Aspects of Mono- and Multiple Dominant Follicle Development in the Human Ovary

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Aspects of Mono- and Multiple Dominant Follicle Development in the Human Ovary.
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Aspecten van de ontwikkeling van één of meerdere dominante follikels in het humane ovarium

PROEFSCHRIFT

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Ter nagedachtenis aan mijn vader

Voor Stijn en Wiebe

Contents

List of Abbreviations

AD	androstenedione
AMH	anti-Müllerian Hormone
ANOVA	analysis of variance
ART	assisted reproductive technique(s)
AUC	area under the curve
BMI	body mass index (=weight/height^2)
CD	cycle day
CRA	clomiphene citrate resistant anovulation
d	day(s)
DF	dominant follicle
DHEAS	dehydroepiandrosterone sulphate
E_2	17β-oestradiol
e.g.	exempli gratia (for example)
ESHRE	European Society for Human Reproduction and Embryology
ET	embryo transfer
FAI	free androgen index (=Tx100/SHBG)
FSH	follicle-stimulating hormone
GnRH	gonadotrophin-releasing hormone
GTT	glucose tolerance test
hCG	human chorionic gonadotrophin
ICSI	intracytoplasmic sperm injection
i.e.	id est (it is)
IGF	insulin-like growth factor
im	intramuscular
IU	international unit
IUI	intrauterine insemination
iv	intravenous
IVF	in-vitro fertilization
LH	luteinizing hormone
OHSS	ovarian hyperstimulation syndrome
OPU	ovum pick up
P	progesterone
PCO	polycystic ovaries
PCOS	polycystic ovary syndrome
PF	pre-ovulatory follicle
recFSH	recombinant human FSH
RIA	radioimmunoassay
sc	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SHBG	sex-hormone binding globulin
SPSS	Statistical Package for the Social Sciences

SRY	sex-determining region of the Y chromosome
T	testosterone
TDF	testes-determining factor
TSH	thyroid-stimulating hormone
TVS	transvaginal sonography
WHO	world health organization
yrs	years

Chapter 1

Introduction and objectives

1.1. General introduction

Reproduction is one of the most essential aspects of life. Understandably, couples confronted with unwanted childlessness often experience social and psychological problems (Downey *et al.*, 1989; Whiteford and Gonzalez, 1995). Although the debate continues as to whether subfertility should be considered as a disease, the major impact of involuntary childlessness on quality of life should not be underestimated. According to the World Health Organization (WHO), health is defined as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (Constitution of the WHO, 1948) and therefore infertility constitutes an unhealthy state. Reproductive medicine and the counselling of subfertile couples contribute to general health and should therefore not be trivialized as luxury medicine.

Infertility is an absent ability to conceive or produce offspring, usually defined as a failure to conceive within a year of regular, unprotected intercourse (Sciarra, 1994). Sterility on the other hand is a complete inability to conceive without assisted reproduction, which only occurs in 3-5% of all couples (Greenhall and Vessey, 1990). Because the probability of success is still considerable after one year of unprotected intercourse (Figure 1.1), the term subfertility seems most accurate in describing a condition of diminished natural fertility. Irrespective the cause (female, male, combined or unexplained), subfertility is a problem of the couple. Decreased fertility of a given individual may be well compensated for by enhanced fertility of the partner. These couples may generate offspring without difficulty and therefore never visit a doctor.

Approximately 90% of normally fertile couples will eventually conceive within 1 year of unprotected intercourse. Within 2 years almost 100% of these normally fertile couples will have conceived (Evers, 2002). However, 10-15% of couples of childbearing age will have difficulties conceiving (primary infertility), or conceiving the number of children they want (secondary infertility), and seek specialist fertility care at least once during their reproductive lifetime (Hull *et al.*, 1985).

Although the percentage of individuals with subfertility in the western world remains constant (about 10-15%), the number of couples seeking specialist fertility care is growing (Evers, 2002). The growing demand on assisted reproductive technology (ART) is mainly age-driven. An increasing number of women delay having children until an age when natural female fertility is declining (van Noord-Zaadstra *et al.*, 1991), contributing to the incidence of subfertility, which may therefore rise in the near future (te Velde and Pearson, 2002). The prevailing concept of female reproductive age assumes that the decline of both quantity and quality of the oocytes/follicles determines the age dependent loss of female fertility (te Velde and Pearson, 2002). Whether an increased aneuploidy of oocytes, a raised chance of exposure to sexually transmitted diseases and/or continually falling sperm counts contribute to the age related decline in fertility (Swan *et al.*, 2000; Dunson *et al.*, 2004), remains to be confirmed. The socially compelled delay in childbearing not only affects fertility and the increased demand on ART. Perhaps even more important is its demographic

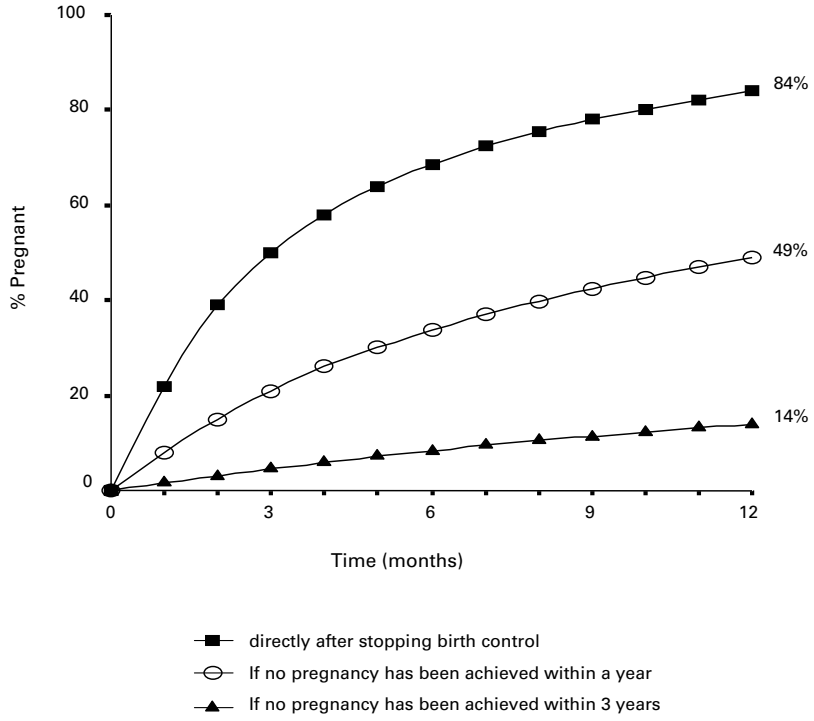

Figure 1.1 Chances for conception per cycle. Probability of conceiving for periods up to 12 months, directly after stopping birth control measures, after 1 year of regular, unprotected intercourse and after 3 years of regular, unprotected intercourse. (te Velde *et al.*, 2000).

consequence of a decreased number of offspring per family. The minimum number of children needed to maintain a population demographically equals 2.1 per family. In many western countries, this number of children per family has declined beneath this minimum (von Cube, 1986).

During the last decades, reproductive medicine has undergone an explosive development (Table 1.1). Although knowledge on human reproduction and success rates of fertility treatment have improved over the years, many couples stay childless despite treatment. Next to unsuccessful treatment, a new problem has arisen. In attempts to increase pregnancy outcome, fertility treatments practised today have become complex, time-consuming, expensive and not without risk. Problems related to fertility therapy, especially ovarian stimulation, include short term complications (like discomfort and ovarian hyperstimulation syndrome), multiple gestation and uncertainties regarding long-term health consequences. Awareness is growing throughout the world that the rate of multiple pregnancies -especially triplet and higher-order pregnancies- following ovarian (hyper)stimulation is no more longer accepted (Edwards *et al.*, 1996; Templeton and Morris, 1998; Fauser *et al.*, 1999; Templeton, 2000; Gleicher *et al.*, 2000; Fauser *et al.*, 2002; Heijnen *et al.*, 2004). In contrast to an increased complexity of fertility treatment, a shift towards milder, more physi-

ological stimulation protocols is needed. These developments will ultimately improve the balance between success, complications and the overall cost-effectiveness of ART. In order to be able to improve fertility treatment, i.e. improvement of success rate as well as reduction in complications, increasing knowledge regarding the physiology of ovarian follicle development and interference with single dominant follicle selection is needed.

Table 1.1 Historical overview of key milestones in Reproductive Medicine

Time (AD)	Name	Subject
98-138	Soranus of Ephesus	Earliest known description of the ovaries
1400	Gian Matteo de Gradi	First used term "ovary" instead of "female testes"
1452-1519	Leonardo da Vinci	Anatomy of human uterus and ovaries
1515-1564	Andreas Vesalius	Description of the hypophysis as "glandula pituitaria cerebri excipiens"
1543	*Humani Corporis Fabrica*	Recognition of follicles in the ovary
1534	Volcher Coiter	Description of the corpus luteum
1561	Gabriele Falloppio *Observationes Anatomicae*	Description of the Fallopian tubes
1641-1673	Reinier de Graaff	Description of "the female testis" (used term "ovary") and "egg-containing" (Graafian) follicle. First account of ovulation.
1827	Carl Ernst von Baer	Description of the human ovary; founder of embryology
1863	Eduard Pfluger	Theory of menstruation
1866	James Marion Sims	Successful artificial insemination in a patient with a history of 9 years of infertility
1876	Oscar Hertwig	Demonstration that fusion of the nuclei of ovum and spermatozoa is essential for fertilization
1878	Schenk	First attempt at in vitro fertilization in rabbits and guinea pigs
1883	Edouard van Beneden	Demonstration that both ovum and sperm reduce their chromosome count by half
1929 - 1939	George W. Corner, Willard M. Allen	Discovery of "progestin" (progesterone) in the corpus luteum. Discovery that deprivation of progesterone and oestrogen causes menstruation
1900	Walter Heape	Investigation of menstrual cycle in animals and introduction of term "estrus"
1921	Evans and Long	Demonstration that administration of anterior pituitary hormone extract to rats affects growth and the estrus cycle
1923	Doisy and Allen	Valuable work on hormones in ovulation
1927-1933	Smith and Engle, Zondek, Fevold *et al.*	Recognition of 2 pituitary hormones (LH and FSH) as the main regulators of ovarian function and oocyte maturation
1930	Marrian	Isolation of estriol from human pregnant urine and study on steroid biochemistry and sex hormones
1934	Leonard and Smith	Description that urine of pregnant women contains predominantly LH of placental origin and urine of climacteric women can induce follicle growth in the ovary
1935	Stein and Leventhal	First description of PCOS

Mid 1940's		Initial (unsuccessful) experiments in treating anovulatory patients with gonadotrophins (purified from pregnant mare serum)
1944	Rock and Menkin	Successful fertilization of human eggs in vitro
1952	Harris	Demonstration that releasing factors are liberated from nerve endings in the hypothalamus tracts into capillaries of portal vessels to the anterior pituitary
1954	Bunge and Sherman	Report of first human pregnancy from stored frozen spermatozoa
1957	Borth *et al.*	Extraction of FSH-like substance from urine of postmenopausal women with gel-electrophoresis
1958, 1966	Gemzell *et al.*	Successful treatment of anovulation with human pituitary gonadotrophins
1959	Chang	Delivery of healthy rabbits after in vitro fertilization and transfer of the four-cell embryos into the fallopian tubes of recipient rabbits.
1969	Edwards and Steptoe	Report of in vitro fertilization of human oocytes which had been matured in vitro
1975	Coy *et al.*	Creation of potent gonadotrophin releasing- hormone agonists
1975	Edwards and Steptoe	Treatment of infertile women with human menopausal gonadotrophin (or clomiphene citrate) and chorionic gonadotrophin and collection of preovulatory eggs for IVF
1976	de Jong	Detection of inhibin in ovarian follicular fluid
1977	Yalow	Development of radioimmunoassay (RIA) to measure minute amounts of biologically active substances
1978	Brown	Description of the FSH threshold concept
1978	Steptoe and Edwards	First successful IVF: Louise Brown
1979	Hackeloer *et al.*	Introduction of pelvic sonography
1982	Zeilmaker	First successful IVF in the Netherlands (Rotterdam)
1982, 1983	Mettler *et al.*, Garcia *et al.*	Use of human menopausal gonadotrophin for ovarian hyperstimulation in IVF.
1983	Zeilmaker	First birth from frozen thawed embryo in IVF
1984	Meldrum *et al.*	Transvaginal sonographic monitoring of follicle growth
1986	American Fertility Association	Publication of guidelines for donor screening in artificial insemination
1987	Fraser and Baird	Prevention of premature LH surge in IVF by using gonadotrophin releasing-hormone analogues.
1988	Abdalla and Leonard	Birth following zygote intrafallopian transfer
1998	Nestler	Induction of spontaneous ovulation in PCOS patients by the use of insulin sensitizing drug metformin
2000	Organon, ASTA Medica	Introduction of 3rd generation GnRH antagonists (ganirelix; cetrorelix)
2002	Mitwally	Description of aromatase inhibitors for ovulation induction in PCOS patients

Chronology based on "Dates in Obstetrics & Gynecology" (Lee, 2000)

1.2. Ovarian physiology

1.2.1. Ovarian development

During early embryo development, the gonads derive from the coelomic epithelium and underlying mesenchymal tissue, together with the primitive germ cells derived from the primitive ectoderm. During the second month of fetal life these indifferent gonads differentiate into testes under influence of testes-determining factor (TDF), a gene product encoded by SRY (sex-determining region of the Y chromosome). In absence of TDF, gonadal differentiation will take place in the female direction. At 6-8 weeks, the first signs of ovarian differentiation are reflected in a rapid mitotic multiplication of the immature germ cells, reaching a maximum of 6-7 million oogonia by 16-20 weeks (Baker, 1963). Oogonia are transformed into oocytes as they enter the first meiotic division and they subsequently arrest in this first meiotic prophase. This process of first meiotic division and transformation into oocytes starts at 11-12 weeks and is completed at birth. A resting oocyte at this stage is surrounded by a single layer of flattened granulosa cells, the primordial follicle.

1.2.2. Early follicle development

From the fourth month of fetal life, when the maximum number of germ cells is reached (approximately 6-7 million), a continuous flow of follicles leaving this pool of primordial follicles can be observed. The magnitude of depletion of the primordial follicle pool is dependent on age and is most pronounced during fetal development, leaving approximately 2 million oocytes/primordial follicles at birth. Reproductive life starts with 400 to 500.000 follicles at menarche. Thereafter, loss of follicles takes place at a fixed rate of approximately 1000 follicles per month, accelerating beyond the age of 35, until the store of primordial follicles will finally be exhausted, around menopause.

Once resting primordial follicles are stimulated to grow, they can either reach full maturation and ovulate or become atretic. Under normal conditions, only about 400 follicles reach the mature preovulatory stage and ovulate in the reproductive life span of a woman. Hence, loss of follicles due to atresia (apoptosis or programmed cell death) seems to be the normal fate of follicles, rather than ongoing growth and ovulation. At any point of the menstrual cycle, follicles at different stages of development with different sizes are present in the ovary. At each developmental stage, follicles can become atretic. Despite increasing knowledge on factors regulating initiation of growth of primordial follicles (like AMH as inhibiting factor (Durlinger *et al.*, 2002a; Durlinger *et al.*, 2002b)), the exact regulating mechanism is still unknown. Follicles entering the growth phase enlarge, both by proliferation and differentiation of granulosa cells and an increase in the size of the oocyte. Subsequently, these follicles transform through different developmental stages (primordial follicle, primary follicle, secondary/preantral follicle) until the development of an antral-cavity, the early antral phase (Figure 1.2). During early pre-antral follicle development, follicle-stimulating hormone

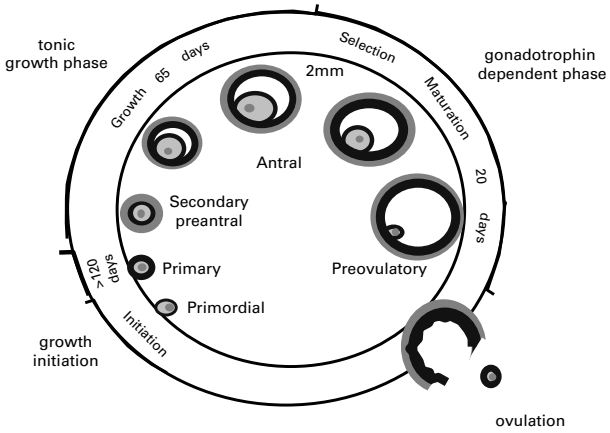

Figure 1.2 Development from primordial to preovulatory follicle and ovulation (Gougeon, 1996).

(FSH) receptors become detectable on granulosa cells. It remains controversial whether or not pre-antral follicle development is totally independent from FSH (Oktay *et al.*, 1997). Once antrum formation has occurred, FSH is required for ongoing follicle maturation, although the exact time of initiation of gonadotrophin-dependent follicle maturation is still uncertain.

1.2.3. Advanced follicle development: the "threshold/window concept"

From a size of 2 mm, follicles gradually become more sensitive to FSH, as a result of the increased number of FSH receptors on their granulosa cells (Hillier, 1994). Although very low levels of gonadotrophins are sufficient for follicles to grow up to 2-5 mm (Govan and Black, 1975), stimulation by FSH is an absolute requirement for development of large preovulatory follicles. The final stages of development prior to ovulation will last for about 2 weeks, during which follicle size increases from 5 mm to a preovulatory size of about 20 mm. Although high levels of FSH, usually occurring during the luteo-follicular transition, give rise to continued growth of a limited number (cohort) of follicles, only one follicle is selected to gain dominance and subsequently ovulate.

The "threshold/window concept" has been proposed as the mechanism underlying single dominant follicle selection. As a result of the demise of the corpus luteum and the subsequent decrease in oestradiol (Le Nestour *et al.*, 1993) and inhibin biosynthesis (Roseff *et al.*, 1989), FSH serum levels rise at the end of the luteal phase of the menstrual cycle (Hall *et al.*, 1992). As soon as FSH surpasses a certain level, referred to as the FSH threshold (Brown, 1978; Schoemaker *et al.*, 1993), during the luteo-follicular transition, a cohort of small antral follicles will be recruited and is rescued from atresia. This cohort of follicles will gain gonadotrophin dependence and is stimulated for further development. At the end of the luteal phase, the number of recruitable follicles is believed to be between 10 and 20 follicles for both ovaries. Due to negative feedback from oestradiol (Zeleznik *et al.*, 1985) and inhibin B (Groome *et al.*, 1996),

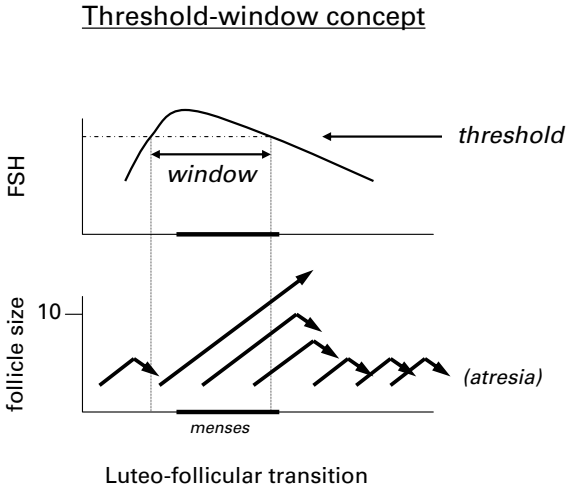

Figure 1.3 The threshold/window concept. Decremental FSH concentration seems essential for single dominant follicle selection.

produced by these growing follicles, FSH concentrations start to decrease in the mid to late follicular phase. This decremental FSH limits the time that the FSH concentration is above the FSH threshold and seems essential for single dominant follicle selection. Despite this decline in FSH, the most mature follicle continues its growth by increased sensitivity for FSH stimulation. All other recruited follicles lack sufficient FSH stimulation and enter atresia. The "FSH gate" (Baird, 1987) or "FSH window" (Fauser *et al.*, 1993) concept adds the element of time to the FSH threshold theory and emphasizes the importance of a transient increase of FSH above the threshold level in order to gain single dominant follicle selection (Figure 1.3).

1.3. Disturbed folliculogenesis

Anovulation represents a major cause of female reproductive dysfunction and can be identified in 18-25% of couples presenting with infertility (Laven *et al.*, 2002). Menstrual cycle disturbances (oligomenorrhoea or amenorrhoea) are usually a reflection of hormonal dysbalance and disturbed folliculogenesis. Whether and how frequently these occasional bleedings are associated with preceding ovulatory cycles is unknown. Obviously, induction of ovulation is required in these anovulatory patients to achieve follicular maturation, subsequent ovulation and ultimately conception.

The clinical approach to ovulation induction in patients with ovarian dysfunction requires understanding of the causes of anovulation. Patients presenting with oligomenorrhoea or amenorrhoea are usually categorized into 3 main categories, referred to as WHO (World Health Organization) classification group 1, 2 and 3 (Rowe *et al.*, 2001).

WHO class 1: Hypogonadotrophic hypo-oestrogenic anovulation (hypothalamic-pituitary dysfunction)

Women in this group have low serum FSH and negligible endogenous oestrogen activity, suggesting a central origin of the disease, i.e. decreased hypothalamic secretion of gonadotrophin-releasing hormone (GnRH) or pituitary unresponsiveness to GnRH. In these patients, growth of follicles is arrested at a stage where further development becomes dependent on stimulation by gonadotrophins. If FSH levels arise above the FSH threshold, due to administration of exogenous gonadotrophins, ovarian response should be normal. These women account for 5-10% of the anovulatory disorders. Causes include stress- or exercise related amenorrhoea, anorexia nervosa and Kallman's syndrome (GnRH deficiency).

WHO class 2: Normogonadotrophic normo-oestrogenic anovulation (pituitary-ovarian "dysbalance")

These women present with FSH and oestradiol levels within the normal range and represent the majority (60-85%) of patients with anovulatory disorders (Laven *et al.*, 2002). Characteristically in these patients, follicle development is initiated from the primordial stage until the antral stage at which follicle development ceases and selection of a dominant follicle and subsequent ovulation do not occur. This abnormal condition may be caused by disturbed intraovarian regulation of FSH action, and therefore response to exogenous FSH may be different from normal (Fauser, 1994). WHO class 2 women frequently suffer from polycystic ovary syndrome (PCOS), a heterogeneous group historically characterized by polycystic ovaries, combined with oligomenorrhoea, obesity and hirsutism (Stein and Leventhal, 1935). More recently diagnosis of PCOS was merely based on clinical symptoms and biochemical parameters (elevated serum LH levels, androgen levels and/or insulin levels). Clinical, morphological, biochemical, endocrine, and, more recently, molecular studies have identified an array of underlying abnormalities and added to the confusion concerning the pathophysiology of the disease. Several different genetic and environmental influences might lead to a similar clinical picture (phenotype) (Laven *et al.*, 2002). Despite the vast literature regarding the aetiology and classification of PCOS, not until recently (2003) a widespread consensus on diagnostic criteria and long-term health risks related to PCOS was formed (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004a; The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004b). Since PCOS is a syndrome, no single diagnostic criterion is sufficient for clinical diagnosis. The new consensus diagnostic criteria include (2 out of 3): 1. oligo- or anovulation; 2. clinical and/or biochemical signs of hyperandrogenism; 3. polycystic ovaries. Disorders that mimic the PCOS phenotype must be excluded (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004a; The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004b). Women with PCOS are at increased risk of developing type 2 diabetes, especially in obese woman or women with a positive family history for type 2 diabetes (Dunaif *et al.*, 1987; Ehrmann *et al.*, 1999; Legro *et al.*, 1999a; Legro *et al.*, 1999b). Although epidemiological evidence is limited, PCOS may also be associated with an increased risk for cardiovascular disease (Conway *et al.*, 1992; Wild, 2002; Legro, 2003) and with increased risk

for endometrial cancer as a result of chronic anovulation with unopposed oestrogen exposure of the endometrium (Hardiman *et al.*, 2003).

WHO class 3: Hypergonadotrophic hypo-oestrogenic anovulation (ovarian dysfunction)
10-30% of the patients with anovulatory disorders presents with high levels of gonadotrophins and low levels of oestrogen, suggesting the presence of a non-functioning ovary. Causes include gonadal dysgenesis (usually due to genetic abnormalities), autoimmune disorders or premature ovarian failure, a condition in which the follicle pool is depleted prior to the time of normal menopause.

1.4. Assisted reproduction

1.4.1. Management of subfertility

Approach of subfertility problems and the chances to conceive spontaneously or after assisted reproductive therapy (ART) depend strongly on the underlying disorder, the age of the woman and the duration of subfertility. Briefly, 4 distinct subsets of subfertility disorders exist: male subfertility, ovulation disturbances, mechanical dysfunction (e.g. uterine abnormalities, tubal dysfunction) and unexplained subfertility (Hull *et al.*, 1985; Collins *et al.*, 1995). The diagnosis of unexplained subfertility can only be made after appropriate diagnostic infertility work-up and an absence of any abnormalities. Unexplained subfertility therefore constitutes a heterogeneous group, depending on the extent of testing.

Therapeutic approaches often include manipulation of the ovary by fertility drugs. Ovulation induction, usually the first-choice treatment in anovulation, aims at restoring normal ovarian function, thus aiming at monofollicular growth. In contrast, ovarian (hyper)stimulation, as a part of ART, aims at multifollicular growth. The purpose of ovarian hyperstimulation is to increase pregnancy chances by the existence of more oocytes at the site of fertilization.

1.4.2. Ovulation induction

The clinical approach in ovulation induction in patients with ovarian dysfunction depends on the cause of the anovulatory disorder. In absence of normo-ovulatory function, treatment is focused on restoring ovulatory function by mimicking physiological hormonal balance. In patients with WHO class 1 anovulation treatment must be focused on restoring or substituting pituitary function, because of the central origin of the problem. If FSH levels rise above the FSH threshold (due to the administration of exogenous FSH or pulsatile GnRH), ovarian response should be normal.

