infant follow-up trial NCT 00702988) was an open-label, randomized trial to investigate the dose–response relationship of a single injection of corifollitropin alfa (60, 120 and 180 μg) versus daily 150 IU of rFSH to initiate multiple follicular growth in a COS protocol for IVF or ICSI (the Corifollitropin Alfa Dose-finding Study Group, 2008).

(iv) NCT 00702351 (pregnancy and infant follow-up trial NCT 00702520) was an uncontrolled pilot trial to evaluate whether a single dose of 100 or 150 μg corifollitropin alfa (for patients weighing ≤60 and >60 kg, respectively) is able to induce multiple follicular growth during the first week of COS for IVF or ICSI using a long GnRH agonist protocol (Fatemi *et al.*, 2010).

(v) NCT 00697255 (pregnancy and infant follow-up trial NCT 00702338) was a pilot study to evaluate if a single or repeated dose (maximum three) of 15 μg of corifollitropin alfa followed by a low daily dose of either hCG or rFSH can induce monofollicular growth in women with WHO Group II anovulatory infertility (Sterrenburg *et al.*, 2009).

(vi) NCT 00696878 (pregnancy and infant follow-up trial NCT 00702234) was an open-label, uncontrolled trial, which evaluated the safety and tolerability of repeated cycles (up to three per patient) with a single injection of 150 μg corifollitropin alfa for the first 7 days of COS ($N = 682$) in a GnRH antagonist protocol (Norman *et al.*, 2011).

Data collection

Information was collected prospectively on pregnancy outcome, mode of delivery and neonatal characteristics (gestational age, gender, weight, length, head circumference and Apgar score). All adverse events (AEs) and serious AEs (SAEs) in the infants were recorded after assessment by the infant's physician at birth and at follow-up 4–12 weeks post-partum. All SAEs were coded using the Medical Dictionary for Regulatory Activities (MedDRA, version 12.0) to enable analysis by organ system.

Any congenital abnormality was recorded as a SAE. All AEs and SAEs were adjudicated by an independent medical expert as being either major or minor congenital malformations according to the broad classification. Major malformations were defined as any congenital malformation that causes functional impairment or requires surgical correction (broad definition; Bonduelle *et al.*, 2002). In this follow-up project, the definition of major malformation also included inguinal hernia for children born after 36 weeks of gestation, patent ductus arteriosus (if the ductus was still patent after 3 months for children born at term or after 6 months for children born before 36 weeks of gestation), atrium septum defect type I (type ostium primum), hypospadias if the meatus was not glandular, pyloric stenosis, inherited diseases and chromosomal anomalies. Minor malformations were defined as any congenital malformation not classified as major.

Statistical analysis

Characteristics of live born infants were summarized by treatment group using means and SDs for continuous variables and frequencies and percentages for categorical variables.

All statistical analyses were performed for the two RCTs unless stated otherwise. Continuous characteristics were compared between treatment groups using an analysis of variance correcting for protocol and pregnancy type (singleton versus multiple). Categorical characteristics were compared using the Cochran–Mantel–Haenszel test stratified for protocol and pregnancy type. *P*-values were reported only if <0.05.

AEs classified as major congenital malformation were coded using MedDRA. More specifically, these AEs were 'mapped' to preferred terms (PTs), which, in turn, were mapped to high-level group terms (HLGTs) to enable further analysis by organ class. The incidence of AEs classified as major congenital malformation was summarized by HLGT, PT and treatment group.

Corifollitropin alfa to rFSH odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for major (respectively, any) congenital malformation were obtained using the Cochran–Mantel–Haenszel method stratified for protocol and pregnancy type. With 440 and 381 fetuses in the corifollitropin alfa and rFSH group, respectively, there was close to 80% power to detect a doubling of the incidence of major malformations (i.e. from 5 to 10%), using a 0.05 two-sided significance level.

Results

Comparative follow-up data from RCTs

Evolution of pregnancies

From the RCTs, 342 women with ongoing pregnancies at 10 weeks who had received corifollitropin alfa during the first 7 days of COS and 312 women in the comparator arm who had received rFSH were enrolled in the follow-up study. The evolution of ongoing pregnancies and number of live born infants in the corifollitropin alfa and rFSH arms of the RCTs are presented in Table II. In total, 302 (88.3%) mothers with 383 (87.0%) infants in the corifollitropin alfa

Table II Ongoing pregnancies, fetuses and infants born following the Phase III RCTs (pooled) and after intervention in all corifollitropin alfa intervention trials (pooled).

	Follow-up Phase III RCTs: Engage and Ensure		Follow-up of all Phase II and III trials
	Corifollitropin alfa [*n* (%)]	rFSH [*n* (%)]	Corifollitropin alfa [*n* (%)]
Pregnancies[a]			
Ongoing pregnancies ≥10 weeks	342	312	677
Singleton pregnancies ≥10 weeks	247 (72.2)	243 (77.9)	522 (77.1)
Multiple pregnancies ≥10 weeks	95 (27.8)	69 (22.1)	155 (22.9)
Ongoing pregnancies ≥20 weeks	336 (98.2)	306 (98.1)	662 (7.8)
Singleton pregnancies at 20 weeks	243 (71.1)	239 (76.6)	512 (75.6)
Multiple pregnancies at 20 weeks	93 (27.2)	67 (21.5)	150 (22.2)
Pregnancies resulting in ≥1 live born	332 (97.1)	304 (97.4)	655 (96.8)
Fetuses and live born infants[a]			
Fetuses at 10 weeks after embryo transfer	440	381	838
Fetuses with known outcome	438 (99.5)	376 (98.7)	833 (99.4)
Fetuses lost between 10 and 20 weeks	6 (1.4)	4 (1.0)	15 (1.8)
Fetuses lost ≥20 weeks	8 (1.9)	2 (0.5)	12 (1.4)
Live born infants	424 (96.4)	370 (97.1)	806 (96.2)
From singleton pregnancy	241 (54.8)	237 (62.2)	507 (60.5)
From multiple pregnancy	183 (41.6)	133 (34.9)	299 (35.7)
Infants with follow-up completed	383 (87.0)	339 (89.0)	735 (87.7)

rFSH, recombinant FSH. All percentages were based on the number of ongoing pregnancies at 10 weeks.
[a]Enrolled in the follow-up trial.

group and 280 (89.7%) mothers with 339 (89.0%) infants in the rFSH group completed the follow-up including the examination of their newborns at 4–12 weeks after birth.

At week 10 of gestation, there were 247 (72.2%) and 243 (77.9%) singletons in the corifollitropin alfa and rFSH group, respectively, whereas the incidence of multiple pregnancies was 95 (27.8%) and 69 (22.1%), respectively, and this difference was not statistically significant. There were three triplet pregnancies in the corifollitropin group which all resulted from the transfer of excellent (Grade 1) embryos; in one case only one embryo was replaced on Day 5 which resulted in one gestational sac, in one case two excellent embryos were replaced on Day 5 resulting in three gestational sacs and in the last case two embryos were replaced at Day 3 and resulted in two gestational sacs.

In total, 6 (1.4%) and 4 (1.0%) ongoing pregnancies terminated at 10–20 weeks in the corifollitropin alfa and rFSH groups, respectively. The incidence of intrauterine death/stillbirth (≥20 weeks of gestation) was 1.9% ($n = 8$) in corifollitropin alfa-treated patients and 0.5% ($n = 2$) in rFSH-treated patients. The incidence was 0.8% in singleton pregnancies and 3.2% in multiple pregnancies after treatment with corifollitropin alfa and, respectively, 0.8 and 0.0% after treatment with rFSH. This resulted in 332/342 (97.1%) pregnancies with a live born infant in the corifollitropin alfa group and 304/312 (97.4%) in the rFSH group. From the 440 fetuses at 10 weeks after embryo transfer in the corifollitropin alfa group, there were 424 (96.4%) live born infants and from 381 fetuses in the rFSH arm there were 370 (97.1%) live born infants.

Neonatal outcome of live born infants

In both treatment groups, most infants were delivered by elective or emergency Caesarean section (54 and 50% in the corifollitropin alfa and rFSH groups, respectively). The percentage of infants delivered vaginally and requiring obstetric assistance via induction, forceps delivery and vacuum extraction was similar in both treatment groups. Gestational age, gender, birthweight, length, head circumference and Apgar scores of live born infants at birth are summarized in Table III. Data are also given separately for singleton and multiple births (see also Supplementary data, Table S1 and Figure S1 for data by trial and treatment group differences and OR). There were no notable differences in these characteristics between the corifollitropin alfa and rFSH groups and none of the differences were statistically significant.

The overall incidence of AEs in live born infants was similar between treatments, 47.4% in the corifollitropin alfa group and 50.8% in the rFSH group ($P = 0.35$). The frequency of infant premature birth (gestational age ≤37 weeks) was similar in both maternal treatment groups (27.8 and 25.7% of live born infants in the corifollitropin alfa and rFSH maternal groups, respectively) and the proportions of infants with low birthweights and very low birthweights or with low Apgar scores were comparable, as shown in Table IV, where none of the differences were statistically significant (see also Supplementary data, Table S2 and Figure S2 for data by trial and treatment group OR). Data are also given separately for singleton and multiple births. Neonatal deaths were reported for 8/424 (1.9%) and 6/370 (1.6%) of live born infants in the corifollitropin alfa and rFSH groups, respectively.

Table III Characteristics of live born infants born after intervention in the Phase III RCTs (pooled) and after intervention in all corifollitropin alfa intervention trials (pooled).

	Follow-up data of Phase III RCTs: Engage and Ensure		Follow-up of all Phase II and III trials
	Corifollitropin alfa (n = 424)	rFSH (n = 370)	Corifollitropin alfa (n = 806)
Female sex [n (%)]	210 (49.5), 424	190 (51.4), 370	397 (49.3), 806
Gestational age (weeks)[a]	37.8 (3.2), 424	38.2 (2.8), 370	38.0 (3.2), 806
Singletons	39.4 (1.9), 242	39.4 (2.1), 237	39.3 (2.2), 507
Multiples	35.6 (3.2), 183	36.2 (2.7), 132	35.7 (3.3), 299
Birthweight, all (g)	2860 (755), 424	2928 (716), 370	2929 (776), 806
Singletons	3297 (534), 241	3247 (586), 237	3303 (594), 507
Multiples	2284 (603), 183	2364 (552), 132	2295 (623), 299
Length at birth, all (cm)	48.2 (4.1), 370	48.6 (4.1), 333	48.4 (4.1), 712
Singletons	50.1 (3.0), 220	50.0 (3.5), 217	49.9 (3.3), 464
Multiples	45.4 (3.8), 150	46.0 (3.9), 116	45.5 (3.9), 248
Head circumference, all (cm)	33.6 (2.2), 303	33.5 (2.6), 272	33.7 (2.2), 576
Singletons	34.4 (1.7), 184	34.2 (2.2), 184	34.3 (1.9), 381
Multiples	32.4 (2.4), 119	32.1 (2.9), 88	32.5 (2.2), 195

Data as mean (SD), n unless otherwise stated.
[a]Enrolled in the follow-up trial.

Congenital malformations

The incidence of any or major congenital malformation (according to the broad definition) detected among live born infants is presented in Table V (see also Supplementary data, Table S3 and Figure S3 for data by trial and treatment group OR and Supplementary data, Table S4). The incidence of any malformation was 16.3% in the corifollitropin alfa maternal treatment group and 17.0% in the rFSH treatment group and statistical analysis showed no significant difference between the treatment groups (OR, 0.94; 95% CI, 0.65–1.37). The incidence of major malformations was 4.0 and 5.4% in the corifollitropin alfa and rFSH groups, respectively. Statistical analysis showed no significant difference in major malformations between the treatment groups (OR,

Table IV Premature live births, infants with low birthweight and low Apgar scores at birth in the Phase III RCTs (pooled) and all corifollitropin alfa intervention trials (pooled).

	Follow-up Phase III RCTs: Engage and Ensure		Follow-up of all Phase II and III trials
	Corifollitropin alfa [n (%), N]	rFSH [n (%), N]	Corifollitropin alfa [n (%), N]
Premature births[a], all	118 (27.8), 424	95 (25.7), 370	208 (25.8), 806
Singletons	20 (8.3), 241	22 (9.3), 237	52 (10.3), 507
Multiples	98 (53.6), 183	72 (54.5), 132	156 (52.2), 299
Birthweight ≤1500 g, all	22 (5.2), 424	14 (3.8), 370	38 (4.7), 806
Singletons	2 (0.8), 241	6 (2.5), 237	6 (1.2), 507
Multiples	20 (10.9), 183	8 (6.1), 132	32 (10.7), 299
Birthweight ≤2500 g, all	129 (30.4), 424	91 (24.6), 370	216 (26.8), 806
Singletons	16 (6.6), 241	16 (6.8), 237	39 (7.7), 507
Multiples	113 (61.7), 183	74 (56.1), 132	177 (59.2), 299
Birthweight <10th percentile, all	60 (14.2), 424	52 (14.1), 370	93 (11.5), 806
Singletons	15 (6.2), 241	21 (8.9, 237	31 (6.1), 507
Multiples	45 (24.6), 183	30 (22.7), 132	62 (20.7), 299
Apgar score (5 min) <7, all	8 (2.1), 377	5 (1.5), 340	17 (2.3), 749
Singletons	3 (1.4), 219	3 (1.4), 221	6 (1.3), 477
Multiples	5 (3.2), 158	2 (1.7), 119	11 (4.0), 272

[a]Gestational age <37 weeks.
n, number of live born infants.

Table V Incidence of minor and major congenital malformations in live born infants in the Phase III RCTs (pooled).

	Corifollitropin alfa ($n = 424$)[a]	rFSH ($n = 370$)[b]
Major malformations	4.0% ($n = 17$)	5.4% ($n = 20$)
Singletons	2.9% ($n = 7$)	5.1% ($n = 12$)
Multiples	5.5% ($n = 10$)	6.1% ($n = 8$)
Minor malformations only	12.3% ($n = 52$)	11.6% ($n = 43$)
Singletons	12.4% ($n = 30$)	9.3% ($n = 22$)
Multiples	12.0% ($n = 22$)	15.9% ($n = 21$)
Any malformation	16.3% ($n = 69$)	17.0% ($n = 63$)
Singletons	15.4% ($n = 37$)	14.3% ($n = 34$)
Multiples	17.5% ($n = 32$)	21.8% ($n = 29$)
OR (95% confidence interval), major malformations[c]	0.71 (0.36–1.38)	
OR ratio (95% confidence interval), any malformation[c]	0.94 (0.65–1.37)	

[a]241 infants from singleton pregnancies and 183 from multiple pregnancies.
[b]237 infants from singleton pregnancies and 133 from multiple pregnancies.
[c]Stratified by protocol and pregnancy type.

0.71; 95% CI, 0.36–1.38). In singleton births, the rates of major malformations were 2.9 and 5.1% in the corifollitropin alfa and rFSH groups, respectively, and in multiple births these rates were 5.5 and 6.1%, respectively. The incidence of congenital malformations in all fetuses with cardiac activity at 10 weeks after embryo transfer (including fetuses of pregnancies that were spontaneously or medically terminated) was similar to that of live born infants (any malformation, 16.1% in the corifollitropin alfa maternal treatment group and 17.6% in the rFSH treatment group; major malformations, 4.3 and 6.3% in the corifollitropin alfa and rFSH groups, respectively). Hydrops fetalis and congenital hydrocephalus were reported for spontaneously or medically terminated fetuses in the corifollitropin alfa group. In the rFSH group, anencephaly and ventricular septal defect were reported in one fetus and hydrops fetalis, trisomy 21 and renal aplasia were reported in three subsequent cases.

All major malformations in live born infants in the RCTs are listed in Table VI showing data for the corifollitropin alfa group and rFSH group separately (see also Supplementary data, Table S5 for major malformations by MedDRA high-level group and PT). In both maternal treatment groups, the reported major malformations were most frequently cardiac and vascular congenital disorders (2.4% for the corifollitropin alfa group and 2.7% for the rFSH group) and gastrointestinal tract congenital disorders (0.9% as major for the corifollitropin alfa group and 1.1% for the rFSH group). Antral septal defects were the only specific major malformations occurring in >1% of infants in either maternal treatment group (0.7% in the corifollitropin alfa group and 1.6% in the rFSH group).

In the corifollitropin alfa and rFSH groups, respectively, the most commonly reported congenital minor malformations were cardiac and vascular disorders (3.1 and 4.9%), musculoskeletal and connective tissue disorders (2.6 and 2.2%), gastrointestinal tract disorders (2.1 and 1.1%), eye disorders (1.2 and 1.4%), skin and subcutaneous tissue disorders (1.2 and 1.1%) and renal and urinary tract disorders (0 and 1.1%).

Combined follow-up data after corifollitropin alfa treatment including all Phase II and III trials

Evolution of pregnancies

Pooling all Phase II and III trials (including Engage and Ensure), 677 pregnant women with 838 fetuses with cardiac activity at 10 weeks after embryo transfer and 806 live births were followed up for safety evaluation, as shown in Tables I and II. In total, 602 (88.9%) mothers with 735 (87.7%) infants completed the follow-up, including the examination of their newborns at 4–12 weeks after birth.

Ongoing pregnancies ≥10 weeks included 522 (77.1%) singleton and 155 (22.9%) multiple pregnancies. In total, 15 (1.8%) ongoing pregnancies terminated at 10–20 weeks and the incidence of intrauterine death/stillbirth (≥20 weeks of gestation) was 1.4% ($n = 12$). This resulted in 655 (96.8%) pregnancies with at least one live born infant. From 838 fetuses at 10 weeks after embryo transfer following corifollitropin alfa treatment, there were 806 (96.2%) live born infants.

Neonatal outcome of live born infants

Gestational age, gender, birthweight, length, head circumference and Apgar scores of all live born infants are also summarized in Table III. The overall incidence of AEs was 43.2% (35.7% in singleton pregnancies and 55.9% in multiple pregnancies). The incidence of neonatal deaths was 2.0%.

Congenital malformations

The incidence of any congenital malformation (according to the broad definition) detected among all live born infants was 14.5% (13.0% in singletons and 17.1% in multiples, see Table VII) and 15.0% in all fetuses with heart beat activity at 10 weeks.

For the nine fetuses that were spontaneously or medically terminated, the following congenital malformations were reported: kidney malformation, Ebstein's anomaly, hydrops fetalis and congenital hydrocephalus, trisomy 21, trisomy 18, cystic lymphangioma, talipes. For one fetus multiple congenital malformations were reported: solitary kidney, cleft palate, renal dysplasia, skull malformation, congenital pulmonary artery anomaly and pulmonary malformation.

Major malformations (according to the broad definition) were reported in 4.5% of live born infants, 2.8 and 7.4% in singletons and multiple births, respectively. From all fetuses with cardiac activity at 10 weeks after embryo transfer, there were 5.4% major malformations. The major malformations in live born infants from all corifollitropin alfa intervention trials are listed in Table VI; the most frequently recorded major malformations did occur in the organ system classes of cardiac and vascular disorders, and gastrointestinal tract disorders (see also Supplementary data, Table S5 for expansion of categories).

Discussion

In this study, the infant follow-up data from two large RCTs showed that the health of 424 live born infants conceived after treatment

Table VI Major congenital malformations in live born infants according to the broad definition (by Medical Dictionary for Regulatory Activities HLGT) observed in the RCTs (pooled) and all corifollitropin alfa intervention trials (pooled).

Category	Follow-up of Phase III RCTs: Engage and Ensure		Follow-up of all Phase II and III trials
	Corifollitropin alfa (n = 424), n (%)	rFSH (n = 370), n (%)	Corifollitropin alfa (n = 806), n (%)
Blood and lymphatic system disorders congenital	0	0	3 (0.4)
Cardiac and vascular disorders congenital	10 (2.4)	10 (2.7)	15 (1.9)
Chromosomal abnormalities and abnormal gene carriers	0	2 (0.5)	2 (0.2)
Congenital and hereditary disorders	0	1 (0.3)	1 (0.1)
Eye disorders congenital	1 (0.2)	0	1 (0.1)
Gastrointestinal tract disorders congenital	4 (0.9)	4 (1.1)	8 (1.0)
Immune system disorders congenital	0	1 (0.3)	0
Infections and infestations congenital	0	0	1 (0.1)
Musculoskeletal/connective tissue disorders congenital	1 (0.2)	1 (0.3)	3 (0.4)
Neurologic disorders congenital	2 (0.5)	0	3 (0.4)
Renal and urinary tract disorders congenital	1 (0.2)	0	1 (0.1)
Reproductive tract and breast disorders congenital	2 (0.5)	2 (0.5)	3 (0.4)
Respiratory disorders congenital	1 (0.2)	0	3 (0.4)
Soft tissue neoplasms benign	0	1 (0.3)	0
Hypothalamus and pituitary gland disorders	1 (0.2)	0	2 (0.2)
Lipid metabolism disorders	0	1 (0.3)	0
Hearing disorders	1 (0.2)	0	2 (0.2)
Cardiac valve disorders	0	1 (0.3)	0
Cardiac arrhythmias	1 (0.2)	0	1 (0.1)
Myocardial disorders	0	1 (0.3)	0
Coronary artery disorders	1 (0.2)	0	1 (0.1)
Penile and scrotal disorders (not infections or inflammations)	0	1 (0.3)	0
Fatal outcomes	1 (0.2)	2 (0.5)	1 (0.1)
Haematology investigations (including blood groups)	0	1 (0.3)	0
Abdominal hernias and other abdominal wall conditions	0	0	1 (0.1)
Reproduction tract disorders NEC	0	0	1 (0.1)

NEC, not elsewhere classified.

with a single dose of corifollitropin alfa during the first 7 days of COS was no different from that of 370 live born infants conceived after COS with daily rFSH. There were no treatment-related differences in neonatal characteristics or in the incidence of major or minor malformations. The live birth rates, mode of delivery, number of premature births and premature births with low birthweights, congenital malformations and stillbirths or neonatal deaths were similar between the treatment groups. The safety data from the two large RCTs are supported and strengthened by the infant follow-up data, from 806 infants, following all trials to date with maternal corifollitropin alfa treatment. The incidence of major malformations in live born infants in the RCTs (4.0% in the corifollitropin alfa group and 5.4% in the rFSH group) was comparable with the incidence when all trials with corifollitropin alfa intervention were included (4.5%).

The incidence and type of major malformations with the corifollitropin alfa therapy in the current study was comparable with the 5% incidence reported in a large study including 1000 fetuses following maternal COS with rFSH in a GnRH antagonist (ganirelix) protocol using the same methodology and definitions (Bonduelle *et al.*, 2010).

The definition of a major malformation, which may vary between studies, influences its absolute incidence which then may vary between 1 and 10% (Rimm *et al.*, 2004). In addition to the definition,

Table VII Incidence of minor and major congenital malformations in all Phase II and III trials with corifollitropin alfa intervention.

	All pregnancies	Singleton pregnancy	Multiple pregnancy
Live born infants	$n = 806$	$n = 507$	$n = 299$
Major malformations	4.5% ($n = 36$)	2.8% ($n = 14$)	7.4% ($n = 22$)
Minor malformations only	10.0% ($n = 81$)	10.3% ($n = 52$)	9.7% ($n = 29$)
Any malformation	14.5% ($n = 117$)	13.0% ($n = 66$)	17.1% ($n = 51$)

the number of observations, the duration of follow-up after birth, the incidence of multiple pregnancies and the thoroughness of the examination are other determining factors. Care should be taken when comparing the incidence of major malformations between different prospective studies or even population surveillance data, as the latter is often hampered by underreporting (Simpson, 1996).

Taking into account any malformation, thus both major and minor malformations, the overall incidence was 16–17% and similar in both treatment groups. This incidence may be considered relatively high but is known to be related to the thoroughness of paediatric examination as well as the awareness of physicians participating in a prospective trial to collect malformations after exposure to a new fertility drug (Leppig *et al.*, 1987).

In the current RCTs, the incidence of multiple pregnancies tended to be higher in patients treated with corifollitropin alfa (43.2%) than in patients treated with rFSH (35.9%), whereas the average number (maximal two) of embryos replaced was similar in both arms of the trials. This difference included three triplets in the corifollitropin alfa arm versus none in the rFSH arm. The occurrence of a monozygotic triplet after replacement of a single embryo is rare, and in general, the occurrence of monozygotic multiples is thought to be related to prolonged culture (Chang *et al.*, 2009; Knopman *et al.*, 2010).

The incidence of intrauterine death/stillbirths at $\geq$20 weeks and neonatal deaths in women treated with corifollitropin alfa for COS (1.5 and 2.0%, respectively, in the current analysis of all trials) was comparable with previously reported data in a large cohort of women undergoing COS for ICSI or IVF (stillbirths of 1.5% and neonatal deaths of 1.1%; Bonduelle *et al.*, 2002) and in the GnRH antagonist arm of the 2010 Bonduelle study (stillbirths of 1.5% and neonatal deaths of 1.2%; Bonduelle *et al.*, 2010).

The data from the current prospective RCTs are reassuring. Despite previous reassuring reports on obstetric and neonatal complications and congenital abnormalities after ART (Ericson and Källén, 2001; Boerrigter *et al.*, 2002; Anthony *et al.*, 2002; Bonduelle *et al.*, 2002; Nygren *et al.*, 2007), the debate continues about the safety of ART for children, and the ways in which safety is evaluated. Concerns about the limitations of commonly used methods of assessment (including lack of appropriate comparison (control) groups, failure to take into account potential confounding variables and differences in the criteria used to evaluate anomalies in the ART population versus the general population) have prompted calls for more rigorous evaluation, including prospective surveillance of congenital abnormalities (Simpson, 1996) and a hesitancy to dismiss observed increases in the risk of adverse outcomes found in literature reviews (Kurinczuk, 2003; Hansen *et al.*, 2005; Reefhuis *et al.*, 2009).