In patients with WHO class 2 anovulation, treatment is focused on increasing ovarian responsiveness to FSH. Treatment with anti-oestrogenic drugs, especially clomiphene citrate (CC), represents the first line treatment strategy. Rising serum FSH levels due to CC interference with oestrogen negative feedback may be held respon-

sible for stimulating follicle growth (Kerin *et al.*, 1985). However, other mechanisms of action have also been proposed (Adashi, 1996). Treatment with CC is cheap with relatively minor complications. However, the majority of patients fail to conceive following CC therapy (Imani *et al.*, 1998; Imani *et al.*, 1999).

Administration of exogenous FSH preparations is held to be the second line modality for ovulation induction, although alternative treatment options such as insulin sensitizing agents or laparoscopic ovarian electrocautery have been developed. The aim of this treatment modality is to approach normal conditions as closely as possible, i.e. maturation and ovulation of a single dominant follicle and subsequent singleton pregnancy. However, the abnormal condition in WHO class 2 patients may be caused by disturbed intraovarian regulation of FSH action, and therefore response to exogenous FSH may be different from normal (Fauser and van Heusden, 1997). Although gonadotrophin ovulation induction is associated with higher ovulation and conception rates than CC treatment, one of the major problems is multifollicular development, resulting in multiple pregnancies and OHSS. In order to reduce the above mentioned complications and improve efficiency of treatment, various dose regimens, such as fixed, intermittent or flexible incremental doses have been tested. However, these step-up regimens ignore the concept that the FSH window should be surpassed for a limited period of time, sufficient to allow a single follicle to gain dominance (Brown, 1978; Fauser, 1994). The gonadotrophin step-down protocol (Schoot *et al.*, 1995; van Santbrink *et al.*, 1995a) may more closely mimic FSH serum concentrations during the follicular phase of the normal menstrual cycle, subsequently restricting the period of time that the FSH concentration is above the threshold. It has been shown that the step-down protocol may result in an increased number of monofollicular cycles, thus reducing the risk of OHSS and multiple pregnancy (van Santbrink and Fauser, 1997). More recently, new approaches have been introduced to improve ovulation induction outcome in WHO 2 class anovulatory patients. Patient selection on the basis of initial screenings may indicate patients at risk prior to ovulation induction therapy (Imani *et al.*, 2002; Mulders *et al.*, 2003). On the other hand, insulin sensitizing agents, such as metformin (Nestler *et al.*, 1998; Lord *et al.*, 2003), or aromatase inhibitors (Mitwally and Casper, 2001; Mitwally and Casper, 2003) have been introduced to facilitate ovulation.

1.4.3. Ovarian (hyper)stimulation

In contrast to ovulation induction, ovarian (hyper)stimulation aims at multifollicular development, in order to generate multiple oocytes for fertilization in vivo (IUI) or in vitro (IVF, IVF/ICSI). Ovarian hyperstimulation can be achieved by the administration of exogenous FSH, effectively interfering with decremental FSH concentrations. By extending the FSH window, i.e. the time the FSH concentration is above the FSH threshold, more follicles are selected and escape from atresia (Figure 1.4).

In conventional hyperstimulation protocols, large amounts of exogenous FSH are administered during the entire follicular phase. This marked and continued elevation of FSH overrules single dominant follicle selection by extending the FSH window, increasing serum FSH concentrations far above the threshold for prolonged periods

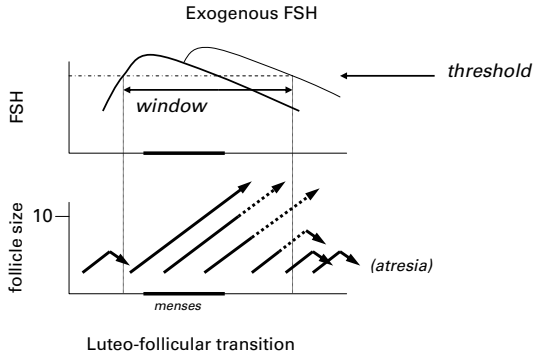

Figure 1.4 The threshold/window concept. Interference with decremental FSH concentrations by the administration of exogenous FSH causes multifollicular development.

and subsequently induces growth of large numbers of dominant follicles. Recently, serious concerns have been expressed concerning the stimulation of large numbers of follicles for assisted reproduction (Edwards *et al.*, 1996; Fauser *et al.*, 1999). Considering the risks, side-effects and the high costs of ovarian hyperstimulation and multiple gestation, current approaches for ovarian stimulation regimens should be re-evaluated (Hughes *et al.*, 1998; Macklon and Fauser, 2000; de Jong *et al.*, 2000a). It has been shown that a moderate, but continued elevation of FSH concentration during the mid to late follicular phase is capable of disrupting single dominant follicle selection and induces ongoing growth of multiple follicles (Schipper *et al.*, 1998b). Mild interference with decremental FSH by extending the FSH window may therefore be a more physiological approach to ovarian hyperstimulation.

1.5. Study objectives

Ovarian response to stimulation, within the scope of ovulation induction or ovarian hyperstimulation, usually shows strong inter-individual variability. This may result in either an inadequate or a too strong response in a significant proportion of women. In case of an inadequate response, the treatment is ineffective with still potential iatrogenic damage. In case of a too strong response, serious health threads may occur, such as OHSS or (high order) multiple pregnancies. In order to develop and optimize (milder) hyperstimulation protocols for assisted reproduction, a more profound understanding of gonadotrophin dependent follicle development and interference with single dominant follicle growth is needed. Previous research from our own group regarding follicle growth (Pache *et al.*, 1990) and the FSH threshold/window concept (van Santbrink *et al.*, 1995b; Schipper *et al.*, 1998b) has generated some novel concepts in this field.

This thesis is focused on various aspects of mono- and multiple dominant follicle development, aiming to generate more insight into (patho)physiological mechanisms concerning dynamics of follicle growth in the human ovary and interference with single dominant follicle selection.

The first objective was to gain additional insight in the significance of the timing of initiation of exogenous FSH on follicle recruitment and selection (influencing the closure of the FSH window). A randomized study in 40 normo-ovulatory women was carried out to address this issue (Chapter 2.1). A second study in normo-ovulatory volunteers undergoing cycle intervention with exogenous FSH was performed to gain more insight in ovarian-pituitary regulation mechanisms by studying the relationship between inhibins and follicle development. The hypothesis was that serum inhibin levels may be useful as a clinical marker for the number of follicles to develop after ovarian hyperstimulation (Chapter 2.2).

After these experiments in normo-ovulatory volunteers, the gained knowledge of timing of initiation of exogenous FSH was tested in a clinical setting. The FSH threshold/window concept was used as a basis of a novel, milder approach to ovarian hyperstimulation in IVF. In a randomized controlled trial in normo-ovulatory IVF patients, this concept was compared to 2 other ovarian hyperstimulation protocols (Chapter 3.2).

The current knowledge on mono- and multi-follicular development may also be useful for the treatment of anovulatory patients. The introduction of new compounds such as insulin sensitizing drugs in the treatment of WHO 2 anovulation, along with the availability of new drugs like GnRH antagonists offer possible new approaches to the treatment of WHO 2 anovulatory patients. To generate more understanding of the pathophysiology in disturbed folliculogenesis in PCOS patients with elevated LH levels, a randomized study was performed. The use of newly available GnRH antagonists may normalise elevated LH serum levels in these patients, which might increase ovarian responsiveness to FSH and even restore normal ovarian function in some women (Chapter 4.2). The last objective was to explore the potential additional effect of insulin sensitizing drugs on ovarian responsiveness during exogenous gonadotrophin induction of ovulation. A placebo controlled double blind randomized study was performed in insulin resistant normogonadotrophic anovulatory patients to test the hypothesis that metformin may improve ovarian sensitivity to stimulation by exogenous FSH (Chapter 4.3).

All these objectives were studied using frequent transvaginal sonography (TVS) combined with hormone estimations in peripheral blood.

Chapter 2

Follicle dynamics and endocrine aspects after intervention in the normal menstrual cycle

2.1. Low-dose exogenous FSH initiated during the early, mid or late follicular phase can induce multiple dominant follicle development

2.1.1. Introduction

Due to demise of the corpus luteum and the subsequent decrease in oestradiol (E_2) (Le Nestour *et al.*, 1993) and inhibin biosynthesis (Roseff *et al.*, 1989), follicle-stimulating hormone (FSH) serum levels start to increase during the late luteal phase of the menstrual cycle (Hall *et al.*, 1992). As soon as FSH levels surpass the "threshold" for ovarian stimulation (Brown, 1978; Schoemaker *et al.*, 1993) during the luteo-follicular transition a cohort of small antral follicles is recruited and stimulated to ongoing growth (Fauser and van Heusden, 1997). These recruited follicles are of comparable potential to gain dominance and to continue their development. As a result of negative feedback from inhibin B (Groome *et al.*, 1996) as well as E_2 (Zeleznik *et al.*, 1985) produced by this cohort of growing follicles, FSH levels decrease during the mid to late follicular phase effectively limiting the number of days where FSH is above the threshold (referred to as the FSH window) (van Santbrink *et al.*, 1995b). Decremental follicular phase FSH appears to be essential for the selection of a single dominant follicle (with a diameter beyond 9 mm) from the recruited cohort (Hodgen, 1982; Zeleznik *et al.*, 1985; van Santbrink *et al.*, 1995b). Despite this decline in FSH levels, the most mature (dominant) follicle escapes from atresia and continues its growth by increased sensitivity for stimulation by FSH (presumably due to up regulation by intra-ovarian factors (Erickson and Danforth, 1995; Fauser and van Heusden, 1997) and the induction of luteinizing hormone (LH) receptors (Sullivan *et al.*, 1999)). Remaining follicles from the recruited cohort lack sufficient stimulation by FSH and enter atresia.

A brief but distinct elevation of FSH levels above the threshold in the early follicular phase does not affect dominant follicle development, although a transient increase in the number of small antral follicles could be observed (Schipper *et al.*, 1998b). On the contrary, a moderate but continued elevation of FSH levels during the mid to late follicular phase extending the FSH window does interfere with single dominant follicle selection and induces ongoing growth of multiple follicles (Schipper *et al.*, 1998b). This confirms previous observations in the monkey showing that interference with decremental FSH can override the selection of a single dominant follicle (Zeleznik *et al.*, 1985).

It is known from ovarian stimulation for in vitro fertilisation (IVF) or intra-uterine insemination (IUI), that a marked and continued elevation of FSH during the entire follicular phase will induce growth of large numbers of dominant follicles of different size (Hillier *et al.*, 1985). These stimulation protocols overrule single dominant follicle selection by extending the FSH window by serum FSH concentrations far above the threshold. Recently, serious concerns have been expressed concerning the stimulation of growth of large numbers of follicles for assisted reproduction (Edwards *et al.*, 1996; Fauser *et al.*, 1999). Considering the risks, side effects and the high costs of ovarian hyperstimulation and multiple gestation, current approaches for ovarian stimulation

regimens should be re-evaluated (Liu and Yen, 1983; Hughes *et al.*, 1998; Macklon and Fauser, 2000; de Jong *et al.*, 2000a). Additional insight into the significance of the timing of initiation of exogenous FSH on follicle recruitment and selection may help to further develop and optimise milder ovarian stimulation protocols for assisted reproduction.

2.1.2. Materials and methods

Subjects and study design

This study was approved by the local Ethics Review Committee. A total of 40 healthy subjects was selected from responders to advertisements in local newspapers and interviews on the local radio and television. Written informed consent was obtained from each participant, and all subjects were paid for their participation.

Inclusion criteria were: 1) Age between 19 and 35 years. 2) History of regular menstrual cycles (cycle lengths between 25-32 days) and no use of oral contraceptive pills or other medical or hormonal treatment for at least 3 months prior to study initiation. 3) Body mass index (BMI) between 19 and 27 kg/m^2. 4) Midluteal progesterone (P) concentrations - assessed 7 days before expected menses - above 25 nmol/l. 5) No prior treatment for infertility. 6) Willingness to use contraceptive measures (intra uterine device, condoms or prior tubal ligation) or to abstain from intercourse during the study period. Detailed oral and written information concerning the importance of contraception was given before and during the study period.

All subjects were studied during a single menstrual cycle. At the onset of menses, subjects were assigned to one of three interventions using a computer-generated randomisation schedule, assigned via numbered sealed envelopes. Group cycle day (CD) 3 received a daily fixed dose of 1 ampoule (75 IU) recombinant FSH (recFSH, Gonal-F®, Serono Benelux BV, The Hague, The Netherlands), starting on CD 3 until the administration of human chorionic gonadotrophin (hCG). Group CD 5 and group CD 7 received a similar daily dose recFSH, but started on CD 5 or CD 7, respectively. RecFSH was administered subcutaneously by self-injection at 22.00 h. Participants were instructed by qualified nurses. As soon as the largest follicle reached a diameter of 18 mm or more, a single dose of 5,000 IU hCG (Profasi®, Serono Benelux BV) was administered intramuscularly at 22.00 h to induce ovulation.

Monitoring consisted of transvaginal sonography (TVS) and blood sampling was performed between 8.00 and 10.00 h, every 2 days starting on CD 3. As soon as the largest dominant follicle reached a diameter of at least 15 mm, TVS and blood sampling were performed on a daily basis until the day of hCG. Finally, TVS and blood sampling were repeated on day hCG+2 and hCG+8. The day of initiation of the following menstrual period was recorded. TVS was performed by a single observer (F.H.), using a 6.5-megahertz transvaginal transducer (EUB-420, Hitachi Medical Corp., Tokyo, Japan). Follicle diameter was calculated as the mean diameter (measured in two dimensions when < 9 mm, and in 3 dimensions if at least one diameter was ≥ 9 mm) as published previously (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b).

Hormone assays

Blood samples were centrifuged within 2 hours after withdrawal and stored at –20 °C until assayed. Serum FSH, LH, and P levels were measured by chemiluminescent immunoassay (Immulite, Diagnostic Products Corporation (DPC), Los Angeles, CA, USA) in single assays. Addition of various doses of recFSH to serum without FSH yielded a curve parallel to that of the standard. Recovery of recFSH was 55.5 ± 3.7(SD, n=4)%. Serum E_2 concentrations were measured in duplicate using radio immunoassay kits (RIA) provided by DPC, as described previously (Fauser *et al.*, 1991). Dimeric inhibin A and inhibin B levels were also determined in duplicate using an immuno-enzymometric assay (Serotec, Oxford, UK), as described previously (Groome *et al.*, 1996; Schipper *et al.*, 1998b). Intra- and interassay coefficients of variation were less than 5% and 7% for FSH, less than 5% and 6% for LH, less than 10% and 10% for P, less than 5% and 7% for E_2, less than 8% and 15% for inhibin A and less than 8% and 14% for inhibin B, respectively. All samples from one subject were run in the same assay.

Data analysis

Results are presented as the median and range. Comparisons of outcome measures between the 3 randomised groups were performed using the Kruskal-Wallis *H* test for continuous data and using the Chi-square test for binary variables. Two group comparisons (between single and multiple dominant follicle selection) were performed using the Mann-Whitney *U* test. Comparisons of means of values in time between two groups were performed using analysis of variance (ANOVA). Correlation coefficients given are Pearson's. *P* values are two-sided and 0.05 was considered the limit for statistical significance. A dominant follicle was defined as a follicle with a mean diameter of 10 mm or more (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b). A pre-ovulatory follicle was defined as a follicle with a mean diameter of 15 mm or more. This distinction seems clinically important, since not all follicles of 10 mm or more necessarily develop into pre-ovulatory follicles. Arbitrarily, P concentration (day of hCG) ≥ 3.2 nmol/l (1.0 ng/ml) was considered as premature luteinisation (Harada *et al.*, 1995; The ganirelix dose-finding study group, 1998). The four subjects showing premature ovulation were not included in the analysis of hormone serum concentrations on the day of hCG. Data were analysed using the commercially available software package SPSS, Inc. (Chicago, IL, USA).

2.1.3. Results

Baseline characteristics and follicle development

Forty normo-ovulatory women entered the study protocol. There were no dropouts during the study. All subjects were ovulatory in the intervention cycle, as assessed by TVS and elevated serum P levels in the midluteal phase. With regard to the distribution of age, BMI, cycle length and baseline serum levels of FSH, E_2 and inhibin B, no significant differences were found between the 3 groups (Table 2.1). Parity was equally distributed over the 3 groups (nullipara *vs.* multipara in group CD 3, CD 5 and CD 7: 54% *vs.* 46%, 54% *vs.* 46% and 57% *vs.* 43%, respectively).

Table 2.1 Clinical, endocrine and menstrual cycle characteristics (median and range) in 40 normo-ovulatory volunteers receiving exogenous FSH (75 IU/d) starting on cycle day (CD) 3, 5 or 7.

	CD 3	CD 5	CD 7
n	13	13	14
Age (yrs)	29 (21-35)	29 (22-34)	28 (20-35)
BMI (kg/m^2)	22 (19-27)	23 (19-27)	22 (19-27)
Cycle length (d)	28 (26-31)	28 (25-31)	28 (26-30)
Cycle day 3			
FSH (IU/l)	6.5 (2.8-13.5)	6.3 (3.3-9.8)	7.7 (5.4-13.1)
E_2 (pmol/l)	127 (64-220)	175 (91-404)	137 (109-264)
Inhibin B (ng/l)	87 (21-192)	130 (12-213)	114 (57-179)
Day of hCG			
FSH (IU/l)	5.8 (4.5-9.6)	6.1 (5.5-10.9)	6.9 (4.7-20.4)
LH (IU/l)	6.8 (1.5-19.1)	4.6 (1.0-21.1)	7.9 (0.3-57.2)
E_2 (pmol/l)	945 (660-2840)	1106 (470-2302)	985 (682-1610)
P (nmol/l)	4.3 (2.5-5.4)	3.6 (1.4-27.3)	4.1 (2.4-8.7)
Inhibin A (ng/l)	51 (28-224)	69 (27-143)	73 (38-195)
Follicle number (≥10 mm)	2.5 (1-6)	2 (1-7)	1 (1-5)
Follicle number (≥12 mm)	1.5 (1-6)	2 (1-5)	1 (1-4)
Follicle number (≥15 mm)	1.5 (1-4)	1 (1-3)	1 (1-3)
Number of subjects with multiple DF[a*]	9 (69%)	10 (77%)	5 (36%)
Number of subjects with multiple PF[b]	7 (54%)	6 (46%)	4 (29%)
Total amount recFSH (IU)[**]	750 (600-1125)	525 (375-825)	450 (75-900)
Follicular phase length (d)	13 (12-19)	13 (11-17)	14 (9-20)
Luteal phase length (d)	12 (9-15)	12 (8-16)	13 (11-17)
P (nmol/l)[c]	53 (23-287)	44 (18-109)	61 (17-96)

[a] DF = dominant follicle (diameter ≥ 10 mm)
[b] PF = pre-ovulatory follicle (diameter ≥ 15 mm)
[*] $P = 0.07$ (Chi-square test)
[**] $P = 0.001$ (Kruskal-Wallis H test)
[c] midluteal

A daily dose of 75 IU of recFSH was able to interfere with single dominant follicle selection in 24 subjects (60%) in all 3 groups. There was no difference in multifollicular growth (defined as at least 2 follicles ≥ 10 mm) or the development of multiple pre-ovulatory follicles (defined as a follicle ≥ 15 mm) between the 3 groups, although a tendency was seen towards less multifollicular growth in group CD 7 ($P = 0.07$) (Table 2.1). Group CD 7 versus all other subjects (groups CD 3 and CD 5) did show a statistical difference in the development of multiple dominant follicles (36% *vs.* 73%; $P = 0.02$). Figure 2.1 shows the mean follicle number in both ovaries during the

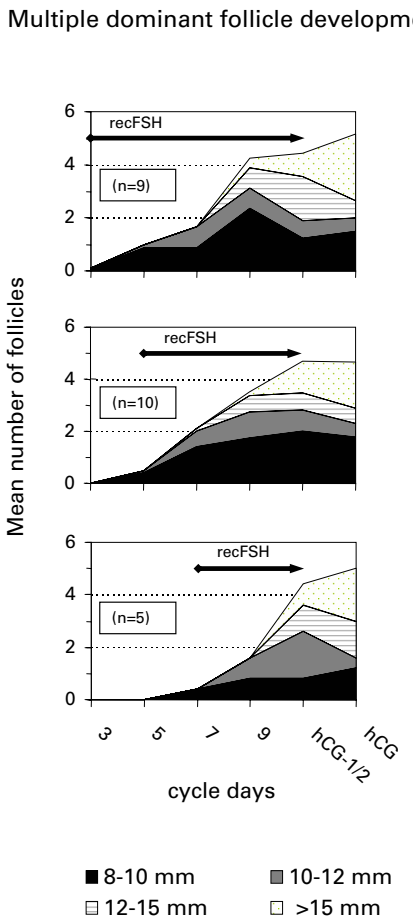

Figure 2.1 Number of follicles (≥ 8 mm) during the follicular phase in 24 normo-ovulatory women receiving fixed low daily doses (75 IU) of exogenous FSH (starting on either cycle day 3, 5 or 7), who developed multiple dominant follicles (≥ 10 mm) on the day of hCG administration. *Areas* represent the mean number of follicles in both ovaries on a given day in all subjects, with shaded areas representing different size classes. The time scale on the *x*-axis is divided into cycle days (day of onset menses = cycle day 1) and days prior to administration of hCG.

follicular phase in subjects presenting with multiple dominant follicle development, in each group.

Table 2.2 shows the comparison of women presenting with single (n=16) or multiple (n=24) dominant follicle development following the administration of recFSH. With regard to age, BMI, cycle length and initial (CD 3) hormone levels, no differences were found between the two groups. There was no difference in parity between the groups with mono- or multifollicular growth (nullipara *vs.* multipara: 62% *vs.* 38% and 50% *vs.* 50%, respectively). The lower the BMI, the more follicles developed (Figure 2.2: $r = -0.44$, $P = 0.007$). None of the subjects with a BMI ≥ 24 kg/m^2 (n=10)

Table 2.2 Clinical, endocrine and menstrual cycle characteristics (median and range) in 40 normo-ovulatory women presenting with either single (n=16) or multiple (n=24) dominant follicle (≥ 10 mm) development following exogenous recFSH starting on either cycle day 3, 5 or 7.

	Dominant follicle		*P*[a]
	Single	Multiple	
n	16	24*	
Age (yrs)	29 (20-34)	29 (21-35)	-
BMI (kg/m^2)	23 (19-27)	22 (19-27)	-
Cycle length (d)	28 (26-31)	28 (25-31)	-
Cycle day 3			
FSH (IU/l)	7.0 (2.8-12.2)	7.2 (3.3-13.5)	-
E_2 (pmol/l)	137 (93-264)	153 (64-404)	-
Inhibin B (ng/l)	114 (26-179)	91 (12-213)	-
Day of hCG			
FSH (IU/l)	6.6 (4.5-20.4)	6.0 (4.5-15.0)	-
LH (IU/l)	7.4 (2.0-49.3)	4.6 (0.3-57.2)	-
E_2 (pmol/l)	793 (470-1353)	1176 (536-2840)	0.02
P (nmol/l)	4.1 (2.4-27.3)	4.3 (1.4-11.7)	-
Inhibin A (ng/l)	50 (28-143)	82 (27-224)	<0.05
Follicle number (≥10 mm)	1	3 (2-7)	-[b]
Follicle number (≥12 mm)	1	2 (1-6)	<0.001
Follicle number (≥15 mm)	1	2 (1-4)	<0.001
Midluteal			
FSH (IU/l)	2.5 (0.2-6.0)	0.9 (0.1-7.3)	0.004
LH (IU/l)	4.2 (0.1-8.5)	0.8 (0.1-13.8)	0.03
E_2 (pmol/l)	482 (158-914)	694 (286-2486)	0.002
P (nmol/l)	50.2 (16.6-90.5)	54.9 (22.8-287)	0.08
Inhibin A (ng/l)	55 (9-122)	79 (37-205)	0.01
Menstrual cycle characteristics			
Follicular phase length (d)	14 (9-19)	13 (11-20)	-
Luteal phase length (d)	13 (8-17)	11 (9-14)	0.002

* 9 from group CD 3, 10 from group CD 5 and 5 from group CD 7

[a] Mann-Whitney *U* test

[b] Based on this criterion both groups were separated

showed multiple pre-ovulatory follicles. BMI was significantly higher in women presenting with single pre-ovulatory follicle development (median BMI 23 kg/m^2 [range 19-27] *vs.* 22 kg/m^2 [range 19-24], $P = 0.03$). There was no correlation between age and the amount of follicles developing ($r = 0.03$, $P = 0.88$). The growth rate of the largest follicle in subjects presenting with single- or multiple dominant follicle selection was similar (Figure 2.3).

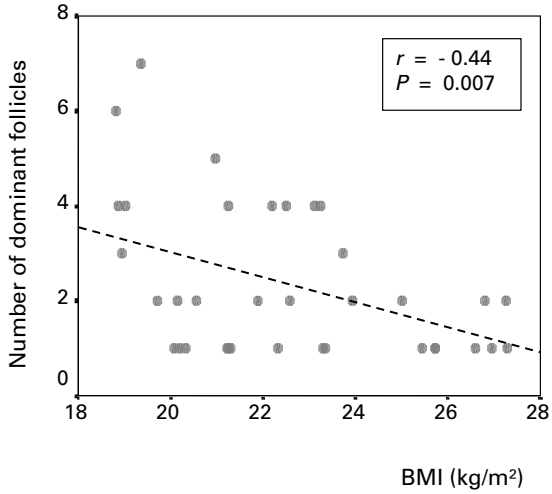

Figure 2.2 Distribution of the number of follicles (≥ 10 mm) observed in both ovaries on the day of hCG administration related to BMI in 40 normo-ovulatory women. Subjects received low daily doses of exogenous FSH during the follicular phase (starting on either cycle day 3, 5 or 7). Pearson's correlation: $r = -0.44$, $P = 0.007$.

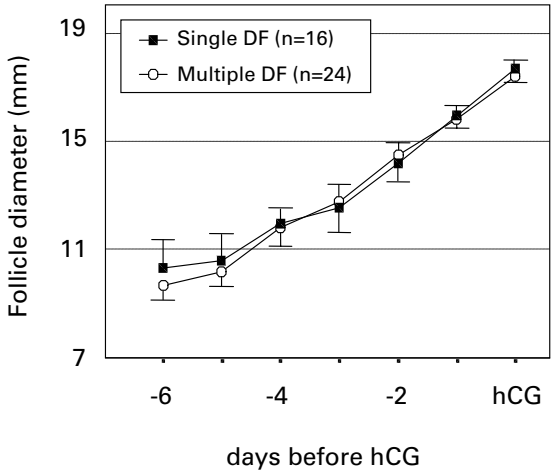

Figure 2.3 Diameter (mean ± SEM) of the largest dominant follicle in the follicular phase of intervention cycles in 40 normo-ovulatory women receiving low daily doses of exogenous FSH, starting on either cycle day 3, 5 or 7. The *lines* represent the subjects presenting with single- or multiple dominant follicle development (≥ 10 mm). The scale on the *x*-axis is expressed as days prior to late follicular phase administration of hCG. DF = dominant follicle(s).

Serum hormone levels

Figure 2.4 shows the hormone concentrations during the follicular phase (separate for women presenting with single or multiple dominant follicles), comparing group CD 3, CD 5 and CD 7. In group CD 7, FSH levels of the subjects with multifollicular growth

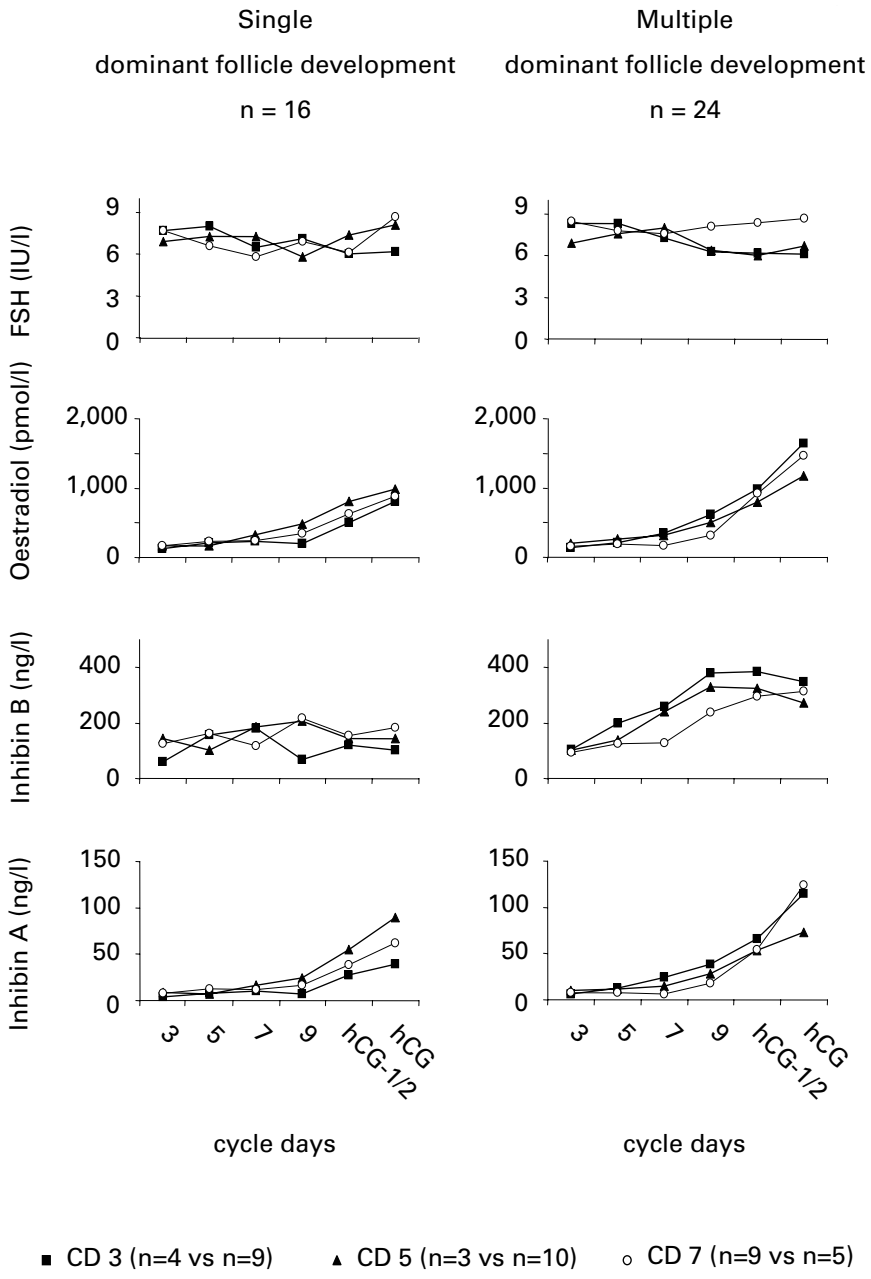

Figure 2.4 Follicular phase FSH, oestradiol and inhibin B and A concentrations in 40 normo-ovulatory women receiving low daily doses of exogenous FSH, starting on either cycle day 3, 5 or 7. The *panels* represent the subjects who developed single (*left panels*) or multiple (*right panels*) dominant follicles (≥ 10 mm) on the day of hCG administration. The time scale on the *x*-axis is divided into cycle days (day of onset menses = cycle day 1) and days prior to administration of hCG. Data are shown as the mean values.

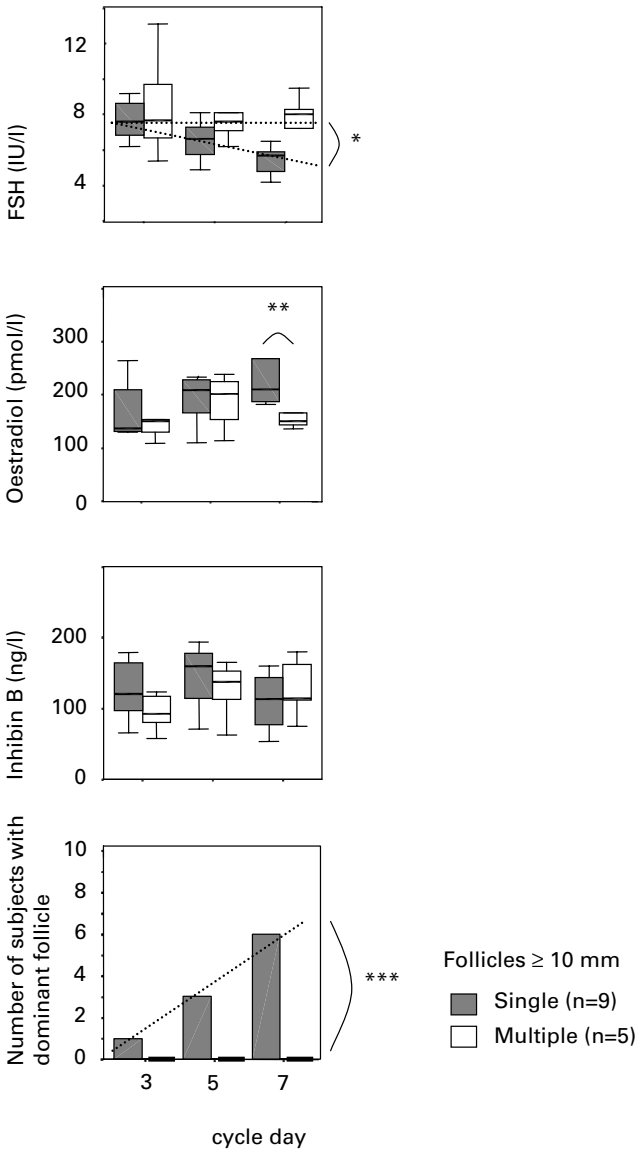

Figure 2.5 FSH, oestradiol and inhibin B concentrations and the number of women presenting with a dominant follicle at various days during the early to midfollicular phase of 14 normo-ovulatory women receiving low daily doses of exogenous FSH starting on cycle day 7. Each panel shows the difference between the subjects presenting with single- or multiple dominant follicle development (≥ 10 mm). *Boxes* indicate 25th and 75th percentiles, with the *horizontal line* representing the median value. *Whiskers* span the range between the 5th and 95th percentiles of the data. *Decremental FSH concentrations in women presenting with monofollicular growth and plateau FSH in multifollicular growth ($P = 0.02$, ANOVA). **Oestradiol concentration on cycle day 7 significantly different ($P = 0.03$, Mann-Whitney *U*-test) ***($P = 0.006$, ANOVA).

did not decrease during the early to mid follicular phase, whereas the subjects exhibiting monofollicular growth presented with decremental FSH concentrations (Figure 2.5, $P = 0.02$). In group CD 7, E_2 levels were lower on cycle day 7 if multiple dominant follicles developed (Figure 2.5: median E_2 151 pmol/l [range 137-253] *vs.* 210 pmol/l [range 183-983], $P = 0.03$). In group CD 3 en CD 5 no differences were seen in FSH and E_2 levels at the day of initiation of recFSH between the subjects who showed mono- and multifollicular growth (group CD 3: median FSH 6.4 IU/l [range 6.2-13.5] *vs.* 7.9 IU/l [range 2.8-12.2] and median E_2 138 pmol/l [range 64-220] *vs.* 126 pmol/l [range 93-156]; group CD 5: median FSH 7.7 IU/l [range 5.7-9.5] *vs.* 6.9 IU/l [range 6.4-8.5] and median E_2 176 pmol/l [range 106-1163] *vs.* 176 pmol/l [range 144-196]. In all groups (CD 3, CD 5 and CD 7) inhibin B levels at the day of initiation of recFSH were similar between subjects showing mono- or multifollicular growth (median inhibin B in group CD 3, CD 5 and CD 7: 62 ng/l [range 26-91] *vs.* 90 ng/l [range 21-192], 80 ng/l [range 76-151] *vs.* 115 ng/l [range 51-412] and 113 ng/l [range 53-160] *vs.* 114 ng/l [range 75-180], respectively).

Serum hormone levels on the day of hCG, comparing subjects presenting with single- and multiple dominant follicle selection are presented in Table 2.2. Differences in E_2 and inhibin A levels on the day of hCG were even more pronounced in subjects presenting with single compared to multiple pre-ovulatory follicles (median E_2 759 pmol/l [range 470-1464] *vs.* 1516 pmol/l [range 839-2840], $P < 0.001$; median inhibin A 49 ng/l [range 27-143] *vs.* 119 ng/l [range 43-224], $P < 0.001$). Premature luteinisation (defined as a P concentration on the day of hCG ≥ 3,2 nmol/l) was observed in 80,6% of all subjects, distributed over all groups (CD 3, CD 5 and CD 7: 89%, 70% and 70%, respectively) and both in single- or multiple dominant follicle development (75% *vs.* 82%). E_2 levels on the day of hCG were comparable between the subjects presenting with or without premature luteinisation (median E_2 1106 IU/l [range 470-2840] *vs.* 985 IU/l [range 536-1541]).

Midluteal serum hormone levels between women exhibiting single and multiple dominant follicle selection are presented in Table 2.2. The differences in midluteal serum concentrations were more pronounced in single versus multiple pre-ovulatory follicle development (median FSH 2.6 IU/l [range 0.2-7.3] *vs.* 0.7 IU/l [range 0.1-1.7], $P<0.001$; median LH 4.1 IU/l [range 0.1-8.5] *vs.* 0.4 IU/l [range 0.1-13.8], $P = 0.006$; median E_2 497 pmol/l [range 158-969] *vs.* 729 pmol/l [range 323-2486], $P = 0.001$; median inhibin A 61 ng/l [range 9-122] *vs.* 91 ng/l [range 37-205], $P = 0.01$). Midluteal serum hormone levels between the three randomisation groups were similar (data not shown).

Cycle characteristics

Reported previous cycle length of the subjects correlated positively with the cycle length in the intervention cycle ($r = 0.44$, $P = 0.005$; data not shown). The intervention cycle was shorter compared to the normal cycle length (median length 26 days [range 17-33] *vs.* 28 days [range 25-33], $P < 0.001$). Four subjects showed premature ovulation (follicle collapse before the dominant follicle reached a diameter of 18 mm) and did not receive hCG. The difference in the length of the intervention cycle and the normal cycle

remained when corrected for subjects showing premature ovulation (median length 26 days [range 21-33] *vs.* 28 days [range 25-31], $P < 0.001$).

The length of the follicular phase (from onset of menses until 2 days after hCG administration or until day of spontaneous ovulation) comparing the 3 groups, is presented in Table 2.1. Within group CD 3 and CD 5 no difference was seen in the length of the follicular phase comparing women with mono- or multifollicular growth (median follicular phase 13 days [range 12-14] *vs.* 14.5 days [range 12-19] in group CD 3 and 13 [range 11-16] *vs.* 16 days [range 11-17] in group CD 5). In group CD 7, the subjects exhibiting multifollicular growth had a significant longer follicular phase (median follicular phase 15 days [range 14-20] *vs.* 12 days [range 9-14], $P = 0.004$). In this group, the mean diameter of the lead follicle on the day of initiation of recFSH (CD 7) was significantly larger in the group showing monofollicular growth compared to the group with multifollicular growth (median size lead follicle 10.6 mm [range 8,1-17,6] *vs.* 7.8 mm [7,2-8,8]).

The luteal phase (from 2 days after hCG administration or from the day of spontaneous ovulation until the start of the next menstrual period) in the subjects with multiple dominant follicle selection was significantly shorter compared to the subjects with single dominant follicle development (Table 2.2, $P = 0.002$). Two subjects were excluded for calculation of the luteal phase length, since these subjects conceived during the intervention cycle.

2.1.4. Discussion

During the luteo-follicular transition, a cohort of small antral follicles at a given stage of development is recruited to gain gonadotrophin dependence and continued growth. Around the mid follicular phase, the most mature follicle gains dominance over other follicles in the cohort. This dominant follicle continues its growth despite decremental FSH concentrations, whereas the remaining follicles from the recruited cohort enter atresia, due to insufficient stimulation by FSH (Fauser and van Heusden, 1997). Decreasing FSH levels and subsequent closure of the FSH window (Baird, 1987; Fauser *et al.*, 1993) seems essential for single dominant follicle selection. This concept adds the element of time to the FSH threshold theory and emphasises the importance of a transient increase of FSH above the threshold level for single dominant follicle selection. Interference with this decremental FSH - and hence extending the FSH window - can override the selection of a single dominant follicle as previously shown in primates (Zeleznik *et al.*, 1985) and the human (Schipper *et al.*, 1998b). In case the dominant follicle is removed in the late follicular phase in monkeys, the other follicles in the cohort can no longer be rescued and recruitment of a new cohort of follicles occurs (Goodman and Hodgen, 1979). The exact moment at which single dominant follicle selection has become irreversible remains to be established.

The present study shows that the administration of low doses of exogenous FSH starting on CD 3, 5 or as late as CD 7, can overrule single dominant follicle selection in the majority of women. In subjects who did not respond the amount of exogenous FSH might have been insufficient to elevate the FSH concentration long enough above the FSH threshold for the remaining non-dominant follicles from the recruited cohort.

A negative correlation was found between BMI and the amount of dominant follicles developed. Lower bodyweight women received a higher FSH dose per kg. However, no correlation was found between BMI and FSH concentrations in the mid or late follicular phase (data not shown). Individual differences in metabolic clearance rate (Diczfalusy and Harlin, 1988) and distribution volume of FSH (Chong *et al.*, 1986; Mannaerts *et al.*, 1993), related to body weight may be involved. Moreover, the influence of weight on induction of ovulation or IVF has previously been stressed (Chong *et al.*, 1986; Lashen *et al.*, 1999). Women presenting with multiple dominant follicles exhibit higher mid to late follicular phase inhibin B levels and higher late follicular phase E_2 and inhibin A concentrations. Serum FSH levels were not distinctly different suggesting differential ovarian responsiveness to FSH being the predominant factor determining mono- or multifollicular response. On the other hand, an immunoassay was used to assess FSH levels (combining endogenous and exogenous FSH) and therefore differences in *in vivo* bioactivity may not be disclosed (Mannaerts *et al.*, 1991; Rose and Gaines-Das, 1998).

Surprisingly we did not find a statistical difference in the number of women with irreversible single dominant follicle selection between the three groups. However, there was a tendency of a lower percentage of women presenting with multiple dominant follicle development when FSH was initiated on CD 7 (36 % *vs.* 69 % and 77%). A larger number of subjects is required to establish whether this tendency represents a true difference. There was a significant difference when CD 7 initiation was compared with the two other groups (CD 3 and CD 5) together ($P = 0.02$). The subjects who showed multiple dominant follicle selection after intervention in the mid to late follicular phase had a longer follicular phase and no signs of selection of the dominant follicle at the day of initiation of exogenous FSH. These results confirm the FSH window hypothesis: Administration of low doses of exogenous FSH will induce multifollicular development, unless selection of the dominant follicle has occurred (defined as the appearance of a follicle ≥ 10 mm), coinciding with a rise in E_2 and a decrease in FSH concentrations.

We observed a substantial number of subjects presenting with premature luteinisation (a rise in serum P concentration on or before the day of hCG administration, which was based on ultrasound criteria only) in all intervention cycles. The definition used for premature luteinisation and the cut-off levels used to define a P rise differ from study to study. Cut-off levels for a subtle rise in P on the day of hCG differ between 0.5 ng/ml (1.59 nmol/l) (Schoolcraft *et al.*, 1991) and 1.5 ng/ml (4.77 nmol/l) (Sengoku *et al.*, 1994). The occurrence of premature luteinisation in our study was not dependent on the day of initiation of exogenous FSH, the occurrence of single or multiple dominant follicle selection or late follicular phase E_2 concentrations (data not shown). The mechanism underlying a subtle P rise during the late follicular phase after ovarian stimulation, the incidence and the implications for outcome of IUI or IVF are not yet clear. Some studies associate premature luteinisation in IVF with poor oocyte quality, decreased fertilisation rates, poor embryo quality and impaired implantation (Schoolcraft *et al.*, 1991; Silverberg *et al.*, 1991; Harada *et al.*, 1995), while other studies suggest no difference in pregnancy outcome (Edelstein *et al.*, 1990; Hofmann *et al.*, 1996;

Ubaldi *et al.*, 1996). Studies regarding the effects of premature luteinisation on clinical outcome in IUI are also contradictory (Sengoku *et al.*, 1994; Manzi *et al.*, 1995).

Although we found a correlation between the reported normal cycle length and the length in the intervention cycle, the intervention cycle was shorter. This phenomenon remained if we corrected for subjects showing a premature ovulation. There is no reason to believe that the administration of exogenous FSH will accelerate the growth of the lead follicle (Pache *et al.*, 1990). However, the administration of hCG might shorten the follicular phase in some women since ovulation was triggered as soon as the lead follicle reached a diameter of 18 mm. In a spontaneous cycle the median pre-ovulatory follicle size is 21 mm, with a range of 18-30 mm (van Santbrink *et al.*, 1995b). A decreased luteal phase length in the subjects presenting with multiple dominant follicle development may represent an additional explanation for the shorter intervention cycle.

The length of the luteal phase was significantly reduced in all cycles with multiple dominant follicle development. A short luteal phase in cycles stimulated with gonadotrophins for IVF has previously been documented (Laatikainen *et al.*, 1988; de Jong *et al.*, 2000b). However, it remains unclear if the reduction in luteal phase length is a consequence of gonadotrophin therapy, co treatment with GnRH analogues, hCG or the follicle puncture procedure (Smitz *et al.*, 1990). In the current study, the short luteal phase was independent of the administration of hCG or the amount of exogenous FSH administered. Midluteal phase hormone levels in intervention cycles presenting with single dominant follicle development were comparable with non-intervention cycles in normo-ovulatory women (Macklon and Fauser, 2000). Although midluteal E_2 and inhibin A levels were significantly higher in cycles with multiple follicle development, midluteal P was similar. As midluteal gonadotrophin serum levels were significantly lower in cycles with multiple dominant follicle development, high E_2 and inhibin A might act luteolytic through negative feedback mechanisms. In vitro, E_2 was found to inhibit gonadotrophin-stimulated P synthesis by luteal cells (Hahlin *et al.*, 1986). Other studies suggest a luteolytic action of estrogens mediated via prostaglandins (Auletta *et al.*, 1976) or arachidonic acid (Fisch *et al.*, 1994). In cycles with multiple dominant follicle development, high initial P production may fall rapidly during the luteal phase, which in turn reduces the length of the luteal phase.

In conclusion, our findings are supportive of the FSH window concept. Subtle interference with decremental FSH by low-dose exogenous FSH can induce multiple dominant follicle development. Provided that no dominant follicle selection has occurred, initiation of FSH administration as late as cycle day 7 is sufficient to interfere with single dominant follicle selection. Multiple follicle development per sé induces changes in the length and endocrine profile of the luteal phase. This information seems relevant for the design of mild ovarian stimulation protocols for IUI or IVF.

2.2. Relationship between inhibin A and B, oestradiol and follicle growth dynamics during ovarian stimulation in normo-ovulatory women

2.2.1. Introduction

Inhibins are principally produced in the ovary by granulosa cells and selectively inhibit follicle-stimulating hormone (FSH) secretion by the pituitary (Burger, 1993). Inhibins are dimeric glycoproteins produced by the gonads consisting of an α subunit linked through disulfide binding with either a β_A or β_B subunit. The resulting $\alpha\beta_A$ heterodimer is referred to as inhibin A, whereas the $\alpha\beta_B$ protein constitutes inhibin B (de Kretser *et al.*, 2002). Inhibin A seems to be the predominant form produced during the late follicular and luteal phase of the normal menstrual cycle, whereas inhibin B is the predominant form during the early and mid follicular phase of the cycle (de Kretser *et al.*, 2002; Laven and Fauser, 2004).

Recent studies in normo-ovulatory women have shown convincingly that inhibin B is predominantly secreted by granulosa cells of pre-antral and small antral follicles and hence its concentration increases during the luteo-follicular transition (Roberts, 1993). Inhibin B levels are highest during the mid follicular phase and decline during the late follicular phase (Groome *et al.*, 1996; Schipper *et al.*, 1998a). A transient rise in inhibin B levels coincides with the mid-cyclic luteinizing hormone (LH) and FSH surge. Thereafter inhibin B levels decline further to a nadir in the mid-luteal phase (Muttukrishna *et al.*, 1994; Groome *et al.*, 1996). Declining inhibin A levels during the late luteal phase seem to be the predominant regulator of rising FSH serum levels during the luteo-follicular transition and hence contribute to the dynamic changes within a menstrual cycle (Brannian *et al.*, 1992; Stouffer *et al.*, 1994; Molskness *et al.*, 1996). In contrast, high inhibin B concentrations during the early follicular phase are responsible for the decline in FSH serum levels closing the FSH window and assuring single dominant follicle selection (Groome *et al.*, 1994). This specific differential pattern of inhibin A and B secretion is established during early puberty and remains constant throughout reproductive life (Sehested *et al.*, 2000).

Since the size of the cohort of recruited follicles seems related to the size of the primordial follicle pool (Scheffer *et al.*, 1999), inhibin B may constitute a suitable marker of ovarian ageing (Soules *et al.*, 1998; Burger, 2000). Moreover, inhibin B serum levels have been suggested to predict poor response to ovarian hyperstimulation in in-vitro fertilization (IVF) patients (McLachlan *et al.*, 1986). Unfortunately, more detailed analyses concluded that early follicular phase inhibin B serum levels are only of limited value in predicting response during ovarian hyperstimulation (Seifer *et al.*, 1997; Tinkanen *et al.*, 1999; Creus *et al.*, 2000; Dumesic *et al.*, 2001). Dynamic inhibin B testing with FSH stimulation (Lockwood *et al.*, 1996; Dzik *et al.*, 2000) or gonadotrophin releasing hormone (GnRH) agonist administration (Ravhon *et al.*, 2000) appears to correlate better with ovarian response. A good correlation was also observed between inhibin B concentrations during ovarian hyperstimulation and the number of oocytes retrieved (Eldar-Geva *et al.*, 2000; Fawzy *et al.*, 2002).

Most, if not all, of the aforementioned studies however, used GnRH agonists to suppress endogenous gonadotrophins and consequently baseline inhibin B levels are decreased (Welt *et al.*, 1997). Hence, results from these studies might not be readily applicable to stimulation protocols used for ovulation induction or minimal ovarian hyperstimulation in conjunction with intrauterine insemination during which generally GnRH analogues are not applied. The current study was designed to study the relationship between serum concentrations of inhibin A and B and the number of developing follicles during various ovarian hyperstimulation protocols without previous down regulation using a GnRH agonist.

2.2.2. Materials and methods

Subjects

This study was approved by the local Ethics Review Committee. A total group of 63 healthy volunteers was selected from responders to advertisements in local newspapers and interviews on the local radio and television. Written informed consent was obtained from each participant, and all subjects were paid for participation, as previously published (Schipper *et al.*, 1998b; Hohmann *et al.*, 2001).

Inclusion criteria were: 1) a history of regular menstrual cycles (cycle length 25-32 days); 2) age 19-35 years; 3) body mass index (BMI) 18-27 kg/m2; 4) mid luteal progesterone concentrations (assessed 7 days prior to expected menses) amounting to at least 18 nmol/l and 5) no previous use of medication or oral contraceptives during at least 3 months prior to the study. Patients with a past history of any endocrine disease or infertility were excluded. All participants either used non-steroidal contraception (intrauterine devices, condoms or prior tubal ligation) or refrained from sexual intercourse during the study period.

Interventions

The first 23 subjects were studied during a natural cycle, followed by an intervention cycle (study 1, group A and B). The remaining 40 volunteers were studied during a single intervention cycle (study 2, group C, D, E) (Schipper *et al.*, 1998b; Hohmann *et al.*, 2001).

The natural cycle was assessed by means of daily transvaginal ultrasound scans (TVS) and daily blood sampling starting on day 12 after the assessed LH surge in the pre study cycle and concluding on the day of ovulation as assessed by TVS. Normal ovulation was confirmed by assessment of elevated progesterone (P) levels (>18 nmol/l) 6 or 7 days later (van Santbrink *et al.*, 1995b). The LH surge in the pre study cycle was determined using a urinary LH self-test (Clear-plan One Step®, Unipath Ltd., Bedford, UK) starting 10 days after the onset of the previous menses.