In conclusion, the current neonatal follow-up data, including comparative data of 440 fetuses and overall data of 838 fetuses conceived after maternal treatment with corifollitropin alfa, further support the safety of this new treatment option in IVF.

Authors' roles

M.B., B.M., A.L., C.B., D.P. and P.D. took part in the analysis and interpretation of data, writing the manuscript and in the final approval of the version to be published.

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Conflict of interest

M.B. has received funding from Merck International, MSD Belgium, IBSA and Ferring International. A.L. has been a speaker for Merck, Inc. and was an investigator in the Phase III trials. B.M. and D.P. are employees of MSD.

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6.3 Discussion

The safety profile of fertility drugs applied for the treatment of infertile, but otherwise healthy women needs to be favorable without any risk for the treated patient or her off-spring. A careful comparative evaluation of all (serious) adverse events, including any case of OHSS, any hypersensitiviy or allergic reaction and any congenital malformation, should be performed to conclude on the risk/benefit of any fertility drug or regimen.

In (potential) normal responders, the ovarian response following corifollitropin alfa treatment is on average higher than following treatment with 150 to 200 IU follitropin-β, simply because the selected doses of corifollitropin alfa provide a nearly maximal ovarian response, whereas the ovarian response to follitropin-β increases with the (starting) dose administered. Preventive measures for OHSS in high responders are similar for corifollitropin alfa and follitropin-β and include coasting (from stimulation day 8 onwards), withholding or lowering the dose of hCG, triggering final oocyte maturation with a GnRH agonist, and/or freezing all embryos [Humaidan et al 2010; Devroey et al 2011]

The pooled OHSS analysis of the two randomized controlled trials [Devroey et al 2009a; Corifollitropin alfa ENSURE study group, 2010] did not indicate a statistical difference and the corifollitropin alfa to follitropin-β Odds Ratio for OHSS was 1.18 (95% CI 0.81-1.71). A logistic regression model for OHSS of any grade including the two studies provided an adjusted Odds Ratio of 0.99 (95% CI 0.67-1.45) using significant covariates including the number of oocytes retrieved. Thus, whereas the corifollitropin alfa regimen may recruit more oocytes than 150 to 200 IU follitropin-β, the application of corifollitropin alfa instead of follitropin-β does not increase the risk of OHSS in normal responders, which was also confirmed by the clinical and statistical comparison of incidences of moderate to severe OHSS [Tarlatzis et al 2012].

The overall incidence of OHSS was about 50% lower in the uncontrolled repeated cycle study of corifollitropin alfa [Norman et al 2011] than in the two randomized controlled phase III studies, partly because of the maximum age was increased from 36 to 39 years. In addition, in the repeated cycle study, patients often received only 150 IU FSH to finish the cycle and did not always receive FSH on the day of hCG, the latter being administered either as urinary or recombinant hCG (~6500 IU).

In total 682 patients started in the repeated cycle study, 375 patients started a second treatment cycle and finally 198 patients underwent 3 consecutive treatment cycles. None of the subjects had moderate or severe drug-related hypersensitivity reactions and only two related cases of mild (injection site) rash were reported following repeated treatment with corifollitropin alfa. None of the subjects developed antibodies against corifollitropin alfa as assessed in blood samples taken after complete drug clearance 2 weeks after embryo transfer or after cycle discontinuation. Absence of hypersensitivity reactions and antibodies

against corifollitropin alfa is reassuring and implies that IVF patients can be treated repeatedly with corifollitropin alfa without concerns of immunogenicity [Norman et al 2011].

The safety analyses by Bonduelle et al [2012] included 677 ongoing pregnancies and 806 live born infants after corifollitropin alfa intervention and 333 ongoing pregnancies with 393 live born infants following daily rFSH treatment in the reference group. Evaluation of the incidence of major and minor congenital malformations in live born infants did not reveal any relevant differences between the treatment groups. Incidences of major congenital malformations were 4.0% and 5.4% after corifollitropin alfa and rFSH treatment in the phase III randomized controlled trials, respectively. These figures are in line with those reported in a large prospective ganirelix follow-up trial including about 1000 fetuses with the same methodology applied [Bonduelle et al, 2010]. In this trial the reported incidence of major congenital malformations was 4.9% in the ganirelix treatment group and 4.3% in the historical GnRH agonist control arm. The incidence of major congenital malformations for all phase II and III trials was 4.5%, both in the corifollitropin alfa follow-up and in the ganirelix follow-up group.

When comparing these incidences with other studies one should acknowledge that the absolute incidence of major malformations may vary largely between 1 and 10% [Rimm et al 2004]. Determining factors are 1) the definition of a major malformation 2) the number of observations, 3) the duration of the follow-up after birth, 4) the incidence of multiple pregnancies and 5) the extensiveness of examination. Therefore the incidence of major malformations cannot be compared between different studies or surveillance data bases [Simpson, 1996] and it is pivotal for each follow-up study to have its own control group.

In conclusion, during phase III trials the safety of a single dose of corifollitropin alfa in comparison to daily rFSH for the first 7 days of stimulation was examined in (potential) normal responders up to 36 years of age treated with ganirelix from stimulation day 5 onwards. The risk of OHSS was not increased in these young IVF patients, but since the ovarian response is slightly higher following treatment with corifollitropin alfa in comparison to daily treatment with 150 to 200 IU rFSH, careful monitoring of the ovarian response is indicated to take timely preventive measures in unexpected high responders. Corifollitropin alfa appeared to be well-tolerated both in single treatment cycles and in repeated treatment cycles and displayed no signs of immunogenicity. To date, the comparative safety data of infants born following treatment with corifollitropin alfa supports the safety of this new treatment option for IVF patients and their offspring.

Chapter 7

Phase IV Trials and Retrospective Analyses of Phase III Trials

Chapter 7 Phase IV Trials and Retrospective Analyses of Phase III Trials

7.1 Introduction

Phase IV trials are post-marketing trials following drug authorization by main regulatory agencies. The purpose of phase IV studies may be to further examine the drug's effect in various patient populations or to extend approved indications or the treatment posology. Phase IV studies may also involve specific safety studies (surveillance) to detect any rare or long-term adverse effects over a much larger patient population and longer time period than during phase I-III trials.

Following launch and drug introduction, clinicians will start integrating new drugs into their routine clinical practice and may address outstanding scientific questions by prospective randomized trials in their own clinic. Sponsored phase IV studies performed under GCP guidelines may be performed within certain areas of interest. In addition, retrospective pooled analyses of completed phase III trials may provide new research leads which should subsequently be confirmed in prospective phase IV trials.

The global introduction of GnRH antagonists from 1999 onwards revolutionized ovarian stimulation for assisted reproduction but there were many unknowns with respect to this new treatment regimen. For comparative reasons, phase III randomized controlled studies of ganirelix had included (potential) normal responders, did not allow any pretreatment, started stimulation on cycle day 2 or 3, and used a fixed treatment regimen of ganirelix starting treatment at day 6 of stimulation. The interest for additional clinical research was large as comparative phase III trials had indicated that the chance of pregnancy was slightly lower following GnRH antagonist treatment than following GnRH agonist treatment [Al-Inany et al 2007]. This observation led to many subsequent randomized controlled studies, retrospective pooled analyses and systematic reviews for meta-analysis.

The initial sponsored phase IV studies of ganirelix addressed the impact of different rFSH starting doses [Out et al 2004], the impact of ganirelix treatment on endometrial development [Simon et al 2005], the impact of oral contraceptive pretreatment [Rombauts et al 2006], the impact of ganirelix treatment during ovarian stimulation for intra-uterine insemination [Lambalk et al 2006], and the application of a GnRH agonist for triggering final oocyte maturation [Itskovitz et al 2000; Fauser et al 2002].

Many prospective, randomized, controlled studies of ganirelix were performed by Professors Kolibianakis and Devroey in Brussels between 2000 and 2006, and this research contributed significantly to our current knowledge of the optimal GnRH antagonist regimen [Tarlatzis et al 2006; Devroey et al 2009b; Devroey et al 2011]. Additional systematic reviews

for meta-analyses also provided further insight on the probability of pregnancy and live birth [Kolibianakis et al 2006; Al-Inany et al 2011].

In parallel to the large phase III trial of corifollitropin alfa [Devroey et al 2009a] a prospective phase IV trial [Xpect trial, Nyboe Andersen et al 2011] was designed to identify predictive factors for ovarian response in a rFSH/GnRH antagonist protocol with or without oral contraceptive (OC) pretreatment. The patient population and treatment regimen in the non-OC group was highly similar to the reference group of the phase III corifollitropin alfa trial. Data from the latter trial were analysed to identify common predictors of too low or too high ovarian response and the phase IV data of the Xpect trial were used to validate the predictive model.

The ongoing debate on the possible need for additional LH activity during ovarian stimulation with rFSH was addressed by a retrospective LH analysis to examine the impact of relatively low or high endogenous LH during stimulation in the large phase III corifollitropin alfa study [Doody et al 2010; Doody et al 2011]. These data were to be confirmed in a large combined LH analysis of individualized data of 1764 patients treated with daily rFSH and ganirelix in 6 different trials [Griesinger et al 2011]. The application of a central laboratory in these randomized trials, made the trials ideal for a pooled LH analysis of individual patient data.

The development of corifollitropin alfa on one hand and the development of mild stimulation on the other hand, both for (potential) normal responders, raised the question as to whether too high and too low ovarian response could affect the chance of pregnancy following fresh embryo transfer. One of the main disadvantages of too profound stimulation could be the increased incidence of pre-ovulatory progesterone rises which are associated with a lower pregnancy rate [Bosch et al 2010; Doody et al 2011]. Thus, retrospective analyses were designed to examine the relation between ovarian response and ongoing pregnancy rates [Fatemi et al 2013].

The development of corfollitropin alfa in a GnRH antagonist protocol was an obvious choice when aiming at maximal reduction of interventions prior to IVF treatment. However, at the time of the dose-finding study of corifollitropin alfa using a GnRH antagonist protocol, it was unknown whether corifollitropin alfa applied in a long GnRH agonist protocol would provide a similar ovarian response and success rate. Assumptions were based on the known differences between GnRH antagonist and long GnRH agonist protocols using daily rFSH including a longer duration of stimulation following treatment with the long GnRH agonist protocol [The European Orgalutran study group, Borm and Mannaerts, 2000]. To confirm that the selected corifollitropin alfa doses would also be sufficient to replace the first 7 days of rFSH using a long GnRH agonist protocol, a proof of concept study was designed in which both strengths of corifollitropin alfa were subsequently tested in a small subset of good prognosis patients treated with a long protocol of daily 0.1 mg triptorelin [Fatemi et al 2010].

7.2 Results

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ORIGINAL ARTICLE ***Reproductive endocrinology***

Predictive factors of ovarian response and clinical outcome after IVF/ICSI following a rFSH/GnRH antagonist protocol with or without oral contraceptive pre-treatment

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BACKGROUND: Prediction of ovarian response prior to the first controlled ovarian stimulation (COS) cycle is useful in determining the optimal starting dose of recombinant FSH (rFSH). However, potentially predictive factors may be subject to inter-cycle variability and many patients are pre-treated with oral contraceptives (OC) for scheduling purposes. Our objective was to determine predictive factors of ovarian response for patients undergoing COS with rFSH in a gonadotrophin-releasing hormone antagonist protocol and to determine the inter-cycle variability of these factors.

METHODS: In this multinational trial, 442 patients were randomized to receive either OC treatment or no treatment prior to their first COS cycle. For candidate predictive factors, patient characteristics were collected at screening, and endocrine and sonographic data were collected during the early follicular phase of the two subsequent cycles. A treatment regimen of 200 IU rFSH and 0.25 mg ganirelix was applied during the second cycle. Predictive factors of ovarian response and of too low (<6 oocytes) or too high (>18 oocytes) ovarian responses were determined using stepwise linear regression and stepwise logistic regression, respectively.

RESULTS: Anti-Müllerian hormone (AMH) and basal FSH were statistically significant predictors of the number of oocytes retrieved and of an excessive ovarian response. For low ovarian response, AMH was the only significant predictive factor. In the non-OC group, the predictive value was higher than in the OC group and higher at the early follicular phase of the stimulation cycle than of the previous cycle. The inter-cycle variation for AMH was low compared with the inter-cycle variation of other hormones. Between the two groups, there were no differences in the number or quality of embryos obtained or transferred, but the implantation rate was significantly lower in the OC group (24.1 versus 30.1%, $P = 0.03$), resulting in an ongoing pregnancy rate of 26.3% compared with 35.7% in the non-OC group ($P = 0.05$).

CONCLUSIONS: The best predictive model of ovarian response was in the non-OC group and included both AMH and basal FSH determined at the early follicular phase of the stimulation cycle. In the proceeding cycle, AMH alone had sufficient predictive value since it was not affected by inter-cycle variability or OC pretreatment.

Clinical trial identifier: **NCT00778999**.

Key words: GnRH antagonist / follicle-stimulating hormone / anti-Müllerian hormone / antral follicle count

[†] The list of Xpect investigators is available in the Appendix.

Introduction

Predicting the ovarian response is helpful in determining the optimal starting dose of recombinant FSH (rFSH), especially prior to the first controlled ovarian stimulation (COS) cycle. Selection of the starting dose based on the predicted ovarian response increases the proportion of patients with a normal ovarian response and decreases the need for dose adjustments during stimulation (Popovic-Todorovic et al., 2003b). Previously published data indicate that several factors, including age, BMI, menstrual cycle length, basal FSH and antral follicle count (AFC) (Popovic-Todorovic et al., 2003a; Fauser et al., 2008; Verhagen et al., 2008; Olivennes et al., 2009), are clinically relevant predictors of oocyte yield, which may be used in a multivariate model or as a single test for the assessment of ovarian response. Often, such tests of ovarian response have assessed the prediction of poor ovarian response rather than the prediction of hyper-response (Broekmans et al., 2006). More recently, it has been suggested that anti-Müllerian hormone (AMH) is a better marker in predicting ovarian response to COS than age, FSH, estradiol (E_2) and inhibin B. The performance of AMH as a predictor of poor ovarian response is anticipated to be very similar to AFC (Broer et al., 2009); however, AFC should be assessed during the early follicular phase to minimize the effect of intra-cycle fluctuations (Broekmans et al., 2010), whereas serum AMH levels are generally thought to remain stable throughout the menstrual cycle (La Marca et al., 2007). The dose–response relationship between AMH and ovarian response to FSH explains why AMH levels have been shown to be useful in both the prediction of poor response and hyper-response. As such, patients with high AMH levels could have an increased risk of developing ovarian hyperstimulation syndrome (OHSS).

To date, however, the role of predictive factors of ovarian response has most frequently been studied in patients treated with a long gonadotrophin-releasing hormone (GnRH) agonist protocol. Predictive factors for GnRH antagonist protocols are lacking. In addition, it is unknown whether predictive factors remain stable between menstrual cycles. Therefore, the accuracy of these predictions may be affected by inter-cycle variability. Pre-treatment regimens, such as the use of oral contraceptives (OC) [often used to assist in scheduling assisted reproductive technology (ART) cycles], may also influence some of the factors used as predictors.

The primary objective of this randomized, open-label, multicentre clinical trial was to identify factors capable of predicting ovarian response in patients undergoing their first treatment cycle with a daily dose of 200 IU rFSH in a GnRH antagonist protocol. In the present study, patients were randomized into two groups with or without OC pre-treatment, to investigate the predictive value in both groups separately. Randomization to OC versus non-OC treatment was performed since OC pre-treatment is more often applied by US clinics than by European clinics, which prevents bias due to regional differences. The inter-cycle variability of endocrine and sonographic factors was assessed by measuring those factors twice, during the early follicular phase of two successive menstrual cycles.

Materials and Methods

The Xpect trial was a randomized, open-label, multicentre clinical trial involving eight centres in the USA and six centres in Europe (one in Denmark and Germany, and two in Spain and Turkey) conducted between October 2006 and July 2008. The study protocol was approved by the independent medical ethics committee or institutional review board for each centre and was conducted in accordance with the Declaration of Helsinki, International Conference on Harmonisation guidelines for Good Clinical Practice and current regulatory requirements. Written informed consent was provided by all patients.

Study population

Women aged 18–39 years with BMI of $\leq$32 kg/m^2, a menstrual cycle length of 24–35 days, access to ejaculatory sperm, an indication for COS and IVF and/or ICSI and who were scheduled for their first COS cycle and were willing and able to give written informed consent were eligible to enrol in the study. Patients were excluded from the study due to a history of an endocrine abnormality, less than two ovaries or any other ovarian abnormality (including endometrioma >10 mm visible on ultrasound), presence of unilateral or bilateral hydrosalpinx, any clinically relevant pathology affecting the uterine cavity (upon discretion of the investigator), fibroids $\geq$5 cm, a history of recurrent miscarriage (three or more) and/or FSH or LH levels >12 IU/l in the early follicular phase.

Study design

The trial was designed to identify endocrine and/or sonographic predictors of ovarian response, including too high (>18 oocytes) and suboptimal (<6 oocytes) ovarian response. Subjects were randomized to receive a fixed daily dose of 200 IU rFSH in a GnRH antagonist protocol either with OC pre-treatment (OC group) or without any OC pre-treatment (non-OC group). Randomization was done by central remote allocation using an interactive voice response telephone system, which allocated each subject to a treatment group. Randomization was stratified for centre and age ($\leq$32 and >32 years).

The study design is summarized in Fig. 1. The OC group received OC (30 μg ethinyl E_2/150 μg desogestrel; Marvelon, NV Organon, The Netherlands) for 14–21 days and started daily rFSH [follitropin beta (Puregon/Follistim AQ Cartridge, NV Organon, The Netherlands)] 5 days after stopping OC treatment (stimulation day 1) provided a withdrawal bleeding had occurred. In the absence of bleeding, the start of COS was delayed up to the first day of full bleeding. If bleeding did not occur within 7 days, the subject was withdrawn from the study. The non-OC group started daily rFSH on Day 2 or 3 of their next menstrual cycle (stimulation day 1). In both groups, a single subcutaneous injection of 200 IU rFSH was initiated on stimulation day 1 and continued daily up to and including the day of triggering of final oocyte maturation by urinary hCG. Based on the observed follicular response, the rFSH dose from stimulation day 6 onwards could be reduced but only in such cases where the investigator considered there was a risk of development of OHSS. The maximum total duration of stimulation was 19 days. Starting on stimulation day 5, all patients received 0.25 mg ganirelix daily. The criteria for giving hCG was the development of three or more follicles $\geq$17 mm. Approximately 34–36 h after induction of oocyte maturation, oocyte pick up followed by IVF or ICSI was performed. At embryo transfer, 3 or 5 days after oocyte pick up, a maximum of two embryos (subjects aged $\leq$36 years) or three embryos (subjects aged >36 years) could be replaced. All patients received daily progesterone ($\geq$600 mg/day vaginally or $\geq$50 mg/day intramuscularly) for luteal phase support for $\geq$6 weeks in case of pregnancy or until menses or up to a negative pregnancy test performed $\geq$14 days after embryo transfer.

Assessments of potential predictive factors

At screening, the following demographic data were collected: age, BMI (kg/m^2), cycle length, age at menarche, duration of infertility, smoking

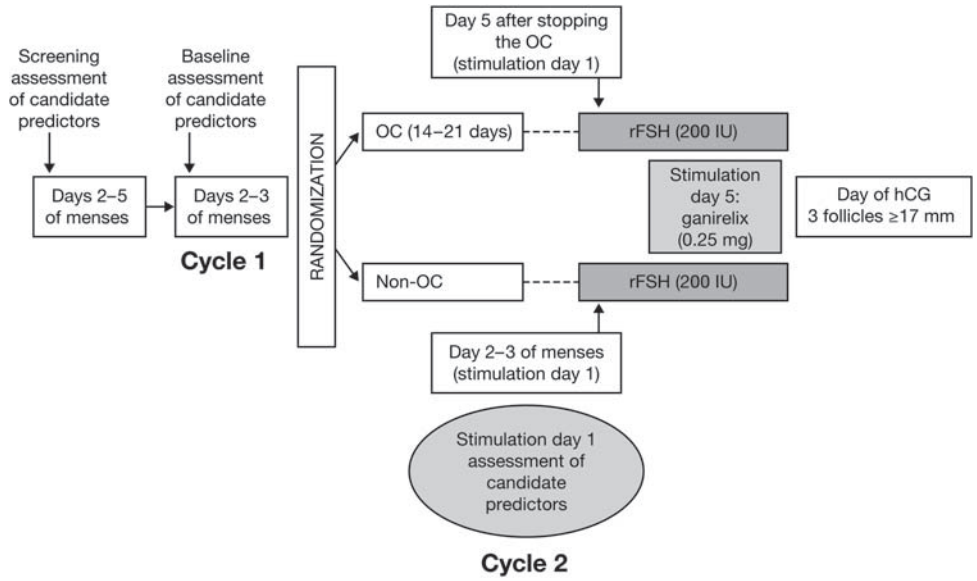

Figure 1 Study design. hCG, human chorionic gonadotrophin; OC, oral contraceptive; rFSH, recombinant follicle-stimulating hormone.

(yes/no) and alcohol use (yes/no). In addition, candidate predictors were assessed in two subsequent cycles: ovarian volume, AFC, basal FSH, LH, testosterone, progesterone, E_2, inhibin B and AMH. In the non-OC group, these assessments were made at randomization on cycle day 2 or 3 (referred to as 'cycle 1') and at stimulation day 1 on cycle day 2 or 3 (referred to as 'cycle 2'). In the OC group, these assessments were made at randomization on cycle day 2 or 3 and at stimulation day 1 at least 5 days after the last OC pill intake.

Assessments during assisted reproduction

Ultrasonographic investigation was performed during rFSH treatment at stimulation day 5 prior to the start of ganirelix treatment, on stimulation day 8, then daily up to and including the day of hCG. The number and quality of embryos retrieved was assessed 3 days after oocyte pick-up. Two weeks after embryo transfer, a pregnancy test (serum or urinary hCG) was performed. Vaginal and/or abdominal ultrasonographic investigation was performed to confirm pregnancy at 5–6 weeks after embryo transfer, and at 10 weeks after embryo transfer to confirm ongoing pregnancy.

Assays

Validated immunoassays were performed at a central laboratory (Essex Pharma Development GmbH, Germany) to measure serum hormone levels of FSH, LH, inhibin B, E_2 and progesterone. Levels of FSH, LH, E_2 and progesterone were determined by time-resolved fluoroimmunoassay (AutoDelfia® immunofluorometric assay, PerkinElmer Life and Analytical Sciences, Brussels, Belgium) with a coefficient of variation of $<10\%$. Detection limits were 0.25 IU/l, 0.6 IU/l, 49.9 pmol/l and 0.38 ng/ml for FSH, LH, E_2 and progesterone, respectively. Serum inhibin B levels were determined by using a validated immunoassay by Diagnostic Systems Laboratories (DSL; Webster, TX, USA) with a coefficient of variation of $<10\%$ and a detection limit of 10.0 pg/ml. All serum samples of a subject were measured with the same inhibin B lot; in total, two lots were applied. Serum AMH levels were determined at a central laboratory (Analytisch Biochemisch Laboratorium BV, The Netherlands) using a validated enzyme-linked immunosorbent assay (DSL, Webster, TX, USA; values presented in nanogrammes per millilitre) with a detection limit of 0.1 ng/ml. All serum samples of a subject were measured with the same AMH lot. However, in this trial serum AMH was measured by three different lots; control samples indicated considerable variability among lots. Around 30% of the AMH measurements were not reported because either the serum sample was received unfrozen at the laboratory, or the sample was analysed after the guaranteed stability period of AMH in human serum.

Clinical outcome parameters

The primary parameter for ovarian response was the number of oocytes obtained and the secondary efficacy parameters were the number of follicles $\geq$11 mm at Day 8 and the number of follicles $\geq$11 mm at day of hCG. In addition, the following outcome parameters are reported: duration of stimulation, total rFSH dose, number of embryos, number of embryos transferred, implantation rate, clinical pregnancy rate and ongoing pregnancy rate. Implantation rate was calculated per patient as the number of gestational sacs observed compared with the number of embryos transferred.

Statistical analysis

In the present prognostic study, the following 16 baseline characteristics were planned to be evaluated for ovarian response prediction: age, age at menarche, menstrual cycle length, duration of infertility, alcohol use (Y/N), smoking (Y/N), BMI, ovarian volume, AFC ($<$11 mm) and serum FSH, LH, E_2, progesterone, inhibin B, AMH and testosterone. For the planned sample size, it was expected that up to five predictive factors were selected in each logistic regression model. Assuming that for each predictive factor at least 10 events are required (Moons et al., 2009), a total of around 50 events are needed. A total sample size of 200 randomized subjects per treatment group was planned, with an additional 20 subjects (10%) to compensate for discontinued subjects.

All analyses were performed in SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) and were based on the intent-to-treat (ITT) population. The ITT population consisted of all randomized subjects who received at least one dose of rFSH. All analyses were performed 'per started cycle': if a treated subject did not reach a certain stage in IVF treatment, then zero values were imputed. For example, if a particular subject did not have oocyte retrieval, then the number of oocytes was set to zero and the pregnancy outcome was set to 'not pregnant'. Hormone values below the lower limit of quantification (LLOQ) were set to 0.5 times the LLOQ for all calculations. Serum progesterone was omitted from the analyses because 139 subjects (66.5%) in the OC group and 93 subjects (46.7%) in the non-OC group had at least one serum progesterone sample level below the LLOQ in the early follicular phase of cycles 1 or 2.

The Spearman rank correlation coefficient (ρ) was calculated to assess the correlation between cycles 1 and 2 for each of the candidate predictive factors. The inter-cycle variability of the candidate predictors was estimated by fitting an analysis of variance model to the data of cycles 1 and 2, including subject as a covariate for each of the candidate predictors separately. Patients with hormone values below the LLOQ on both assessment days were excluded from the analyses to prevent overestimation of the correlation and inter-cycle variability.

Stepwise linear regression was applied to identify the predictive factors of the number of oocytes retrieved and the predictive factors of the number of follicles $\geq$11 mm on Day 8 and on day of hCG, respectively. Stepwise logistic regression was applied to identify significant factors for prediction of high ovarian response (>18 oocytes) and low ovarian response (<6 oocytes), respectively. The significance level of the candidate predictive factors to enter the model was set to 0.15 and to stay in the model, it was set to 0.10. After selection of the candidate predictive factors using stepwise selection, the final model selects those prognostic factors with statistical significance, i.e. $P \leq 0.05$. The goodness-of-fit of the normal regression models was quantified by the coefficient of determination R^2. Receiver operating characteristic (ROC) curves were calculated and the area under the curves (AUC) was used to assess the discriminative power of the logistic regression models.

Regression models for cycle 1 include the candidate predictive factors measured at screening and in cycle 1. Regression models for cycle 2 include the candidate predictive factors measured at screening and in cycle 2. Cycle 1 models were fitted for the combined treatment groups (OC, non-OC), and treatment group was included as a candidate predictive factor, while cycle 2 models were fitted per treatment group. Only additive models (i.e. without potential interactions of factors) were considered. The regression models were not adjusted for the AMH inter-lot variability, i.e. AMH lot was not added as a factor to the list of candidate predictive factors to be included in the models. Three subjects were excluded from the regression analyses because they discontinued the trial prior to oocyte retrieval for reasons other than too low ovarian response. However, one subject who discontinued the trial due to risk of OHSS was excluded from the linear regression analyses but included in the logistic regression analyses as a high responder (>18 oocytes). Because AMH appeared to be a strong predictive factor for ovarian response in this study, subjects without AMH measurements (around 30% of the total sample size) were not included in the regression analyses.

To explore the potential predictive factors of ongoing pregnancy, the data of the two treatment groups were combined, and the treatment group was included in the regression models as a candidate predictive factor.

Difference in implantation rate between the two treatment groups was evaluated using a cumulative logit model with proportional odds property for the implantation rate, including the factors: treatment group, age class as stratified (≤32 and >32 years) and region (Europe, USA). Difference in ongoing pregnancy rate between the two treatment groups was estimated using a generalized linear model with identity link for the ongoing pregnancy rate including the factors: treatment group, age class as stratified and region.

Results

Patient demographics and disposition

The present manuscript focuses on the prediction of ovarian response. In addition, inter-cycle variability of the candidate predictors and the impact of OC pre-treatment on the values of the candidate predictors are addressed.

A total of 442 subjects were randomized—223 subjects to the OC group and 219 subjects to the non-OC group (Fig. 2). Altogether, 34 subjects did not receive rFSH, including 14 subjects in the OC group and 20 subjects in the non-OC group. In the OC group, 19 subjects did not receive hCG for final oocyte maturation compared with 27 subjects in the non-OC group. A total of 380 subjects (86.0%) had embryo transfer, 195 subjects (87.4%) in the OC group and 185 subjects (84.5%) in the non-OC group. Of the subjects who started stimulation, 6.7% (14/209) of subjects in the OC group and 7.0% (14/199) in the non-OC group did not have embryo transfer.

The two treatment groups were similar with respect to mean age, BMI, age at menarche, duration of infertility, cycle length, alcohol use and smoking (Table I).

Inter-cycle variability

Candidate endocrine and sonographic predictive factors were assessed in the early follicular phase at randomization (cycle 1) and at stimulation day 1 (cycle 2) (Table II). Table II shows the median and the 5th and 95th percentiles of the endocrine (serum FSH, LH, E_2, testosterone, inhibin B and AMH) and sonographic (AFC, ovarian volume) factors on the day of randomization (cycle 1) and on stimulation day 1 (cycle 2). In addition, it shows the correlation between cycles 1 and 2 and the cycle-to-cycle variation of the candidate predictive factors. In the non-OC group, the correlation between cycles 1 and 2 was the highest for AMH ($\rho = 0.88$) and testosterone ($\rho = 0.84$), which appeared to be less influenced by inter-cycle variability than basal FSH, LH, E_2, inhibin B, AFC and ovarian volume (Table II). The inter-cycle variability is illustrated in Fig. 3 for AFC, FSH and AMH. The figure illustrates the larger inter-cycle variability of serum FSH and basal AFC compared with AMH.

For the OC group, five days after the last OC intake, at stimulation day 1, circulating FSH, LH and E_2 levels were comparable to those at the start of OC pre-treatment, whereas the AFC and ovarian volume were still slightly reduced. As in the non-OC group, the highest correlation between cycles 1 and 2 was found for AMH ($\rho = 0.90$) and testosterone ($\rho = 0.79$). In comparison with the non-OC group, a lower correlation between cycles 1 and 2 was found for FSH, E_2 and inhibin B (Table II).

Clinical outcome

There was no difference between the two treatment groups in terms of duration of stimulation, total dose of rFSH administered, or the number and size of follicles recruited (Table III and Fig. 4).

Clinical outcomes, including the number of oocytes, the total number of embryos, number of embryos transferred and the pregnancy rates are presented in Table III. The mean number of oocytes

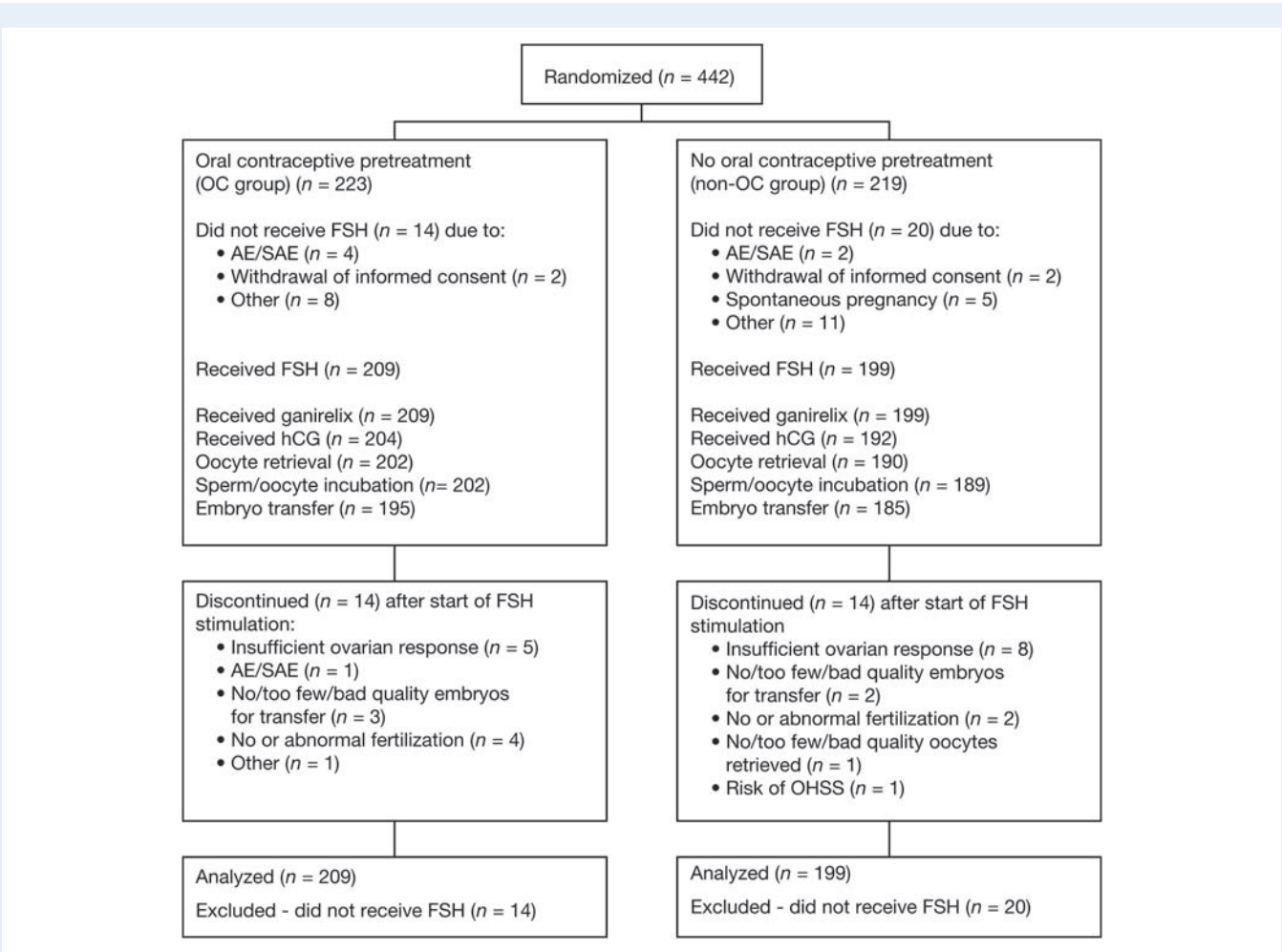

Figure 2 Subject disposition. AE, adverse event; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotrophin; OC, oral contraceptive; OHSS, ovarian hyperstimulation syndrome; SAE, serious AE.

Table 1 Potential predictive factors assessed at screening.

	OC (*n* = 209)	Non-OC (*n* = 199)	Overall (*n* = 408)
Age, years, mean (SD)	31.8 (3.7)	31.6 (4.1)	31.7
BMI, kg/m^2, mean (SD)	24.2 (3.6)	23.6 (3.4)	23.9
Age at menarche, years, mean (SD)	12.6 (1.4)	12.9 (1.5)	12.7
Duration of infertility, years, mean (SD)	3.9 (3.2)	3.7 (3.0)	3.8
Cycle length, days, mean (SD)	28.6 (1.8)	28.5 (1.8)	28.6
Alcohol use, *n* (%)	86 (41.1)	86 (43.2)	41.7%
Smoking, *n* (%)	32 (15.3)	34 (17.1)	16.2%

retrieved was 12.4 (SD = 6.7) and 12.1 (SD = 7.7) in the OC and non-OC groups, respectively. Fertilization rates were 66.9% in the OC group and 62.9% in the non-OC group. The mean number of good quality embryos obtained was 4.4 (±3.6) for the OC group and 4.8 (±4.9) for the non-OC group. An equal mean number of 1.9 embryos was replaced in both treatment groups; implantation rates were 23.4 and 30.4% in the OC and non-OC groups, respectively ($P = 0.03$). In the OC group, three subjects (5.2%) with a vital pregnancy experienced a miscarriage compared with six (7.7%) in the non-OC group. The ongoing pregnancy rate per started stimulation cycle was 26.3 and 35.7% in the OC and non-OC groups, respectively ($P = 0.05$, adjusted for age class and region).

There were in total five cases of OHSS, one moderate and one severe case in the OC group and three moderate cases in the non-OC group.

Predictive factors

Predictive factors for number of oocytes retrieved

Cycle 1 data were less predictive for the number of oocytes retrieved than cycle 2 data. AMH was the only predictive factor that appeared in all models regardless of the cycle or treatment group. In the cycle 1 model, the goodness-of-fit measure R^2 was 0.33, whereas in cycle 2, these values were 0.41 and 0.46 in the OC group and in the non-OC group, respectively. Predictive factors in cycle 1 were AMH

Table II Assessments of hormones and ultrasound scan.

	Cycle 1, median, (P5, P95)	Cycle 2, median, (P5, P95)	Inter-cycle variation, (SD)	Correlation, ρ (95% CI)
Non-OC group				
Serum AMH (ng/ml)	1.84 (0.4, 4.3), $n = 136$	1.91 (0.5, 4.5), $n = 137$	0.45	0.88 (0.83–0.91)
AFC	12.0 (4, 22), $n = 196$	11.0 (3, 22), $n = 198$	3.16	0.67 (0.58–0.74)
Serum FSH (IU/l)	6.16 (3.8, 9.8), $n = 189$	6.67 (4.2, 10.4), $n = 173$	1.33	0.63 (0.53–0.71)
Serum E_2 (pmol/l)	110.5 (59, 212), $n = 189$	100.6 (59, 196), $n = 173$	34.5[a]	0.54 (0.42–0.64)
Serum inhibin B (pg/ml)	49.8 (13, 108), $n = 189$	47.9 (14, 95), $n = 173$	18.2	0.53 (0.42–0.63)
Serum T (nmol/l)	1.04 (<0.4, 2.1), $n = 189$	1.11 (0.4, 2.6), $n = 173$	0.26	0.84 (0.79–0.88)
Serum LH (IU/l)	4.58 (2.1, 8.5), $n = 189$	4.97 (2.2, 9.1), $n = 173$	1.27	0.56 (0.45–0.66)
Total ovarian volume (ml)	11.98 (5.6, 24.3), $n = 190$	10.92 (5.4, 22.7), $n = 194$	4.27	0.48 (0.36–0.58)
OC group				
Serum AMH (ng/ml)	2.16 (0.5, 6.3), $n = 143$	2.11 (0.6, 4.6), $n = 145$	0.51	0.90 (0.87–0.93)
AFC	11 (5, 23), $n = 208$	10 (5, 21), $n = 207$	2.89	0.67 (0.59–0.74)
Serum FSH (IU/l)	6.16 (4.2, 10.1), $n = 202$	7.28 (3.2, 14.0), $n = 179$	2.42	0.42 (0.29–0.53)
Serum E_2 (pmol/l)	103.9 (63, 208), $n = 202$	101.3 (<50, 255), $n = 179$	51.8[b]	0.25 (0.10–0.38)
Serum inhibin B (pg/ml)	49.6 (13, 97), $n = 202$	53.1 (<10, 140), $n = 179$	30.4	0.35 (0.21–0.47)
Serum T (nmol/l)	1.15 (0.4, 2.6), $n = 202$	0.91 (<0.4, 2.5), $n = 179$	0.31	0.79 (0.73–0.84)
Serum LH (IU/l)	4.57 (2.4, 7.6), $n = 202$	4.81 (1.7, 9.4), $n = 179$	1.48	0.48 (0.35–0.58)
Total ovarian volume (ml)	10.34 (4.8, 20.2), $n = 200$	8.87 (4.3, 18.4), $n = 201$	4.03	0.52 (0.41–0.61)

AFC, antral follicle count; AMH, anti-Müllerian hormone; CI, confidence interval; E_2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; OC, oral contraceptive; T, testosterone; ρ, Spearman correlation coefficient.
[a]Four extreme values (E_2>600 pmol/l) were omitted for calculation of inter-cycle variation.
[b]One extreme value (E_2>600 pmol/l) was omitted for calculation of inter-cycle variation.

($P < 0.001$), FSH ($P < 0.001$), AFC ($P = 0.003$) and age at menarche ($P = 0.009$). Predictive factors in cycle 2 were AMH ($P < 0.001$) and FSH ($P < 0.001$) in the non-OC group and AMH ($P < 0.001$), FSH ($P = 0.003$), ovarian volume ($P = 0.02$) and age ($P = 0.04$) in the OC group.

Predictive factors for low (<6 oocytes) and high (>18 oocytes) ovarian response

Table IV presents the final logistic regression models of low and high ovarian response, respectively. The best predictive models were obtained for the non-OC group just prior to the start of stimulation (cycle 2) both for low and high ovarian response (Table IV).

To discriminate low ovarian response (<6 oocytes) from normal or high ovarian response (≥6 oocytes), the cycle 2 model of the non-OC group included serum AMH ($P < 0.001$), smoking ($P = 0.04$) and serum FSH ($P = 0.05$) as significant factors (AUC = 0.90; Fig. 5A). In the OC group, the final cycle 2 model for low ovarian response included only AMH as a predictive factor (AUC = 0.84). When, in the non-OC group, only AMH was included in the cycle 2 model, the AUC was 0.88, whereas this value was 0.84 in cycle 1 for the combined groups.

To discriminate high ovarian response (>18 oocytes) from normal or low ovarian response (≤18 oocytes), the cycle 2 model of the non-OC group included serum AMH ($P = 0.001$) and serum FSH ($P = 0.002$) as significant factors (AUC = 0.86; Fig. 5B). In the OC group, the cycle 2 model for high ovarian response included AMH and FSH as predictive factors (AUC = 0.78). When only AMH was included in the cycle 2 model, the AUC was 0.82 in the non-OC group and 0.74 in the OC group, whereas this value was 0.77 in cycle 1 for the combined groups.

AMH levels in subjects with low, normal and high ovarian response

Using cycle 2 data of subjects in the non-OC group, AMH showed a positive association with ovarian response (see Fig. 6). The median AMH levels were 0.88 ng/ml in the low responders ($n = 16$), 1.92 ng/ml in the normal responders ($n = 90$) and 3.32 ng/ml in the high responders ($n = 25$). FSH showed a negative association with ovarian response. Median serum FSH levels were 8.07 ($n = 17$), 6.69 ($n = 116$) and 5.41 ($n = 33$) IU/l in the low, normal and high responder groups, respectively.

Predictive factors for number of follicles (≥11 mm)

For the number of follicles at stimulation day 8, the final model included AFC ($P < 0.001$), serum FSH ($P < 0.001$) and serum AMH ($P = 0.001$) as significant predictive factors ($R^2 = 0.54$). For the number of follicles at the day of hCG, the final model included AFC ($P < 0.001$), FSH ($P < 0.001$), serum AMH ($P = 0.001$) and age of menarche ($P = 0.002$) as significant predictive factors ($R^2 = 0.52$). These models were based on the cycle 2 data of the non-OC group. The follicular response increased with lower FSH and with higher AFC, AMH and age of menarche.

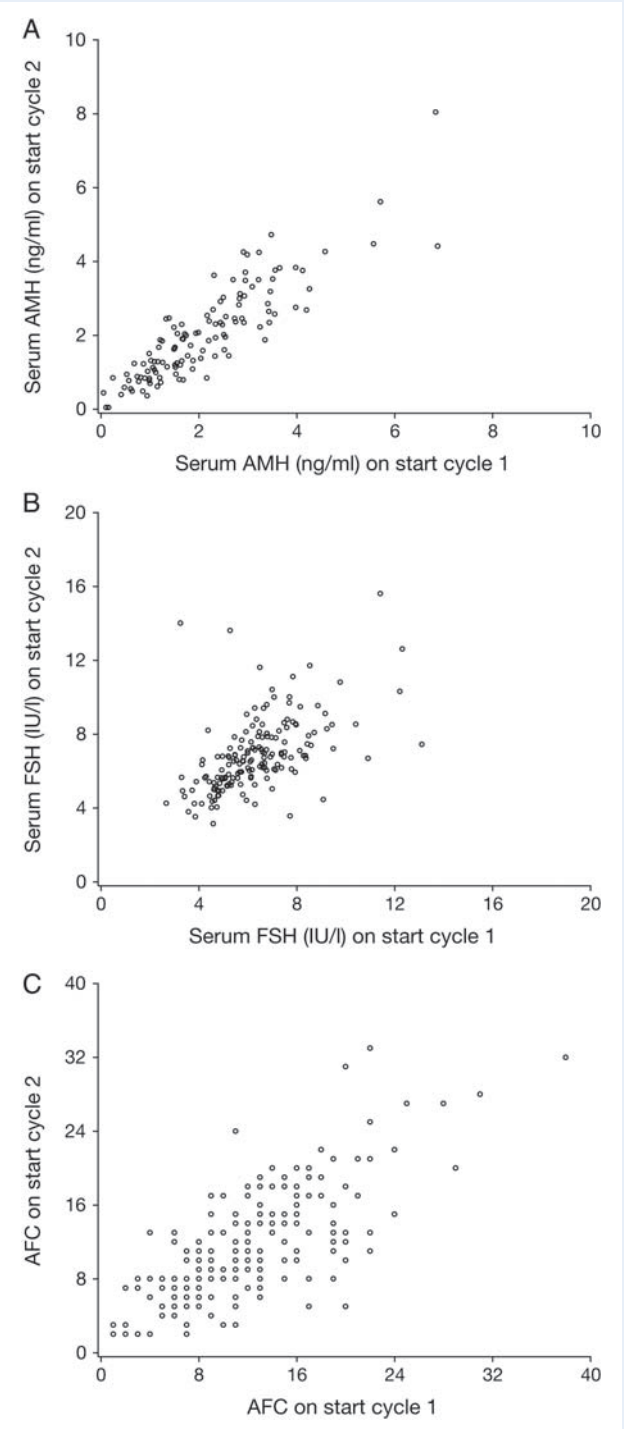

Figure 3 Serum AMH level (ng/ml) (**A**), serum FSH level (IU/l) (**B**) and AFC (**C**) on start cycle 2 versus start cycle 1 in the non-OC group (ITT). AFC, antral follicle count, AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; ITT, intent to treat; OC, oral contraceptive.

Predictive factors for ongoing pregnancy

The overall ongoing pregnancy rate per started cycle is presented in Table III. The final model at randomization (cycle 1) of the combined OC and non-OC groups included FSH ($P = 0.02$), ovarian volume ($P = 0.03$), inhibin B ($P = 0.04$) and smoking ($P = 0.05$) as predictive factors, while at stimulation day 1 (cycle 2), it only included FSH ($P = 0.01$) as a predictive factor. Note that the treatment group was not a statistically significant factor in these models ($P > 0.05$). The area under the ROC curve was 0.66 and 0.59 at cycles 1 and 2, respectively, which was considered to be low in the sense that (ongoing) pregnancy cannot be reliably predicted for individual subjects.

Table III Clinical outcome per started cycle.

	OC ($n = 209$)	Non-OC ($n = 199$)
Duration of stimulation, days, median (P5, P95)[a]	10.0 (8, 12)	9.0 (7, 12)
Total rFSH dose, IU, median (P5, P95)	1800.0 (1300, 2400)	1600.0 (1200, 2200)
Follicles ≥ 11 mm at Day 8, mean (SD)	9.9 (5.4)	10.6 (5.7)
Follicles ≥ 11 mm at day of hCG, mean (SD)[a]	13.2 (5.9)	12.6 (5.9)
Serum E_2 at the day of hCG, pmol/l, median (P5, P95)[a]	4624 (1585, 11120)	4954 (1897, 9982)
Oocytes, mean (SD)	12.4 (6.7)	12.1 (7.7)
Mature oocytes, mean (SD)	9.9 (5.7)	9.4 (6.4)
2PN fertilized oocytes, mean (SD)[b]	7.7 (4.9)	7.4 (5.7)
Embryos, mean (SD)[b]	8.5 (5.0)	8.5 (6.3)
Good quality embryos, mean (SD)[b]	4.4 (3.6)	4.8 (4.9)
Embryos transferred, mean (SD)[c]	1.9 (0.4)	1.9 (0.5)
Implantation rate, %[c,d]	24.1	30.1
Biochemical pregnancy rate, *n* (%)	71 (34.0)	101 (50.8)
Clinical pregnancy rate, *n* (%)	62 (29.7)	86 (43.2)
Ongoing pregnancy rate, *n* (%)[e]	55 (26.3)	71 (35.7)

ET, embryo transfer; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; OC, oral contraceptive; 2PN, pro-nucleate; rFSH, recombinant follicle-stimulating hormone.
[a]Restricted to subjects with hCG administration for final oocyte maturation.
[b]Restricted to subjects with IVF and/or ICSI.
[c]Restricted to subjects with ET.
[d]$P = 0.03$, adjusted for region (Europe, USA) and age category (≤32 and >32 years).
[e]$P = 0.05$, adjusted for region (Europe, USA) and age category (≤32 and >32 years).

Discussion

In this prospective trial examining potential predictive factors of ovarian response in a GnRH antagonist protocol, AMH appeared to be an important predictor for the number of oocytes retrieved, whereas AFC was one of the identified predictors for the number

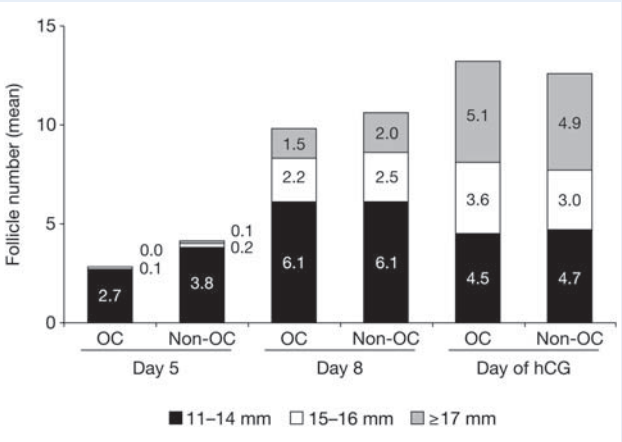

Figure 4 Mean number of follicles per stimulation day, treatment group and follicle size category.

of follicles ≥11 mm on the day of hCG. However, it should be noted that the AFC variability in this multicentre trial may have been increased due to the different operators performing the ultrasound measurements. While both factors are direct indicators of ovarian reserve and highly linked to each other, AMH is produced by both antral and pre-antral follicles, while the latter are not included in the AFC (La Marca et al., 2010).