Study 1 (Group A and B): For the subjects who were followed during a natural cycle, a second series of daily TVS and blood sampling started at day 10 (LH+10) after the LH surge in the control cycle. Women were randomly assigned to group A or B as published previously (Schipper, 1998b). All participants received exogenous urinary FSH (Metrodin-HP®, Serono Benelux BV, The Hague, The Netherlands) from the same batch. Group A received a single dose of 375 IU urinary FSH sc at day LH+14,

effectively increasing the FSH concentrations above the presumed threshold without affecting the length of the FSH window (Fauser and van Heusden, 1997). Group B received a similar total dose in 5 consecutive injections of 75 IU urinary FSH sc starting on day LH+19, thereby preventing decremental serum FSH concentrations in the mid- to late-follicular phase and thus widening the FSH window. FSH was administered shortly after the daily blood withdrawal. Daily TVS as well as blood sampling were continued until the day of sonographically assessed ovulation. Normal ovulation was confirmed by assessment of elevated P levels 7 days later.

Study 2 (Group C, D and E): The remaining 40 subjects were randomly assigned to groups C, D and E, as published previously (Hohmann, 2001). All these subjects received a daily fixed dose of 75 IU recombinant FSH (recFSH; Gonal-F®, Serono Benelux BV) starting either on day 3, 5 and 7, respectively, until the day of human chorionic gonadotrophin (hCG) administration. As soon as the largest follicle reached a diameter of 18 mm or more a single dose of 5,000 IU hCG (Profasi®, Serono Benelux BV) was administered im at 22.00 h to induce ovulation. TVS as well as blood sampling was performed on a two daily basis starting on cycle day 3 until the largest follicle reached a diameter of 15 mm or more. Thereafter, ultrasound scans and blood sampling were performed daily until sonographically assessed ovulation. Ovulation was confirmed by blood sampling and TVS 8 days after hCG administration.

Sonographic imaging was performed using a 6.5 MHz transvaginal transducer (model EUB-415/420; Hitachi Medical Corporation, Tokyo, Japan). The ovaries were localized and scanned as described previously (Pache *et al.*, 1990). Follicle diameter was calculated as the mean diameter (measured in 2 dimensions when < 9 mm and measured in 3 dimensions when > 9 mm), as published previously (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b). A dominant follicle was defined as a follicle with a diameter of 10 mm or more, whereas a pre-ovulatory follicle should measure 15 mm or more. This distinction is clinically important since not all follicles measuring 10 mm or more reach the pre-ovulatory stage. Sonographically assessed ovulation was defined as a decrease in size of > 50% of the largest follicle (≥ 18 mm).

Hormone assays

Blood samples were obtained by venepuncture and processed within 2 hours after withdrawal. Serum was stored at –20 °C until assayed. Serum levels of LH and FSH were measured using luminescence based immuno assays (Immulite, Diagnostic Products Corp., Los Angeles, CA, USA) whereas serum E_2 levels were measured using coated tube radioimmunoassays provided by the same supplier. Standards used in the gonadotrophin assays were based on WHO 2nd IRP 78/549 and WHO 1st IRP 68/40 for FSH and LH respectively. Sensitivities of the assays were 0.1 U/l for FSH and LH, and 10 pmol/l for E_2. Intra- and interassay coefficients of variation were less than 5% and 6% for LH, less than 5% and 7% for FSH and less than 5% and 7% for E_2, respectively.

Dimeric inhibin A and B levels were assessed using an immuno-enzymometric assay obtained from Serotec (Oxford, UK), as described previously (Schipper *et al.*, 1998a). The detection limit of the assay, defined as the amount of inhibin equivalent with the signal of the blank + 3 SDs of this signal, was 3.4 ng/l for both inhibin A and B. Intra- and inter-assay coefficients of variation were less than 8% and 15% for

inhibin A and less than 8% and 14% for inhibin B respectively. All samples from one subject were run within the same assay.

Data analysis

The early follicular phase was defined as day^{LH+14} – day^{LH+18} for the natural cycle and group A and B (interventions study 1) and as cycle day (CD) 1 – CD 5 for group C, D and E (interventions study 2). The mid- and late follicular phase were defined as day^{LH+19} – day^{LH+23} and day^{LH+24} – day$^{LH\ surge}$ (natural cycle, group A and B) and CD 6 – CD 10 and CD 11 – day^{hCG} (group C, D and E), respectively. Data are presented as means and SD if distributed normally or as median and ranges if distributed otherwise. The early-, mid- or late follicular phase value of a subject was chosen to be the mean value in each phase, in order to prevent overrating of individual subjects in the calculations. Student's-*t* test statistics was performed on normally distributed data. Whenever a non-parametric distribution was found, groups were compared using the Mann-Whitney *U*- test. Comparisons of outcome measures between more than 2 groups were performed using the Kruskal-Wallis *H*-test for continuous data and using the χ^2 test for binary variables. Comparisons of data over time between groups were done using analysis of variance (ANOVA). Correlation coefficients given are Pearson's. *P*-values are two-sided and $P < 0.05$ was considered to indicate statistical significance. Data were analysed using a commercially available software package (SPSS, Chicago, Illinois, USA).

2.2.3. Results

Baseline characteristics

Sixty-three normo-ovulatory women entered the study protocol. In the first study, 11 and 12 subjects were assigned to group A and B, respectively. In the second study, 13, 13 and 14 subjects were allocated to group C, D and E, respectively. With regard to the distribution of age, cycle length and baseline endocrine serum concentrations of E_2 and inhibin B no statistical differences existed between groups (Table 2.3). FSH and inhibin A were comparable between groups A and B. Comparing group C, D and E also no significant differences were found.

Hormone concentrations

Differences in mean early-, mid- and late follicular phase levels of inhibin B, inhibin A, E_2 and FSH between the natural cycle and the intervention groups A, B, C, D and E are depicted in Figure 2.6. In the natural cycle, inhibin B levels showed a significant rise from the early follicular phase to their highest levels in the mid follicular phase ($P < 0.01$). Inhibin A and E_2 levels increased significantly in the late follicular phase ($P < 0.001$ and $P < 0.001$, respectively). In group A, FSH serum concentrations showed a significant decrease from the early to mid follicular phase ($P = 0.001$), with a subsequent decrease in inhibin B concentrations from the early to late follicular phase ($P < 0.05$). Group B, C and D (all starting exogenous FSH in the early follicular phase or beginning of the mid follicular phase) showed all an increase in inhibin B levels from the early to mid follicular phase ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively)

Table 2.3 Baseline characteristics (median and range) in 63 healthy female normo-ovulatory volunteers, participating in the study with various intervention regimens with exogenous FSH.

	Intervention Group				
	Study 1		Study 2		
	A *(n= 11)*	B *(n=12)*	C *(n=13)*	D *(n=13)*	E *(n=14)*
Age *(yrs)*	28 (25 - 33)	28 (20 - 34)	29 (21 - 35)	29 (22 - 34)	28 (20 - 35)
Cycle length *(days)*	28 (27 – 30)	29 (27 – 30)	28 (26 – 31)	28 (25 – 31)	28 (26 – 30)
FSH * *(IU/l)*	5.1 (3.9 – 6.0)	5.1 (3.6– 7.7)	6.5 (2.8 – 13.5)	6.3 (3.3 – 9.8)	7.7 (5.4 – 13.1)
E_2* *(pmol/l)*	156 (102 – 230)	143 (106 – 259)	127 (64 – 220)	175 (91 – 404)	137 (109 – 264)
inhibin A * *(ng/l)*	5 (2 - 9)	5 (2 - 6)	5 (4 – 9)	7 (3 – 52)	8 (4 – 11)
Inhibin B * *(ng/l)*	98 (29 – 221)	154 (68 – 234)	87 (21 – 192)	130 (12 – 213)	114 (57 – 179)

***** All baseline endocrine parameters were assessed on cycle day 3 (pre-intervention cycle for group A and B, intervention cycle for group C, D and E)

without significant changes in FSH concentrations. Although inhibin B levels in group E showed an increase from the early to late follicular phase ($P < 0.01$), the increase started later with a significant increase from mid- to late follicular phase ($P < 0.05$). In all 5 intervention groups inhibin A and E_2 levels increased during the follicular phase, showing a significant increase between early- and mid follicular phase levels, except group A (inhibin A and E_2 levels: $P < 0.001$ and $P < 0.001$, $P < 0.001$ and $P < 0.001$, $P = 0.001$ and $P < 0.001$, and $P < 0.05$ and $P < 0.001$ for group B, C, D and E, respectively), and between mid- and late follicular phase levels ($P < 0.001$ and $P < 0.001$, $P < 0.001$ and $P < 0.001$, $P < 0.01$ and $P < 0.01$, $P < 0.001$ and $P < 0.01$, and $P = 0.001$ and $P = 0.001$, for group A, B, C, D and E, respectively).

In the early follicular phase, group A showed a significant rise in FSH concentration ($P < 0.001$), with a subsequent rise in inhibin B concentration ($P < 0.001$) compared to the natural cycle. Also group C showed an increased FSH ($P < 0.001$) and inhibin B ($P < 0.01$) concentration compared to the natural cycle. In the mid follicular phase serum hormone levels in group A were comparable to those concentrations in the natural cycle. In group B, C and D however, inhibin B- and A, E_2 and FSH concentrations were all increased compared to the natural cycle ($P = 0.01$, $P < 0.01$, $P < 0.001$ and $P < 0.01$ for group B; $P < 0.05$, $P < 0.001$, $P < 0.05$ and $P < 0.001$ for group C; and $P = 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.001$ for group E, respectively). Mid follicular inhibin B in group E was comparable to the natural cycle, whereas inhibin A, E_2 and FSH levels were increased ($P < 0.001$, $P < 0.05$ and $P < 0.001$, respectively).

No differences were observed comparing the natural cycle and group A in the late follicular phase. The other intervention groups showed late follicular inhibin B levels

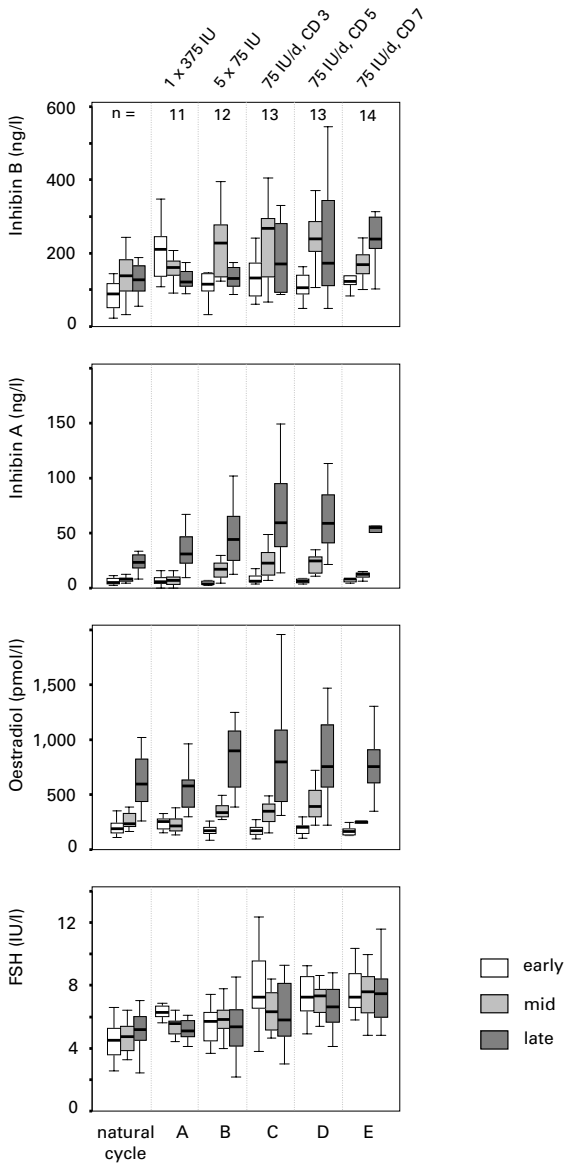

Figure 2.6 Box and whisker plots depicting mean concentrations during the early-, mid- and late follicular phase of inhibin B and A, oestradiol as well as FSH in 63 normo-ovulatory women, comparing the natural cycle (n = 23) and 5 intervention cycles with exogenous FSH. Group A received 375 IU in a single dose in the early follicular phase (n = 11), group B received 5 consecutive doses of 75 IU in the mid follicular phase (n = 12), group C (n = 13), group D (n = 13) and group E (n = 14) received daily doses of 75 IU commencing on either cycle day (CD) 3, CD 5 or CD 7, respectively, until administration of hCG in the late follicular phase. *Boxes* indicate 25th and 75th percentiles, with the *horizontal line* representing the median value. *Whiskers* span the range between the 5th and 95th percentiles of the data.

comparable to the natural cycle, except for group E, which showed a significant rise in inhibin B ($P < 0.001$). These intervention groups all showed a significant rise in late follicular inhibin A levels compared to the natural cycle ($P < 0.01$, $P < 0.001$, $P < 0.001$ and $P < 0.001$ for group B, C, D and E, respectively). No differences were found in E_2 concentrations, except for a slight increase in group B ($P < 0.05$).

Follicle growth and serum inhibin concentrations

In the natural cycle the rise in serum inhibin B levels coincided with a similar increase in the number of small antral follicles with diameters ranging from 5-11 mm (Pearson's correlations between inhibin B and follicles ranging 5-9 mm and 9-11 mm: r = 0.423, $P < 0.001$ and r = 0.316, $P = 0.01$, respectively). Inhibin A increased only after a follicle of 15 mm or larger was detected during the late follicular phase (Pearson's correlations between inhibin A and follicles ranging 15 - 17 mm: r = 0.357, $P < 0.05$ and r = 0.427, $P < 0.01$, respectively).

Interventions C, D and E all resulted in a significant increase ($P < 0.01$) in the number of follicles measuring 10 mm or more. In total, 24 subjects (60%) from groups C, D and E, showed multifollicular growth (defined as > 1 follicle measuring > 10 mm at the day of hCG administration). In multifollicular cycles, mid follicular inhibin B levels were significantly elevated compared to monofollicular cycles (median mid follicular inhibin B 245 ng/l [range 75-830] *vs.* 147 ng/l [range 57-291] for multi- and monofollicular cycles, $P = 0.001$). In the late follicular phase median inhibin B, -A and E_2 levels were elevated in multifollicular cycles compared to the monofollicular cycles (median late follicular inhibin B 230 ng/l [range 50-902] *vs.* 113 ng/l [range 76-298],

Table 2.4 Pearson's correlations (r) between median early-, mid- and late follicular phase levels of inhibin B, inhibin A, FSH and E_2 with the number of follicles of different size categories in the late follicular phase[§] of various intervention cycles in 63 normo-ovulatory women.

	# follicles 5 – 10 mm	# follicles 10 – 15 mm	# follicles 15 – 20 mm
Inhibin B (ng/l)			
Early	0.260 *	-0.256 *	0.022
Mid	0.293 *	0.352 **	0.539 ***
Late	0.128	0.536 ***	0.636 ***
Inhibin A (ng/l)			
Early	-0.103	-0.054	0.049
Mid	0.135	0.084	0.424 ***
Late	0.106	0.378 **	0.755 ***
FSH (IU/l)			
Early	-0.158	0.012	0.147
Mid	-0.044	0.202	-0.073
Late	-0.162	0.018	-0.042
E_2 (pmol/l)			
Early	-0.056	-0.099	-0.039
Mid	0.162	0.044	0.336 **
Late	0.086	0.360 **	0.737 ***

[§] day LH surge (group A and B); day of hCG (group C, D and E)
Level of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

$P < 0.05$; inhibin A 75 ng/l [range 14-185] *vs.* 39 ng/l [range 14-57], $P < 0.01$ and E_2 886 pmol/l [range 221-2188] *vs.* 664 pmol/l [range 310-807], $P < 0.05$ respectively).

Table 2.4 shows the relation between median early-, mid- or late follicular phase levels of inhibin B, -A, FSH and E_2 with the number of follicles of different size categories in the 5 intervention cycles. Only early follicular inhibin B concentration correlated slightly with the number of small follicles at the end of the follicular phase. In contrast, in the mid follicular phase inhibin B levels, as well as inhibin A levels, correlated with the number of developing larger follicles. In the late follicular phase a strong correlation was found between inhibin B, inhibin A and E_2 levels with the number of larger follicles at the end of the follicular phase.

2.2.4. Discussion

The present study in normo-ovulatory women confirms that inhibin B is indeed mainly produced by small antral follicles and hence inhibin B levels increase during the early- to mid-follicular phase. Thereafter, inhibin B levels decline during the late-follicular phase. Inhibin A is predominantly produced by the pre-ovulatory follicle and serum concentrations are therefore high during the late-follicular phase. An increase in FSH stimulation during the early follicular phase induces an increased growth of small antral follicles and hence elevated inhibin B concentrations. If this increase in FSH is long enough to extend the FSH window (the period during which FSH levels are above the threshold for ovarian stimulation (Baird, 1987; Fauser and van Heusden, 1997)), the intervention can result in ongoing multifollicular growth and hence an increase in both inhibin B as well as inhibin A. However, if single dominant follicle selection is not affected, serum inhibin B returns to normal and inhibin A remains comparable to serum levels in the natural cycle.

In the natural cycle serum inhibins showed the expected pattern. After the initial rise in FSH a number of small antral follicles is recruited and subsequently inhibin B is produced. Thereafter the dominant follicle is selected and smaller follicles from the recruited cohort become atretic. Hence, inhibin B levels decrease (de Kretser *et al.*, 2002; Laven and Fauser, 2004). Inhibin A and E_2 levels increase after the dominant follicle selection (Groome *et al.*, 1994; Muttukrishna *et al.*, 1995; Lockwood *et al.*, 1996). Inhibin B serum concentrations in the natural cycle showed a biphasic pattern in the mid- to late-follicular phase. This pattern correlated highly with the number of follicles measuring up to 11 mm, whereas the number of follicles larger than 11 mm were relatively constant in this phase of the cycle suggesting that inhibin B mainly originates from follicles up to 11 mm. A similar relationship has been reported recently in menstrual cycles of normo-ovulatory women (Burger *et al.*, 1998).

In the current study, inhibin B levels were increased in all intervention cycles during which exogenous FSH was administered. Moreover, in those cycles with multi-follicular development the highest levels were recorded. In case the FSH window is effectively broadened more small follicles will emerge from the recruitable pool (Schipper *et al.*, 1998b). Since inhibin B is mainly produced by small antral follicles up to 12 mm it might constitute a marker for multiple follicle development (Bukman and Heineman, 2001). In the current study inhibin B serum concentrations during the early- and mid

follicular phase were indeed significantly correlated with the number of small developing follicles. Moreover, mid- and late follicular phase inhibin B levels were higher in those cycles which resulted in multi-follicular development, reflected in significant correlations in mid- and late follicular phase inhibin B levels and the number of larger follicles at the end of the follicular phase (day LH surge or day of hCG administration). Apparently, mid- and late follicular phase inhibin B concentrations during ovarian (hyper)stimulation constitute a marker for the number of developing follicles (Eldar-Geva *et al.*, 2000; Fawzy *et al.*, 2002).

Prediction of those women that may under- or over-respond to ovarian (hyper) stimulation protocols seems to be of clinical importance. During ovarian stimulation (ovulation induction or ovarian hyperstimulation) it has been shown that the number of follicles measuring 12 mm or more at the day of hCG administration is strongly correlated with the incidence of high order multiple pregnancies (Tur *et al.*, 2001; Dickey *et al.*, 2001). Similarly, E_2 serum concentrations are strongly correlated with adverse outcome (Tur *et al.*, 2001). Since mid follicular inhibin B serum concentrations were correlated both with the number of follicles measuring 10 mm or more as well as with multifollicular development, inhibin B might also constitute a predictor of multiple gestation during ovarian hyperstimulation. Ideally such a predictor should be sufficiently accurate and distinct in time from the moment that stimulation protocols are commenced (Yong *et al.*, 2003). Consequently, based on early follicular phase inhibin B serum levels, treatment might be optimized since women at risk for ovarian hyperstimulation syndrome and multiple pregnancies might be more easily identified prior to treatment. Unfortunately, in the current study as in others, the basal inhibin B serum levels did not correlate with the final pre-ovulatory number of follicles (Seifer *et al.*, 1997; Hall *et al.*, 1999; Tinkanen *et al.*, 1999; Yong *et al.*, 2003). Moreover, early basal as well as stimulated inhibin B levels seem to reflect the potential follicular development of the ovary, but are not significantly associated with the final oocyte number which is mainly determined by the antral follicle count and the basal FSH serum concentration (Yong *et al.*, 2003). Therefore, inhibin B constitutes a poor marker in the risk assessment of women which might over-respond to treatment.

In conclusion, the present data extend our understanding of the relationship between follicle dynamics, serum inhibins and FSH levels during multiple dominant follicle development resulting from ovarian hyperstimulation with exogenous FSH. However, although mid follicular inhibin B does correlate with the number of developing follicles, it does not facilitate the identification of women at risk for multiple follicle development.

Chapter 3

Mild ovarian stimulation for assisted reproduction

3.1. Introduction

Shortly after the first pregnancy following in vitro fertilization (IVF) was reported in a spontaneous cycle, evidence accumulated that overall pregnancy chances per IVF attempt increase significantly when more than one embryo is transferred. Ovarian stimulation protocols have subsequently been developed aiming at ongoing growth of several follicles in order to obtain multiple oocytes for fertilization in vitro and multiple embryos. However, the rapid increase in serum oestradiol levels (as a result of multiple dominant follicle development) frequently induces a premature rise in endogenous LH (Yen, 1977; Liu and Yen, 1983), resulting in premature luteinisation or ovulation before final oocyte maturation and oocyte retrieval. Therefore timing of induction of final oocyte maturation by human chorionic gonadotrophin (hCG) was crucial for obtaining good quality oocytes (Laufer *et al.*, 1984). Despite intensive and frequent monitoring by both ultrasound and hormonal measurements, many IVF cycles had to be cancelled due to premature luteinisation or ovulation (Hamori *et al.*, 1987). In order to improve the efficacy of IVF treatment, it was needed to avoid the occurrence of a premature LH rise.

LH and FSH are secreted by the anterior pituitary in response to pulsatile hypothalamic secretion of gonadotrophin releasing hormone (GnRH). Since a continuous administration of GnRH causes a decreased pituitary secretion of LH and FSH, GnRH agonists were developed. GnRH agonist administration leads to a constant stimulation of the pituitary GnRH receptor system due to an extended half-life and an enhanced binding affinity for the GnRH receptor compared to native GnRH. GnRH agonists initially stimulate the release of gonadotrophins ("flare-up phase"), with complete pituitary suppression after 2 to 3 weeks of treatment, due to pituitary desensitization and receptor down-regulation ("down-regulation phase").

Since GnRH agonists are capable of completely suppressing pituitary secretion of endogenous gonadotrophins, these compounds were introduced in IVF stimulation protocols for the prevention of a premature LH rise. Consequently, ovarian stimulation with gonadotrophins could be continued for an extended period of time and more oocytes could be obtained. Clinical pregnancy rates per cycle and per embryo transfer were reported to increase with the routine use of GnRH agonists for IVF (Hughes *et al.*, 1992). However, ovarian stimulation protocols have become extremely complex and expensive, take many weeks and are not without danger. Concerns over complications and health-economics arising from current strategies for ovarian hyperstimulation for IVF have been increasingly expressed (Edwards *et al.*, 1996; Fauser *et al.*, 1999). Novel approaches to ovarian (hyper)stimulation for IVF are needed. The clinical introduction of GnRH antagonists has allowed to re-evaluate the current strategies of profound ovarian stimulation. In contrast to GnRH agonists, GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotrophin release by competitive binding to the GnRH receptor without receptor activation or initial stimulation and a rapid recovery of normal secretion of endogenous LH and FSH (Ditkoff *et al.*, 1991; Hall, 1993). The degree of gonadotrophin suppression depends on the dose of GnRH antagonist administered (Fujimoto *et al.*, 1997; Oberyé *et al.*, 1999a). During ovarian

(hyper)stimulation, administration of GnRH antagonists can be restricted to the time when a premature LH rise can be expected, effectively shortening the duration of the IVF protocol. Cessation of GnRH antagonist treatment ensures a rapid recovery of the pituitary-gonadal axis. Furthermore, a lower total dose of FSH is needed for stimulation, since FSH levels are only partly suppressed with the use of GnRH antagonists. Moreover, since endogenous gonadotrophin secretion can be left undisturbed up to the mid follicular phase, GnRH antagonists offer the opportunity to develop alternative and milder stimulation protocols.

3.2. A randomized comparison of two ovarian stimulation protocols with GnRH antagonist co-treatment for *in vitro* fertilization commencing recombinant FSH on cycle day 2 or 5 with the standard long GnRH agonist protocol

3.2.1. Introduction

Conventional ovarian stimulation protocols aim to stimulate growth of many follicles in order to obtain multiple oocytes for *in vitro* fertilization (IVF) and thus multiple embryos allowing selection for transfer (Templeton and Morris, 1998). Currently applied standard IVF protocols take many weeks, are complex and expensive, and are not without risk. Problems related to ovarian stimulation include emotional stress, abdominal discomfort, short-term complications such as ovarian hyperstimulation syndrome and multiple gestation, and uncertainties regarding long-term health consequences (Fauser *et al.*, 1999). Many of the problems associated with current IVF stimulation regimens relate to the unphysiological approach to ovarian (hyper)stimulation (Edwards *et al.*, 1996). Preceding the administration of high doses of gonadotrophins, pituitary down regulation is normally achieved by prolonged administration of GnRH agonists (the so-called 'long protocol'). Up to the present, IVF practice has focused on optimizing success in terms of pregnancy rate per started IVF cycle. Profound ovarian stimulation is therefore applied, despite the above mentioned side effects, risks and costs. If the balance between risks and benefits of IVF treatment is to improve, a paradigm shift is required in the approach to treatment and in the way success from IVF is defined. Increasing knowledge regarding the physiology of ovarian follicle development and dominant follicle selection (Fauser and van Heusden, 1997), together with the clinical availability of new compounds such as GnRH antagonists (Al Inany and Aboulghar, 2002), have presented the opportunity to develop novel, milder approaches for ovarian stimulation for IVF (Macklon and Fauser, 2000).