Predictive factors, including demographic, sonographic and endocrine factors, are often obtained prior to the treatment cycle, and are used to determine the best rFSH-starting dose (Olivennes et al., 2009; Popovic-Todorovic et al., 2003a; Popovic-Todorovic et al., 2003b) or the best treatment regimen, i.e. GnRH antagonist versus long GnRH agonist protocol (Nelson et al., 2009). However, the findings of the current study indicated that several of these potential predictive factors, including AFC, FSH and inhibin B, were affected by cycle-to-cycle variability. Accordingly, in the current study, the best predictive models were obtained when using predictors measured at stimulation day 1 of the actual treatment cycle.

Of the candidate predictive factors, serum AMH was found to have the lowest inter-cycle variability, a finding that is consistent with previous studies indicating that serum AMH samples may be taken at any time point prior to the actual IVF treatment cycle (McIlveen et al., 2007). Moreover, this study confirmed that AMH levels remain unmodified due to OC pre-treatment as previously described for pituitary-suppressed patients using GnRH analogues or OC (La Marca et al., 2010). Although our final predictive models often indicated more significant predictive factors than AMH, our data also demonstrate that AMH alone is a reliable predictor of ovarian response. An added value of baseline FSH in the predictive model was observed only in the non-OC group when determined at the early follicular phase of the stimulation cycle.

The predictive factors identified for ovarian response in the current GnRH antagonist protocol were similar to previous reported factors for a long GnRH agonist protocol as reviewed by Broekmans et al. (2006) and Verhagen et al. (2008). However, with the exception of AMH, the cut-off values for high or low ovarian response of those factors may be different under pituitary suppression, and predictive models cannot be extrapolated from antagonist to agonist protocols or *vice versa* (Pinto et al., 2009).

Table IV Final logistic regression models of low ovarian response (<6 oocytes) and high ovarian response (>18 oocytes), respectively.

Cycle	Treatment group	AUC: Model including AMH only	AUC: Model including all predictive factors	Identified predictive factors
Low ovarian response (<6 oocytes)				
1	OC + non-OC combined	0.84	0.85	AMH ($P < 0.001$) Smoking ($P = 0.05$)
2	OC	0.84	0.84	AMH ($P < 0.001$)
	Non-OC	0.88	0.90	AMH ($P < 0.001$) Smoking ($P = 0.04$) FSH ($P = 0.05$)
High ovarian response (>18 oocytes)				
1	OC + non-OC combined	0.77	0.80	AMH ($P < 0.001$) AFC ($P = 0.03$) FSH ($P = 0.03$)
2	OC	0.74	0.78	AMH ($P < 0.001$) FSH ($P = 0.03$)
	Non-OC	0.82	0.86	AMH ($P < 0.001$) FSH ($P = 0.002$)

AUC, area under the curve of the receiver operating characteristics (ROC) curve.

Previous studies have arbitrarily defined an appropriate ovarian response as retrieval of 5–14 oocytes (Popovic-Todorovic et al., 2003a). Initially, AMH measurements were applied to predict poor ovarian response, and different AMH assays and definitions of poor response were reported ranging between a cut-off value of two and six oocytes. In the largest prospective study by Nelson et al. (2007), poor response was defined as two or less oocytes, whereas excessive response was 21 or more oocytes (Nelson et al., 2007). In the present study, an appropriate response was defined as the retrieval of at least six oocytes and a maximum of 18 oocytes. The rationale for this definition is the observation that pregnancy rates decrease only when <6 oocytes are retrieved (Sharma et al., 2002). In accordance with this, the large National Dutch study of van der Gaast et al. (2006) showed that the increase in pregnancy rates in relation to the number of oocytes increased markedly from one to five oocytes, whereas the increase continued but was more moderate above six

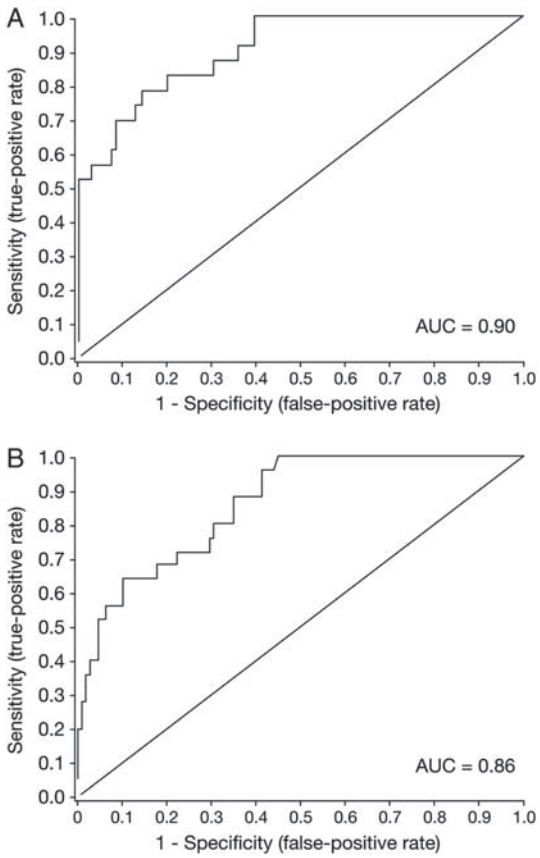

Figure 5 Receiver operating characteristic (ROC) curve of the stimulation cycle prediction model for low ovarian response [<6 oocytes, (**A**) and high ovarian response (>18 oocytes, (**B**))] in the non-OC group. AUC, area under the curve.

oocytes (van der Gaast et al., 2006). Regarding the upper limit of appropriate response, the risk of OHSS increases greatly when more than 18 antral follicles or oocytes are obtained (Papanikolaou et al., 2006).

In a study by Olivennes et al. (2009), a wide range of FSH doses from 75 to 225 IU/day were applied using a dosing algorithm, including four predictive factors, i.e. age, FSH, BMI and AFC. Overall, in this rFSH dosing trial, a median number of nine oocytes were retrieved and the average per FSH dose group varied between 8 and 12 oocytes. This study gives additional evidence that predictive factors may be used to dose in order to obtain appropriate responses.

As reviewed by La Marca et al. (2010), a total of nine earlier studies have compared AMH with AFC in order to predict ovarian response. Five of the studies found that AFC and AMH had a correlation similar to the number of oocytes retrieved, whereas four other studies indicated that AMH was either less good or better. In accordance with our findings, Nelson et al. (2007) showed that AMH was the best predictor compared with age and FSH, but they did not include AFC measurements. More recently in a prospective, non-randomized trial, the same group demonstrated that AMH levels could be successfully applied as single parameter to determine the starting dose of FSH as well as the application of a GnRH antagonist instead of GnRH agonist protocol (Nelson et al., 2009).

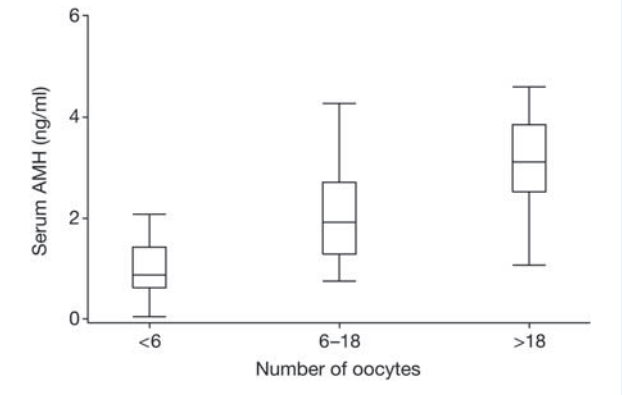

Figure 6 Serum anti-Müllerian hormone (AMH) levels in low, average and high responders. Restricted to cycle 2 data of subjects in the non-OC group who had oocyte pick-up. Figure presents box-plots with median values and the 5th, 25th, 75th and 95th percentiles of serum AMH.

Like the current trial, a recent prospective study showed a significant correlation between basal AMH concentrations and extremes of response in an unselected population of women undergoing their first cycle of ovarian stimulation for IVF (Nardo et al., 2009). Unlike Nelson and colleagues, they included AFC in ROC analyses and demonstrated that AMH is a superior predictor of excessive ovarian response, which enables clinicians to identify women at risk of OHSS better than AFC.

In the current study, some difficulties were encountered during the analyses of serum AMH levels, which may have influenced the results. AMH was measured with three different lots from the same commercial kit, but application of in-house control samples indicated a large inter-lot variability. When tested, the inclusion of the factor AMH lot had a statistically significant impact on the model for the number of oocytes retrieved ($P = 0.01$; non-OC group, cycle 2). As both samples from individual subjects were tested within the same lot, the inter-lot variability did not affect the estimation of the inter-cycle variation in the OC and non-OC groups. However, because of this variability, only the predictive ability of the candidate predictive factors were evaluated and no thorough validation of the models was performed to provide model prediction errors.

In general, the choice of possible AMH cut-off values to predict low, normal or high ovarian response requires consideration when assessment of any inter-lot variability is not part of the assay validation. Methodological problems with the DSL assay have been reported before (Bersinger et al., 2007; La Marca et al., 2010; Streuli et al., 2009) but should be resolved with the launch of the AMH Gen II Elisa (Beckman Coulter Inc., Brea, CA, USA). If so, serum AMH levels of low, normal and high responders may show less overlap and the predictive value of AMH may further increase.

The current study was not designed to detect a clinical relevant difference in ongoing pregnancy rate between OC and non-OC treatment. Randomization to OC versus non-OC treatment was performed since OC pre-treatment is more often applied by US clinics than by European clinics, which prevents bias due to regional differences.

Nevertheless, due to the randomized design of the study, the comparison between the two treatment groups is valid and showed a significantly lower implantation rate and correspondingly lower ongoing pregnancy rate after OC pre-treatment. The possible impact of OC pre-treatment in a GnRH antagonist protocol was first reported in 2006 (Huirne et al., 2006; Kolibianakis et al., 2006; Rombauts et al., 2006) just prior to the start of the current study. Although OC pre-treatment was thought to improve follicular homogeneity and increase the number of oocytes and good quality embryos, a tendency for lower implantation rates was observed in OC recipients. A first meta-analysis (Griesinger et al., 2008) of randomized controlled trials confirmed this trend, and a second meta-analysis after completion of the current trial indicated that the probability of an ongoing pregnancy per randomized subject was significantly lower in those receiving OC ($P = 0.02$) (Griesinger et al., 2010).

Although OC pre-treatment is a convenient way for clinics to schedule oocyte retrievals, it reduces the advantages of a GnRH antagonist protocol by extending the duration of treatment and the amount of FSH required to reach the same criteria of hCG (Rombauts et al., 2006), especially when stimulation is started shortly after OC discontinuation. In the current study, the time interval between the last dose of OC intake and the start of stimulation was 5 days, and all patients had onset of menses before starting COS. Circulating hormone levels measured on stimulation day 1 indicated that 5 days after the last dose of OC, pituitary suppression due to OC pre-treatment was mainly reversed. Despite this, the present study shows that the overall predictive value of sonographic and endocrine tests were less good after OC pre-treatment. The latter facilitated scheduling of oocyte retrieval and did not affect ovarian response, number of oocytes or embryo quality, compared with the results obtained from non-OC subjects. However, it appears that the implantation rate is reduced by OC pre-treatment, whereas no effect was observed on the number or quality of oocytes and embryos. Further research is required to examine whether OC treatment may have any carry-over effect on the endometrial development in the following stimulation cycle.

In conclusion, serum AMH and basal FSH were significant factors for the prediction of the number of oocytes retrieved as well as for the prediction of high responders (>18 oocytes). In low responders (<6 oocytes), only AMH was a significant factor. AFC, basal FSH and AMH were significant factors for the prediction of the number of follicles ≥11 mm on the day of hCG. AMH showed less cycle-to-cycle variation than basal FSH and AFC. The use of AMH or AFC, as a single test or in combination, for the prediction of ovarian response will prevent cycle cancellations due to too low or too high ovarian response and reduce the risk of OHSS. The final challenge will be to construct a reliable and simple algorithm that will enable clinicians to choose for each patient the best treatment protocol, which should prospectively reduce cancellation rates and therefore improve the success rates per started cycle.

Authors' roles

A.N.A. was the lead investigator of this multicentre trial and contributed to the trial design and interpretation of data. H.W. was primarily responsible for the data analyses. K.G. contributed to the trial design and interpretation of data and B.M. was responsible for the scientific content of the trial protocol and report. All four authors contributed equally to the intellectual content of this manuscript.

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Conflict of interest

A.N.A. has been an investigator for trials initiated by Merck-Serono and Ferring. H.W., K.G. and B.M. are employees of Merck, Sharp and Dohme & Co.

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Appendix

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ARTICLE

No association between endogenous LH and pregnancy in a GnRH antagonist protocol: part I, corifollitropin alfa

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Kevin Doody, MD, received his medical degree and served a residency in obstetrics and gynaecology at Baylor College of Medicine in Houston. After completing his residency, Dr Doody received subspecialty training at UT Southwestern Medical Center. He completed his fellowship in reproductive endocrinology/infertility in 1989. He is board certified in obstetrics and gynaecology and subspecialty board certified in reproductive endocrinology/infertility. Dr Doody has been repeatedly honoured as one of Fort Worth's Top Docs, one of Texas's Super Doctors and one of the Best Doctors in America. Dr Doody was also recognized as Microsoft Physician of the Year 2004.

Abstract The relationship between endogenous LH concentrations and ongoing pregnancy rates among normogonadotrophic patients undergoing ovarian stimulation in a gonadotrophin-releasing hormone antagonist protocol were examined. In the Engage trial, 1506 patients received corifollitropin alfa (150 μg) or daily recombinant FSH (rFSH) (200 IU) for the first 7 days of stimulation with 0.25 mg ganirelix from stimulation day 5. Patients were retrospectively stratified by serum LH percentiles (<25th, 25th–75th and >75th) on stimulation day 8 and day of human chorionic gonadotrophin administration. Odds ratios (OR) with and without adjustment for predictive factors for ongoing pregnancy were estimated. LH concentration was not associated with pregnancy rates in either treatment arm, in contrast to ovarian response and serum progesterone. With adjustment for these predictors and age, OR (95% confidence interval) for ongoing pregnancy on stimulation day 8 for LH categories <P25 versus ≥P25, >P75 versus ≤P75 and <P25 versus >P75 were 0.75 (0.53–1.06), 1.26 (0.87–1.83) and 0.70 (0.46–1.09) in the corifollitropin alfa arm and 0.80 (0.54–1.17), 1.28 (0.87–1.87) and 0.73 (0.46–1.16) in the rFSH arm respectively. There was also no significant difference in pregnancy rates between LH categories on day of human chorionic gonadotrophin administration with either treatment.

KEYWORDS: endogenous LH, Engage trial, GnRH antagonist, pregnancy rates

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Introduction

The role of LH during ovarian stimulation in women less than 37 years old is not completely understood and contradictory evidence exists as to whether or not profound suppression of endogenous LH affects IVF/intracytoplasmic sperm injection (ICSI) outcomes (Balasch et al., 2001; Esposito et al., 2001; Humaidan et al., 2002; Merviel et al., 2004; Westergaard et al., 2000). Patients with hypogonadotrophic hypogonadism (World Health Organization group I) require the administration of a daily low amount (75 IU) of exogenous LH activity, as recombinant LH, human chorionic gonadotrophin (HCG) or human menopausal gonadotrophin, for adequate follicular and endometrial development (Burgués, 2001; Filicori et al., 1999; The European Recombinant Human LH Study Group, 1998).

Research regarding the need for LH add-back in normogonadotrophic patients should distinguish between long gonadotrophin-releasing hormone (GnRH) agonist protocols, which induce profound pituitary suppression prior to stimulation, and GnRH antagonist protocols, in which normal to partially suppressed endogenous LH concentrations are observed during stimulation. Most published studies on LH requirement are based on retrospective analyses of low endogenous LH concentrations during stimulation using a long GnRH agonist protocol (Balasch et al., 2001; Fleming et al., 1998; Westergaard et al., 2000). In both GnRH agonist and antagonist cycles, supplementation with LH activity enhances androgen and oestrogen biosynthesis. Patients receiving LH/HCG supplementation have higher oestradiol concentrations per growing follicle than patients stimulated with FSH only (Bosch et al., 2008; Smitz et al., 2007; Tarlatzis et al., 2006). In addition, it has been shown that the use of exogenous HCG may reduce or even replace the requirements for FSH administration during the last days of stimulation (Blockeel et al., 2009; Filicori et al., 2005).

Previous studies using GnRH antagonist protocols showed that low endogenous LH concentrations during recombinant FSH (rFSH) stimulation (Bosch et al., 2005; Kolibianakis et al., 2004; Merviel et al., 2004) or supplementation with recombinant LH (Baruffi et al., 2007; Griesinger et al., 2005) or human menopausal gonadotrophin (Bosch et al., 2008) do not affect pregnancy rates. One prospective study and LH analysis of patients treated with an rFSH/GnRH antagonist protocol suggested that those patients with the lowest endogenous LH concentrations on stimulation day 8 had the highest pregnancy rate (Kolibianakis et al., 2004). A recent univariate analysis indicated that low endogenous LH concentrations as measured on stimulation days 1, 5 and 8 in 750 patients treated with daily rFSH in a GnRH antagonist protocol did not adversely affect ongoing pregnancy rates (Doody et al., 2010).

The present retrospective analysis of a large, randomized controlled trial (Devroey et al., 2009) examines the association between endogenous LH concentrations and ongoing pregnancy rates in corifollitropin alfa and rFSH treatment groups.

Materials and methods

Ongoing pregnancy rates relative to endogenous serum LH concentrations during ovarian stimulation were retrospectively analysed from the Engage trial, the details of which have been reported previously (Devroey et al., 2009). Briefly, women aged 18–36 years with bodyweight from >60 kg to ≤90 kg and a regular menstrual cycle received a single dose of 150 μg corifollitropin alfa (Elonva; N.V. Organon, The Netherlands) or daily 200 IU rFSH (Puregon/Follistim pen; N.V. Organon) for the first 7 days of ovarian stimulation. From stimulation day 8 onwards, treatment in both groups was continued as needed with a daily s.c. dose of ≤200 IU rFSH up to and including the day of HCG administration. GnRH antagonist ganirelix (0.25 mg, Orgalutran/Ganirelix Acetate Injection; N.V. Organon) was administered once daily s.c. starting on stimulation day 5 up to and including the day of HCG injection. Urinary HCG (10,000 IU or 5000 IU) was administered to induce final oocyte maturation when three follicles ≥17 mm were observed by ultrasound scan or the next day. Oocyte retrieval was performed 34–36 h later, followed by either IVF or ICSI (Devroey et al., 2009).

Blood samples were drawn in the morning just prior to GnRH antagonist and gonadotrophin injections and the serum was immediately stored at −20°C until analysis of FSH, LH, oestradiol and progesterone concentrations.

Validated immunoassays were performed to measure serum concentrations of FSH, LH, oestradiol and progesterone at stimulation days 1, 5, 8 and day of HCG administration. All hormone measurements were determined at one central laboratory (Waltrop, Germany) using a time-resolved fluoroimmunoassay (AutoDelfia immunofluorometric assay; PerkinElmer Life and Analytical Sciences, Brussels, Belgium). Detection limits for serum FSH, LH, oestradiol and progesterone were 0.25 IU/l, 0.6 IU/l, 50 pmol/l and 1.3 nmol/l (0.4 ng/ml) respectively. The intra- and inter-assay variabilities of these assays were <5% and <10%, respectively.

Statistical analysis

All analyses were performed for the intention-to-treat (ITT) population. The ITT population includes all randomized subjects who received one or more dose(s) of corifollitropin alfa or rFSH. Subjects were grouped according to the treatment they were randomized to. Ongoing pregnancy rates were analysed per started cycle: subjects in the ITT group whose IVF cycles were cancelled were included and considered not pregnant in the statistical analyses.

Patients were stratified by LH percentiles to examine the LH effect on ongoing pregnancy rates. They were divided into three groups: below the 25th LH percentile (<P25), between the 25th and 75th LH percentiles (P25–P75) and above the 75th LH percentile (>P75). LH concentration was analysed as a three-level class variable and not as a continuous variable because almost a quarter of the LH measurements on stimulation day 8 and day of HCG were below the lower limit of quantification. Patients without LH measurements were excluded from the analyses.

Overall differences in baseline characteristics, stimulation characteristics and ovarian response between the <P25, P25–P75 and >P75 groups of patients were tested using either analysis of variance (ANOVA; for comparing means) or the Kruskal–Wallis test (for comparing medians).

The effect of treatment (corifollitropin alfa, rFSH) on the LH concentrations on stimulation days 5 and 8 and day of HCG was tested using the Mann–Whitney *U*-test.

Differences in ongoing pregnancy rates between the <P25, P25–P75 and >P75 groups of patients were analysed using PROC GENMOD in SAS version 9.1 (SAS Institute, Cary, NC, USA). A logistic regression model between ongoing pregnancy rate and LH concentration (<P25, P25–P75, >P75) was fitted for each combination of treatment group (corifollitropin alfa, rFSH) and day (stimulation day 8 and day of HCG). Separate models for the treatment groups were applied because the results for the rFSH group will be included in the combined analysis described in part II of this article (Griesinger et al., 2011). *P*-values of the LH effect on ongoing pregnancy rate were derived using the likelihood ratio test. Maximum likelihood estimates of odds ratios (OR) and associated two-sided 95% confidence intervals (CI) were computed for <P25 versus ≥P25 (low versus not-low LH), >P75 versus ≤P75 (high versus not-high LH) and <P25 versus >P75 (low versus high LH). The *P*-values and estimated OR and CI were computed with and without adjustment for predictive factors of ongoing pregnancy.

Age was selected as one of the predictive factors of ongoing pregnancy as it is well known that the chance of pregnancy is age related. Stepwise logistic regression analysis was applied to identify the other predictive factors per treatment group and stimulation day. Candidate predictive factors entered in the model were age, serum FSH concentration on stimulation day 1, antral follicle count (AFC) on stimulation day 1 and number of oocytes retrieved. Progesterone concentration and number of follicles ≥11 mm on stimulation day 8 were added to the above factors to identify the predictive factors for ongoing pregnancy for OR adjustment at stimulation day 8, while progesterone concentration and number of follicles ≥11 mm on day of HCG were added to identify the predictive factors for OR adjustment on day of HCG. The significance level of the candidate predictive factors to enter the model was set to 0.15 and to stay in the model was set to 0.10. The final model selects the predictive factors with statistical significance, i.e. $P \leq 0.05$. The predictive factors were added as independent covariates to the model to find the adjusted estimates of LH effect. Only additive models were considered to identify predictive factors.

Results

Baseline characteristics by LH category

Table 1 presents the baseline characteristics of patients treated with either corifollitropin alfa or rFSH for the first 7 days of ovarian stimulation, divided into three groups: low (<P25), medium (P25–P75) and high (>P75) serum LH concentrations on stimulation day 8. Patients with higher serum LH concentrations were more likely to demonstrate markers of diminished ovarian reserve (AFC, FSH). These

Table 1 Demographic and other baseline characteristics by treatment group and LH on stimulation day 8.

Characteristic	*LH percentile category*			*P-value*
	<P25	*P25–P75*	*>P75*	
Corifollitropin alfa (*n*)	216[a]	316	176	–
Age (years)	31.3 ± 3.3	31.4 ± 3.4	31.9 ± 3.3	NS
Body mass index (kg/m^2)	24.5 ± 2.6	24.9 ± 2.9	25.1 ± 2.7	≤0.05
Bodyweight (kg)	68.2 ± 7.0	68.9 ± 7.9	69.3 ± 7.6	NS
Serum LH on day 1 (IU/l; median (P5, P95))	4.0 (2.1, 7.0)	4.5 (2.4, 7.9)	4.9 (2.5, 9.8)	≤0.05
Serum FSH on day 1 (IU/l; median (P5, P95))	6.1 (3.9, 8.9)	6.3 (4.5, 9.6)	6.9 (4.5, 13.1)	≤0.05
Antral follicles <11 mm	12.8 ± 5.0	12.7 ± 4.3	11.4 ± 4.2	≤0.05
Duration of infertility (years)	3.1 ± 2.3	3.5 ± 2.5	3.4 ± 2.6	NS
Primary infertility	55.6	51.9	55.1	NS
Secondary infertility	44.4	48.1	44.9	NS
Recombinant FSH (*n*)	169	340	169	–
Age (years)	30.9 ± 3.4	31.6 ± 3.1	32.0 ± 3.4	≤0.05
Body mass index (kg/m^2)	24.6 ± 2.7	24.9 ± 2.8	25.1 ± 2.5	NS
Bodyweight (kg)	68.1 ± 7.3	68.5 ± 7.4	68.8 ± 7.0	NS
Serum LH on day 1 (IU/l; median (P5, P95))	4.2 (2.1, 7.0)	4.4 (2.3, 8.0)	4.8 (2.7, 8.6)	≤0.05
Serum FSH on day 1 (IU/l; median (P5, P95))	6.4 (3.9, 9.5)	6.4 (4.4, 9.9)	6.5 (4.9, 10.9)	≤0.05
Antral follicles <11 mm	12.4 ± 4.4	12.6 ± 4.6	12.1 ± 4.4	NS
Duration of infertility (years)	3.1 ± 2.2	3.3 ± 2.3	3.2 ± 2.0	NS
Primary infertility	52.7	53.5	50.9	NS
Secondary infertility	47.3	46.5	49.1	NS

Values are mean ± standard deviation or %, unless otherwise stated.
≤0.05 indicates unequal means or medians among the three LH categories.
NS = not statistically significant; <P25 = patients below the 25th LH percentile; P25–P75 = patients between the 25th and 75th LH percentiles; >P75 = patients above the 75th LH percentile.
[a]On stimulation day 8, >25% of patients treated with corifollitropin alfa had a value below the lower limit of quantification and were all included in the <P25 category.