During the luteo-follicular transition in the normal menstrual cycle, FSH concentrations rise and surpass the threshold stimulating a cohort of small antral follicles to grow (Fauser and van Heusden, 1997). Around the mid follicular phase, the most mature follicle gains dominance over other cohort follicles (Pache *et al.*, 1990). This dominant follicle continues its growth despite decremental FSH concentrations (van Santbrink *et al.*, 1995b), whereas the remaining follicles from the recruited cohort enter atresia, due to insufficient stimulation by FSH. This decreasing FSH level and

subsequent closure of the FSH 'gate' (Baird, 1987) or 'window' (Fauser *et al.*, 1993) appears essential for single dominant follicle selection.

Multifollicular growth is established in current IVF protocols by generating FSH serum concentrations far above the threshold from the early follicular phase for an extended period. However, single dominant follicle selection may be disrupted by the administration of low doses of exogenous FSH during the mid to late follicular phase, effectively preventing the physiological decrease in FSH concentrations (Schipper *et al.*, 1998b; Hohmann *et al.*, 2001). The clinical introduction of GnRH antagonists in IVF (Borm and Mannaerts, 2000; Albano *et al.*, 2000; Olivennes *et al.*, 2000; Fluker *et al.*, 2001; The European and Middle East Orgalutran Study Group, 2001) allows ovarian stimulation to be commenced in the undisturbed menstrual cycle. Extending the FSH window by administering low dose exogenous FSH from the mid to late follicular phase may indeed be effective in IVF (de Jong *et al.*, 2000a), resulting in a shorter and more patient-friendly treatment cycle. However, it remains unclear whether the reduced number of oocytes obtained following mild stimulation may impair outcome (Fauser *et al.*, 1999). In order to address these issues, we carried out a prospective randomized study comparing stimulation characteristics and IVF outcomes of the standard long GnRH agonist protocol for ovarian stimulation with two GnRH antagonist protocols commencing FSH in the early or mid follicular phase.

3.2.2. Materials and methods

Subjects and study design

This study was approved by the local Ethics Review Committee. Written informed consent was obtained from each participant. Between November 1999 and May 2001, 169 patients with an indication for IVF with or without intra-cytoplasmic sperm injection (ICSI) were recruited. After assignment to IVF or IVF/ICSI (those patients with a total motile sperm count < 1.0×10^6), randomization was performed to one of the three treatment groups using a computer-generated randomization schedule, assigned via numbered sealed envelopes.

Inclusion criteria were: 1) age between 20 and 38 years; 2) body mass index (BMI; body weight divided by the square of body height) between 19 and 29 kg/m^2; 3) history of regular menstrual cycles, ranging from 25 to 35 days; 4) no relevant systemic disease, severe endometriosis or uterine and ovarian abnormalities; 5) no more than 3 previous IVF cycles and 6) no previous IVF cycle with a poor response or ovarian hyperstimulation syndrome. Group A was treated with the GnRH agonist triptoreline (Decapeptyl®, Ferring BV, Hoofddorp, The Netherlands) 1 mg/day sc, starting one week before the expected menses (usually cycle day 21). After down regulation was achieved (serum E_2 level < 150 pmol/l) ovarian stimulation was commenced with a fixed daily dose of 150 IU sc recombinant FSH (recFSH) (Gonal-F®, Serono Benelux BV, The Hague, The Netherlands). Groups B and C were treated with the GnRH antagonist cetrorelix (Cetrotide®, ASTA Medica, Amsterdam, The Netherlands), 0.25 mg/day sc, commencing when the largest follicle had reached a diameter of 14 mm, as published previously (de Jong *et al.*, 2000a). RecFSH was initiated on cycle day 2 (group B) or 5 (group C). Triptoreline and cetrorelix were continued up to and including the day of

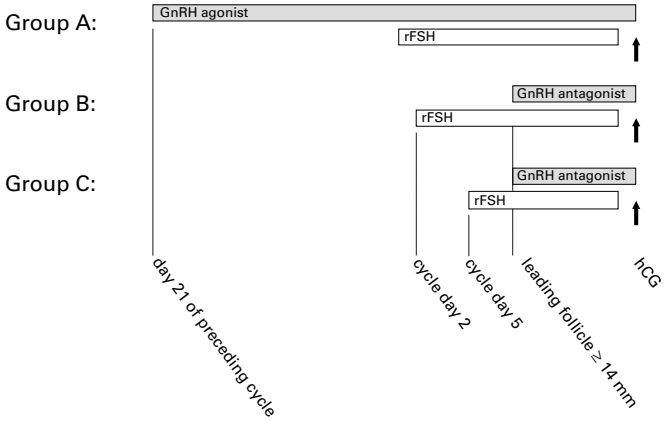

Figure 3.1 Schematic presentation of the three studied stimulation regimens: a GnRH agonist long protocol and two late follicular phase GnRH antagonist protocols with start of a fixed dose of recFSH on cycle day 2 or cycle day 5.

human chorionic gonadotrophin (hCG) administration. When the leading follicle had reached a diameter ≥ 18 mm and at least 3 follicles had reached a diameter ≥ 15 mm, recFSH was stopped and a single sc bolus of 10,000 IU hCG (Pregnyl®, NV Organon, Oss, The Netherlands) was administered, 35 hours before the planned time of oocyte retrieval. All follicles ≥ 12 mm were aspirated. Subsequently, IVF with or without ICSI was performed and a maximum of 2 embryos was transferred 3-5 days thereafter, as described previously (Huisman *et al.*, 2000). All embryos were scored on the day of embryo transfer (ET) between 8.00 h and 10.00 h. Luteal support in the form of intravaginal progesterone (P) (Progestan®, NV Organon), 3 times daily 200 mg, was given from the day of oocyte retrieval until a urine pregnancy test was performed 17 days later. A schematic description of the applied treatment regimens is given in Figure 3.1.

Assessments

Baseline blood sampling and transvaginal sonography (TVS) were performed on cycle day 2 or 3 of the pre-treatment cycle in group A and on cycle day 2 of the treatment cycle in groups B and C. Monitoring of response during the treatment cycle consisted of TVS and blood sampling (cycle day 2, 5, 8; day of hCG administration; day of oocyte retrieval and 7 days thereafter) for hormonal analysis (E_2, FSH, LH, inhibin B and P). Additional TVS monitoring was performed as clinically indicated.

Embryo scoring was carried out on the day of embryo transfer (3, 4 or 5 days after oocyte retrieval) blinded to the stimulation protocol. Scoring was based on developmental stage and morphology, using previously described criteria (Veeck, 1998; Huisman *et al.*, 2000). Scoring criteria for day 3 embryos included cell number, regularity of blastomeres, fragmentation and morphological aspects such as granulation. Criteria for day 4 embryos included the degree of embryo compaction and the presence of separated cells or fragments. Day 5 embryos were scored using criteria including embryonic

stage, cavitation, inner cell mass and cell morphology. When no fragmentation was evident and the developmental stage was appropriate for their age, embryos were described as 'high grade' and scored as embryo score 1. Embryos showing developmental delay and more than 50% fragmentation were described as 'low grade' and scored as embryo score 4. Embryos of better and worse intermediate quality were scored as embryo score 2 and 3, respectively.

In case of a positive urine pregnancy test (biochemical pregnancy), an ultrasound scan was carried out at 5-6 weeks after oocyte retrieval to determine the viability of the pregnancy. A second ultrasound was performed at 12 weeks of gestation to confirm an ongoing pregnancy (positive heart beat).

Hormone assays

Blood samples were centrifuged within 2 hours after withdrawal and serum was stored at –20 °C until assayed. Serum FSH, LH, and P levels were assessed by chemiluminescent immunoassay (Immulite, Diagnostic Products Corporation (DPC), Los Angeles, CA, USA). Serum E_2 levels were measured using radio immunoassay kits provided by DPC, as described previously (Fauser *et al.*, 1991). Dimeric inhibin B levels were determined using an immuno-enzymometric assay (Serotec, Oxford, UK), as described previously (Groome *et al.*, 1996). Intra- and interassay coefficients of variation were less than 5% and 7% for FSH, less than 5% and 6% for LH, less than 10% and 10% for P, less than 5% and 7% for E_2 and less than 8% and 14% for inhibin B, respectively.

Data analysis

The power calculation for this study was based on E_2 levels on the day of hCG, since E_2 provides a measure of ovarian response and correlates with the number of follicles and oocytes. Previous studies from our own group have indicated that late follicular phase mean E_2 levels between 2,800 and 4,500 pmol/l do not represent clinically important differences in ovarian response (Beckers *et al.*, 2000; de Jong *et al.*, 2000a), whereas concentrations below this range are associated with poor outcome. In order to detect whether a given stimulation protocol was associated with reduced ovarian response compared with the other stimulation protocols, represented by E_2 levels below 2,800 pmol/l (with 90% power, and *P*-values of 0.05), at least 120 patients (40 per group) were needed. Eligibility for analysis following inclusion- or protocol violations was decided by a third party, blinded for the randomization protocol.

Results are presented as the median and range, unless otherwise indicated. Comparisons of outcome measures between the three randomized groups were performed using the Kruskal-Wallis *H*-test for continuous data and using the χ^2-test for binary variables. Two group comparisons were performed using the Mann-Whitney *U*-test. Pearson's correlation coefficients were calculated. *P*-values are two-sided and 0.05 was considered the limit of statistical significance.

Data were analyzed using the commercially available software package SPSS, Inc. (Chicago, IL, USA).

3.2.3. Results

Subjects and baseline characteristics

Of the 169 patients randomized, 13 patients failed to start IVF treatment within the study period. 14 patients were excluded for analysis because of inclusion (n = 4) and protocol (n = 10) violations (4 of whom conceived). The remaining 142 patients (45, 48 and 49 patients for group A, B and C, respectively) were included in the data analysis.

With regard to the distribution of age, BMI, and baseline serum concentrations for FSH and inhibin B no significant differences were found between the three groups (Table 3.1). There was no difference between the three groups in cycle length (median cycle length in groups A, B and C: 28 d [range 25-35], 28 d [range 25-33] and 28 d [range 25-33], respectively; $P = 0.69$) or the duration of infertility (median duration in groups A, B and C: 3.5 yrs [range 0.6-12.0], 3.2 yrs [range 0.5-8.1] and 3.3 yrs [range 0.3-14.0], respectively; $P = 0.19$). Moreover, no difference was observed in the percentage with primary infertility (for groups A, B and C: 71%, 73% and 65%, respectively; $P = 0.70$) or the percentage of patients undergoing IVF/ICSI (for groups A, B and C: 22%, 15% and 18%, respectively; $P = 0.64$). In the IVF (without ICSI) patients no difference was found in the sperm quality of the partner (median total motile sperm count per ejaculate for groups A, B and C: 45×10^6 [range 1.1-420.0], 40.0×10^6 [range 0.3-220.0] and 38.8×10^6 [range 2.0-180.0], respectively; $P = 0.94$), the incidence of males with a total motile sperm count < 5,0 x 10^6 (χ^2 test, $P = 0.57$; data not shown), or the distribution of causes of infertility (χ^2 test, $P = 0.55$; data not shown).

Outcome

Clinical outcome parameters comparing groups A, B and C are shown in Table 3.1 and Figure 3.2. A high cancellation rate because of failure to meet criteria for oocyte

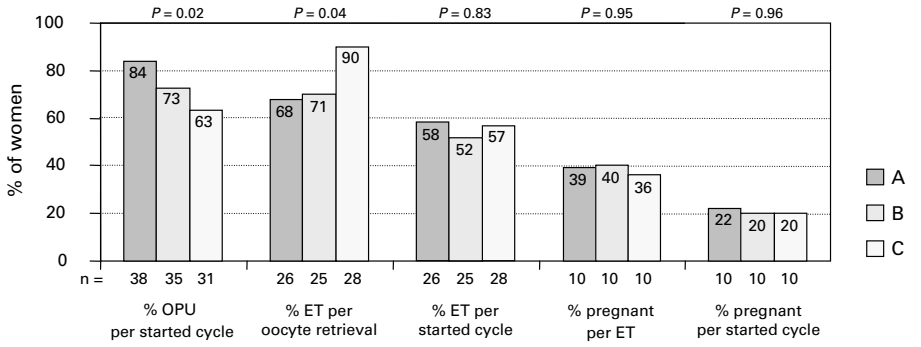

Figure 3.2 Percentage of women undergoing oocyte retrieval per started IVF cycle, percentage of women undergoing ET per oocyte retrieval and per started cycle, pregnancies per ET and pregnancies per started IVF cycle, comparing a GnRH agonist long protocol (group A) with two GnRH antagonist protocols with start of exogenous FSH on cycle day 2 (group B) or cycle day 5 (group C). Comparisons were performed using χ^2 test.

retrieval was observed in group C. Low response leading to cancellation of the cycle in this group correlated with an increased age and higher early follicular phase FSH concentrations (median age 35 yrs [range 28-39] *vs.* 33 yrs [range 24-39], $P = 0.001$, and median FSH 8.0 IU/l [range 2.9-29.4] *vs.* 5.8 IU/l [range 2.1-21.0], $P = 0.02$, for low and normal response patients, respectively). In patients with successful stimulation

Table 3.1 Patient characteristics and clinical IVF outcome (median and range) of a randomized comparison between a GnRH agonist protocol and two GnRH antagonist protocols commencing recFSH on cycle day 2 or cycle day 5, respectively.

	GnRH agonist long protocol (n=45)	GnRH antagonist recFSH day 2 start (n=48)	GnRH antagonist recFSH day 5 start (n=49)	*P**
Included patients (*N* = 142):				
Age (yrs)	33 (25-39)	33 (26-38)	33 (24-39)	0.36
BMI (kg/m^2)	23.0 (19.6-28.1)	24.2 (19.7-28.4)	22.9 (19.7-29.0)	0.08
$FSH_{day\ 2/3}$ (IU/l)	5.5 (1.0-10.8)	6.3 (2.0-16.0)	6.3 (2.1-29.4)	0.38
Inhibin $B_{day\ 2/3}$ (ng/l)	124 (21-292)	104 (11-260)	104 (18-727)	0.12
Patients undergoing oocyte retrieval (*N* = 104):				
n (% per started cycle)	38 (84%)	35 (73%)	31 (63%)	0.02
Cycle day start cetrorelix [a]	---	8 (5-12)	10 (8-14)	0.007
Day hCG				
FSH (IU/l)	6.5 (2.8-11.3)	6.6 (4.6-9.3)	6.8 (3.5-12.7)	0.66
LH (IU/l)	1.0 (0.1-2.6)	0.7 (01.-5.3)	1.5 (0.1-5.8)	0.09
E_2 (pmol/l)	3,407 (850-10,347)	2,555 (653-8,045)	3,193 (901-14,992)	0.23
P (nmol/l)	2.5 (0.6-6.1)	2.8 (0.8-6.1)	3.1 (0.7-7.3)	0.38
No. of follicles (≥ 10 mm) $_{day\ hCG}$	10 (4-21)	8 (3-27)	8 (3-22)	0.07
No. of follicles (≥ 15 mm) $_{day\ hCG}$	5 (3-17)	5 (3-11)	4 (3-10)	0.20
No. of oocytes retrieved	9 (1-25)	8 (2-31)	7 (1-27)	0.57
No. of embryos	4 (0-16)	4 (0-13)	3 (0-19)	0.99
Fertilization rate per subject (%)	50 (0-100)	54 (0-100)	68 (0-100)	0.12
No. of pregnancies (%)[b]	10 (22%)	10 (20%)	10 (20%)	0.96
No. of ongoing pregnancies (%)[c]	8 (18%)	8 (17%)	8 (16%)	0.98
No. of twin pregnancies (%[d])	3 (38%)	2 (25%)	3 (38%)	0.83

* Kruskal-Wallis *H*-test for continuous data; χ^2-test for binary variables.

[a] Largest follicle ≥ 14 mm

[b] Defined as a positive urine hCG test (percentage per started cycle)

[c] Defined as positive heart beat on ultrasound at 12 weeks of gestation (percentage per started cycle)

[d] Percentage of ongoing pregnancies

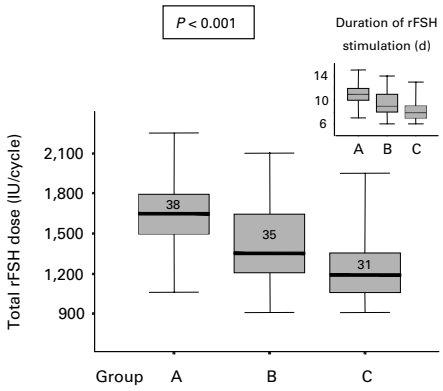

Figure 3.3 Duration of recFSH stimulation (in days) and the total dose of recFSH used (in IU per cycle) in 104 patients undergoing oocyte retrieval, comparing three different ovarian stimulation protocols for IVF: a GnRH agonist long protocol (group A) *vs.* two GnRH antagonist protocols, starting recFSH on either cycle day 2 (group B) or cycle day 5 (group C). As a fixed dose of recFSH (150 IU/day) was used, the total amount of recFSH used was directly related to the length of stimulation. *Boxes* indicate 25th and 75th percentile, with the *horizontal line* representing the median value. *Whiskers* span the range observed. Comparisons were performed using the Kruskal Wallis *H* test.

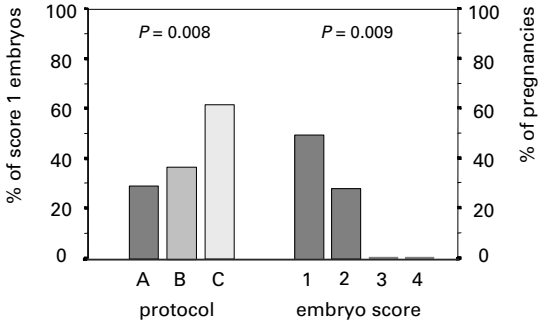

Figure 3.4 Bar diagrams representing the percentage of embryos with a score of 1 (see *Subjects and Methods* for definitions) after oocyte retrieval in 104 patients, comparing a GnRH agonist long protocol (group A; n = 38) with two GnRH antagonist protocols commencing recFSH on cycle day 2 (group B; n = 35) or cycle day 5 (group C; n = 31; *left panel*) and the pregnancy rate per embryo score of the best embryo transferred in 79 patients undergoing ET (*right panel*). Comparisons were

resulting in oocyte retrieval, the total amount of recFSH used and the duration of FSH stimulation decreased from group A to group C (Figure 3.3) (median total amount of recFSH used for groups A, B and C: 1650 IU [range 1050-2250], 1350 IU [900-2100] and 1200 IU [900-1950], respectively; $P < 0.001$). Group B and C showed a decrease of used recFSH of 18% and 27% respectively *vs.* group A. This difference in total amount of recFSH was more pronounced when the cancelled cycles were included according to an intention-to-treat analysis (median total amount of recFSH used for

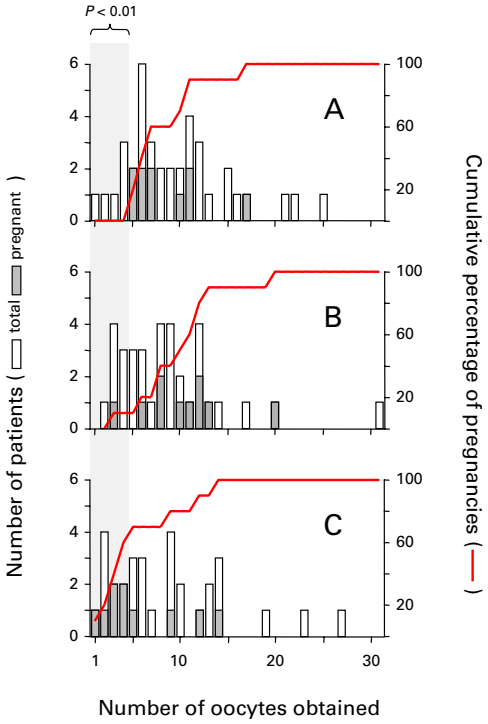

Figure 3.5 Relation between the number of oocytes retrieved and pregnancy, comparing a GnRH agonist long protocol (group A; n = 38) with two GnRH antagonist protocols with start of recFSH on cycle day 2 (group B; n = 35) or cycle day 5 (group C; n = 31). *Bars* represent the total number of patients (□) and pregnant patients separately (■) per number of oocytes retrieved. *Lines* represent the percentage of pregnant women, cumulative over the number of oocytes obtained. The *shaded area* represents patients in whom four or fewer oocytes were obtained and for whom the chance of pregnancy is significantly different comparing groups A, B and C (by χ^2 test, $P < 0.01$).

groups A, B and C: 1650 IU [range 1050-2250], 1425 IU [900-2100] and 1050 IU [450-1950], respectively; $P < 0.001$). Group B and C showed a decrease of 14% and 36% respectively *vs*. group A. Although none of the participants reaching criteria for oocyte retrieval demonstrated a premature LH surge (LH > 10 IU/l), three patients (2 from group B and 1 from group C) ovulated after hCG administration prior to oocyte retrieval. In 1 patient (group C) embryo transfer was not carried out due to an imminent ovarian hyperstimulation syndrome.

The number of follicles on the day of hCG administration, the number of oocytes retrieved and the number of embryos in patients where oocytes could be retrieved are given in Table 3.1. After oocyte retrieval, the best embryo was more frequently scored as 1 in group C (Figure 3.4) (groups A, B and C: 29%, 37% and 61%, respectively; $P = 0.008$). For the overall group the quality of the best embryo available for transfer was directly related to the pregnancy rate (Figure 3.4) ($P = 0.009$), independent from the use of a GnRH agonist or antagonist (implantation rate when embryo score of best

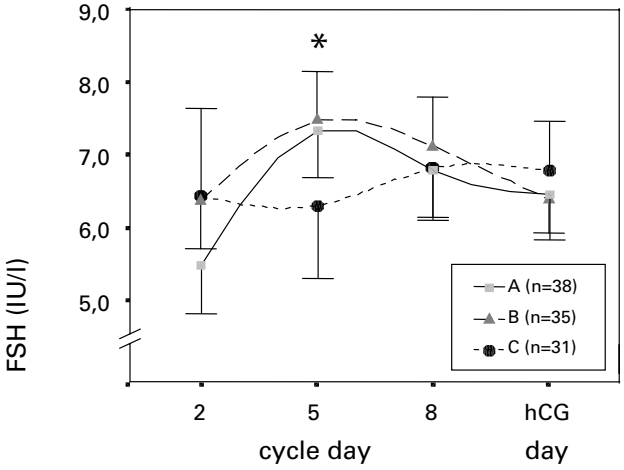

Figure 3.6 FSH serum concentrations (mean ± SEM) in the follicular phase of an IVF stimulation cycle in 104 patients undergoing oocyte retrieval, comparing a GnRH agonist long protocol (group A) with two GnRH antagonist protocols, with start of recFSH on cycle day 2 (group B) or cycle day 5 (group C). The time scale on the *x*-axis is divided into cycle days (group A: day start of recFSH = cycle day 1; groups B and C: day of onset menses = cycle day 1) and day of administration of hCG. FSH on cycle day 5 is statistically different comparing groups A, B and C.

embryo is 1 or 2: 40% for GnRH agonist versus 39 % for GnRH antagonist; $P = 1.0$). Fewer cycles in group C were cancelled after oocyte retrieval because of total fertilization failure or abnormal embryo development (groups A, B and C: 32%, 29% and 7%, respectively; $P = 0.03$). For IVF without ICSI, the occurrence of total fertilization failure was related to sperm quality (median total motile sperm count 47.5×10^6 [range 2.0-420.0] *vs*. 17.0×10^6 [range 0.3-75.0], respectively; $P = 0.008$). Abnormal embryo development was not related to sperm quality (data not shown). The median day of embryo transfer was not different comparing the 3 groups ($P = 0.93$; data not shown). There was no statistically significant difference in the quality of the best embryo transferred between the three groups (percentage of embryo score 1 of best embryo for groups A, B and C: 42%, 52% and 64%, respectively; $P = 0.11$).

Figure 3.5 shows the number of pregnant or non pregnant patients per number of oocytes retrieved, comparing groups A, B and C. In 6 out of 10 pregnant women in group C 4 or less oocytes were retrieved, whereas this occurred in none of the pregnant women in group A and in just 1 patient in group B ($P < 0.01$). With regard to (ongoing) pregnancy rate per started cycle no differences were found between the groups (Table 3.1 and Figure 3.2). The overall pregnancy rates of the 142 study patients were: 21% per started cycle, 29% per oocyte retrieval and 38% per embryo transfer.

Endocrinology

Table 3.1 shows E_2 levels on the day of hCG in patients reaching oocyte retrieval. Figure 3.6 shows the FSH serum concentrations during the follicular phase of the treatment cycle in all patients reaching criteria for oocyte retrieval, comparing groups A,

B and C. In groups A and B, FSH concentrations increased between cycle day 2 and 5 (median FSH day 2 *vs*. day 5: 5.1 IU/l [range 2.6-9.9] *vs*. 7.3 IU/l [range 3.5-13.0], $P < 0.001$ for group A and 6.2 IU/l [range 2.0-10.9] *vs*. 6.9 IU/l [range 3.8-14.4], $P = 0.02$ for group B) and decreased between cycle day 5 and day of hCG (median FSH day 5 *vs*. day of hCG: data presented above and in Table 3.1: $P = 0.04$ for group A and $P = 0.02$ for group B). However, in group C, FSH concentrations remained constant during the follicular phase (median FSH day 2: 5.9 IU/l [range 2.1-21.0] *vs*. day 5: 5.7 IU/l [range 3.2-15.9], $P = 0.98$, *vs*. day of hCG: 6.8 IU/l [range 3.5-12.7], $P = 0.08$).

3.2.4. Discussion

The concept of extending the FSH window to induce the development of multiple dominant follicles for IVF by administering exogenous FSH from the mid follicular phase onwards, combined with the use of GnRH antagonists, constitutes a novel strategy for mild ovarian stimulation. Since GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotrophin release (Christin-Maitre *et al.*, 2000; Al Inany and Aboulghar, 2002), these compounds offer the opportunity to commence the IVF treatment cycle in an undisturbed menstrual cycle. This study shows that the stimulating capacity of endogenous FSH can be exploited during the early follicular phase for the cyclic recruitment of a cohort of early antral follicles. Commencing exogenous FSH during the mid follicular phase results in multiple dominant follicle development despite a substantial reduction in the amount of exogenous FSH required.

In the GnRH antagonist day 5 group, significantly more cancellations due to 'low response' were observed compared to the GnRH agonist and GnRH antagonist day 2 groups. We observed a significant difference in age and baseline FSH, between the cancelled patients and those who met criteria for oocyte retrieval in the GnRH antagonist day 5 group. This may indicate that a subgroup of women likely to have a low response to mild stimulation can be identified before onset of the IVF. In contrast to previous reports, this difference in age and FSH between low and normal response patients was not found in the GnRH agonist or the GnRH antagonist day 2 groups. This is likely to be due to the relatively low number of patients involved. Compared to previous studies (The European and Middle East Orgalutran Study Group, 2001; Nygren and Andersen, 2001; ASRM/SART registry, 2002), we observed a relatively high cancellation rate (16% and 27%) prior to oocyte retrieval in both the GnRH agonist and the GnRH antagonist day 2 groups. This may be explained by differences in patient selection for IVF and differences in treatment protocols since we used a fixed dose of exogenous FSH. In addition, cancellation criteria in minimal stimulation protocols may need to be revised due to the observed pregnancies in women with a low oocyte yield.

For those patients in the GnRH antagonist day 5 group who met criteria for oocyte retrieval, the duration of stimulation was shorter compared to both other groups, resulting in a 27% reduction of the total amount of exogenous FSH required. The 14% reduction in total amount of exogenous FSH used comparing the GnRH antagonist day 2 *vs*. the GnRH agonist group was comparable to differences observed in previous

studies (Albano *et al.*, 2000). A reduction in exogenous FSH may affect ovarian response, usually assessed by the number of follicles that have developed and the E_2 levels during the late follicular phase. A reduced ovarian response will result in less oocytes after retrieval and probably a reduction in the number of resulting embryos. Given the close correlation between the number of embryos from which to select and chance of pregnancy (Templeton and Morris, 1998), pregnancy rates may suffer from less profound stimulation protocols. We did observe a tendency towards the development of less dominant follicles (≥ 10 mm) in the GnRH antagonist day 5 group. However, since no difference was found between the development of larger follicles (≥ 15 mm), mainly contributing to the production of E_2 (van Dessel *et al.*, 1996), no difference was observed in E_2 levels on the day of hCG administration between the three studied stimulation protocols. Thus, in those patients achieving multifollicular growth after extending the FSH window, a normal ovarian response is seen. The concern that fewer oocytes will be obtained after milder stimulated cycles resulting in fewer pregnancies is therefore not supported by our findings. Despite a tendency towards the development of less dominant follicles, no difference was seen in the number of oocytes retrieved.

The median fertilization rate per subject did not differ between the three groups and consequently the median number of embryos obtained was comparable. However, since more patients in the GnRH agonist and GnRH antagonist day 2 groups were cancelled because of a total fertilization failure or abnormal embryo development, patients in the GnRH antagonist day 5 group presented with a better chance of undergoing an embryo transfer compensating for the observed increased cancellation rate. Compared to previous studies reporting transfer rates after oocyte retrieval between 80-95% (Nygren and Andersen, 2001; ASRM/SART registry, 2002), 68-90% of the patients participating in the current study underwent an embryo transfer. This difference may be explained by the relatively small proportion of patients undergoing ICSI (18%), next to differences in patient inclusion, laboratory performance and the small numbers. When the criteria for oocyte retrieval were met in the current study, the chance of subsequently producing good quality embryos was significantly increased in the GnRH antagonist day 5 group. Milder stimulation may result in selection of good quality oocytes, which may result in better quality embryos. Although embryo quality is not the only factor determining implantation rate, embryo score is predictive of pregnancy (Hunault *et al.*, 2002). Our study provides further confirmation that high quality embryos are more likely to implant and result in pregnancy. Despite a better chance of retrieving high quality embryos, the patients in the GnRH antagonist day 5 group demonstrated a comparable pregnancy rate per embryo transfer. It has been suggested that the use of GnRH antagonists is associated with reduced implantation rates compared to conventional protocols (Al Inany and Aboulghar, 2002). This could not be confirmed in the current study, which may be related to the flexible GnRH antagonist protocol applied.

Overall, the pregnancy rate per started IVF cycle was comparable between the three groups, with a pregnancy rate of 21% per started cycle for all patients together. Despite our relatively high cancellation rates during the stimulation phase or after oocyte retrieval, these results are comparable to the percentages reported elsewhere. European studies report pregnancy rates per started cycle varying between 16-26%,

with a mean of 21% (Nygren and Andersen, 2001). American studies tend to report higher percentages of pregnancies per started cycle (delivery rate per started cycle of 25%), but at the cost of extended, complex and expensive stimulation protocols and increased chances for higher order multiple pregnancies due to the transfer of more than 2 embryos (ASRM/SART registry, 2002). Many studies from individual centres only mention pregnancy rates per oocyte retrieval or per embryo transfer, which are in the same range as the overall pregnancy rates observed in the current study (29% per oocyte retrieval and 38% per embryo transfer).

A low response during ovarian stimulation is currently believed to represent ovarian aging and poor oocyte quality (Nikolaou *et al.*, 2002; Beckers *et al.*, 2002; de Boer *et al.*, 2002). However, a low number of oocytes after mild stimulation may constitute a normal response, resulting in high quality oocytes and embryos. In our study, the response of 4 or less oocytes after profound ovarian stimulation (group GnRH agonist or GnRH antagonist day 2) observed in 19% of the patients, was indeed associated with impaired pregnancy outcome (only 7% of these patients conceived). However, after mild stimulation, the presence of 4 or less oocytes (observed in 29% of the patients) was associated with a good chance of pregnancy (67% of these patients conceived). This indicates that a low number of oocytes obtained after minimal stimulation may represent a selection of oocytes more likely to result in pregnancy. The low total dose of exogenous FSH may only stimulate the most mature follicles to ongoing growth allowing a degree of selection of oocytes to occur.

The advantages of mild ovarian hyperstimulation for IVF are being increasingly recognized (Edwards *et al.*, 1996; Edwards *et al.*, 1997; Fauser *et al.*, 1999). The reduction in the duration of ovarian stimulation (less injections) combined with less side-effects (Borm and Mannaerts, 2000) diminishes patient discomfort and reduces costs. Milder stimulation may require less monitoring since short-term complications and long-term risks are expected to be reduced, although this remains to be established in larger series of patients. Despite these advantages, a lower ovarian response after milder stimulation resulting in an increased cancellation rate prior to oocyte retrieval, has been put forward as an argument against the use of these protocols and restrained its introduction into clinical practice. However, despite the higher rate of cancellations in the GnRH antagonist day 5 group, the overall outcome did not differ between the three groups. This raises the question as to whether this higher incidence of 'low' ovarian response and thus higher cancellation rate prior to oocyte retrieval, is in fact detrimental for overall IVF outcome. The cancelled patients in the GnRH antagonist day 5 group may represent those with a poor chance of a high quality embryo transfer with impaired chances to conceive in IVF. Since cancellation after mild stimulation can partly be predicted by age and early follicular FSH concentrations (two markers of ovarian reserve), low response after mild stimulation may allow patient selection prior to oocyte retrieval, avoiding subsequent procedures that are unlikely to lead to pregnancy. Moreover, cancellation of a cycle after a short stimulation should be viewed differently from cancellation after a prolonged stimulation period. This earlier selection of poor prognosis patients may improve overall health economics of IVF for the majority of patients.

The finding that a low number of oocytes obtained after minimal stimulation is associated with good pregnancy chances, indicates that a large number of oocytes is not required for a successful IVF program. Although the criteria for oocyte retrieval did not differ between the protocols in this study, in retrospect these findings suggest the need for an adjustment of minimal criteria for oocyte retrieval after milder stimulation. A physiological reduction in the number of oocytes generated following mild ovarian stimulation distinctly differs from a pathological reduction associated with ovarian aging. The clinical introduction of GnRH antagonists allows a more physiological approach to ovarian stimulation. Moreover, the trend towards single embryo transfer avoiding multiple pregnancies (Gerris *et al.*, 1999; Martikainen *et al.*, 2001), reduces further the need for high numbers of oocytes and embryos. The present study demonstrates the clinical applicability of the concept of extending the FSH window for ovarian stimulation in IVF. However, the full clinical potential of the described mild stimulation protocol requires confirmation in larger multi-centre studies.

Chapter 4

Alternative approaches to disturbed follicle growth

4.1. Introduction

Ovulation induction, usually the first choice of treatment in anovulation, aims at monofollicular growth. The clinical approach in ovulation induction in patients with ovarian dysfunction depends on the cause of the anovulatory disorder. In absence of normo-ovulatory function, treatment is focused on restoring ovulatory function by mimicking a physiological hormonal balance.

Anovulation can be classified in the clinic on the basis of hormone assays. Most anovulatory patients (approximately 80%) present with serum FSH and oestradiol levels within the normal range (WHO class 2). In these patients treatment is focused on increasing responsiveness to FSH. However, the WHO class 2 group of patients is a very heterogeneous group, which shows a marked variability in ovarian response to ovulation induction. In order to avoid a failure of response or a hyperresponse, treatment modalities in normogonadotrophic anovulation should become more patient tailored. The introduction of new compounds as insulin sensitizing drugs for ovulation induction and the availability of new drugs like GnRH antagonists offer new possible approaches to the treatment of WHO 2 anovulatory patients.

4.2. LH suppression following different low doses of the GnRH antagonist ganirelix (Orgalutran®) in polycystic ovary syndrome

4.2.1. Introduction

Normogonatrophic normo-oestrogenic anovulation, including polycystic ovary syndrome (PCOS), is the most common anovulatory disorder in women and represents a major cause of female infertility. Approximately 40% of women with normogonadotrophic anovulatory infertility may present with LH levels above the normal range (van Santbrink *et al.*, 1997; Laven *et al.*, 2002). Elevated LH, mainly caused by an increased LH pulse amplitude (Santen and Bardin, 1973; Waldstreicher *et al.*, 1988), is believed to be involved in ovarian dysfunction by stimulating ovarian androgen synthesis, which in turn induces follicle atresia (Givens *et al.*, 1974; Gilling-Smith *et al.*, 1997). Patients presenting with elevated LH levels during the follicular phase have been reported to have a less favourable outcome from fertility therapy, especially in vitro fertilization (IVF) (Stanger and Yovich, 1985; Shoham *et al.*, 1993). Data concerning ovulation induction outcome are conflicting. Elevated LH levels were associated with a less favourable outcome in several studies (Homburg *et al.*, 1988), whereas others could not confirm this association with either ovulation (Imani *et al.*, 1999; Imani *et al.*, 2000) or pregnancy outcome (Rai *et al.*, 2000; Eijkemans *et al.*, 2003; Mulders *et al.*, 2003). Accordingly, gonadotrophin releasing hormone (GnRH) agonists have previously been applied to suppress LH either next to exogenous gonadotrophins (Dodson, 1989; Schoot *et al.*, 1992) or preceding pulsatile GnRH (Filicori *et al.*, 1989). Reported clinical results have been conflicting, but a recent Cochrane analysis (Bayram

et al., 2004b) failed to establish a beneficial effect of GnRH agonist co-treatment on ovulation induction outcome. In addition, an unsuccessful attempt in 2 patients has been published aiming at profound inhibition of endogenous gonadotrophin output by high dose GnRH antagonist (Nal-Glu) and subsequent stimulation of gonadotrophin release by pulsatile GnRH (Dubourdieu *et al.*, 1993).

Ganirelix represents a third generation GnRH antagonist. Initial studies demonstrated potent and acute suppression of gonadotrophin and steroid levels in normo-ovulatory women (Nelson *et al.*, 1995). For the prevention of a premature LH rise during ovarian hyperstimulation in IVF, a daily dose of 0.25 mg ganirelix was established to be the lowest effective dose (The ganirelix dose-finding study group, 1998). Although some studies suggested that pituitary sensitivity for steroid feedback may be different in PCOS (Patel *et al.*, 2004), the administration of different doses of a single injection of GnRH antagonist in PCOS showed a dose dependent decrease in LH (Hayes *et al.*, 1998; Elkind-Hirsch *et al.*, 2003), similar to that in normo-ovulatory controls (Oberyé *et al.*, 1999a). If repeated low doses of the GnRH antagonist ganirelix are capable of suppressing elevated LH levels in PCOS and if this lower serum LH might induce normal follicular growth is unknown. The present study was designed to establish whether different low doses of the GnRH antagonist ganirelix are capable of normalising LH levels in PCOS patients with elevated LH levels, which subsequently might reverse the anovulatory status of these patients.

4.2.2. Materials and methods

Subjects

This study was approved by the local Ethics Review Committee. Between January 2001 and February 2003, all patients attending the outpatient clinic of the Erasmus Medical Center with an amenorrhea or an oligomenorrhoea of at least 35 days fulfilling the inclusion criteria as outlined below and elevated LH levels were approached to volunteer for participation in this study. After screening, 24 participants were selected. Written informed consent was obtained from each participant and all were paid for travel expenses and inconvenience related to participation.

Inclusion criteria were: 1) oligomenorrhoea (bleeding intervals between 35 days and 6 months) or amenorrhoea (bleeding interval longer than 6 months); 2) elevated LH ($\geq$ 7.0 IU/l, in at least 2 independent samples) (van Santbrink *et al.*, 1997); 3) normal FSH (1-10 IU/l); 4) spontaneous or progestagen induced withdrawal bleeding; 5) age at time of screening between 20 and 40 years; 6) body mass index (BMI; body weight divided by the square of body height) between 20 and 30 kg/m^2 and body weight between 50 and 90 kg; 7) normal serum glucose, prolactin and thyroxin levels; 8) no use of medication interfering with pituitary-ovarian feedback mechanisms or pharmacokinetics of study medication and 9) both ovaries present (and visible at ultrasound).

Study design

The first day of the study was chosen between 14 to 18 days after the onset of a spontaneous menses or progestagen induced withdrawal bleeding in order to avoid

under-representation of elevated LH levels after menses or withdrawal bleeding, due to negative feedback action of steroids at the pituitary level (van Hooff *et al.*, 1999). Participants arrived at 7.30 h at the clinic, after fasting since 24.00 h the previous day. An intra-venous catheter was inserted in a vein on the forearm. Repetitive venous blood sampling was performed starting at 8.00 h and lasting for 8 hours (until 16.00 h) with 20 minute intervals. After the first blood sample (8.00 h) patients received breakfast. Between 8.00 h and 10.00 h randomization was performed to one of the three different doses of ganirelix using a computer-generated randomization schedule, assigned via numbered sealed envelopes. In the morning a transvaginal sonography (TVS) was performed. At 10.00 h, immediately after the blood withdrawal, patients received a sc injection with the assigned dose of ganirelix [0.125 mg (group A), 0.250 mg (group B) or 0.500 mg (group C)] in the upper thigh. Lunch was served between 12.00 and 13.00 h. At the end of the first day a last blood sample was taken at 22.00 h and participants could go home.

Following this first day, participants visited the clinic the following 7 days (visit 2-8). All visits, blood sampling through venous puncture was performed at a fixed time (10.00 h). At visit 2 to 7, immediately after blood withdrawal, participants received their daily injection of ganirelix sc (dose according to their randomization). TVS was performed every 2 to 3 days.

Hormone assays

Blood samples were centrifuged within 2 hours after withdrawal and serum was stored at –20 °C, until assayed. Serum levels of LH, FSH, and progesterone (P) were assessed using luminescence based immuno assays (Immulite, Diagnostic Products Corp., Los Angeles, CA, USA). Serum E_2, testosterone (T), androstenedione (AD) and sex hormone-binding globulin (SHBG) levels were measured using coated tube radioimmunoassays provided by the same supplier. Intra- and interassay coefficients of variation were less than 5% and 6% for LH, less than 5% and 7% for FSH, less than 10% and 10% for P, less than 5% and 7% for E_2, less than 3% and 5% for T, less than 8% and 11% for AD, and less than 4% and 5% for SHBG, respectively. Dimeric inhibin A and inhibin B levels were assessed using an immuno-enzymometric assay obtained from Serotec (Oxford, UK), as described previously (Groome *et al.*, 1994). The detection limit of the assay, defined as the amount of inhibin equivalent with the signal of the blank + 3 SDs of this signal, was 3.4 ng/l. Intra- and inter-assay coefficients of variation were less than 8% and 15% for inhibin A and less than 8% and 14% for inhibin B, respectively.

Data analysis

The power calculation for this study was based on suppression of the median LH. In a group of 48 normo-ovulatory controls, a LH of 6.9 IU/l represented the upper limit in the early/mid follicular phase (cycle day 3-5) (van Santbrink *et al.*, 1997). LH levels above this upper limit were chosen to be elevated. Using this cut-off, 47% of women suffering from anovulatory infertility (WHO group 2), exhibit elevated LH levels (van Santbrink *et al.*, 1997). The mean and standard deviation in the patient group with elevated LH levels were taken as a reference for pre-treatment values. The distribution of these data was non-normal and became normal after log-transformation. Hence the

most suitable outcome measure in this case was the median LH-level. For suppression of the LH concentration from the median pre-treatment value (11.3 IU/l) to normal (7.0 IU/l), with a power of 80% and an alpha of 0.025 (0.05 two-sided), 24 patients were needed (8 patients per group).

Results are presented as the median and range, unless otherwise indicated. Comparisons of outcome measures between the three randomized groups were performed using the Kruskal-Wallis *H*-test for continuous data and using the χ^2-test for binary variables. Two group comparisons were performed using the Mann-Whitney *U*-test. Pearson's correlation coefficients were calculated. *P*-values are two-sided and 0.05 was considered the limit of statistical significance. Data were analyzed using the commercially available software package SPSS version 11 (SPSS Inc.,Chicago, IL, USA).

4.2.3. Results

Subjects and baseline characteristics

After screening, 24 subjects were selected and randomised to one of the 3 dose groups. After randomisation, group A consisted out of 9 subjects whereas groups B and C consisted out of 7 and 8 subjects, respectively. Five subjects were excluded for further analysis, since they presented with a dominant follicle at the start of the study, defined as a follicle or cyst visible at ultrasound and measuring more than 15 mm or having E_2

Table 4.1 Baseline characteristics in three different dosage groups of the GnRH antagonist ganirelix (*N* = 19).

Characteristics*	**Group A 0,125 mg**	**Group B 0,250 mg**	**Group C 0,500 mg**
n	6	6	7
Age (yrs)	28.2 (22.0-39.7)	28.7 (26.3-37.9)	24.8 (20.8-30.9)
BMI (kg/m^2)	25.0 (21.5-30.3)	26.4 (20.3-30.9)	25.6 (20.1-30.1)
LH (IU/l)	15.2 (6.6-22.3)	11.2 (6.7-17.1)	11.7 (9.9-18.2)
FSH (IU/l)	6.5 (4.4-10.7)	4.7 (4.1-6.2)	4.4 (3.6-9.4)
E_2 (pmol/l)	193 (157-261)	216 (133-329)	221 (126-361)
P (nmol/l)	1.4 (0.6-8.6)	1.3 (0.8-1.9)	1.5 (0.6-2.9)
Inhibin A (ng/l)	2 (0-18)	3 (1-5)	3 (1-7)
Inhibin B (ng/l)	88 (60-131)	136 (80-203)	142 (59-420)
T (nmol/l)	1.3 (1.0-2.6)	1.5 (1.1-1.7)	1.8 (0.8-2.6)
AD (nmol/l)	9.0 (4.7-11.6)	11.3 (9.6-15.2)	8.6 (6.9-16.2)
DHEA (nmol/l)	32 (4-85)	40 (26-55)	30 (19-61)
DHEAS (µmol/l)	4.0 (0.4-7.0)	4.3 (2.8-6.2)	4.6 (1.8-12.7)
SHBG (nmol/l)	35.6 (23.4-50.9)	22.8 (17.8-46.4)	36.9 (24.8-63.9)
FAI (100 x T/SHBG)	3.2 (2.4-11.1)	6.5 (3.7-7.5)	4.9 (1.6-7.1)
Total follicle number	50 (38-73)	63 (54-82)	66 (32-78)

Data are expressed as median and range

* Predose values on treatment day 1 (t = 10.00 h)

serum levels exceeding 500 pmol/l. Consequently, groups A, B and C consisted thereafter out of 6, 6 and 7 subjects. Hence a total of 19 patients remained for data analysis.

Baseline characteristics of the three different dosage groups are given in Table 4.1. All patients presented with polycystic ovaries on TVS and all did meet the criteria for PCOS according to the 2003 Rotterdam PCOS consensus (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004a; The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004b). Briefly, baseline values were comparable between the three groups. Moreover, the excluded 5 subjects were not significantly different from those individuals who were analysed as far as baseline characteristics were concerned (data not shown), except for E_2 (median serum level 587 pmol/l) as well as P (median serum level 23 nmol/l), which were significantly ($P < 0.001$ and $P < 0.01$, respectively) different compared to groups A, B and C.

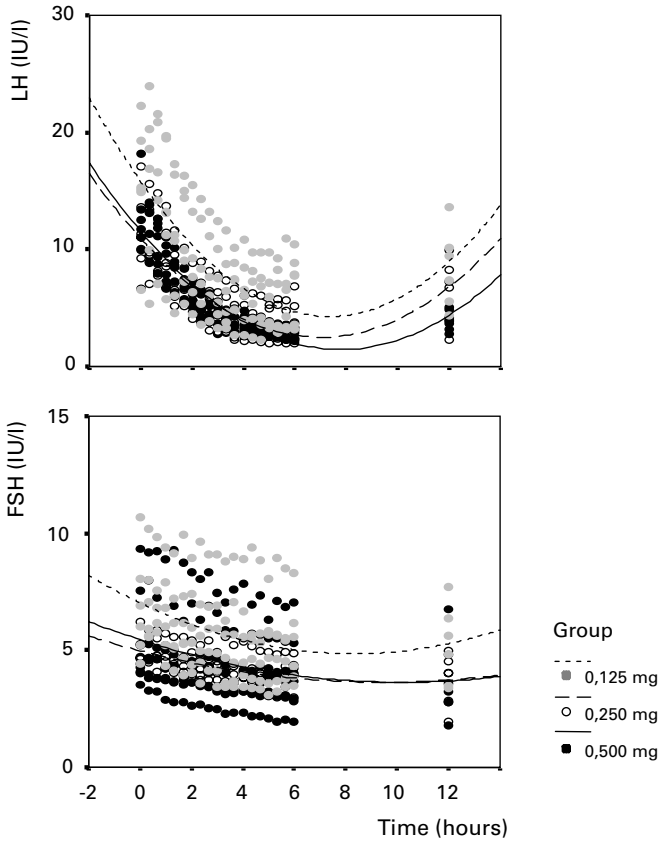

Figure 4.1 LH (*upper panel*) and FSH (*lower panel*) serum concentrations during the first day of GnRH administration in 19 subjects. The scale on the *x*-axis is expressed as hours after a single injection of the GnRH antagonist ganirelix (0.125 mg, 0.250 mg or 0.500 mg). The decline and subsequent increase show a quadratic pattern. Due to differences in initial patient characteristics the decline and subsequent increase are not dose dependent.

Intervention protocols

Data concerning repetitive serum LH and FSH measurements during the first day ganirelix was administered are given in Figure 4.1. Median predose LH concentrations, although increased, were comparable in all 3 groups (Table 4.1, $P = 0.20$). Following ganirelix administration, median serum LH concentrations decreased significantly ($P < 0.001$) within 6 hours to 4.7 IU/l (range 2.3-9.2), 2.7 IU/l (range 1.9-5.3) and 2.5 IU/l (range 0.4-3.4) for group A, B and C, respectively. The lowest LH serum concentration was achieved in the highest dosage group ($P < 0.02$). The decrease in LH serum levels after 6 hours in groups A (9.3 IU/l), B (8.2 IU/l) and C (9.1 IU/l) was comparable ($P = 0.64$) (Figure 4.2). In group A a 49% reduction in median serum LH was established after 6 hours, whereas this reduction amounted to 69% and 75% in groups B and C, respectively.

The time interval needed to reach the lowest LH serum level was also significantly ($P < 0.05$) different between the three groups. Group C individuals needed on average,

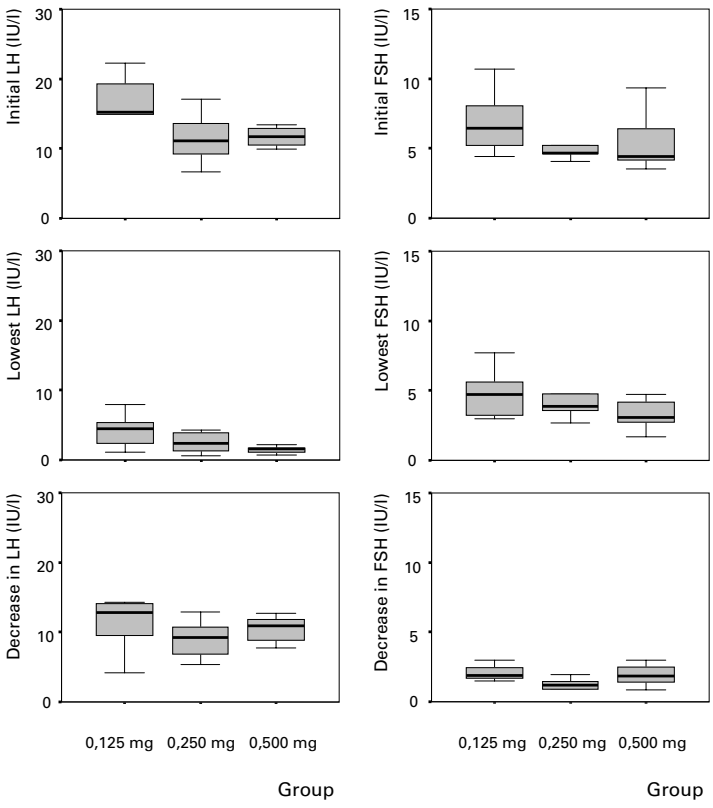

Figure 4.2 Box and whisker plots depicting the changes in LH (*left panels*) and FSH (*right panels*) in three different dosage groups during the first day of GnRH antagonist administration in 19 subjects. *Boxes* indicate 25th and 75th percentiles, with the *horizontal line* the median value. *Whiskers* span the range between the 5th and 95th percentiles of the data.