Table 2 LH concentrations in LH percentile categories per treatment group and stimulation day.

Stimulation day	*LH percentiles*				
	P5	*P25*	*P50*	*P75*	*P95*
Corifollitropin alfa					
Day 1	2.28	3.39	4.48	5.82	8.11
Day 5	<0.6	1.18	2.04	4.19	12.5
Day 8	<0.6	<0.6	0.96	1.58	3.07
Day of HCG	<0.6	<0.6	1.00	1.77	3.53
Recombinant FSH					
Day 1	2.30	3.38	4.41	5.64	7.95
Day 5	<0.6	0.93	1.46	2.31	6.60
Day 8	<0.6	0.91	1.57	2.66	5.27
Day of HCG	<0.6	0.75	1.39	2.57	4.92

Values are IU/l.
HCG = human chorionic gonadotrophin.
Median LH concentrations differed ($P \leq 0.05$) between the two treatment groups on stimulation day 5, stimulation day 8 and day of HCG.

patients were older and had lower AFC and higher FSH (stimulation day 1) than patients with medium or low serum LH concentrations. There was no difference between the three groups with respect to the cause of infertility (data not shown), the duration of infertility or the incidence of primary infertility (**Table 1**).

Serum LH concentrations during treatment with corifollitropin alfa or daily rFSH

Table 2 presents the serum LH percentiles (P5, P25, P50, P75 and P95) in each treatment group at stimulation days 1, 5 and 8 and day of HCG administration. Serum LH concentrations were higher at stimulation day 5 in the corifollitropin alfa group than in the rFSH group, whereas the reverse was observed at stimulation day 8 and day of HCG administration (**Figure 1**). The difference in median LH concentration between the corifollitropin alfa group and the rFSH group was significant ($P \leq 0.05$) on stimulation day 5, stimulation day 8 and day of HCG, respectively (**Table 2**). At stimulation day 8, the P50 (median) value was 0.96 IU/l in the corifollitropin alfa group and 1.57 IU/l in the rFSH group. The P25 values were <0.6 IU/l and 0.91 IU/l and the P75 values were 1.58 IU/l and 2.66 IU/l, respectively.

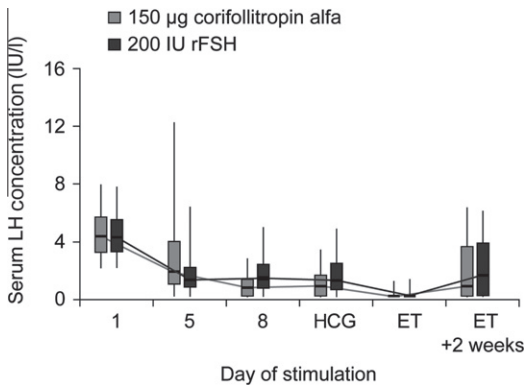

Figure 1 Box plot of serum LH concentrations during and after stimulation with corifollitropin alfa and rFSH. Treatment with the gonadotrophin-releasing hormone antagonist was started for all patients at stimulation day 5. Solid lines = median; boxes = P25—P75; whiskers = P5—P95; ET = embryo transfer; HCG = human chorionic gonadotrophin; rFSH = recombinant FSH.

Stimulation characteristics and ovarian response per LH category

The main stimulation characteristics in the trial per LH category at stimulation day 8 for both the corifollitropin alfa and rFSH arms are shown in **Table 3**. In both treatment groups, patients with lower LH required a slightly longer duration of stimulation and slightly more daily rFSH (50—80 IU) from stimulation day 8 onwards to reach the same HCG criteria. In both treatment groups, fewer follicles ≥11 mm were observed at stimulation day 8 and day of HCG in patients with higher LH concentrations (**Table 4**). Despite this, serum oestradiol and progesterone concentrations of patients in the higher percentile of endogenous LH were higher than in patients with lower LH concentrations. Fewer oocytes were retrieved in patients with higher LH concentrations.

There was no difference in the total number of embryos or the number of good-quality embryos obtained among the low, medium and high LH concentration groups of patients in each treatment group. The number and quality of embryos transferred were very similar among all the different subsets (data not shown).

Identification of predictive factors

Predictive factors of ongoing pregnancy in addition to age were identified using stepwise logistic regression to adjust the estimated OR at stimulation day 8 and day of HCG, respectively. The identified predictive factors to adjust the estimated OR at stimulation day 8 were: (i) serum progesterone on stimulation day 8 (corifollitropin alfa group: OR 0.80, 95% CI 0.68—0.95, $P = 0.01$; rFSH group: OR 0.87, 95% CI 0.75—1.00, $P = 0.05$): a higher progesterone concentration on stimulation day 8 was associated with a lower pregnancy rate in both treatment groups; (ii) number of follicles ≥11 mm on stimulation day 8 (corifollitropin alfa group only: OR 1.03, 95% CI 1.00—1.05, $P = 0.03$): a higher number of follicles was associated with a higher pregnancy rate; and (iii) number of oocytes retrieved (rFSH group only: OR 1.03, 95% CI 1.01—1.06, $P = 0.02$): a higher number of oocytes was associated with a higher pregnancy rate.

On the day of HCG trigger, none of the candidate predictive factors were statistically significant. Age was borderline significant in the rFSH group on day of HCG administration (OR 0.96, 95% CI 0.91—1.00).

Ongoing pregnancy rates according to LH percentiles

In both the corifollitropin alfa and rFSH groups, the ongoing pregnancy rates were similar for patients with low (<P25), medium (P25—75) or high (>P75) endogenous LH

Table 3 Stimulation characteristics by treatment group and LH on stimulation day 8.

Characteristic	*LH percentile category*			P-*value*
	<P25	*P25–P75*	*>P75*	
Corifollitropin alfa (*n*)	216	316	176	–
Total dose of rFSH (IU)	443.6 ± 316.0	407.3 ± 298.9	395.3 ± 317.8	NS
Total dose of rFSH from day 8 onwards (IU)	442.7 ± 315.2	407.3 ± 298.9	376.0 ± 281.4	≤0.05
Duration of stimulation (days)	9.8 ± 1.6	9.6 ± 1.4	9.3 ± 1.8	≤0.05
Recombinant FSH (*n*)	169	340	169	–
Total dose of rFSH (IU)	1811.8 ± 292.5	1742.9 ± 252.3	1728.6 ± 249.5	≤0.05
Total dose of rFSH from day 8 onwards (IU)	424.3 ± 277.1	353.9 ± 244.0	337.5 ± 238.4	≤0.05
Duration of stimulation (days)	9.6 ± 1.3	9.3 ± 1.1	9.2 ± 1.1	≤0.05

All values are mean ± standard deviation.
$P \leq 0.05$ indicates unequal means among the three LH categories.
NS = not statistically significant; rFSH = recombinant FSH; <P25 = patients below the 25th LH percentile; P25–P75 = patients between the 25th and 75th LH percentiles; >P75 = patients above the 75th LH percentile.

Table 4 Ovarian response by treatment group and LH on stimulation day 8.

Ovarian response	*LH percentile category*			P-*value*
	<P25	*P25–P75*	*>P75*	
Corifollitropin alfa (*n*)	216	316	176	–
Follicles on day 8				
≥11 mm	13.8 ± 6.9	13.1 ± 6.6	10.9 ± 6.0	≤0.05
≥15 mm	5.1 ± 5.2	5.2 ± 4.5	4.5 ± 3.9	NS
≥17 mm	2.2 ± 3.6	2.1 ± 2.6	2.0 ± 2.5	NS
Hormones on day 8				
Oestradiol (pmol/l)	2466 (598, 7046)	3068 (1013, 8368)	3397 (818, 10,570)	≤0.05
Progesterone (nmol/l)	1.7 (1.3, 3.2)	1.8 (1.3, 3.5)	2.1 (1.3, 3.9)	≤0.05
Follicles on day of HCG				
≥11 mm	16.6 ± 7.5	16.2 ± 7.0	13.6 ± 7.2	≤0.05
≥15 mm	10.3 ± 5.5	9.6 ± 4.7	8.1 ± 4.4	≤0.05
≥17 mm	6.1 ± 4.1	5.7 ± 3.0	4.8 ± 2.8	≤0.05
Hormones on day of HCG				
Oestradiol (pmol/l)	3743 (1380, 9212)	4881 (1875, 12,111)	6129 (2000, 13,689)	≤0.05
Progesterone (nmol/l)	2.6 (1.3, 7.4)	2.8 (1.3, 5.4)	3.0 (1.4, 5.5)	≤0.05
Oocytes	14.9 ± 8.1	14.1 ± 7.6	12.3 ± 9.2	≤0.05
Recombinant FSH (*n*)	169	340	169	–
Follicles on day 8				
≥11 mm	11.8 ± 6.5	11.8 ± 6.0	10.8 ± 5.0	NS
≥15 mm	4.9 ± 4.4	5.3 ± 4.0	5.0 ± 3.8	NS
≥17 mm	2.0 ± 2.5	2.6 ± 2.8	2.7 ± 2.8	≤0.05
Hormones on day 8				
Oestradiol (pmol/l)	2096 (690, 7524)	3162 (1158, 8936)	3854 (1453, 9982)	≤0.05
Progesterone (nmol/l)	2.1 (1.3, 4.6)	2.4 (1.4, 4.6)	2.7 (1.5, 5.1)	≤0.05
Follicles on day of HCG				
≥11 mm	14.9 ± 6.7	13.7 ± 6.1	12.6 ± 5.5	≤0.05
≥15 mm	9.3 ± 4.5	8.7 ± 4.0	7.8 ± 3.8	≤0.05
≥17 mm	5.9 ± 3.6	5.5 ± 2.7	5.5 ± 2.9	NS
Hormones on day of HCG				
Oestradiol (pmol/l)	3354 (1171, 8588)	4588 (1967, 10,093)	6092 (2567, 11,010)	≤0.05
Progesterone (nmol/l)	2.7 (1.4, 6.0)	3.0 (1.5, 5.4)	3.2 (1.5, 5.4)	≤0.05
Oocytes	13.2 ± 6.9	12.9 ± 7.0	11.5 ± 6.0	≤0.05

Values are mean ± standard deviation or median (P5, P95).
$P \leq 0.05$ indicates unequal means or medians among the three LH categories.
HCG = human chorionic gonadotrophin; NS = not statistically significant; <P25 = patients below the 25th LH percentile; P25–P75 = patients between the 25th and 75th LH percentiles; >P75 = patients above the 75th LH percentile.

Table 5 Ongoing pregnancy rate per started cycle, treatment group, stimulation day and LH.

Stimulation day	*LH percentile category*	*Ongoing pregnancy rate*	
		n/total	*% (95% CI)*
Corifollitropin alfa			
Day 8	<P25	77/216	35.6 (29.3–42.4)
	P25–P75	125/316	39.6 (34.1–45.2)
	>P75	68/176	38.6 (31.4–46.3)
Day of HCG	<P25	75/208	36.1 (29.5–43.0)
	P25–P75	126/307	41.0 (35.5–46.8)
	>P75	73/170	42.9 (35.4–50.7)
Recombinant FSH			
Day 8	<P25	60/169	35.5 (28.3–43.2)
	P25–P75	125/340	36.8 (31.6–42.1)
	>P75	65/169	38.5 (31.1–46.2)
Day of HCG	<P25	63/175	36.0 (28.9–43.6)
	P25–P75	132/352	37.5 (32.4–42.8)
	>P75	74/174	42.5 (35.1–50.2)

CI = confidence interval; HCG = human chorionic gonadotrophin; <P25 = patients below the 25th LH percentile; P25–P75 = patients between the 25th and 75th LH percentiles; >P75 = patients above the 75th LH percentile.

Table 6 Logistic regression model for ongoing pregnancy in LH percentile categories per treatment group and day.

Treatment group	*<P25 versus ≥P25*	*>P75 versus ≤P75*	*<P25 versus >P75*
Stimulation day 8			
Corifollitropin alfa	0.86 (0.62–1.21)	1.05 (0.74–1.49)	0.88 (0.58–1.33)
	0.75 (0.53–1.06)[a]	1.26 (0.87–1.83)[a]	0.70 (0.46–1.09)[a]
rFSH	0.91 (0.63–1.32)	1.10 (0.77–1.59)	0.88 (0.57–1.37)
	0.80 (0.54–1.17)[b]	1.28 (0.87–1.87)[b]	0.73 (0.46–1.16)[b]
Day of HCG			
Corifollitropin alfa	0.78 (0.55–1.10)	1.20 (0.84–1.71)	0.75 (0.49–1.13)
	0.77 (0.55–1.08)[c]	1.22 (0.85–1.74)[c]	0.74 (0.49–1.12)[c]
Recombinant FSH	0.84 (0.59–1.21)	1.27 (0.89–1.82)	0.76 (0.49–1.17)
	0.79 (0.55–1.14)[c]	1.35 (0.94–1.93)[c]	0.70 (0.45–1.09)[c]

Values are estimated odds ratios (95% confidence interval).
The LH effect was not statistically significantly different ($P > 0.05$) for any of the stimulation days or treatment arms, unadjusted or adjusted for predictive factors.
HCG = human chorionic gonadotrophin.
[a]Adjusted for age and progesterone and number of follicles ≥11 mm on stimulation day 8.
[b]Adjusted for age, progesterone on stimulation day 8 and oocytes.
[c]Adjusted for age.

concentrations on stimulation day 8 and day of HCG administration (Table 5). The effect of LH concentration on stimulation day 8 and day of HCG was not statistically significant in these treatment groups (Table 6). The estimated OR for <P25 versus ≥P25, >P75 versus ≤P75 and <P25 versus >P75 groups were not statistically significantly different from 1.0 for any of the stimulation days or treatment arms (Table 6), regardless of whether the estimated OR was unadjusted or adjusted for predictive factors.

Discussion

In the 1506 normogonadotrophic women who received either corifollitropin alfa or rFSH during the Engage study, there was no relationship between endogenous LH concentrations and ongoing pregnancy rates. The LH analyses showed that, with a GnRH antagonist protocol to prevent premature LH surges during ovarian stimulation, the

ongoing pregnancy rate was not influenced by either lower or higher endogenous LH concentrations on stimulation day 8 or day of HCG administration. This observation suggests that neither implantation rate nor early miscarriage rates are affected by the amount of circulating LH activity.

On stimulation day 8, the P25 value was <0.6 IU/l in the corifollitropin alfa group and 0.91 IU/l in the rFSH group. These values are below 1.2 IU/l, the cut-off value below which recombinant LH is indicated for ovulation induction in anovulatory women with profound LH deficiency (The European Recombinant Human LH Study Group, 1998). These findings are in agreement with other studies that used GnRH antagonist protocols (Bosch et al., 2005; Merviel et al., 2004), which also showed that low LH values were not associated with decreased pregnancy rates. Although it is known that high concentrations of GnRH antagonist may reduce clinical pregnancy rates (Huirne et al., 2005; The Ganirelix Dose-finding Study Group, 1998), this reduction of fertility does not appear to be mediated through LH deficiency.

In the current trial of young women aged 18–36 years, serum LH concentrations on stimulation day 8 varied from undetectable concentrations (<0.6 IU/l) to 3 IU/l in the corifollitropin alfa group and 5 IU/l in the daily rFSH group. This variability was partly related to the woman's age and the ovarian reserve as women with lower serum LH concentrations had a higher ovarian response. In contrast to GnRH-agonist protocols, GnRH antagonist needs to be administered only during the period of stimulation when a LH rise becomes imminent. In the current study, GnRH antagonist was started for all patients on stimulation day 5 and the observed difference in endogenous LH concentrations at the end of the follicular phase may be related to the larger recruited cohort of follicles in the corifollitropin alfa group compared with the rFSH group. This observation is in line with the lower endogenous LH on day of HCG in patients treated with a fixed daily dose of 200 IU rFSH as compared with a fixed daily dose of 150 IU rFSH (Out et al., 2004).

Endogenous LH concentrations on stimulation day 8 showed a negative association with the number of follicles. This relationship is due to the difference in ovarian reserve in the three LH categories, but it is noted that both serum oestradiol and progesterone showed a positive association, which confirms that steroidogenesis is to a certain extent driven by endogenous LH concentrations regardless of the number of antral follicles or oocytes recovered (The Ganirelix Dose-finding Study Group, 1998).

The stepwise logistic regression analyses indicated that higher progesterone concentrations on stimulation day 8 reduce the ongoing pregnancy rate, while an increased number of follicles on stimulation day 8 (corifollitropin alfa group) or oocytes (rFSH group) increase the ongoing pregnancy rate. Thus, a higher oocyte yield and lower progesterone concentrations are associated with a higher chance of pregnancy. However, serum progesterone concentrations increase during the late follicular phase with the number of growing follicles. This finding supports the concept of an optimal range of oocytes, below and above which outcomes are compromised (van der Gaast et al., 2006). This would imply that pregnancy rates may be improved as long as the number of oocytes is still below the optimal range, whereas overstimulation may increase serum progesterone concentrations during the late follicular phase, which is known to compromise the chance of implantation (Bosch et al., 2010). Further data analysis is required to explore the impact of high progesterone concentrations during stimulation on pregnancy outcome in a GnRH antagonist protocol, which has significantly lower progesterone concentrations than a GnRH agonist protocol.

On the day of HCG administration, the stepwise regression analysis indicated that only age was a significant factor – this may be due to the large variability in the number of follicles and progesterone concentrations at this time. In the current investigation, as neither low nor high endogenous LH concentrations impacted ongoing pregnancy rates in either direction, the data suggest that the amount of endogenous LH was sufficient to support follicular function and implantation in both treatment groups during ovarian stimulation prior to IVF or ICSI. Also, the estimated OR for ongoing pregnancy of low LH (<P25) versus not low LH (≥P25), high LH (>P75) versus not high LH (≤P75) and low LH ($P < 25$) versus high LH (>P75) were not statistically significant for any of the treatment groups and treatment days investigated.

In conclusion, LH analysis of the Engage trial showed that ongoing pregnancy rates were not affected by the extent of LH suppression as measured at stimulation day 8 and day of HCG administration. These findings support previous GnRH antagonist studies that indicated that clinical outcome is not compromised in the absence of exogenous LH supplementation.

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ARTICLE

No association between endogenous LH and pregnancy in a GnRH antagonist protocol: part II, recombinant FSH

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Abstract The association between endogenous LH concentrations during ovarian stimulation in a gonadotrophin-releasing hormone (GnRH) antagonist protocol and pregnancy likelihood was examined in a large combined analysis of individualized patient data obtained after treatment with recombinant FSH and a GnRH antagonist prior to IVF/intracytoplasmic sperm injection. Data from 1764 patients from six randomized controlled trials were pooled for retrospective analysis. Ongoing pregnancy and miscarriage rates for patients stratified by LH percentiles were assessed. Patients in the lowest LH quartile (<P25) were younger with a higher predicted ovarian reserve and response compared with patients in the highest quartile (>P75). With adjustment for identified predictive factors of pregnancy, estimated odds ratios (95% confidence interval) for ongoing pregnancy for LH categories <P25 versus ≥P25, >P75 versus ≤P75 and <P25 versus >P75 were 0.96 (0.75–1.22), 1.13 (0.88–1.45) and 0.89 (0.66–1.21) on stimulation day 8, and 0.96 (0.76–1.21), 1.03 (0.82–1.30) and 0.95 (0.72–1.26) on the day of human chorionic gonadotrophin, respectively. No significant differences in pregnancy or miscarriage rates between the LH categories were observed. Endogenous LH concentrations have no association with the likelihood of ongoing pregnancy in women undergoing ovarian stimulation using a recombinant FSH/GnRH antagonist protocol.

KEYWORDS: combined analysis, endogenous LH, GnRH antagonist, pregnancy rates

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Introduction

The association between endogenous LH concentrations and clinical outcome in a recombinant FSH (rFSH) gonadotrophin-releasing hormone (GnRH) antagonist protocol has not been studied extensively but should be understood before advocating clinical management decisions based on endogenous LH concentrations.

The first studies on the relationship between low endogenous LH concentrations and clinical outcome in GnRH antagonist protocols were published in 2004, by Merviel et al. (2004), who found no impact of low endogenous LH (≤0.5 IU/l) on clinical pregnancies in 270 patients following ovarian stimulation for IVF, and by Kolibianakis et al. (2004), who reported that profound LH suppression (≤0.5 IU/l) on stimulation day 8 in a study of 116 women was associated with a higher chance of achieving an ongoing pregnancy.

Previous dose-finding studies have indicated that high doses of GnRH antagonists may induce very profound LH suppression and reduce the probability of clinical pregnancy (Huirne et al., 2005; The Ganirelix Dose-finding Study Group, 1998). However, low serum LH concentrations, defined by percentile analysis in 110 patients treated with 0.25 mg GnRH antagonist, were not shown to be associated with the probability of pregnancy (Bosch et al., 2005).

A recent publication (Doody et al., 2010) reported no association between endogenous LH concentrations measured on stimulation days 1, 5 or 8 and ongoing pregnancy rates in 750 patients treated with daily rFSH and 0.25 mg ganirelix in the Engage trial (Devroey et al., 2009). The analysis of this trial was then extended with a study of endogenous LH concentrations measured on stimulation day 8 and on the day of human chorionic gonadotrophin (HCG) administration, and the probability of pregnancy in both the rFSH and corifollitropin alfa treatment arms of the study (approximately 750 patients in each treatment arm) (Doody et al., 2011, part I of this study). In this study, endogenous LH concentrations ranging between <0.6 IU/l and 5 IU/l were not associated with the chance of ongoing pregnancy, whereas serum progesterone concentration on stimulation day 8 and ovarian response (follicles on stimulation day 8/oocytes) appeared to be significant predictors of ongoing pregnancy. Moreover, this analysis demonstrated that patients with lower serum LH concentrations tend to be younger, with a higher ovarian reserve and a higher ovarian response than patients with higher LH concentrations. In the study by Kolibianakis et al. (2004), patients with lower serum LH concentrations during stimulation had a higher chance of pregnancy but were also slightly younger and had statistically significantly lower endogenous FSH concentrations on stimulation day 1.

The present study examined the association of endogenous LH concentrations during the follicular phase with ongoing pregnancy rates in large data sets derived from six randomized trials with a total of 1764 patients treated with rFSH in a GnRH antagonist (ganirelix) protocol prior to IVF or intracytoplasmic sperm injection (ICSI). Identified predictors from the rFSH arm of the Engage trial (Doody et al., 2011, part I) were included as covariates in a combined analysis of the six trials using individual patient data stratified by LH concentrations determined within each trial by a central laboratory.

Materials and methods

Ongoing pregnancy rates and miscarriage rates relative to endogenous serum LH concentrations during ovarian stimulation were assessed from the following six trials, all of which included a GnRH antagonist (ganirelix) treatment arm with a daily dosage of 0.25 mg: (i) Engage (Devroey et al., 2009); (ii) Ensure (Corifollitropin alfa Ensure Study Group, 2010); (iii) Xpect (NCT identifier NCT00778999, Nyboe Andersen et al., in press); (iv) Ganirelix EU (The European Orgalutran Study Group, 2000); (v) Ganirelix ME (The European and Middle East Orgalutran Study Group, 2001); and (vi) Ganirelix NA (The North American Ganirelix Study Group, 2001). Patients were normogonadotrophic women with an indication for ovarian stimulation prior to IVF or ICSI. In all six trials, only data from the rFSH/ganirelix arms were used for the current analyses.

Engage trial (rFSH arm only)

Women aged 18–36 years with bodyweight from >60 kg to ≤90 kg received daily 200 IU rFSH (Puregon/Follistim pen; Organon, The Netherlands) up to and including the day of HCG administration. From stimulation day 8, the dose of rFSH was adjusted if necessary, according to the ovarian response. The GnRH antagonist ganirelix (0.25 mg, Orgalutran/ganirelix acetate injection, Organon) was administered once daily s.c. starting on stimulation day 5 upto and including the day of HCG injection. Urinary HCG (10,000 IU or 5000 IU) was administered i.m. to induce final oocyte maturation (Devroey et al., 2009).

Ensure trial (rFSH arm only)

Women aged 18–36 years with bodyweight ≤60 kg received daily 150 IU rFSH (Puregon/Follistim pen) upto and including the day of HCG administration. From stimulation day 8, the dose of rFSH was adjusted if necessary, according to the ovarian response. The GnRH antagonist ganirelix (0.25 mg) was administered once daily s.c. starting on stimulation day 5 upto and including the day of HCG injection. Urinary HCG (10,000 IU) was administered i.m. to induce final oocyte maturation (Corifollitropin alfa Ensure Study Group, 2010).

Xpect trial (excluding the oral contraception pretreatment arm)

Women aged 18–39 years with body mass index (BMI) ≤32 kg/m^2 received daily 200 IU rFSH (Puregon/Follistim pen) upto and including the day of HCG administration, with dose adjustment as necessary after stimulation day 6. On stimulation day 5, the GnRH antagonist ganirelix (0.25 mg) was administered daily s.c. upto and including the day of HCG administration. To induce final oocyte maturation, 5000–10,000 IU HCG was administered (NCT identifier NCT00778999; Nyboe Andersen et al., in press).

European ganirelix trial (ganirelix arm only)

Women aged 18–39 years with BMI 18–29 kg/m^2 received daily 150 IU rFSH (Puregon/Follistim pen) from stimulation day 1 upto the day of HCG, with dose adjustment as necessary after stimulation day 6. From day 6 of rFSH treatment, the GnRH antagonist ganirelix (0.25 mg) was administered daily s.c. upto and including the day of HCG administration. Urinary HCG (10,000 IU, Pregnyl; Organon) was administered to induce final oocyte maturation (The European Orgalutran Study Group, 2000).

European and Middle East ganirelix trial (ganirelix arm only)

Women aged 18–39 years with BMI 18–29 kg/m^2 received daily 150 IU rFSH (Puregon/Follistim pen) from stimulation day 1 upto the day of HCG, with dose adjustment after day 6 depending on the ovarian response as monitored by ultrasound scan. From day 6 of rFSH treatment, the GnRH antagonist ganirelix (0.25 mg) was administered daily s.c. upto and including the day of HCG administration. Urinary HCG (10,000 IU, Pregnyl) was administered to induce final oocyte maturation (The European and Middle East Orgalutran Study Group, 2001).

North American ganirelix trial (ganirelix arm only)

Women aged 18–39 years with BMI 18–29 kg/m^2 received daily 225 IU rFSH (Puregon/Follistim pen) from stimulation day 1 upto the day of HCG, with dose adjustment after day 6 depending on the ovarian response. From day 6 of rFSH treatment, the GnRH antagonist ganirelix (0.25 mg) was administered daily s.c. upto and including the day of HCG administration. Urinary HCG (10,000 IU, Pregnyl) was administered to induce final oocyte maturation (The North American Ganirelix Study Group, 2001).

General trial criteria

In all six trials, patients with an irregular menstrual cycle were excluded. Stimulation was always started on days 2 or 3 of menses and final oocyte maturation by HCG was triggered when at least three follicles $\geq$17 mm were observed by ultrasound scan. Patients had a transvaginal ultrasound-guided oocyte retrieval 34–36 h after 10,000 IU urinary HCG (Pregnyl) administration, followed by either IVF or ICSI.

In the Engage, Ensure and Xpect trials, luteal phase support with progesterone (at least 600 mg/day vaginally or at least 50 mg/day i.m.) was started on the day of oocyte retrieval and continued for at least 6 weeks. In the other three trials, luteal support was given according to the clinic's routine practice. Ongoing pregnancy rates were calculated based on the presence of at least one fetus with heart activity at least 10 weeks after embryo transfer. The miscarriage rate was calculated for subjects with a clinical pregnancy defined as the presence of at least one gestational sac.

Hormone assessments were made in blood samples drawn in the morning just prior to GnRH antagonist and gonadotrophin injections and the serum immediately stored at −20°C until analysis.

Validated immunoassays were performed to measure serum concentrations of FSH, LH, oestradiol and progesterone at stimulation days 1, 5 and 8 and day of HCG. In all trials except for the Ganirelix NA trial, these hormones were determined at one central laboratory (Waltrop, Germany) using a time-resolved fluoroimmunoassay (AutoDelfia immunofluorometric assay; PerkinElmer Life and Analytical Sciences, Brussels, Belgium). In the Ganirelix NA trial, serum LH was measured by the Immulite 1000 LH assay (DPC, Los Angeles, CA) and oestradiol and progesterone at a central laboratory (Quest Diagnostics, USA).

Statistical analysis

All analyses included the intent-to-treat (ITT) groups comprising subjects randomized to rFSH treatment who started stimulation. Per trial, patients were stratified according to the 25th and 75th percentiles (P25 and P75) of serum LH concentrations, resulting in three groups of patients <P25 (low LH), P25–P75 (medium LH) and >P75 (high LH). Patients without LH measurements were excluded from these groups.

Overall differences in baseline characteristics, stimulation characteristics and ovarian response between low (<P25), medium (P25–P75) and high (>P75) serum LH concentrations on stimulation day 8 were presented and tested for statistically significant differences between LH categories using either analysis of variance (ANOVA) for comparing means or the Kruskal–Wallis test for comparing medians. A *P*-value $\leq$0.05 was considered statistically significant. No multiplicity correction was applied (*P*-values were not corrected for multiple testing in order to control the overall type I error rate of 0.05). The <P25, P25–P75 and >P75 groups of patients of the separate trials on stimulation day 8 were pooled per LH category for this purpose.

Differences in ongoing pregnancy rates between low (<P25), medium (P25–P75) and high (>P75) LH concentrations were estimated per trial and overall (combined). The individual patient data of the six trials were fitted to a logistic regression model using PROC GENMOD in SAS version 9.1 (SAS Institute, Cary, NC, USA). The ongoing pregnancy rate was modeled as a function of trial (six-level class variable), LH category (three-level class variable, i.e. <P25, P25–P75 and >P75) and several identified predictive factors of ongoing pregnancy. The predictive factors were identified using the data of the rFSH arm from the Engage trial (Doody et al., 2011, part I). The miscarriage rate was modeled as a function of trial and LH category for estimation of the differences in miscarriage rate between the <P25, P25–P75 and >P75 groups of patients per trial and overall. Separate models of ongoing pregnancy rate and miscarriage rate were obtained for the LH categories on stimulation day 8 and day of HCG, respectively.

Trial by LH interaction was first added to the model to determine the heterogeneity of the LH effect across the six trials. If the *P*-value for interaction was >0.10, then the effect across the six trials was considered homogeneous and the model without interaction was applied to provide estimates per trial and overall. If the *P*-value for heterogeneity was <0.10, then estimates are not provided.

Table 1 Mean baseline characteristics of all patients included in the combined LH analysis presented by LH on stimulation day 8.

Variable	*LH percentile category*			*P-value*
	<P25 (n = 436)	*P25–P75 (n = 891)*	*>P75 (n = 437)*	
Age (years)	31.2 ± 3.5	31.6 ± 3.6	31.8 ± 3.7	0.02[a]
Body mass index (kg/m^2)	23.3 ± 3.0	23.7 ± 3.0	23.9 ± 3.1	0.01[a]
Bodyweight (kg)	64.4 ± 9.2	64.7 ± 9.0	65.1 ± 9.3	NS[a]
Serum LH on day 1	4.1 (2.0, 7.2)	4.6 (2.4, 7.9)	5.0 (2.7, 9.4)	<0.01[b]
Serum FSH on day 1	6.1 (3.5, 10.0)	6.4 (4.2, 10.5)	6.7 (4.5, 11.6)	<0.01[b]

Values are mean ± standard deviation or median (P5, P95). NS = not statistically significant; <P25 = patients below the 25th LH percentile; P25–P75 = patients between the 25th and 75th LH percentiles; >P75 = patients above the 75th LH percentile.
[a]Analysis of variance.
[b]Kruskal–Wallis test.

In case of homogeneity ($P > 0.10$), *P*-values of the overall LH effect based on the likelihood ratio test were provided and maximum likelihood estimates of odds ratios (OR) and associated two-sided 95% confidence intervals (CI) of <P25 versus ≥P25 (low versus not low LH), >P75 versus ≤P75 (high versus not high LH) and <P25 versus >P75 (low versus high LH) per trial and overall were presented. For the ongoing pregnancy-rate model, the *P*-values and estimated OR and CI with and without adjustment for the predictive factors are presented.

Results

Patient population

In total, the LH analysis included 1764 patients. Age, BMI, bodyweight and serum FSH and LH concentrations on stimulation day 1 per LH percentile category on stimulation day 8 (<P25, P25–P75 and >P75) are presented in **Table 1**. Patients with higher serum LH concentrations on stimulation day 8 were older and also had slightly higher baseline FSH and LH concentrations than patients with lower serum LH concentrations at stimulation day 8.

Serum LH concentrations on stimulation day 8

Serum LH concentrations per percentile (P5, P25, P50, P75 and P95) in each trial are presented in **Table 2**. On stimulation day 8, the P50 (median) value ranged from 1.32 IU/l to 2.10 IU/l between trials. The P25 values varied between 0.78 IU/l and 1.30 IU/l and the P75 values ranged from 1.97 IU/l to 3.25 IU/l.

Table 2 LH concentrations in LH percentile categories on stimulation day 8 by trial.

Trial	*LH percentiles*				
	P5	*P25*	*P50*	*P75*	*P95*
Engage	<0.6	0.91	1.57	2.66	5.27
Ensure	<0.6	0.81	1.60	2.71	5.52
Xpect	<0.6	1.04	1.79	3.25	6.16
Ganirelix EU	<0.6	0.86	1.32	2.23	4.94
Ganirelix ME	<0.6	0.78	1.32	1.97	4.63
Ganirelix NA	0.50	1.30	2.10	2.90	4.90

Stimulation characteristics and ovarian response per LH category

The duration of stimulation, the total dose of rFSH and endocrine parameters per LH percentile category are presented in **Table 3**. Patients with lower LH concentrations required 0.5 days longer stimulation and on average 100 IU rFSH more than patients with higher LH concentrations to reach the same criteria for HCG administration.

On stimulation day 8 and day of HCG, patients with higher LH concentrations had a lower number of follicles ≥11 mm whereas their serum oestradiol and progesterone concentrations were higher compared with patients with lower LH concentrations. In line with the lower number of follicles, fewer oocytes were recovered in patients with higher LH concentrations: the estimated difference was 1.9 oocytes compared with patients with lower LH concentrations.

Ongoing pregnancy rates per LH category and trial

Per trial, the ongoing pregnancy rates in the <P25, P25–P75 and >P75 groups of patients on stimulation day 8 and day of HCG are shown in **Table 4**.

Estimated LH effects on ongoing pregnancy

Neither the overall LH effect nor the trial by LH interaction effect (heterogeneity) were statistically significant based on the LH categories on stimulation day 8 or day of HCG, with or without adjustment of the predictive factors (**Table 5**).

The estimated OR for ongoing pregnancy rates by LH category on stimulation day 8 and day of HCG administration are given in **Table 6**. In patients with low LH concentrations, the estimated overall OR for ongoing pregnancy rate of <P25 versus ≥P25 on stimulation day 8 was 0.96 (95% CI 0.75–1.22), when adjusted for trial, age, number of oocytes retrieved and serum progesterone concentration

Table 3 Stimulation characteristics and ovarian response of all patients included in the combined LH analysis presented by LH on stimulation day 8.

Characteristic/response	*LH percentile category*			*P-value*
	<P25 (n = 436)	*P25–P75 (n = 891)*	*>P75 (n = 437)*	
Duration of stimulation (days)	10.0 ± 1.7	9.8 ± 1.7	9.5 ± 1.5	<0.01
Total dose of rFSH (IU)	1759 ± 522	1729 ± 469	1652 ± 457	<0.01
Follicles on day 8				
≥11 mm	10.4 ± 5.8	10.0 ± 5.6	9.5 ± 5.2	0.02
≥15 mm	4.9 ± 4.5	5.1 ± 4.0	4.9 ± 3.9	NS
≥17 mm	2.0 ± 2.6	2.4 ± 2.7	2.6 ± 2.9	0.01
Hormones on day 8				
Oestradiol (pmol/l)	2007 (573, 7670)	2936 (635, 8973)	3854 (998, 9982)	<0.01
Progesterone (nmol/l)	1.7 (<1, 4.2)	1.9 (<1, 4.3)	2.3 (1.1, 4.7)	<0.01
Follicles on day of human chorionic gonadotrophin				
≥11 mm	13.1 ± 6.3	12.2 ± 6.1	11.0 ± 5.8	<0.01
≥15 mm	8.8 ± 4.3	8.2 ± 4.1	7.2 ± 3.9	<0.01
≥17 mm	5.6 ± 3.1	5.2 ± 2.8	4.9 ± 2.7	<0.01
Hormones on day of human chorionic gonadotrophin				
Oestradiol (pmol/l)	3622 (1240, 9564)	4991 (1809, 11,671)	5975 (2019, 13,212)	<0.01
Progesterone (nmol/l)	2.3 (1.2, 5.8)	2.6 (1.2, 5.3)	2.9 (1.3, 5.9)	<0.01
Oocytes	11.9 ± 6.8	11.1 ± 6.7	10.0 ± 6.1	<0.01

Values are mean ± standard deviation or median (P5, P95). rFSH, recombinant FSH; NS = not statistically significant.

Table 4 Ongoing pregnancy rate per started cycle, by trial and LH on stimulation day 8 and the day of HCG.

Trial	*rFSH start dose (IU)*	*Ongoing pregnancy rate by LH category*		
		<P25	*P25–P75*	*>P75*
Stimulation day 8				
Engage	200	60/169 (35.5)	125/340 (36.8)	65/169 (38.5)
Ensure	150	12/30 (40.0)	20/61 (32.8)	10/30 (33.3)
Xpect	200	17/42 (40.5)	33/87 (37.9)	13/41 (31.7)
Ganirelix EU	150	20/105 (19.0)	44/210 (21.0)	23/105 (21.9)
Ganirelix ME	150	19/49 (38.8)	29/103 (28.2)	13/50 (26.0)
Ganirelix NA	225	15/41 (36.6)	25/90 (27.8)	14/42 (33.3)
Day of human chorionic gonadotrophin				
Engage	200	63/175 (36.0)	132/352 (37.5)	74/174 (42.5)
Ensure	150	10/31 (32.3)	25/65 (38.5)	9/30 (30.0)
Xpect	200	18/45 (40.0)	37/92 (40.2)	11/45 (24.4)
Ganirelix EU	150	21/112 (18.8)	55/224 (24.6)	19/112 (17.0)
Ganirelix ME	150	20/53 (37.7)	29/109 (26.6)	21/53 (39.6)
Ganirelix NA	225	16/46 (34.8)	28/96 (29.2)	17/45 (37.8)

Values are *n*/total (%) unless otherwise stated. rFSH, recombinant FSH.

on stimulation day 8 (**Figure 1**). The estimated overall OR for ongoing pregnancy of <P25 versus ≥P25 on the day of HCG administration was 0.96 (95% CI 0.76–1.21), when adjusted for trial and age. For patients with high LH concentrations, the estimated overall OR for ongoing pregnancy rate of >P75 versus ≤P75 on stimulation day 8 was 1.13 (95% CI 0.88–1.45) when adjusted for trial, age, number of oocytes retrieved and serum progesterone concentration on stimulation day 8. The estimated overall OR for ongoing pregnancy rate of >P75 versus ≤P75 on the day of HCG administration was 1.03 (95% CI 0.82–1.30) when adjusted for trial and age.

In summary, neither low nor high endogenous LH concentrations on stimulation day 8 or day of HCG showed a

Table 5 Logistic regression model for ongoing pregnancy by LH and heterogeneity (trial by LH interaction) per day.

Model	*P-value*	
	LH effect	*Heterogeneity*
Stimulation day 8		
Adjusted for trial	0.79	0.91
Adjusted for trial, age, no. of oocytes retrieved and progesterone concentration on day 8	0.59	0.94
Day of human chorionic gonadotrophin		
Adjusted for trial	0.96	0.17
Adjusted for trial and age	0.94	0.16

Table 6 Logistic regression model for ongoing pregnancy in LH percentile categories per started cycle, per trial and overall on stimulation day 8 and day of human chorionic gonadotrophin.

Trial	*<P25 versus ≥P25*	*>P75 versus ≤P75*	*<P25 versus >P75*
Stimulation day 8			
Engage	0.91 (0.63–1.32)	1.10 (0.77–1.59)	0.88 (0.57–1.37)
Ensure	1.35 (0.57–3.21)	0.88 (0.36–2.12)	1.33 (0.46–3.82)
Xpect	1.28 (0.61–2.65)	0.72 (0.34–1.54)	1.46 (0.59–3.61)
Ganirelix EU	0.86 (0.49–1.52)	1.12 (0.65–1.94)	0.84 (0.43–1.64)
Ganirelix ME	1.71 (0.86–3.40)	0.71 (0.34–1.46)	1.80 (0.77–4.23)
Ganirelix NA	1.32 (0.62–2.78)	1.06 (0.50–2.25)	1.15 (0.47–2.85)
Overall	1.07 (0.85–1.36)[a]	0.99 (0.78–1.25)[a]	1.06 (0.79–1.41)[a]
	0.96 (0.75–1.22)[b]	1.13 (0.88–1.45)[b]	0.89 (0.66–1.21)[b]
Day of human chorionic gonadotrophin			
Engage	0.84 (0.59–1.21)	1.27 (0.89–1.82)	0.76 (0.49–1.17)
Ensure	0.92 (0.38–2.23)	0.79 (0.32–1.94)	1.11 (0.38–3.29)
Xpect	1.43 (0.70–2.93)	0.48 (0.22–1.04)	2.06 (0.83–5.09)
Ganirelix EU	0.89 (0.51–1.56)	0.75 (0.42–1.32)	1.13 (0.57–2.24)
Ganirelix ME	1.24 (0.65–2.39)	1.40 (0.73–2.69)	0.92 (0.42–2.02)
Ganirelix NA	1.07 (0.52–2.17)	1.30 (0.64–2.63)	0.88 (0.37–2.07)
Overall	0.97 (0.77–1.22)[a]	1.03 (0.82–1.30)[a]	0.96 (0.73–1.27)[a]
	0.96 (0.76–1.21)[c]	1.03 (0.82–1.30)[c]	0.95 (0.72–1.26)[c]

Values are estimated OR (95% confidence interval).
[a]Adjusted for trial.
[b]Adjusted for trial, age, number of oocytes retrieved and serum progesterone concentration on day 8.
[c]Adjusted for trial and age.

significant association with ongoing pregnancy likelihood. The estimated OR were not statistically significantly different from 1.0.

Miscarriage rates within trials according to LH category

Miscarriage rates per started cycle within each trial in the <P25, P25–P75 and >P75 groups of patients on stimulation day 8 and day of HCG are shown in **Table 7**.

Estimated LH effects on miscarriage

Trial by LH interaction (heterogeneity) on day of HCG was statistically significant ($P = 0.05$). Therefore, differences in miscarriage rates among the three LH categories on day of HCG could not be estimated.

Trial by LH interaction (heterogeneity) on stimulation day 8 was borderline significant. The overall LH effect on stimulation day 8 was not statistically significant. In patients with low LH concentrations, the estimated overall OR for miscarriage rate of <P25 versus ≥P25 on stimulation day 8 (adjusted for trial) was 1.07 (95% CI 0.59–1.94). For patients with high LH concentrations, the estimated overall OR for miscarriage rate of >P75 versus ≤P75 on stimulation day 8 (adjusted for trial) was 0.87 (95% CI 0.47–1.60).

In summary, neither low nor high endogenous LH concentrations on stimulation day 8 showed an association with the likelihood of miscarriage. The estimated OR were not statistically significantly different from 1.0.

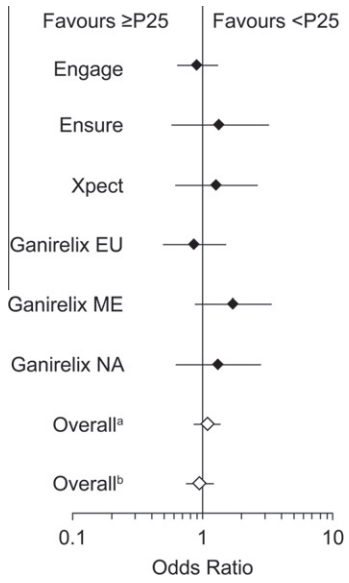

Figure 1 Estimated odds ratios (per trial and overall) of patients in the LH percentile group <P25 versus patients in ≥P25 group for ongoing pregnancy rate per started cycle on stimulation day 8. [a]Adjusted for trial.
[b] Adjusted for trial, age, number of oocytes retrieved and serum progesterone concentration on stimulation day 8.

Discussion

Neither low nor high endogenous LH concentrations were associated with the likelihood of pregnancy in a pooled analysis of individual LH data of 1764 patients from six clinical trials that used rFSH for ovarian stimulation in a GnRH antagonist protocol. In addition to the lack of effect of endogenous LH concentrations on pregnancy rates, the current analyses showed that neither low nor high LH concentrations impacted miscarriage rates.

The validity of the pooled analyses was shown by the applied heterogeneity tests. The LH effects on pregnancy achievement across the six trials were demonstrated to be homogeneous, while the LH effects on miscarriage rates showed some variation across the trials.

The current analyses have the advantage that the LH assessments were carried out for all patients just prior to GnRH antagonist injection (Griesinger et al., 2006) and were analysed by a central laboratory allowing a more consistent analysis of the impact of endogenous LH on clinical outcome. The disadvantage of measuring serum LH concentrations 24 h after GnRH antagonist administration, just prior to the next antagonist injection, is that the absolute values are much higher than the LH nadir, which is reached after 4 h (Oberyé et al., 1999).

It should be noted that the included patient population had normal serum FSH and LH concentrations in the early follicular phase, were relatively young (up to age 39 years) and consisted of (potentially) normal-responder patients. Within this population, the current pooled LH analysis confirmed the observation that patients with lower pre-ovulatory LH concentrations tend to be younger with a higher ovarian reserve, as reflected by their lower baseline FSH and LH at stimulation day 1, resulting in a higher ovarian response than in patients with higher pre-ovulatory LH concentrations (Doody et al., 2011, part I). None of the patients included in this pooled LH analysis were pre-treated with oral contraceptives or any other hormonal preparation within 1 month prior to randomization. The current pooled LH analysis was performed on stimulation day 8 and included all subjects who started stimulation and who provided a blood sample for hormone analysis on the specified day. Importantly, it included all subjects regardless of whether they discontinued prior to HCG administration or before embryo transfer.

Table 7 Miscarriage rate per started cycle for subjects with a clinical pregnancy presented by trial and LH category on stimulation day 8 and day of human chorionic gonadotrophin.

Trial	*rFSH start dose (IU)*	*Miscarriage rate by LH category*		
		<P25	*P25–P75*	*>P75*
Stimulation day 8				
Engage	200	5/169 (3.0)	11/340 (3.2)	4/169 (2.4)
Ensure	150	2/30 (6.7)	2/61 (3.3)	0/30 (0.0)
Xpect	200	6/42 (14.3)	4/87 (4.6)	1/41 (2.4)
Ganirelix EU	150	1/105 (1.0)	7/210 (3.3)	3/105 (2.9)
Ganirelix ME	150	0/49 (0.0)	4/103 (3.9)	4/50 (8.0)
Ganirelix NA	225	2/41 (4.9)	5/90 (5.6)	2/42 (4.8)
Day of human chorionic gonadotrophin				
Engage	200	6/175 (3.4)	9/352 (2.6)	5/174 (2.9)
Ensure	150	1/31 (3.2)	3/65 (4.6)	0/30 (0.0)
Xpect	200	7/45 (15.6)	6/92 (6.5)	1/45 (2.2)
Ganirelix EU	150	3/112 (2.7)	5/224 (2.2)	4/112 (3.6)
Ganirelix ME	150	0/53 (0.0)	8/109 (7.3)	0/53 (0.0)
Ganirelix NA	225	2/46 (4.3)	6/96 (6.3)	2/45 (4.4)

Values are *n*/total (%).

The predictive factors for ongoing pregnancy in addition to age that were applied for adjustment of the estimated OR were derived from a stepwise logistic regression analysis of the rFSH arm of the largest of the six included trials, the Engage trial (Doody et al., 2011, part I). Moreover, these factors (progesterone concentration and number of oocytes retrieved) and age have previously been described to impact pregnancy rates (Bosch et al., 2010; Broekmans et al., 2006).

An alternative approach to identify predictive factors for ongoing pregnancy would be to pool the data of the rFSH/ganirelix arms of the six trials. However, the applied model should take into account possible trial effects on each of the candidate predictive factors. These trial effects could be related to differences in the trial population, hormone assays and the treatment regimen. Accurate adjustment for trial effect in this particular application is difficult to justify and therefore this approach was not considered.

To date, the number of studies addressing the impact of endogenous LH concentrations in GnRH antagonist protocols is limited in contrast to various univariate analyses of the associations between mid-follicular concentrations of LH and pregnancy rates in long GnRH agonist protocols (Balasch et al., 2001; Cabrera et al., 2005; Esposito et al., 2001; Humaidan et al., 2002; Nakagawa et al., 2008; Westergaard et al., 2000), which have been reported with inconclusive results. Due to differences in the duration and extent of LH suppression in long GnRH agonist protocols, caution should be exerted when comparing pregnancy outcomes relative to LH concentrations from trials using different compounds, doses or route of administration of GnRH agonists.

Although the absolute concentrations of endogenous LH in a GnRH antagonist protocol do not affect clinical outcome during ovarian stimulation with rFSH, it may still be that addition of recombinant LH or HCG during stimulation positively affects clinical outcome. In a systematic review and meta-analysis to assess whether the addition of recombinant LH during ovarian stimulation increases live-birth rates, seven randomized clinical trials were identified, five with a long GnRH agonist protocol and two with a GnRH antagonist protocol (Kolibianakis et al., 2007). No significant difference in the probability of live birth was found for patients treated with or without recombinant LH supplementation (OR 0.92, 95% CI 0.65—1.31). This finding held in subgroup analyses that ordered the studies by dose of recombinant LH added, the type of analog used to inhibit premature LH surge, the time recombinant LH was added during the follicular phase and the age of patients analysed.