Table 4.2 Androgen levels 12 hours after the first injection of three different doses of the GnRH antagonist ganirelix ($N = 19$).

	Group A 0,125 mg	**Group B 0,250 mg**	**Group C 0,500 mg**
n	6	6	7
T (nmol/l)	1.0 (0.7-2.9)	1.3 (0.9-1.6)	1.4 (0.5-2.7)
AD (nmol/l)	6.9 (4.3-9.1)	9.3 (8.2-12.2)	6.7 (4.6-16.2)
DHEA (nmol/l)	25 (5-31)	25 (18-54)	28 (12-54)
DHEAS (µmol/l)	4.2 (0.4-6.7)	4.3 (2.5-6.2)	4.4 (1.5-12.3)
SHBG (nmol/l)	37.0 (23.0-49.9)	24.3 (19.9-48.7)	34.8 (24.8-62.5)
FAI (100 x T/SHBG)	2.8 (1.6-12.6)	5.3 (3.3-6.2)	3.7 (1.2-7.8)

Data are expressed as median and range

about 1 hour more to reach the nadir in LH serum levels compared to groups A and B. After an initial decrease a subsequent increase in LH serum concentrations could be observed in all groups (Figure 4.1). At 12 hrs after ganirelix administration, LH serum levels were still reduced by 45%, 37% and 66% from predose values in group A, B and C, respectively, which was significantly lower ($P < 0.01$) in all three groups in comparison to these predose values.

Basically, FSH serum levels showed a similar pattern, although suppression was less profound. Predose FSH levels did not differ between the three groups ($P = 0.18$) and FSH serum concentrations did decrease significantly ($P < 0.001$) with 23%, 19% and 25% in groups A, B and C, respectively. However, the decrease as well as the lowest serum level and the relative drop in FSH levels were not different between the three groups (Figure 4.2). Again, 12 hours after ganirelix administration, FSH levels were still significantly different from predose values ($P < 0.05$) and were reduced by 19%, 14% and 27% respectively. There were no statistically significant differences in the time interval needed to reach maximum suppression of FSH levels.

In all three groups LH as well as FSH serum levels returned to nearly baseline (predose ganirelix) levels within 24 hours after the first drug administration. Predose values of LH and FSH were not significantly different from values measured on the second day prior to the second dose of ganirelix ($P = 0.15$ and $P = 0.16$, respectively). Similarly, there were no differences between the predose values on treatment day 1 and all consecutive values prior to drug administration during the subsequent treatment days ($P = 0.65$ for LH and P 0.84 for FSH, respectively). The area under the curve (AUC), representing the time during which the LH serum level was decreased was comparable in all three groups ($P = 0.127$). The respective AUC in groups A, B and C were 10.4, 8.5 and 9.7 IU·h/l. The corresponding AUC values for FSH amounted 35.0, 25.9 and 27.6 IU·h/l in groups A, B and C respectively. Again these values were comparable between the three groups ($P = 0.246$).

Table 4.2 shows the median values of androgens (T, AD, DHEA, DHEAS) 12 hours after the first injection of ganirelix, which were not significantly suppressed compared to predose values. The suppression of T levels were 23%, 13% and 22% after 12

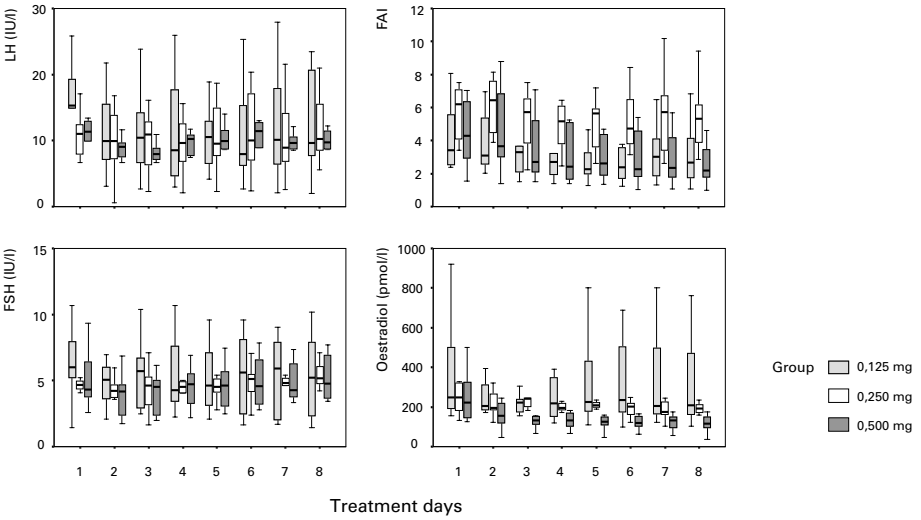

Figure 4.3 Box and whisker plots depicting changes in LH, FSH, FAI and oestradiol serum levels in three different dosage groups of daily GnRH antagonist administrations (0.125 mg, 0.250 mg and 0.500 mg) during the whole treatment period (t = 10.00 h) in 19 subjects. *Boxes* indicate 25th and 75th percentiles, with the *horizontal line* the median value. *Whiskers* span the range between the 5th and 95th percentiles of the data.

hours for group A, B and C respectively, and 8%, 0% and 61% after 7 days for group A, B and C respectively. Only in group C a significant suppression in T was observed after 7 days of GnRH antagonist treatment ($P < 0.05$). Individual data concerning LH, FSH, free androgen index (FAI = Tx100/SHBG) and E_2 levels during the entire course of the experimental period are depicted in Figure 4.3. Briefly, there were no appreciable differences between these values in time during the study period. Similarly there were no significant differences between the three different dosage groups. On the contrary, E_2 values did significantly decrease in time ($P < 0.01$). The decrease in E_2 values was most pronounced in the highest dosage group ($P < 0.05$).

Finally, there was a significant correlation between LH serum values (all 10.00 h values) on one hand and FSH (r = 0.658; $P < 0.001$) and inhibin B (r = 0.243; $P < 0.01$) on the other hand, whereas no correlation was found between LH and E_2 (r = 0.117; $P = 0.152$), inhibin A (r = -0.082; $P = 0.315$) and androgens [T (r = 0.01; $P = 0.826$), AD (r = -0.064; $P = 0.434$), DHEA (r = -0.018; $P = 0.826$), DHEAS (r = -0.067; $P = 0.416$) or FAI (r = -1.280; $P = 0.115$)]. Besides their correlation with LH, FSH serum levels were significantly correlated with E_2 (r = -0.229; $P < 0.01$) and inhibin A (r = -0.363; $P < 0.001$). In addition E_2 levels were significantly correlated with inhibin A (r = 0.7243; $P < 0.001$), inhibin B (r = 0.176; $P < 0.05$), T (r = 0.185; $P < 0.051$) and FAI (r = 0.161; $P < 0.05$). Apart from the correlation of FAI, T and AD with inhibin B (data not shown; $P < 0.01$), all androgens correlated well with each other (data not shown; $P < 0.001$).

4.2.4. Discussion

The present study showed that the repeated daily administration of the GnRH antagonist ganirelix could induce a significant suppression of pituitary LH output and to a lesser extent of FSH serum levels in PCOS patients presenting with elevated endogenous LH concentrations. Pituitary suppression in these women induces LH concentrations within the normal range (i.e. early- to mid follicular phase levels in normo-ovulatory women). A ganirelix dose dependent difference in suppression of endogenous gonadotrophin levels (i.e. a difference in actual decrease) was not observed. The decrease in serum LH and FSH levels was transient and lasted only for up to 12 hours when the antagonist was administered on a daily basis. Due to the transient nature of the gonadotrophin suppression, endogenous predose LH and FSH serum levels were not significantly suppressed in time after repeatedly daily administration. Similarly, androgen levels were not significantly suppressed using this regimen. Oestradiol levels decreased whereas inhibin B levels did not change during the treatment period. Similarly, neither spontaneous follicle development nor ovulations could be observed during repeatedly administration of the GnRH antagonist.

Gonadotrophin serum concentrations in the present study were temporarily but significantly suppressed within 6 to 12 hours after administration of the GnRH antagonist. As to be expected, LH levels were more profoundly suppressed compared to FSH. Similar dose response curves have been observed in normal cycling women (The ganirelix dose-finding study group, 1998; Oberyé *et al.*, 1999a). The magnitude of gonadotrophin suppression (i.e. the percentage of suppresion) was dose dependent, as also reported by other studies (The ganirelix dose-finding study group, 1998; Hayes *et al.*, 1998), whereas in our study the actual decrease in gonadotrophins was comparable between the different doses. The possibility that subtle differences in predose values between our study groups and the time-point at which the lowest LH was reached in each group could underlay these discrepant findings cannot be excluded. However, it has been reported that dose dependency was less evident in normal women using ganirelix after discontinuing oral contraceptive therapy (Oberyé *et al.*, 1999a). Moreover, in PCOS patients a dose dependent decrease in LH and FSH could only be observed at lower dosages of GnRH antagonists (Hayes *et al.*, 1998).

In the current study, serum LH (and to a lesser extent serum FSH) was only suppressed temporarily, in line with the concept of competition between the antagonist and native GnRH at the level of the pituitary GnRH receptor. Following the initial decrease, LH levels gradually increased to pre-treatment levels. Since LH levels were only suppressed during a limited time interval (12 hours), LH was still significantly elevated during a considerable period of time on any given treatment day. Similar pharmacodynamics have been described in normo-ovulatory women (Oberyé *et al.*, 1999a; Oberyé *et al.*, 2000). In PCOS patients, the relative decrease as well as the percentage inhibition of LH was comparable to normo-ovulatory volunteers (Hayes *et al.*, 1998; Elkind-Hirsch *et al.*, 2003). Similarly to LH, FSH levels do increase after an initial decrease following GnRH antagonist treatment (Hayes *et al.*, 1998; Oberyé *et al.*, 1999a; Oberyé *et al.*, 2000).

In case LH is sufficiently suppressed a subsequent fall in androgen levels should be recorded (Hayes *et al.*, 1998). Although a similar reduction in androgen levels in PCOS has been reported (Hayes *et al.*, 1998), we could not confirm this in the present study. The suppression of androgen levels 12 hours after the administration of ganirelix was not significant in the current study. Although we did observe a dose dependent reduction in testosterone levels after prolonged treatment, androgen levels restored to pre-treatment levels after prolonged treatment in the lower dose groups. Since patients in the current study were treated during a longer period of time, a restoration of androgen levels towards pre-treatment elevated levels might have been missed in the former study (Hayes *et al.*, 1998). Indeed, a similar failure to normalize androgen levels in PCOS patients under GnRH treatment alone or in combination with pulsatile GnRH co-treatment has been reported (Dubourdieu *et al.*, 1993).

Although adjuvant GnRH treatment seems to facilitate ovulation induction in PCOS patients (Elkind-Hirsch *et al.*, 2003), there are no reports on return of spontaneous ovulatory cycles after GnRH antagonist treatment in the human. In the current study we did not observe follicle development during the treatment period despite a significant reduction in LH serum concentrations. However, since LH was only suppressed temporarily, the decrease in LH serum concentrations might be insufficient to correct the endocrine environment in PCOS women to such an extent that ovulation is resumed. On the contrary, sustained suppression of endogenous LH levels and concomitant re-establishment of normal GnRH pulsatility and subsequent LH levels did not induce ovulation in PCOS patients (Dubourdieu *et al.*, 1993). These findings are indicative for an inherent ovarian defect in PCOS patients. Hence elevated LH serum concentrations might merely constitute an epi-phenomenon rather than a causal factor in PCOS and normalization of LH levels might not restore normal cyclicity and ovulation (Laven *et al.*, 2002). Since androgen levels did not correlate with LH levels, this might again indicate the limited role of LH in the pathophysiology of PCOS. Indeed, it has been shown that initial LH concentrations do not correlate with ovulation induction outcome, whereas androgens constitute a reliable predictor for ovarian dysfunction as well as treatment outcome (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000). Moreover, the concomitant temporal decrease in FSH might lower endogenous FSH to such an extent that folliculogenesis and dominant follicle selection is insufficiently supported. Serum E_2 levels decreased also in concordance with LH and FSH suppression. Apparently this decrease was not sufficient to interfere with normal oestrogen feedback, as achieved during anti-oestrogen therapy for ovulation induction. However, the limited number of patients might render this study underpowered and the findings should be interpreted with caution.

In conclusion, the present study demonstrates that treatment with a daily low dose of the GnRH antagonist ganirelix effectively normalises endogenous LH in PCOS patients presenting with increased LH secretion. Effective GnRH antagonist doses are similar to doses established to be effective in preventing a premature LH rise during ovarian hyperstimulation for IVF. Moreover, the degree of LH and FSH suppression is very similar to that observed in normal cycling women treated with equal low dosages. However, LH levels are only temporarily suppressed following daily low doses of ganirelix. This transient suppression of elevated endogenous LH and a dose dependent

suppression of testosterone, accompanied by a subtle suppression of endogenous FSH, does not re-establish normal follicle dynamics in PCOS patients. All together these findings illustrate the limited role of elevated LH in the pathogenesis of PCOS.

4.3. Does Metformin modify ovarian responsiveness during exogenous follicle-stimulating hormone ovulation induction in normogonadotrophic anovulation? *A placebo controlled double blind assessment.*

4.3.1. Introduction

One of the major problems associated with gonadotrophin induction of ovulation in patients with normogonadotrophic anovulation is multifollicular development, resulting in multiple pregnancy and ovarian hyperstimulation syndrome (Aboulghar and Mansour, 2003; Fauser and Macklon, 2004). Surpassing the follicle-stimulating hormone (FSH) threshold for inducing follicular growth for too long may result in multifollicular development (Brown, 1978; Fauser and van Heusden, 1997). In order to reduce the above mentioned complications and improve effectiveness of treatment, new approaches have been suggested such as patient selection on the basis of initial screening characteristics (Imani *et al.*, 2002; Mulders *et al.*, 2003) or alternative dose regimens for FSH ovulation induction (van Santbrink and Fauser, 1997). Also new compounds such as aromatase inhibitors (Fisher *et al.*, 2002) or insulin sensitizers (Nestler and Jakubowicz, 1996) are clinically applied attempting to improve treatment outcome.

In normogonadotrophic anovulation (World Health Organisation [WHO] classification group II) including polycystic ovary syndrome (PCOS), hyperandrogenism and insulin resistance are prominent features (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004a; The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004b). Although the role of hyperinsulinemia in PCOS has not yet been entirely elucidated, high insulin concentrations at the ovarian level are proposed to increase androgen production (Gilling-Smith *et al.*, 1994; Dunaif, 1997), increase oestradiol (E_2) synthesis by granulosa cells (Erickson *et al.*, 1990; Coffler *et al.*, 2003) and to cause arrested early antral follicle development (Franks *et al.*, 1999). Hyperandrogenism may be induced by increased luteinizing hormone (LH) serum concentrations stimulating theca cell androgen biosynthesis (Dunaif, 1997), by inducing increased sensitivity of theca cells for stimulation by LH (la Marca *et al.*, 2000) or by dysregulating the FSH-dependent aromatase activity in granulosa cells (Mu *et al.*, 2000; la Marca *et al.*, 2002). Development of clinical hyperandrogenism may also be enhanced by hyperinsulinemia by lowering sex hormone binding globulin (SHBG) production in the liver (Nestler and Jakubowicz, 1997).

It has been postulated that the use of insulin sensitizers, such as metformin, in normogonadotrophic anovulation may improve the endocrine milieu by decreasing both insulin resistance and hyperandrogenism (Lord *et al.*, 2003). These changes may restore menstrual cyclicity and spontaneous ovulation (Velazquez *et al.*, 1997; Morin-

Papunen *et al.*, 1998; Diamanti-Kandarakis *et al.*, 1998), or may render these women more responsive to ovulation inducing drugs such as clomiphene citrate (Lord *et al.*, 2003) or exogenous FSH (de Leo *et al.*, 1999; Yarali *et al.*, 2002). Ovulatory response to clomiphene citrate is enhanced by the addition of metformin in clomiphene resistant (CRA) and obese PCOS patients (Nestler *et al.*, 1998; Lord *et al.*, 2003). In ovulation induction, the degree of hyperinsulinemia is proposed to be the best estimation of requirement for (exogenous) FSH (Homburg *et al.*, 1996). Furthermore, the addition of metformin to FSH ovulation induction is suggested to modulate ovarian response resulting in more monofollicular cycles (de Leo *et al.*, 1999).

These findings suggest that follicular sensitivity for FSH is enhanced by metformin. The aim of the current study is to explore the potential additional effect of metformin on ovarian responsiveness during gonadotrophin induction of ovulation.

4.3.2. Material and methods

Subjects

This study was approved by the local Ethics Review Committee. Between July 1999 and June 2001, all patients attending the outpatient clinic of the Erasmus Medical Center with an amenorrhea or an oligomenorrhoea of at least 56 days fulfilling the inclusion criteria as outlined below were approached to volunteer for participation in this study. After screening, 20 participants were selected. Written informed consent was obtained from each participant. Inclusion criteria were women seeking pregnancy with: 1) age at time of screening between 18 and 37 years; 2) normal serum E_2 and FSH concentrations (as published previously (van Santbrink and Fauser, 1997)) ; 3) severe oligomenorrhoea (interval between bleeding > 56 days) or amenorrhea (interval between bleeding > 6 months) ; 4) insulin resistance (defined as a fasting glucose-insulin ratio < 4.5 mg/10^{-4} U (Legro *et al.*, 1998)); 5) normal serum prolactin and thyroxin levels; 6) no signs of liver- or kidney insufficiency and heart- or vascular disease; 7) failure to ovulate (clomiphene resistant anovulation) or conceive (clomiphene failure) during clomiphene citrate treatment. Patients with overt diabetes mellitus were excluded.

Study design

This is a randomized, double blind, placebo controlled study comparing FSH gonadotrophin induction of ovulation with or without metformin. All participants were randomized for metformin or placebo using sealed and numbered envelopes by the pharmacy of the Erasmus Medical Center. Initial screening was performed within 2 months before inclusion and included a full history (age, menstrual cycle length), laboratory (FSH, LH, E_2, androstenedione [AD], testosterone [T], SHBG, Insulin, Glucose, Prolactin, thyroid stimulating hormone, liver and kidney function) and ultrasound assessment. Ultrasound examinations were performed by a single observer (F.H.) using a 6.5 MHz transvaginal transducer, as published before (van Santbrink and Fauser, 1997).

First phase:

At study day 1, patients visited the clinic after 10-12 hours of fasting, at 8.00 h for a glucose tolerance test (GTT), blood sampling A (i.e. FSH, LH, E_2, AD, T, SHBG), length- and weight measuring, blood pressure, a urine pregnancy test and a transvaginal ultrasound (TVS). In case of a negative pregnancy test, patients started the same day with progestins (Duphaston® 10 mg, 2 dd 1 tablet) for 7 days. Patients returned at day 8 for blood sampling A and a TVS (Figure 4.4). This day patients were randomized for the two different therapies (metformin or placebo). That same day patients started with either metformin (2 x 1 tablet of 850 g daily) or placebo (2 x 1 tablet daily).

Patients returned on day 11-12 and were subsequently monitored (TVS and blood sampling A) every 3-4 days until ovarian response (Figure 4.4). All visits were in the morning between 9.00 h and 11.00 h. Ovarian response was defined as 1 or more follicles with a mean diameter of at least 10 mm. In case of ovarian response and follicular growth, patients were subsequently monitored every 1-2 days until ovulation. One or two days after the day of ovarian response patients returned for a GTT at 8.00 h, and for weight measuring and blood pressure. No FSH was administered in patients ovulatory with metformin or placebo. Metformin or placebo was withheld the day after spontaneous ovulation was detected. One week after ovulation patients returned for a TVS and blood sampling for progesterone. In case menstruation did not occur 14 days after ovulation, a urine-pregnancy test was performed. In case of a positive pregnancy test, a TVS was performed after 7 to 8 weeks (to determine the viability of the pregnancy) and after 12 weeks amenorrhoea (to determine whether the pregnancy was ongoing). In case of absent ovarian response, patients did continue their daily medication and monitoring every 3-4 days until day 36.

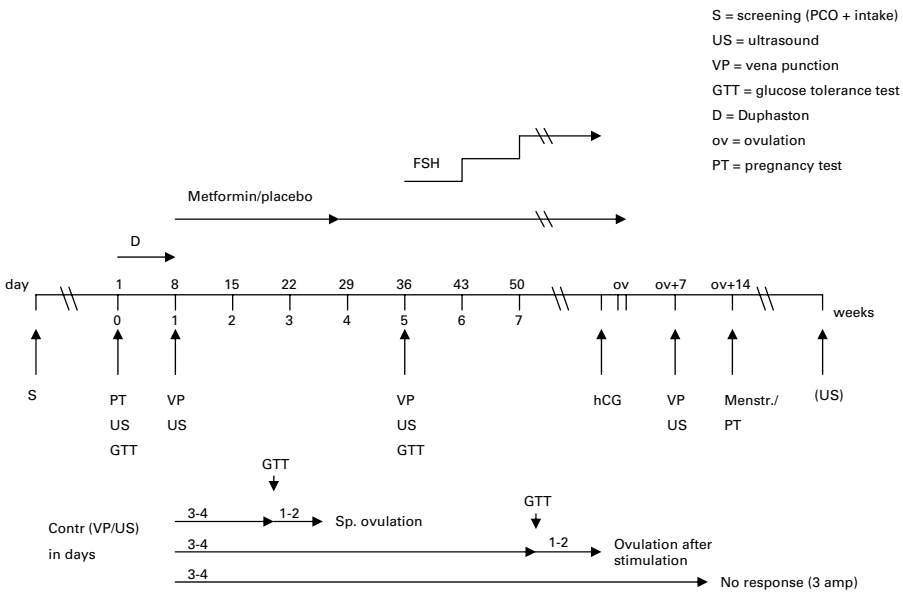

Figure 4.4 Schematic presentation of study design.

Second phase:
In all patients who did not show an ovarian response within 35 days FSH ovulation induction was started. At day 36, patients returned at 8.00 h, after 10-12 hours of fasting, for a GTT, weight measuring, blood pressure, blood sampling A and a TVS. Patients were instructed by the clinical research nurse for subcutaneous self-injections. Patients continued to use metformin- or placebo therapy. From day 36 onwards patients were monitored (TVS and blood sampling A) every 3-4 days until ovarian response. The same day patients started with recombinant FSH (recFSH) stimulation according to a low-dose-step-up regimen (White *et al.*, 1996). The used medication was recFSH (Gonal-F®, Serono Benelux BV, The Hague, The Netherlands), applying a starting dose of 50 IU recFSH sc daily in the morning between 9.00 h and 12.00 h (fixed time for each patient). If no response was observed within 7 days of stimulation, the first dose increase of recFSH was by 25 IU/day. Further dose increase by 37.5 IU/day was performed weekly, if no response was detected. Maximum dosage was 225 IU recFSH a day. In case of no response at maximum dosage the study was ended for this particular subject.

As soon as ovarian response was documented, the patient was monitored every 1-2 days and continued the same dosage of recFSH until the largest follicle reached a mean diameter of at least 18 mm. One or two days after response the patient returned at 8.00 h, after 10-12 hours of fasting, for a GTT, weight measuring and blood pressure. As the largest follicle reached this diameter of 18 mm or more, a dosage of 5,000 IE human chorionic gonadotrophin (hCG; Profasi®, Serono Benelux BV, The Hague, The Netherlands) sc was administered at 11.00 h to induce ovulation. Patients were advised to have intercourse approximately 36 hours after the injection. In case of more than 3 follicles with a diameter of ≥15 mm, no hCG was administered and the use of contraceptives was advised. Two days after the administration of hCG, patients returned for TVS to be examined for indirect signs of ovulation on ultrasound (free fluid, disappearance or decrease of size or changed shape of the dominant follicle) and blood sampling A. The day after ovulation, study-medication (metformin/placebo) was stopped. One week after ovulation, a TVS and blood sampling for progesterone were performed. The date of the next menstruation was recorded. If menstruation had not started yet 14 days after ovulation a urine pregnancy test was performed.

Assay methods
The glucose tolerance test (GTT) was performed after 8–12 hours of fasting. It started at 8.00 h with a fasting blood withdrawal (glucose and insulin) followed by oral intake of 75 gram of glucose. Blood withdrawal followed at 30, 60, 90 and 120 minutes thereafter (Legro *et al.*, 1998). The method of blood withdrawal, the assays used and the intra- and inter-assay coefficients of variation valid for this study have all been described previously (Imani *et al.*, 1999; Imani *et al.*, 2000).

Data analysis
Before initiation of the study, power calculations were performed to determine the required number of patients for the detection of significant differences in the primary study endpoints i.e. duration of the stimulation phase and total amount of used recFSH

to reach ovulation. Randomization was performed by the pharmacy of the Erasmus Medical Center. The patient and the doctor were unaware of the randomization and medication during the entire study. In case of response and ovulation before starting FSH ovulation induction, duration of stimulation and ampoules of FSH needed are defined as zero. Based on a median duration of FSH stimulation of 17 days in case of co-treatment with placebo (van Santbrink *et al.*, 1997) and 14 days in case of metformin (Nestler *et al.*, 1998) the difference was calculated to be apparent with at least 22 patients. Values given are mean ± SD unless stated otherwise. Analysis of the data was performed using the SPSS software package. The *P* values given are 2-sided, and 0.05 was considered the limit of statistical significance. Differences between patient groups were tested using the Student's *t*-test or Chi-square test for paired data on an interval and nominal scale, respectively. A biochemical pregnancy is defined as a positive urine pregnancy test; an ongoing pregnancy is defined as positive heart action on ultrasound after 12 weeks of pregnancy.