In the current investigation, the combined analyses of the individual patient data from six trials confirmed the findings of the analysis of the Engage trial (Doody et al., 2011, part I). Neither low nor high endogenous LH concentrations affected ongoing pregnancy rates, and in principle the amount of endogenous LH was sufficient to support rFSH during ovarian stimulation prior to IVF or ICSI. The validity of these findings at stimulation day 8 rather than day of HCG may be more relevant as the latter analysis excludes patients who were discontinued prior to HCG administration. Furthermore, from a clinical point of view, the results on stimulation day 8 are more relevant, since LH supplementation in those patients with low endogenous LH after GnRH antagonist initiation on stimulation days 5 or 6 of stimulation is still possible.

In conclusion, a GnRH antagonist treatment during ovarian stimulation results in variable amounts of endogenous circulating LH. Combined analyses of individual LH concentrations from 1764 patients from six trials who underwent ovarian stimulation with rFSH in a GnRH antagonist protocol show that ongoing pregnancy rates were not associated with the extent of LH suppression as measured on stimulation day 8 and day of HCG. This is the largest combined analysis to date to evaluate the association of endogenous LH concentrations with the likelihood of pregnancy in a GnRH antagonist protocol controlling for significant covariates shown to influence pregnancy rates. It provides robust evidence from prospective trials measuring serum LH in a central laboratory that endogenous LH concentrations have no association with the chance of ongoing pregnancy in normogonadotrophic women undergoing ovarian stimulation with rFSH. Thus, endogenous LH concentrations cannot serve as a rationale for adding LH activity to a GnRH antagonist ovarian stimulation protocol.

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Corifollitropin alfa in a long GnRH agonist protocol: proof of concept trial

Fifty healthy women, aged 18–39 years with no known risk of ovarian hyperstimulation were treated with 100 or 150 μg corifollitropin alfa (dependent on body weight) in a long GnRH agonist protocol. At these doses, corifollitropin alfa initiated and supported growth of a large cohort of follicles during the first week of ovarian stimulation. (Fertil Steril® 2010;94:1922–4. ©2010 by American Society for Reproductive Medicine.)

The novel fertility hormone corifollitropin alfa (Elonva; NV Organon, Oss, The Netherlands) is a recombinant fusion protein of human FSH and the carboxy terminal peptide of hCG (1). It has a slower absorption rate and an approximately twofold longer half-life than recombinant human FSH (rFSH) (2, 3), but the same pharmacologic activity. Unlike rFSH, which requires daily injections over the course of 1 week, a single injection of corifollitropin alfa is able to initiate and sustain follicular growth for an entire week (4, 5) and is therefore appropriately described as a sustained follicle stimulant. Thus, compared to rFSH, corifollitropin alfa reduces the number of injections needed for ovarian stimulation before IVF or intracytoplasmic sperm injection and may lower the treatment burden of subjects undergoing controlled ovarian stimulation (1).

Corifollitropin alfa has been developed in a GnRH antagonist protocol in two therapeutic strengths of 100 μg for patients weighing ≤60 kg and 150 μg for patients weighing >60 kg. Administration of corifollitropin alfa at these doses has been shown to be safe and effective (6, 7), provides similar exposure to FSH activity, and induces a similar ovarian response (8). The use of a short GnRH antagonist protocol offers a number of advantages over longer GnRH agonist protocols, including shorter treatment cycles, a decreased risk of ovarian hyperstimulation syndrome (OHSS), and an absence of the side effects associated with estrogen-withdrawal (9). In addition, the simple GnRH antagonist protocol is thought to reduce the psychologic stress and the dropout rates of subjects (10, 11), and it is especially suitable for normal and high responders (12).

Unlike GnRH antagonist protocols, a long GnRH agonist protocol causes profound pituitary suppression of endogenous gonadotropins at the start of ovarian stimulation. As a result, daily FSH recruits a larger cohort of follicles in a long GnRH agonist protocol, and the duration of stimulation to reach the same hCG criteria is longer, leading to an increased consumption of FSH (13). Because of this, the current pilot study was conducted to confirm whether the selected 100-μg and 150-μg doses of corifollitropin alfa would support the same 7-day interval of ovarian stimulation in a long GnRH agonist protocol.

The study protocol was reviewed and approved by the Independent Medical Ethics Committee at the Centre for Reproductive Medicine in Brussels. First, a cohort of patients was recruited and treated with a single injection of 150 μg corifollitropin alfa; subsequently, a cohort of patients was recruited and treated with a single injection of 100 μg corifollitropin alfa. Patient inclusion criteria of the trial were largely similar to those previously described by Devroey et al. (6), with body weight criteria depending on the prescribed corifollitropin alfa dose.

Treatment with 0.1 mg triptorelin SC was initiated in the midluteal phase (between cycle days 21 and 24), and this dose was continued up to downregulation (defined by E_2 levels <50 ng/L and progesterone levels <1.48 μg/L). Patients received a single dose of 150 μg or 100 μg corifollitropin alfa SC, followed 1 week later (stimulation day 8) by a daily dose of rFSH (follitropin beta) up to and including the day of hCG administration. All subjects in the first group (150 μg) received the maximum allowed daily rFSH dose of 200 IU from stimulation day 8 to the day of hCG administration, whereas all subjects in the second group (100 μg) received a daily dose of 150 IU from stimulation day 8 to the day of hCG administration. To induce final oocyte maturation, 10,000 IU hCG was given on the first day that three follicles ≥17 mm were observed by ultrasound examination. Oocytes were retrieved after 36 hours, followed by IVF or intracytoplasmic sperm injection. All embryos were transferred on day 5, regardless of the number and quality of embryos available on day 3 (14).

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TABLE 1

Clinical outcome after treatment with corifollitropin alfa in a long GnRH agonist protocol.

	100 μg corifollitropin alfa (n = 25)	150 μg corifollitropin alfa (n = 24)
Follicles (≥11 mm) on day 8	10.7 (5.7)	11.9 (5.0)
Median serum E_2 on day 8 (pmol/L)	3,780	3,712
Follicles (≥11 mm) on day of hCG	17.5 (5.5)	18.3 (6.4)
Median serum E_2 on day of hCG (pmol/L)	10,019	10,221
Number of oocytes, per started cycle	15.4 (6.7)	17.8 (5.1)
Fertilization rate (%)	71.6	69.6
Number of embryos on day 3		
Total	10.0 (5.1)	10.8 (4.9)
Good quality embryos	4.8 (4.8)	4.8 (4.5)
Number of embryos on day 5		
Total	6.9 (4.4)	8.0 (5.1)
Good quality embryos	1.9 (2.0)	1.4 (1.4)
Number of embryos transferred	1.1 (0.3)	1.3 (0.5)
Subjects with SET, n (%)	20 (90.9)	12 (66.7)
Subjects with elective SET, n (%)	9 (40.9)	9 (50.0)
Subjects with DET, n (%)	2 (9.1)	6 (33.3)
Number of embryos cryopreserved	1.3 (2.2)	2.5 (3.3)
Pregnancy rate per started cycle, n (%)		
Biochemical	10 (40.0)	9 (37.5)
Clinical[a]	8 (32.0)	8 (33.3)
Vital[b]	8 (32.0)	8 (33.3)
Ongoing[c]	7 (28.0)	8 (33.3)
Pregnancy rate per embryo transfer, n (%)		
Biochemical	10 (45.5)	9 (50.0)
Clinical[a]	8 (36.4)	8 (44.4)
Vital[b]	8 (36.4)	8 (44.4)
Ongoing[c]	7 (31.8)	8 (44.4)

Note: All values are mean (SD) unless otherwise stated. SET = single embryo transfer; DET = double embryo transfer.

[a] Visualization of at least one gestational sac on ultrasound examination.

[b] Visualization of at least one fetus with heart beat activity on ultrasound examination.

[c] One subject with a vital pregnancy in the 100-μg group was lost to follow-up, and ongoing pregnancy could not be confirmed.

Fatemi. Corifollitropin alfa with GnRH agonist. Fertil Steril 2010.

The primary endpoint of this trial was the occurrence of an inhibin-B drop, as a decline of inhibin-B is typically associated with insufficient support of the cohort of growing follicles (5). Given the exploratory nature of this pilot study, a sample size of 20 evaluable subjects per corifollitropin alfa dose was considered appropriate. No statistical analysis was performed to compare the results between the two dose groups.

Of the 50 subjects who met the study criteria, one patient in the 150-μg group discontinued before receiving corifollitropin alfa because of a spontaneous pregnancy. All the remaining 49 subjects progressed to treatment with hCG and oocyte retrieval. Treatment groups had comparable demographics, with an overall mean age of 31.1 years and a mean body weight of 63.2 kg in the first (150 μg) cohort and 54.0 kg in the second (100 μg) cohort. Serum hormone levels at screening were also similar between the groups, with an overall median FSH level of 7.2 IU/L.

Following profound pituitary suppression with triptorelin and treatment with a single dose of corifollitropin alfa (100 or 150 μg), median serum inhibin-B levels showed a progressive rise. None of the treated patients showed a profound decrease in serum inhibin-B levels after either dose. Additional study results are presented in Table 1.

In a previous large trial of corifollitropin alfa using a GnRH antagonist protocol and similar inclusion/exclusion criteria, the duration of stimulation was 9 days (6). In the current study, the median duration of stimulation was 10.5 and 11 days in the 150 μg and 100 μg dose groups, respectively. Accordingly, additional rFSH was required to complete ovarian stimulation. The total amount of rFSH required from day 8 to reach the criteria for hCG administration was 700 IU (3.5 days) in the 150-μg dose group and 600 IU (4 days) in the 100-μg dose group. Although the amount of FSH required appears to be relatively high in this study, it reflects the prolonged ovarian stimulation observed in long GnRH agonist protocols (13). Furthermore, all patients received a fixed daily dose of 200 or 150 IU FSH to complete ovarian stimulation.

The observed ovarian response in this small pilot trial in terms of number of follicles, serum E_2 levels on the day of hCG, and the number of oocytes retrieved was relatively high compared with previous findings using daily doses of 225 IU rFSH in a long GnRH agonist protocol (15, 16), in which an average of 14 follicles and 12 to 14 oocytes were retrieved. A previous study in female volunteers following pituitary suppression with a high-dose oral contraceptive found that a relatively large cohort of small

follicles was recruited following treatment with corifollitropin alfa (3). These findings suggest that after profound suppression, corifollitropin alfa recruits a larger cohort of follicles than does daily rFSH, likely because of the higher circulating FSH activity during the first days of stimulation (3). Other factors that may have contributed to the relatively high ovarian response include the exclusion of proven poor responders, the use of a fixed dose of rFSH from stimulation day 8 onward, and the fact that all subjects received rFSH on the day of hCG administration.

Consistent with previous studies, application of corifollitropin alfa in a long GnRH agonist protocol was well tolerated (2–6, 17). No subjects were withdrawn for safety reasons, and there were no serious adverse events. Despite the relatively high ovarian response, which is a known risk factor for OHSS (18–20), no subjects developed OHSS.

A relatively high number of mature oocytes and good-quality single-cleavage stage (day 3) embryos were obtained in this study, but there was a relatively low number of good quality single-blastocyst stage (day 5) embryos. This reduction in good-quality embryos did not seem to affect the treatment outcome, which was good in terms of ongoing pregnancy rate, despite the high proportion of Single Embryo Transfer (SET). This finding matches published data from a prospective, randomized, controlled trial in good-prognosis subjects which showed that subjects undergoing single blastocyst transfer experienced significantly higher ongoing pregnancy and delivery rates compared with subjects undergoing day 3 embryo transfer, despite having fewer good-quality supernumerary embryos available for cryopreservation (14, 21).

The current study shows that the corifollitropin alfa dose levels under investigation are able to support follicular growth for the first week of stimulation after profound pituitary suppression; they also seem to cause the recruitment of a larger cohort of follicles than with daily rFSH in a long GnRH agonist protocol. However, in view of the limited sample size, larger, prospective, controlled trials are needed to support the safety and efficacy of 100 and 150 μg corifollitropin alfa in a long GnRH agonist protocol.

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7.3 Discussion

Phase IV studies are essential to obtain additional efficacy and safety information following drug approval. Such studies are especially important for drugs that introduce a new treatment regimen, as comparative registration studies may apply a fixed treatment regimen in a rather selected patient population. Accordingly, the clinical outcome following ganirelix treatment was studied in patients defined as poor responders, high responders, or with polycystic ovary syndrome [Griesinger et al 2006]. There was little knowledge on the prediction of potential low or high ovarian responders treated with a GnRH antagonist protocol which would be especially helpful for patients undergoing their first treatment cycle. A prospective trial including both patients with or without oral contraceptive (OC) pretreatment, indicated that anti-Müllerian hormone (AMH) is the best predictive factor for the prediction of both low and high ovarian response and is not affected by intercycle variability [Nyboe Andersen et al 2011]. The study also demonstrated a significantly lower ongoing pregnancy rate per started cycle for patients pretreated with OC versus patients without OC pretreatment, which was in line with previous reports and made the meta-analysis of randomized controlled trials comparing patients with or without OC pretreatment conclusive [Griesinger et al 2010]. To date, the cause of the decreased pregnancy rate following pretreatment with OCs is not clearly understood, however, one of the explanations could be that the OC causes a carry-over effect on endometrial development. Some clinicians believe that this effect can be overcome by adding LH activity during stimulation. However, this hypothesis can only be examined in a prospective trial in which IVF patients following OC pretreatment are randomized to treatment with daily rFSH only or daily rFSH with a daily dose of hCG.

Whether the degree of LH suppression influences the ongoing pregnancy rates in a GnRH antagonist protocol without any pretreatment was studied in a retrospective comparative analysis of normal responders with low or high endogenous LH treated with daily follitropin-β or corifollitropin alfa [Doody et al 2011] and in a large pooled LH analysis of over 1700 patients [Griesinger et al 2011]. The data provided convincing evidence that the degree of LH suppression during stimulation is not associated with the ongoing pregnancy rate. A large randomized controlled study in patients stimulated with hMG (≈10 IU hCG per 75 IU FSH) and follitropin-β showed no difference in ongoing pregnancy rates [Devroey et al 2012], which indicates that supplementation with LH activity does not improve clinical outcome in this IVF population.

Recent clinical research of corifollitropin alfa has examined the efficacy and safety of this new treatment regimen in older IVF patients [Boostanfar et al 2012b]. Some clinics have explored the efficacy of corifollitropin alfa in older patients or proven poor responders using deviating treatment regimens, including a long GnRH agonist protocol. The first study of corifollitropin alfa in a long GnRH agonist protocol was performed shortly after the dose-finding study, applying the two selected doses of corifollitropin alfa in subsequently 2 small

groups of good prognosis IVF patients [Fatemi et al 2010]. Interestingly, the mean (SD) number of oocytes retrieved was respectively 15.4 (6.7) and 17.8 (5.1) following treatment with 100 µg and 150 µg corifollitropin alfa, an ovarian response that was higher than anticipated. As this first small study was uncontrolled, this observation needs to be confirmed in a prospective controlled study, primarily in low responders, who may benefit from a higher ovarian response resulting in lower cancellation rates and improved pregnany rates.

Chapter 8

General Discussion

Chapter 8 General Discussion

This thesis presents the main scientific data behind the successful clinical development of three innovative drugs for the treatment of infertility. The development of each drug is unique and the considerations leading to the final indication(s) and design of phase I, II and III trials is explained. The introduction of these drugs has provided new treatment options prior to IVF or ICSI with less drug exposure and less interventions (see Figure 9), reducing the treatment-related stress and burden imposed upon infertile couples. Moreover, these short treatment cycles result in fewer treatment cycles per time interval, a faster recovery of the patient following each treatment, and a lower discontinuation rate [Boivin et al 2012].

Infertility can result from female and/or male factors or may be unexplained. With women delaying childbearing, infertility due to aging has become a growing problem [Broekmans et al 2007]. Worldwide more than 5 million infants have been born as a result of ART and a growing percentage of IVF patients are aged 35 years and older [Centers for Disease Control and Prevention, 2011]. New drugs and treatment options that increase success rates by superior efficacy, safety and/or treatment convenience remain warranted. However, drug development is a risky entrepreneurship that requires large investments and multidisciplinary expertise from drug discovery to global introduction. Candidate drugs are usually selected for further development based on their medical need, the estimated feasibility for successful development, patent expiration, and the potential market size.

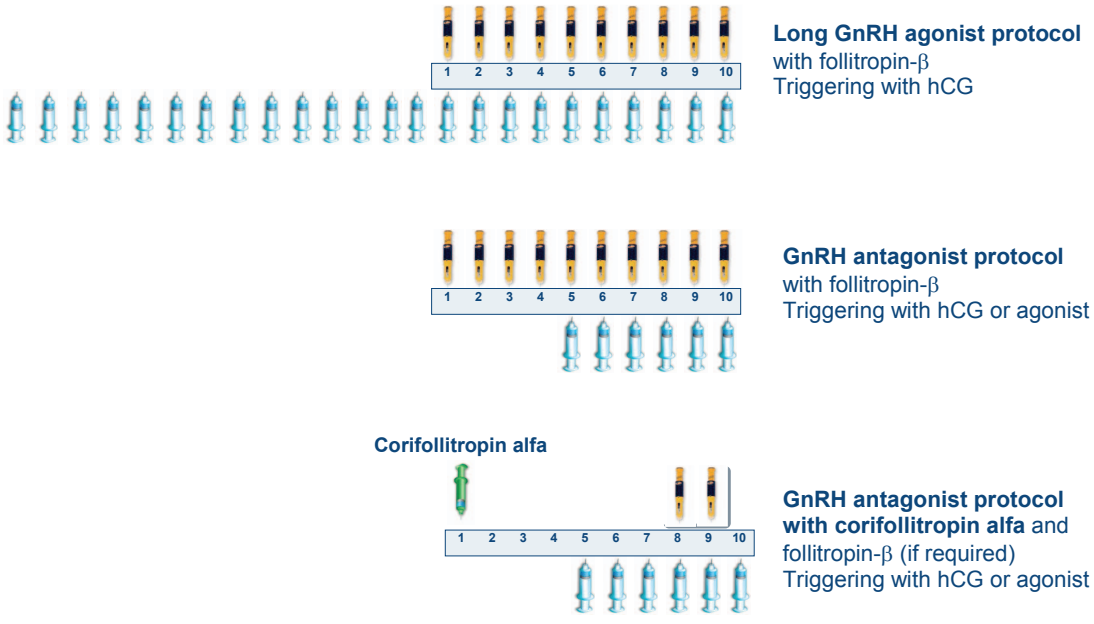
The history of drug development for infertility treatment indicates a tremendous change over the last 15 years [Lunenfeld, 2004]. The first widely available gonadotropin preparations for ovarian stimulation were urinary human menopausal gonadotropins (hMG, Humegon, Pergonal) and urinary human chorionic gonadotrophin (hCG, Pregnyl, Profasi) both requiring deep intramuscular injections. This was followed by purified urinary FSH (uFSH, Metrodin, Metrodin-HP) and purified hMG (Menopur). In 1996, pure recombinant FSH (Puregon/Follistim, Gonal-F) was approved followed later by recombinant hCG (Ovidrel/Ovitrelle) and recombinant LH (Luveris).

The first publication reporting on the application of GnRH agonists during ovarian stimulation was in 1982 [Fleming et al 1982]. By the mid 1990s, GnRH agonists had become the standard of care for IVF patients and significantly improved pregnancy rates. However, the treatment burden with long GnRH agonist protocols is high, since patients require 2 to 3 weeks of pretreatment until down-regulation is established. In contrast, GnRH antagonist is administered only on the days of stimulation when an LH rise becomes imminent. However, it took another 10 years until the third generation of GnRH antagonists was finally approved, as previous generations were hampered by histamine-release inducing properties and depot formation after injection due to "gelling" resulting in unreliable and unpredictable release from the injection site. The global introduction of GnRH antagonists ganirelix (Orgalutran) and cetrorelix (Cetrotide) revolutionized ovarian stimulation for ART by shortening the total

duration of treatment for ovarian stimulation from 3 to 4 weeks to 9 to 10 days. Even more relevant, treatment with a GnRH antagonist protocol was shown to reduce the incidence of OHSS and allowed treatment with a GnRH agonist instead of hCG to trigger final oocyte maturation, thereby eliminating early OHSS [Devroey et al 2011]. However, a single GnRH antagonist protocol has not been adopted generally and a wide variety of protocols are still applied, even though a simple GnRH antagonist protocol was proposed a few years ago [Devroey et al 2009b].

Further reduction of drug interventions was achieved by the development of the first rFSH agonist, corifollitropin alfa (Elonva) in 2010. A single dose of corifollitropin alfa was able to replace the first 7 injections of daily rFSH, which is most beneficial in patients who reach the criterion for triggering final oocyte maturation following this single injection of corifollitropin alfa. Thus, if a normal responder patient was switched from a long GnRH agonist/daily FSH/hMG protocol to a corifollitropin alfa/GnRH antagonist protocol, the number of drug interventions for ovarian stimulation they would receive would be reduced by about 75% (see Figure 9).

Figure 9 **Evolution of treatment options for controlled ovarian stimulation from lengthy and complicated to short and simple as driven by the development of ganirelix and corifollitropin alfa.**



The current thesis explains the development strategy and the main clinical findings of three fertility drugs that were developed sequentially between 1990 and 2010. Prior to phase I studies, preclinical pharmacology, pharmacokinetics and (reproductive) toxicology were performed in various species including rodents and dogs.

Preclinical pharmacology of a new drug should elucidate its mechanism of action including receptor affinity, *in vitro* and *in vivo* bioactivity and specificity. Comparative *in vitro* bioassays and *in vivo* animal models may predict its (specific) activity in comparison to reference drugs with know pharmacological action in humans. The predictive value of any preclinical models depends on the species and primary endpoints under evaluation. For gonadotropins like follitropin-β and corifollitropin alfa, diverse models using rat Sertoli or granulosa cells, or immature hypophysectomized or intact rats, provided valuable knowledge, probably due to the high homology between the rat and human FSH receptor [Minegish et al 1991]. Comparative pharmocokinetics and toxicology were examined in both rodents and dogs, however, repeated exposure and long-term treatment of animals with follitropin-β or corifollitropin alfa induces (neutralizing) antibodies in animals which may complicate the interpretation of findings.

Following preclinical development, first-in-human studies of both follitropin-β and corifollitropin alfa were carried out on volunteers with hypogonadotropic hypogonadism, as the potential immunogenicity of these large glycoproteins was difficult to estimate in preclinical models. Serious complications of immunogenicity are rare but cannot be excluded. Therefore, the ideal subjects for first-in-human studies were hypogonadotropic men or women not wanting procreation. Once the immunogenicity of a biological is proven very low, biosimilars or structure-related molecules may be tested directly in healthy volunteers during first-in-human studies.

Phase II studies of follitropin-β, ganirelix and corifollitropin alfa were very different, mainly because follitropin-β was replacing the available urinary FSH/hMG preparations using the same therapeutic dose and treatment regimen, ganirelix was a fertility drug with a different mechanism of action, and corifollitropin alfa was a recombinant FSH agonist with very different pharmacokinetics from follitropin-β. Both ganirelix and corifollitropin alfa did not only introduce a new fertility drug but also a different treatment regimen. Whereas the pharmacokinetics and therapeutic dose of follitropin-β was confirmed during phase I studies, the therapeutic doses of ganirelix and corifollitropin alfa were still unknown at the start of phase II research. Accordingly, ganirelix was tested in a very broad range of doses during the phase II dose-finding study, whereas corifollitropin alfa was first tested in a pilot study and only thereafter was a dose-finding study designed. Modeling and simulation was required to select the optimal single dose of corifollitropin alfa that would retain the FSH activity above the threshold level to support multifollicular growth for an entire week. This approach was aimed at optimal exposure, preventing overexposure in patients with a relatively low body weight and underexposure in women with a relatively high body weight. In view of its pharmacokinetics, increasing or decreasing the dose of corifollitropin alfa does not respectively increase or decrease the ovarian response which is at the near maximum of its

dose-response curve. The latter knowledge does require a paradigm shift in the thinking of prescribing IVF specialists who are used to adjusting the dose of daily (r)FSH or hMG based on the individual ovarian response.

Phase III studies of follitropin-β, ganirelix and corifollitropin alfa were not so different with respect to their primary efficacy and safety endpoints, although it was only in the randomized controlled trials of corifollitropin alfa that the non-inferiority margins of the treatment difference for both oocytes and pregnancy rates were predefined. With respect to adverse events, OHSS was always the main safety parameter of interest, even though these cases were not considered drug-related during ganirelix trials. Hypersensitivity and immunogenicity were monitored particularly intensively during the clinical development of corifollitropin alfa, as it was yet unknown whether or not the potential immunogenicity of this recombinant fusion molecule could be higher than that recombinant FSH preparations.

The safety profile of new fertility drugs can only be fully evaluated if it includes the health outcome of infants born resulting from the treatment. The collection of this safety information is most cumbersome during phase III programs as infants are often not born at the IVF units of participating investigators.

Drug approval by Health Authorities following phase I through to phase III trials is often the first milestone in clinical research. Phase IIIb and IV trials are required to explore additional treatment options or beneficial treatment in different subsets of patients, which is especially worthwhile with new treatment regimens as with ganirelix and corifollitropin alfa.

With the development of drugs that require fewer injections, patients have a reduced treatment burden and are more likely to continue subsequent treatment cycles when pregnancy is not achieved. This is not only of benefit to patients but also to clinics who will improve their cumulative pregnancy rates. Compounds like ganirelix and corifollitropin alfa have contributed to the development of short treatment regimens with fewer interventions. Development of Low Molecular Weight (LMW) stimulating agents suitable for oral intake [Gerrits et al, 2013] certainly further improve patient convenience, but the development of these oral compounds may be less straightforward than for parenteral fertility drugs.

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Summary chapter 1

Drug development starts with drug discovery and is completed with clinical research to evaluate the efficacy and safety of investigational drugs in humans for the intended indication. This thesis provides an overview of the sequential clinical development of three fertility drugs namely follitropin-β (recombinant human FSH), ganirelix (GnRH antagonist) and corifollitropin alfa (long-acting recombinant FSH agonist). Follitropin-β and corifollitropin alfa are New Biological Entities (NBEs) as they are large recombinant glycoprotein molecules with a three-dimensional structure that is essential for their bioactivity. In contrast, ganirelix is a decapeptide and an New Chemical Entity (NCE). Follitropin-β and corifollitropin alfa are both indicated for the development of multiple follicles in women participating in an ART program. Ganirelix is indicated for the prevention (or inhibition) of premature endogenous LH surges during multiple follicular development. The development of these fertility drugs has dramatically changed treatment prior to ART from being lengthy and complicated to short and simple.

Samenvatting hoofdstuk 1

De ontwikkeling van een nieuw geneesmiddel begint met de ontdekking van een werkzame stof en wordt voltooid met klinisch onderzoek om de werking en veiligheid van deze stof bij de mens te evalueren. Dit proefschrift geeft een overzicht van de opeenvolgende klinische ontwikkeling van 3 geneesmiddelen voor de behandeling van onvruchtbaarheid namelijk follitropin-β (recombinant humaan follikelstimulerend hormoon (rFSH), ganirelix (een Gonadotrofine Releasing Hormoon (GnRH) antagonist) en corifollitropin alfa (een recombinant gonadotrofine met langdurige follikelstimulerende activiteit). Follitropin-β en corifollitropin alfa zijn Nieuwe Biologische Entiteiten (NBEs) omdat het grote recombinante glycoproteinen betreft waarvan de drie-dimensionale structuur essentieel is voor hun werking. In tegenstelling tot follitropin-β en corifollitropin alfa, is ganirelix een decapeptide en wordt ookwel een Nieuwe Chemische Entiteit (NCE) genoemd. Patiënten worden behandeld met follitropin-β of corifollitropin alfa voor de ontwikkeling van meerdere follikels voorafgaand aan *in vitro* fertilisatie (IVF) of intracytoplasmatische sperma injectie (ICSI), terwijl ganirelix gedurende deze behandeling vroegtijdige stijging van het lichaamseigen hormoon luteïniserend hormone (LH) doet voorkomen of afremmen. De ontwikkeling van deze vruchtbaarheidshormonen heeft de behandeling van patiënten drastisch veranderd van langduring en gecompliceerd naar kort en simpel.

Summary chapter 2

Preclinical pharmacology is essential to estimate the bioactivity and specificity of any drug during early development. Pharmacology experiments may elucidate their mechanism of action in the absence or presence of other hormones or drugs. If preclinical animal models

are predictive for human, comparative controlled experiments are giving the best estimates of effective doses in phase I and II studies. *In vitro* FSH bioassays using rat or human Sertoli or granulosa cells have both shown to provide a reliable prediction of the intrinsic bioactivity of follitropin-β and corifollitropin alfa. Initial *in vitro* and *in vivo* experiments of follitropin-β showed that recombinant FSH was very comparable to urinary FSH. *In vivo* experiments in hyphophysectomized rats with follitropin-β with or without concomitant hCG elucidated for the first time the role of FSH and LH activity during folliculogenesis. Due to its much longer circulating half-life, *in vivo* experiments of corifollitropin alfa revealed in different species clear differences in comparison to follitropin-β. In addition, the classical Steelman Pohley bioassay did not provide a reliable estimate of the *in vivo* bioactivity of corifollitropin alfa in humans. Consequently, the early clinical development of corifollitropin alfa was more extensive as reflected by a variety of subsequent phase I and II studies.

Samenvatting hoofdstuk 2

Preklinische farmacologie is belangrijk om de werking en de specificiteit van een nieuw geneesmiddel in een vroeg stadium van ontwikkeling vast te leggen. Farmacologische experimenten kunnen het werkingsmechanisme van stoffen onderzoeken, al dan niet in samenhang met andere geneesmiddelen. Indien preklinische diermodellen voorspellend zijn voor de mens, geven vergelijkende experimenten de beste inschatting van de werkzame dosering voorafgaand aan fase I en II onderzoek. *In vitro* FSH bioassays ontwikkeld met Sertoli of granulosa cellen van rat of mens zijn gebruikt om de intrinsieke bioactiviteit van follitropin-β en corifollitropin alfa te voorspellen. Eerste *in vitro* en *in vivo* experimenten met follitropin-β toonden aan dat de pharmacodynamische eigenschappen van recombinant FSH zeer vergelijkbaar waren met urinair FSH. *In vivo* experimenten met follitropin-β bij gehypofysectomeerde ratten met of zonder hCG behandeling toonden voor het eerst de specifieke rol van FSH en LH gedurende de follikelontwikkeling aan. Vanwege zijn langere halfwaarde tijd, toonden *in vivo* experimenten met corifollitropin alfa in diverse diersoorten duidelijke verschillen aan ten opzichte van follitropin-β. Bovendien bleek de klassieke Steelman Pohley bioassay geen betrouwbare inschatting te geven van de potentie van corifollitropin alfa in de mens. Bijgevolg was de vroege klinische ontwikkeling van corifollitropin alfa ook omvangrijker met meerdere fase I and II studies.

Summary chapter 3

Phase I studies are usually performed in healthy male or female volunteers unless safety aspects require otherwise. Traditionally, first-in-human studies should reveal the maximum tolerated dose in humans by means of single- and/or multiple-rising dose studies. At the same time, these studies will provide single-dose and multiple-dose pharmacokinetics of the investigational drug. Depending on the study design and study population included in phase I

studies, first information on the potency of the drug for the desirable indication may be obtained. The first-in-human phase I studies of follitropin-β and corifollitropin alfa were both including subjects with hypogonadotropic hypogonadism (HH) as the potential immunogenicity could not be estimated in preclinical models. Follitropin-β was tested in a single dose (300 IU) and in multiple doses and provided information on its safety and pharmacokinetics both in HH men and women. In addition, the ability of follitropin-β to stimulate multiple follicular growth in the complete absence of LH activity was documented for the first time. The first-in-human study of corifollitropin alfa was performed in HH men who received up to 4 injections of one low dose (15 μg) of corifollitropin alfa. Thereafter, a second phase I study in healthy pituitary-suppressed volunteers of reproductive age was performed to assess the potency of increasing doses (15-120 μg) of corifollitropin alfa to induce multiple follicular growth and to assess its duration of action.

Samenvatting hoofdstuk 3

Fase I studies maken doorgaans gebruik van gezonde mannelijke of vrouwelijke vrijwilligers tenzij bepaalde veiligheidsaspecten anderzins vereisen. In de allereerste humane studies wordt de veiligheid van een nieuw geneesmiddel bestudeerd door toediening van enkelvoudige en meervoudige oplopende doseringen. Dergelijke studies geven tegelijkertijd informatie over het metabolisme van het nieuwe geneesmiddel. Afhankelijk van de opzet van de studie en de studiepopulatie kan gedurende het fase I onderzoek al eerste informatie verkregen worden over de doseringen die mogelijk werkzaam zijn voor de beoogde indicatie. De eerste humane studies van follitropin-β en corifollitropin alfa includeerden vrijwilligers met hypogonadotroop hypogonadisme (HH) omdat het risico ten aanzien van immuunreacties op basis van preklinische modellen moeilijk is te voorspellen. Follitropin-β werd getest in enkelvoudige en meervoudige doseringen en gaf informatie rondom de bijwerkingen en farmacokinetiek in zowel HH mannen als vrouwen. Bovendien werd vastgesteld in HH vrouwen dat follitropin-β in staat is meervoudige follikelgroei te stimuleren in volledige afwezigheid van LH activiteit. Om het risico op immuunreacties van corifollitropin alfa in de mens te bestuderen zijn in de eerste fase I studie HH mannen behandeld met 4 sequentiele injecties van een lage dosering (15 μg) corifollitropin alfa. Vervolgens werd in een tweede fase I studie bij gezonde vrouwelijke vrijwilligers met onderdrukte hypofyse-activiteit uitgevoerd om het effect van een toenemende, enkelvoudige dosering (15-120 μg) corifollitropin alfa vast te stellen alsmede de duur van deze werking.

Summary chapter 4

Phase II studies are performed to allow final selection of one or more doses for further clinical development. Ideally, a phase II dose-finding should indicate which dose is too low

and which dose is a too high for the intended indication. Since preclinical research and phase I studies indicated that follitropin-β was very similar to urinary FSH, no dose-finding study was required for follitropin-β. Primarily, phase II research of follitropin-β was to address the question as to whether patients treated with different GnRH agonist protocols, especially with a long GnRH agonist protocol, could be treated with pure recombinant FSH lacking LH activity. In contrast, the GnRH antagonist ganirelix was tested in a classical, double-blind, dose-finding study including 6 dose groups (0.0625 – 2 mg). The lowest and highest dose group were discontinued half-way through the study based on the advice of an independent committee. Of the four remaining dosages, 0.25 mg provided optimal suppression to prevent premature LH surges to occur without compromising implantation rates. Corifollitropin alfa was first tested in small groups of patients in 3 doses of 120 to 240 μg, which appeared to provide nearly maximum ovarian response rates. The phase II controlled dose-finding study including 60, 120 and 180 μg demonstrated that the optimal dose for multiple follicular development during the first 7 days of ovarian stimulation is higher than 60 μg and lower than 180 μg. Subsequent dose selection was based on modeling and simulation taking into account the fact that exposure to corifollitropin alfa is inversely related to body weight and that FSH activity had to be retained for an entire week above the threshold required to support multiple follicular development. Finally, two strengths of corifollitropin alfa were selected for further development in phase III studies, namely 150 μg for subjects weighing >60 kg and 100 μg for subjects ≤ 60 kg.

Samenvatting hoofdstuk 4

Fase II studies worden uitgevoerd om de optimale dosering of doseringen van een nieuw geneesmiddel te selecteren voor verdere klinische ontwikkeling. Idealiter zou een fase II studie moeten weergeven welke dosering te laag is of welke dosering te hoog is voor de gewenste indicatie. Aangezien preklinisch en fase I onderzoek duidelijk aangaf dat follitropin-β zeer vergelijkbaar is met urinair FSH, was een klassieke dose-finding studie niet nodig. Het voornaamste doel van het fase II onderzoek was gericht op de vraag of patiënten die behandeld werden met verschillende GnRH agonist protocollen effectief behandeld konden worden met puur recombinant FSH zonder LH activiteit. Daarentegen is de GnRH antagonist ganirelix getest in een klassieke, dubbel-blinde, dose-finding studie met in totaal 6 doseringsgroepen (0.0625 – 2 mg). Halverwege deze studie werden de hoogste en laagste dosering stopgezet door een onafhankelijke adviescommissie. Van de 4 overblijvende doseringen bleek 0.25 mg optimale hypofyse suppressie te geven om vroegtijdige LH stijgingen te voorkomen zonder de kans op implantatie te verminderen. Corifollitropin alfa werd eerst in kleine groepen patiënten getest met 3 doseringen van 120 tot 240 μg, die een bijna maximale ovariële respons bleken te induceren. Een controleerde dose-finding studie met 60, 120 and 180 μg toonde vervolgens aan dat de optimale dosering voor meervoudige

follikelontwikkeling gedurende de eerste 7 dagen van de ovariële stimulatie hoger is dan 60 µg en lager dan 180 µg. De uiteindelijk corifollitropin alfa doseringen werden geselecteerd op basis van pharmacokinetische and pharmacodynamische simulatiemodellen waarbij in het model rekening werd gehouden met de omgekeerde evenredigheid tussen het lichaamsgewicht en de hoeveelheid corifollitropin alfa in de bloedcirculatie. Daarnaast zou de totale FSH activiteit voldoende hoog moet blijven om meervoudige follikelontwikkeling gedurende een hele week te ondersteunen. Op basis hiervan werden uiteindelijk 2 doseringen corifollitropin alfa geselecteerd voor verdere ontwikkeling in fase III studies, namelijk 150 µg voor vrouwen met een lichaamsgewicht >60 kg en 100 µg voor vrouwen met een lichaamsgewicht ≤ 60 kg.

Summary chapter 5

A phase III program includes large, randomized, comparative trials in patients to assess the efficacy and safety of a new drug or treatment regimen in comparison to the current care. Phase III studies should be sufficiently powered to exclude or detect a clinically meaningful difference of the primary endpoint between treatment groups. Thus, the size of each phase III trial is determined by the selected primary efficacy endpoint. Comparison of the pivotal phase III studies of follitropin-β, ganirelix and corifollitropin alfa did not reveal large differences in terms of trial design or primary endpoints. For comparative reasons, studies in IVF patients often only include women with normal regular cycles and treatment regimens are fixed. The number of oocytes and/or the ongoing pregnancy rate per started cycle are most frequently considered as the primary endpoints. Firstly, follitropin-β was compared to urinary FSH and developed in a long GnRH agonist protocol. The outcome of these trials indicated that per started cycle follitropin-β provided more oocytes than urinary FSH with similar (ongoing) pregnancy rates following fresh embryo transfer. Subsequently, the GnRH antagonist ganirelix was compared to a long GnRH agonist protocol. Using follitropin-β for ovarian stimulation, the short ganirelix protocol provided fewer oocytes with a small trend towards lower ongoing pregnancy rates. Finally a single dose of corifollitropin alfa was compared to daily 150 to 200 IU follitropin-β using a ganirelix protocol which demonstrated that corifollitropin alfa provided significantly more oocytes and was proven statistically non-inferior in comparison to follitropin-β in terms of the ongoing pregnancy rate per started cycle following fresh embryo transfer.

Samenvatting hoofdstuk 5

Een fase III programma voor de ontwikkeling van een nieuw geneesmiddel betreft grote, gerandomiseerde, vergelijkende studies in patiënten om de werkzaamheid en veiligheid van het nieuwe geneesmiddel of behandeling te bestuderen in vergelijking met de tot dan toe bekende standaardtherapie. Phase III studies moeten voldoende groot zijn om een klinisch

relevant verschil ten aanzien van het primaire eindpunt tussen de groepen aan te tonen. Met andere woorden, het aantal patiënten in een fase III studie wordt bepaald door het primaire eindpunt van de studie en het verschil dat klinisch relevant wordt geacht. In vergelijkend onderzoek wordt de inclusie van IVF patiënten vaak beperkt tot vrouwen met een normale regelmatige cyclus en hun behandeling beperkt tot een gestandaardiseerd protocol. Het aantal verkregen eicellen en/of het aantal doorgaande zwangerschappen zijn vrijwel altijd het primaire eindpunt van de studie. In eerste instantie werd follitropin-β vergeleken met urinair FSH en ontwikkeld in een lang GnRH agonist protocol. De uitkomsten van deze studies toonden aan dat na behandeling met follitropin-β meer eicellen werden verkregen dan na behandeling met urinair FSH, met gelijke percentages (doorgaande) zwangerschappen na directe terugplaatsing van embryo's. Vervolgens werd de GnRH antagonist ganirelix vergeleken met een lang GnRH agonist protocol. Gebruikmakend van dezelfde dagelijkse dosis follitropin-β werden met het korte ganirelix protocol minder eicellen verkregen met een trend tot lagere percentages doorgaande zwangerschappen. Tenslotte werd een enkelvoudige dosering corifollitropin alfa vergeleken met een dagelijkse dosering van 150 of 200 IU follitropin-β waarbij significant meer eicellen werden verkregen en bewezen werd dat de kans op doorgaande zwangerschappen statistisch niet-inferieur is aan dagelijks follitropin-β na directe terugplaatsing van een of twee embryo's.

Summary chapter 6

Main safety endpoints during phase III trials of follitropin-β, ganirelix and corifollitropin alfa were very similar and focused on the incidence of adverse events including Ovarian Hyperstimulation Syndrome (OHSS), hypersensitivity, immunogenicity, and the health outcome of offspring. The clinical safety research in Chapter 6 is restricted to corifollitropin alfa, which was the last developed product.

With respect to OHSS, special attention was paid to moderate or severe OHSS with early onset of which the incidence is known to increase with the ovarian response and is not related to pregnancy. Even though corifollitropin alfa provided in young IVF patients significantly more oocytes (1 to 2) than 150 to 200 IU follitropin-β, the risk for OHSS was not significantly increased. Immediate drug-related adverse reactions related to hypersensitivity were not observed following (repeated) corifollitropin alfa administration during the phase III program. In addition, none of the exposed patients developed an immune reponse against corifollitropin alfa. Analysis of the health outcome of over 800 live-born infants of women treated with corifollitropin alfa, revealed that both the nature and incidence of major and minor congenital malformations are similar as for live-born infants following treatment of women with follitropin-β.

Samenvatting hoofdstuk 6

Het veiligheidsonderzoek gedurende fase III studies van follitropin-β, ganirelix en corifollitropin alfa was zeer vergelijkbaar tussen studies en betrof bijwerkingen met bijzondere aandacht voor overstimulatie, overgevoeligheidsreacties, immuunreacties, en de gezondheid van de kinderen geboren uit zwangerschappen na behandeling met deze geneesmiddelen. Het klinische veiligheidsonderzoek beschreven in hoofdstuk 6 richt zich met name op corfollitropin alfa, het laatste ontwikkelde vruchtbaarheidshormoon. Met betrekking tot overstimulatie (OHSS) is speciale aandacht noodzakelijk voor de matige tot ernstige OHSS met vroegtijdige verschijnselen, waarvan de incidentie toeneemt met de mate van ovariële respons en die niet gerelateerd is aan een mogelijke zwangerschap. Alhoewel na corifollitropin alfa behandeling in jonge IVF patiënten significant meer eicellen werden verkregen dan na behandeling met 150 of 200 IU follitropin-β, bleek het risico op OHSS niet significant verhoogd te zijn. Onmiddellijke bijwerkingen gerelateerd aan overgevoeligheidsreacties werden niet waargenomen na (herhaalde) toediening van 150 µg corifollitropin alfa gedurende het fase III programma. Bovendien ontwikkelde geen enkele behandelde patiënt een immuunreactie tegen corifollitropin alfa. Analyse van de gezondheid van meer dan 800 kinderen die geboren zijn uit zwangerschappen na behandeling met corifollitropin alfa toonde dat zowel de aard als het percentage van grote en kleine misvormingen vergelijkbaar zijn met die zoals waargenomen in kinderen geboren na behandeling van vrouwen met follitropin-β.

Summary chapter 7

Phase IV studies are performed to obtain additional efficacy and/or safety data following drug approval by Health Authorities. Such studies may include different subsets of patients or treatment posology and may extend drug labeling if studies are adequate and conclusive. Phase IV studies as well as retrospective analyses of fase III data are essential for drugs like ganirelix and corifollitropin alfa which introduce a new treatment regimen. Phase IV studies of ganirelix examined, among others, the impact of ganirelix treatment on endometrial development, the application of ganirelix in patients undergoing ovulation induction for IUI, predictive factors of ovarian response, various starting doses of rFSH, the impact of OC pretreatment and the application of a GnRH agonist for triggering final oocyte maturation.

Predictive models of ovarian response are essential for patients if the anticipated ovarian response would be too low to be beneficial or would be too high to be safe. Prospective Phase IV research indicated that factors like AMH, AFC and FSH are the best predictive factors for ovarian response following stimulation with follitropin-β in a ganirelix protocol. Retrospective LH analyses of phase III trials indicated that there is no asssociation between endogenous LH levels and the chance on pregnancy using a ganirelix protocol with either corifollitropin alfa or follitropin-β for ovarian stimulation. With the introduction of corifollitropin

alfa, additional research will be related to the use of a GnRH agonist for triggering final oocyte maturation in high responders and the use of corifollitropin alfa in a long GnRH agonist protocol for low responders. The first uncontrolled study of the latter regimen indicated a higher ovarian response than anticipated, thus future controlled studies should reveal whether this could be an potential benefit for patients with a relatively low ovarian reserve.

Samenvatting hoofdstuk 7

Fase IV onderzoek wordt uitgevoerd om extra gegevens te verkrijgen over de effectiviteit of veiligheid van een geneesmiddel nadat het reeds is goedgekeurd door gezondheidsauthoriteiten. Dergelijke studies kunnen zich richten op speciale groepen van patiënten of op de behandelingswijze en, indien de uitkomsten positief zijn, kunnen deze gebruikt worden om de bijsluitertekst uit te breiden. Fase IV studies, alsmede retrospectief onderzoek van de fase III gegevens, zijn vooral belangrijk voor geneesmiddellen zoals ganirelix en corifollitropin alfa die een nieuw behandelingsregime introduceren. Fase IV studies met ganirelix bestudeerden o.a. het effect van ganirelix behandeling ten aanzien van de ontwikkeling van het baarmoederslijmvlies, bij ovulatie inductie voorafgaand aan intrauterine inseminatie, predictiefactoren van ovariële respons, diverse startdoseringen van recombinant FSH, voorbehandeling met de contraceptiepil en de toepassing van een GnRH agonist voor de inductie van de finale eicelrijping.

Predictieve modellen ter voorspelling van het aantal te verkrijgen eicellen zijn belangrijk voor vrouwen met een mogelijke te lage of te hoge ovariële respons om respectievelijk effectief of veilig te zijn. Prospectief fase IV onderzoek heeft aangetoond dat factoren zoals AMH, AFC en FSH een goede voorspelling geven van het aantal te verkrijgen eicellen na behandeling met follitropin-β in een ganirelix protocol. Retrospectieve LH analyses van fase III studies gaven aan dat er geen asssociatie is tussen endogeen LH en de kans op zwangerschap na behandeling met ganirelix in combinantie met corifollitropin alfa of follitropin-β voor ovariële stimulatie. Met de introductie van corifollitropin alfa wordt verder onderzoek verwacht, met name naar het gebruik van een GnRH agonist voor de inductie van de finale eicelrijping in patiënten met een (verwachte) hoge ovariële respons en naar het gebruik van corifollitropin alfa in een lang GnRH agonist protocol in patiënten met een (verwachte) lage ovariële respons. In een eerste niet-gecontroleerde studie met corifollitropin alfa in een lang protocol van triptoreline werden namelijk relatief meer eicellen verkregen dan verwacht. Toekomstig gecontroleerd onderzoek met corifollitropin alfa zal moeten aantonen of de kans op zwangerschap per gestarte behandeling voor patiënten met een relatief laag ovariële reserve verbeterd kan worden.

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Acknowledgment

This thesis summarizes the most important research & development executed between 1985 and 2010 which resulted in the sequential registration of 3 fertility drugs. Success has many fathers, especially over such long period.

The basics of assay development and animal pharmacology were taught to me at the Biochemical and Pharmacological R & D Laboratories headed by Ton Schuurs and Lenus Kloosterboer who coached me through the process of first manuscript writing.

In 1990 I switched from preclinical to clinical research and joined the Medical Unit under the supervision of Herjan Coelingh Bennink. His enthusiasm and scientific knowledge were a great example. He did broaden my knowledge and invited me to the many Expert meetings he organized to support the development of follitropin-β and ganirelix. Especially the first phase I studies in volunteers with hypogonadotropic hypogonadism were complicated and brought me into contact with Professors Bart Fauser, Philip Bouchard and Howard Jacobs. In 1992, we published with Professors Paul Devroey and Andre Van Steirteghem in the Lancet about the first established pregnancy following follitropin-β treatment, which was the start of a very long and fruitful collaboration with the VUB in Brussels.

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Curriculum Vitae

Bernadette Mannaerts was born on March 13, 1957 in Tilburg. Following her academic degree in Medical Biology (B5* old style) at the University of Utrecht, she worked at the Veterinary faculty on the morphology and karyotyping of bovine embryos. She started her career at legacy Organon in preclinical research in 1985 at the Biochemical and Pharmacological R&D laboratories, where she guided biological characterisation studies on gonadotrophins, anti-gonadotrophins and GnRH analogues by means of in vitro bioassay methods, immunoassays, chromatofocussing and animal experiments. During that period she performed most of the preclinical pharmacology studies with recombinant FSH (Puregon/Follistim). In 1990, she joined the Medical Research and Development Unit and was responsible for phase I to III trials of Puregon which was first approved in 1996. In 1997, she became the Head of Clinical Projects Infertility and was responsible for the global development of the GnRH antagonist ganirelix. From 2000 to 2002 she was appointed as the Director of Medical Services Infertility to guide the marketing introduction of ganirelix and additional phase IV trials. In 2002 she returned to Global Clinical Research as the Head of Fertility and developed successfully recombinant corifollitropin alfa (Elonva) which was first approved via the Centralized Procedure in Europe in 2010.