4.3.3. Results

Twenty WHO II anovulatory patients were included in the study of which 9 patients were randomized for placebo and 11 for metformin treatment. Initial patient characteristics are shown in Table 4.3. At randomisation, patient characteristics did not significantly differ between groups. All patients were randomised after a progestagen-induced withdrawal bleeding. Patient characteristics after metformin/placebo treatment compared to initial characteristics are shown in Table 4.4. In both groups insulin-glucose ratio did not significantly change, but androgen (both AD and T) serum concentrations did decrease significantly ($P = 0.03$ for AD and $P = 0.01$ for T) in the metformin group only. Also body mass index (BMI = weight/height^2 [kg/m^2]) ($P = 0.001$) decreased and gonadotrophins ($P = 0.02$ for FSH and $P = 0.04$ for LH) increased significantly in the metformin group but not in the placebo group. Two patients (both in the placebo group) presented with an ovarian cyst the day FSH treatment should start and were excluded from further evaluation. One patient was ovulatory on metformin alone (before FSH treatment). Seventeen patients (7 patients in the placebo and 10 patients in the metformin group) received exogenous FSH. Ovulation was confirmed in all patients receiving hCG. Two patients in the metformin group presented with a mild ovarian hyper response (without hospitalization) and hCG was withheld. Differences between placebo and metformin treatment characteristics (Figure 4.5) were assessed in terms of duration of stimulation (33 *vs.* 15 days), total amount of FSH needed (3,238 *vs.* 950 IU), FSH response dose (150 *vs.* 75 IU), pre-ovulatory E_2 serum levels (1,203 *vs.* 615 pmol/L) and monofollicular cycles defined as 1 follicle > 12 mm on the day of hCG (14 *vs.* 75%). Only E_2 serum concentration and monofollicular growth on the day of hCG were statistically significant differences (Figure 4.6). In the metformin group, 3 pregnancies (1 early miscarriage and 2 ongoing singleton pregnancies) occurred while in the placebo group none. Serious gastro-intestinal problems were not reported during the use of study medication.

Table 4.3 Initial characteristics (median and range) of 20 normogonadotrophic, insulin resistant anovulatory patients randomized for receiving either metformin or placebo.

	Placebo	**Metformin**	***P***
n	9	11	ns
Age (yrs)	28 (24-34)	28 (22-32)	ns
Primary infertility (%)	78%	36%	ns*
BMI (kg/m^2)	34 (27-44)	38 (28-51)	ns
Amenorrhoea (%)	44%	36%	ns*
FSH (IU/l)	2.7 (2.0-5.7)	4.0 (1.4-12.2)	ns
E_2 (pmol/l)	205 (112-278)	220 (102-366)	ns
Glucose/insulin ratio	0.19 (0.09-0.24)	0.16 (0.07-0.23)	ns
Testosterone (nmol/l)	2.0 (0.4-3.7)	1.6 (0.8-4.3)	ns
AD (nmol/l)	14.3 (2.4-16.3)	10.0 (5.8-23.1)	ns
FAI (T x 100/SHBG)	13.4 (2.3-33.6)	12.3 (3.8-35.6)	ns

* Chi-square test

Table 4.4 Patient characteristics (mean ± SD) comparing before and after 5 weeks of treatment with either placebo or metformin in 20 normogonadotrophic insulin resistant anovulatory patients.

	Placebo			**Metformin**		
	initial	after	*P**	initial	after	*P**
n	9	9		11	11	
BMI (kg/m^2)	34 ± 5	34 ± 5		37 ± 7	36 ± 7	0.00
FSH (IU/l)	2.4 ± 1.5	3.2 ± 1.4		4.2 ± 1.5	5.7 ± 2.6	0.02
LH (IU/l)	5.8 ± 4.4	6.5 ± 6.2		5.3 ± 3.2	7.4 ± 2.8	0.04
E_2 (pmol/l)	344 ± 313	213 ± 119		256 ± 170	208 ± 107	
Testosterone (nmol/l)	1.9 ± 0.9	2.0 ± 1.1		2.0 ± 1.2	1.7 ± 1.0	0.01
AD (nmol/l)	12.5 ± 6.0	11.4 ± 5.5		14.6 ± 6.9	11.9 ± 3.9	0.03
FAI (T x 100/SHBG)	11.7 ± 10.3	12.1 ± 9.3		15.3 ± 10.4	14.7 ± 11.6	
Glucose/insulin ratio	0.2 ± 0.1	0.2 ± 0.1		0.2 ± 0.1	0.2 ± 0.1	

* student *T*-test

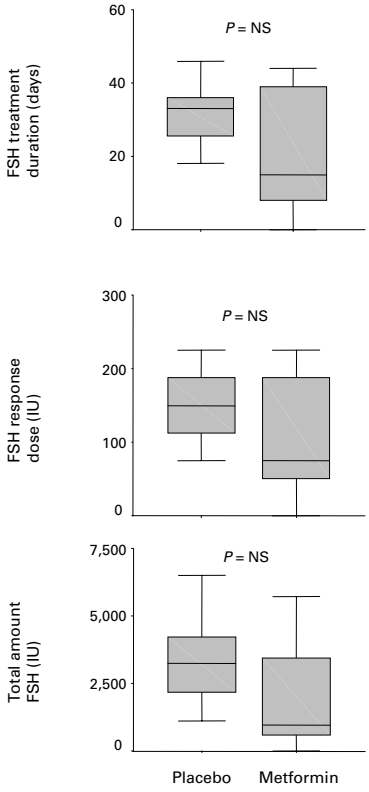

Figure 4.5 Results of FSH ovulation induction using a low-dose step-up protocol in 17 normogonadotrophic insulin-resistant anovulatory subfertile patients after randomisation and co-treatment with metformin (2 × 850 mg daily; n = 10) or placebo (n = 7). *Panels* show the duration of FSH treatment, the total amount of exogenous FSH used and the exogenous FSH response dose. Differences are not statistically significant.

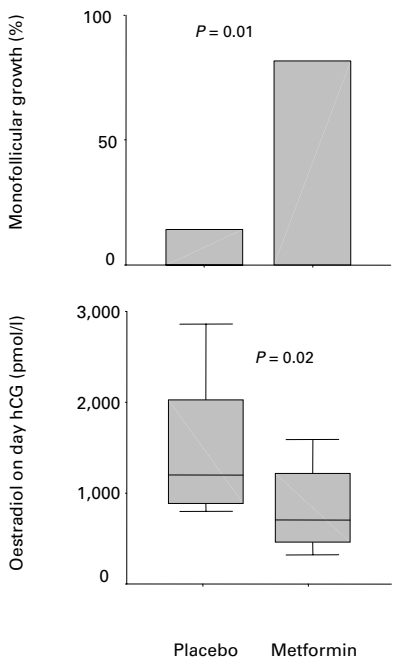

Figure 4.6 Results of FSH ovulation induction using a low-dose step-up protocol in 17 normogonadotrophic insulin-resistant anovulatory subfertile patients after randomisation and co-treatment with metformin (2 × 850 mg daily; n = 10) or placebo (n = 7). The *upper panel* shows the number of patients presenting with a single dominant follicle, whereas the *lower panel* shows the serum oestradiol concentration at day of hCG administration. Differences are statistically significant.

4.3.4. Discussion

The FSH threshold for initiation of gonadotrophin-dependent follicle growth has been recognised as an important entity for many years (Brown, 1978; Fauser and van Heusden, 1997). The period of time the FSH-threshold is surpassed (the FSH-window) determines whether one or more follicles are selected for ongoing growth and ovulation. It is generally thought that in PCOS patients the FSH threshold is higher than in regularly menstruating women. More FSH is required to start follicle growth and this can be managed by enhancing the endogenous FSH production by clomiphene citrate or by giving exogenous FSH.

A crucial question is why the endogenous hormone feed-back system is not responding to this relative shortage of FSH in PCOS patients. One reason might be that PCOS ovaries produce more E_2 and androgens that inhibit FSH release. It is suggested that hyperinsulinemia plays a key role in this, by stimulating cytochrome P450c17 in theca cells (Nestler and Jakubowicz, 1996; Nestler and Jakubowicz, 1997) and aromatase in granulosa cells (Mu *et al.*, 2000; la Marca *et al.*, 2002; Coffler *et al.*, 2003) directly, independent of FSH. This results in enhanced production of T, AD and E_2. The change in endocrine milieu may produce an overreaction of negative feed-back during early follicular growth resulting in an early FSH drop followed by follicle growth arrest and the well-known polycystic image of the ovaries on sonography.

Clomiphene citrate is first choice treatment for WHO II women. About 75% of all treated patients will respond with ovulation and about 45% conceive (Imani *et al.*, 1999). BMI, hyperandrogenism (free androgen index), leptin and hyperinsulinemia (fasting insulin and insulin-like growth factor binding protein-1) were determined to be the strongest predictors for remaining anovulatory during clomiphene citrate treatment (Polson *et al.*, 1989; Imani *et al.*, 1998; Imani *et al.*, 2000). Although hyperinsulinemia seems more profound in obese women (Galtier-Dereure *et al.*, 1997) also lean PCOS women (mean BMI 22 kg/m^2) are shown to have a significant reduction in insulin response to glucose load and androgen serum concentrations (Nestler and Jakubowicz, 1997; Genazzani *et al.*, 2004). The addition of metformin to clomiphene in CRA is recognized as a valuable treatment option before starting with exogenous gonadotrophins (Nestler *et al.*, 1998; Lord *et al.*, 2003).

Less is known about the addition of metformin during gonadotrophin induction of ovulation. Two limited studies described co-administration of metformin to gonadotrophin induction of ovulation in normogonadotrophic anovulatory patients (de Leo *et al.*, 1999; Yarali *et al.*, 2002). The first study (de Leo *et al.*, 1999) included PCOS patients with clomiphene resistant anovulation or failure to conceive and randomised for treatment A or B. Insulin resistance status of included patient was unknown. Treatment A consisted of 2 conventional urinary FSH ovulation induction step-up cycles followed by 1 cycle with addition of metformin (n = 10), treatment B offered metformin pre-treatment followed by a conventional FSH ovulation induction step-up cycle combined with metformin (n = 10). It is concluded that androgens and E_2 serum concentrations were significantly lower in cycles with metformin co-treatment and significantly more cycles with monofollicular growth did occur. The second study (Yarali *et al.*, 2002) selected 32 PCOS patients with normal glucose tolerance and clomiphene

resistant anovulation. These patients were randomised for metformin or placebo and a 6 week pre-treatment period followed. All anovulatory patients were treated with FSH using a low dose step-up protocol (White *et al.*, 1996). No significant differences were determined in ovarian response after metformin or placebo treatment between these groups although significantly lower serum androgens (free testosterone) were described after metformin treatment.

In our study population we included insulin resistant patients only, because this subgroup was expected to react favourable on metformin treatment. Although metformin treatment did not result in a (significant) decrease of insulin resistance, it did cause a significant decrease of androgen serum concentrations and improved the endogenous gonadotrophin-oestrogen balance, as was demonstrated in former studies (de Leo *et al.*, 1999; Yarali *et al.*, 2002). This endocrine shift might explain the following benefits during the FSH treatment phase: although not significant, considerable less FSH and a shorter treatment period were required to grow a dominant follicle. The dosage of FSH on the day a dominant follicle could be recognized by TVS was lower in the metformin group, but not significantly. Above that, on the day hCG was given significant lower E_2 serum levels and significant more cycles with monofollicular (not more than one follicle > 12 mm) growth were established. These results are comparable with former published studies (de Leo *et al.*, 1999).

In conclusion, these results suggest that metformin may improve the endocrine profile (by decreasing hyperandrogenemia) and in that way facilitates monofollicular development during gonadotrophin ovulation induction in WHO 2 patients. This effect may be more pronounced in patients with insulin resistance. As this study comprehends only a small population, larger studies are required to further evaluate the effects of metformin co-treatment in normogonadotrophic FSH ovulation induction, especially on conception and birth rates.

Chapter 5

General discussion and conclusions

General discussion

Over the last 25 years, ovarian stimulation has gradually become more complex, time-consuming and expensive. It generates stress and its side effects and complications have attracted more and more attention. Large numbers of follicles and aspirated oocytes are regarded as criteria of success of ovarian hyperstimulation, since large numbers of oocytes are needed to achieve sufficient numbers of embryos for fresh as well as cryopreserved transfers. Given the close association between the number of embryos eligible for selection and transfer and the chance of pregnancy (Templeton and Morris, 1998), a balanced weighing of success and complications has only gained sufficient attention during the last decade. A large proportion of excess embryos followed by transfer in a subsequent (unstimulated) cycle is often used as justification for excessive ovarian hyperstimulation for IVF. However, a large proportion of cryopreserved embryos will not survive the freezing and subsequent thawing process and those surviving show disappointing pregnancy rates (de Jong *et al.*, 2002). Although success rates have improved over the years, with pregnancy rates around 20-30% per started IVF cycle, the short-term and possible long-term effects on women's and children's health cannot be ignored. Multiple pregnancies, i.e. twins and especially high order multiples, are the major causes of maternal morbidity and fetal morbidity and mortality.

In recent years awareness is growing throughout the world that ovarian hyperstimulation should be re-evaluated (Edwards *et al.*, 1996; Olivennes and Frydman, 1998; Fauser *et al.*, 2002) and that (high order) multiple pregnancies should not be considered an acceptable outcome of ART (Templeton and Morris, 1998; Fauser *et al.*, 1999; Fauser and te Velde, 1999; Templeton, 2000). Moreover, if the balance between risks and benefits of IVF treatment has to be improved, a paradigm shift is required in the approach to treatment and in the way success of IVF is defined. In contrast to rating IVF success in terms of pregnancy rate per started cycle or per embryo transfer, IVF success rate should be expressed in terms of delivery of a singleton term life baby per cycle (Min *et al.*, 2004). The latter definition will facilitate the introduction of single embryo transfer on a larger scale. By expressing results in terms of the delivery of a single healthy baby per IVF treatment or treatment period which may include multiple cycles (Heijnen *et al.*, 2004), the development of patient-friendly stimulation protocols with less stress, discomfort and fewer complications should be further encouraged. Most problems (stress, discomfort, OHSS) associated with the current ovarian hyperstimulation regimens relate to the unphysiological approach to the ovary. Moreover, the pharmacological stimulation ignores the potential detrimental effects on oocyte quality and endometrial receptivity. Increasing knowledge regarding the physiology of ovarian follicle development and dominant follicle selection, together with the clinical availability of new compounds, such as GnRH antagonists, have presented the opportunity to develop novel, milder approaches for ovarian stimulation.

As described before, the FSH threshold/window concept has been proposed as the mechanism underlying single dominant follicle selection. The concept emphasises the importance of a transient increase of FSH above a certain level (the threshold) for a limited time interval (the window) in order to gain single dominant follicle selection.

By administration of exogenous FSH, the window is extended facilitating an increased number of follicles of the recruited cohort to gain dominance (Fauser and van Heusden, 1997). Low dose exogenous FSH is capable to interfere with decremental FSH levels, subsequently inducing multifollicular growth (Schipper *et al.*, 1998b). The first study in this thesis showed that subtle interference with decremental FSH by administration of low-dose exogenous FSH can induce multiple dominant follicle development in the majority (60%) of women. Provided that no dominant follicle selection has occurred yet, initiation of FSH administration as late as cycle day 7 is still capable of interference with single dominant follicle selection. In the subjects without a dominant follicle who did not respond, the amount of exogenous FSH might have been too low to extend the window sufficiently for the selection of multiple dominant follicles. The observed negative correlation for BMI and the number of developing dominant follicles suggests that differences in pharmacokinetics of exogenous FSH are involved. However, differential ovarian responsiveness to FSH may be the predominant factor determining mono- or multifollicular response. As result of multifollicular growth, hormone levels (e.g. oestradiol and inhibin A) are increased in the late follicular phase. The effects of these supraphysiological hormone concentrations on follicle- and oocyte quality remain unclear (Fauser and Devroey, 2003). Moreover, this study showed that multifollicular growth per se has a distinct effect on luteal phase characteristics resulting in changes in the length and endocrine profile of the luteal phase. An abnormal luteal phase in cycles stimulated with gonadotrophins for IVF has previously been documented (Beckers *et al.*, 2000). Recently, it has been suggested that high follicular phase oestrogen levels, as a result of ovarian hyperstimulation, and their concomitant negative feedback on the pituitary level might be involved in the accelerated demise of the corpus luteum (Tavaniotou *et al.*, 2002; Beckers *et al.*, 2003). In clinical practice, luteolysis is prevented by luteal phase hCG support or luteal function replaced by progesterone supplementation. However, the potential detrimental effect of ovarian hyperstimulation on endometrial receptivity has been largely ignored, despite the knowledge that endometrial changes have an impressive negative influence on embryo implantation (Kolibianakis *et al.*, 2002; Fauser and Devroey, 2003; Devroey *et al.*, 2004).

Ovarian response to exogenous gonadotrophins usually shows strong inter-individual variation. Prediction of those women who may under- or overrespond to ovarian (hyper) stimulation protocols seems to be of clinical importance. Although the aforementioned study showed that differences in pharmacokinetics are involved in determining ovarian response, differential ovarian responsiveness to FSH may be the predominant factor determining mono- or multifollicular response. Ovarian sensitivity to FSH is regulated by intra-ovarian factors, like FSH receptor inhibitors (Schipper *et al.*, 1997) and growth factors (Giudice *et al.*, 1996). Growth factors act in an auto- or paracrine fashion through their specific receptors. Within cells, their signal transduction pathways merge with the FSH-activated pathways and subsequently modulate FSH stimulated responses within the cell. Numerous growth factors have been identified which contribute to this regulation of normal ovarian function. The intra-ovarian produced growth factor inhibin acts in a paracrine fashion by stimulating androgen synthesis (Hillier *et al.*, 1991; Hillier *et al.*, 1993) and in an endocrine fashion by selectively suppressing FSH secretion by the pituitary. Inhibin B, recognized as the predominant form of inhibin in

the developing cohort of small (pre) antral follicles (Groome *et al.*, 1994; Groome *et al.*, 1996), is secreted by these follicles and hence inhibin B concentrations generally increase during the early follicular phase. It is suggested that the early follicular phase inhibin B serum concentration is related to the size of the recruited cohort of growing follicles (de Kretser *et al.*, 2002; Laven and Fauser, 2004), and therefore inhibin B might be a suitable marker for follicular response during ovarian stimulation with gonadotrophins. In our study we observed that an increase in FSH by the administration of exogenous FSH during the early follicular phase indeed induces an increased number of small antral follicles and hence elevated inhibin B concentrations. If subsequently the FSH window is extended, ongoing growth of multiple dominant follicles occurs and elevated inhibin B and A levels are observed. However, if single dominant follicle selection is not affected, serum inhibin B levels return to normal and inhibin A remains comparable to serum levels in the natural cycle. Mid- and late follicular phase inhibin B concentrations were therefore elevated in multifollicular cycles and correlated with the number of pre-ovulatory follicles at the end of the follicular phase. Unfortunately, no relation was found between early follicular phase inhibin B and the final number of pre-ovulatory follicles. In previous studies comparable relations were found, but early follicular phase FSH and antral follicle count appeared to be more predictive in determining ovarian response (Yong *et al.*, 2003). Although stimulated inhibin B concentrations reflect the potential follicular development of the ovary, it does not facilitate the identification of women at risk for multiple dominant follicle selection (and subsequent multiple pregnancy). Recently anti-Müllarian Hormone (AMH) has been identified as a strong candidate marker for ovarian reserve in normo-ovulatory women (de Vet *et al.*, 2002). AMH has a stable expression during the menstrual cycle and seems to be a strong predictor for the number of oocytes retrieved in patients undergoing IVF treatment (van Rooij *et al.*, 2002). The clinical introduction of GnRH antagonists facilitated the development of new approaches to ovarian (hyper) stimulation in IVF (Bouchard and Fauser, 2000). As GnRH antagonist action is characterized by an immediate suppression of pituitary gonadotrophin release, treatment can be limited to the days in the mid-to-late follicular phase truly at risk for a premature LH rise. Therefore, these compounds offer the opportunity to commence the IVF treatment within an undisturbed menstrual cycle. In most studies using a GnRH antagonist, ovarian stimulation is initiated in the early follicular phase, on cycle day 2 or 3 (Albano *et al.*, 1996; Albano *et al.*, 1997; Borm and Mannaerts, 2000; The European and Middle East Orgalutran Study Group, 2001). However, single dominant follicle selection can be disturbed by commencing exogenous FSH administration in the mid follicular phase as late as cycle day 7, allowing normal early follicular phase recruitment of a cohort of follicles and utilising the endogenous inter-cycle FSH rise (Schipper *et al.*, 1998b; de Jong *et al.*, 2000a; Hohmann *et al.*, 2001). Extending the FSH window for multifollicular growth by administering exogenous FSH from the mid follicular phase onward constitutes a novel mild approach to ovarian stimulation in IVF. We showed that such a mild protocol results in pregnancy rates per started cycle comparable to those observed after profound ovarian hyperstimulation with GnRH agonist co-treatment, despite a shorter stimulation and a marked reduction in exogenous FSH needed. Although a higher cancellation rate was observed in the mild protocol due to low response, this was compensated by improved embryo quality

concomitant with a higher chance of having an embryo transfer. The cancelled patients were older and their baseline FSH concentrations were elevated, compared to the patients undergoing oocyte pick up, which may indicate that patients likely to show a low response during mild stimulation may be identified at the start of the IVF treatment. Milder stimulation may result in selection of good quality oocytes, perhaps due to more physiological follicular phase hormone concentrations and improved synchronization of the cohort of growing dominant follicles. Good quality oocytes will lead to better embryos, which is a predictive factor for pregnancy (Staessen *et al.*, 1992; Hunault *et al.*, 2002; Hohmann *et al.*, 2003). Preimplantation screening studies show that the majority of embryos obtained after profound ovarian stimulation is chromosomally abnormal or demonstrate mosaicism (Baart *et al.*, 2004). Whether embryos retrieved after mild ovarian stimulation show less chromosomal abnormalities should be further investigated.

A low response during ovarian stimulation is currently believed to represent ovarian aging and poor oocyte quality (Beckers *et al.*, 2002; de Boer *et al.*, 2002). However, a low number of oocytes after mild stimulation may constitute a normal, more physiological response. Indeed, the presence of four or fewer oocytes after mild stimulation was associated with high pregnancy rates, indicating that a "physiological" reduction in the number of oocytes generated after mild ovarian stimulation distinctly differs from the "pathological" reduction associated with ovarian ageing. In this respect, criteria for cancellation during ovarian stimulation should be revised for minimal stimulation protocols. Adjustment of minimal criteria for oocyte retrieval will reduce cancellation rate coinciding with minimal stimulation. Further data regarding oocyte-embryo quality and implantation rates in low response patients after minimal stimulation should prove that minimal ovarian stimulation facilitates early patient selection or indeed generates a better oocyte and subsequent embryo quality as a result of a more physiological ovarian and uterine environment in these patients.

Minimal ovarian stimulation, especially when combined with single embryo transfer, not only improves the balance between success of IVF and risks, but also improves overall health economics. Firstly, IVF treatment will become more patient-friendly (shorter duration of treatment, less injections, less side-effects, shorter oocyte retrieval procedure because of less follicles to puncture) with reduced chances for complications, such as OHSS, multiple gestation or possible long term health effects (e.g. risks for ovarian cancer). Secondly, a reduced number of dominant follicles, combined with hormone concentrations closer to physiology, might select the best oocytes of the cohort with subsequent better embryos. Moreover, a more physiological approach might interfere less with normal endometrial receptivity and hence increase the chances for implantation. Finally, direct- and indirect costs will be reduced (e.g. less medication, less monitoring, shorter duration of oocyte pick up procedure, reduced laboratory costs, less risk for OHSS and hospitalization, reduced multiple pregnancy rates as well as reduced preterm deliveries and a reduction in morbidity).

The prospect of increased implantation rates by selecting chromosomally normal embryos for transfer (Munne, 2003), may improve outcome of single embryo transfer. First steps are taken in combining mild stimulation protocols with single embryo transfer. A minor drop in pregnancy per cycle (Gerris, 2004), should be compensated by comparable overall pregnancy rates per total IVF treatment. Currently, a multicentre

study in terms of health economics and clinical outcome in IVF is being performed, comparing the conventional treatment IVF hyperstimulation protocol with dual embryo transfer during three consecutive cycles with minimal ovarian stimulation with single embryo transfer during 4 consecutive cycles (Heijnen, ongoing research). Preliminary results indicate comparable pregnancy rates with virtually no multiple pregnancy rates.

The aforementioned findings are all supportive for the threshold/window concept and have shown the clinical applicability of this concept for ovarian (hyper)stimulation. Milder ovarian stimulation is a promising alternative for the conventional IVF strategies and supports the current shift towards more physiological stimulation protocols with less side-effects/complications and hence a better cost effectiveness in terms of health economics. If future investigations do prove our hypothesis that milder ovarian stimulation selects the healthiest oocytes of the cohort and diminishes the detrimental effects on endometrial receptivity, the introduction of single embryo transfer on a larger scale will be facilitated which in turn eliminates the epidemic of multiple pregnancies due to ART. Since quantity in terms of number of oocytes not equals quality, a paradigm shift in approach of poor response patients is necessary. Instead of increasing doses of exogenous FSH, these patients may benefit more from mild ovarian stimulation or natural cycle IVF (Tarlatzis *et al.*, 2003; Morgia *et al.*, 2004). The use of markers for prediction of ovarian response (like AMH or antral follicle count) in all patients is questionable. The majority of patients will show a normal response to mild ovarian stimulation, and therefore the assessment of a marker for response does not give any additional information. However, in the patients not showing multifollicular response to mild ovarian stimulation, the assessment of AMH might be useful. Although antral follicle count strongly correlates with AMH, it is more difficult to assess and therefore less preferable. Patients with normal AMH levels may benefit from early follicular phase start of exogenous FSH administration, in similar doses as in the mild stimulation protocol. In contrast, patients with low AMH levels represent the patients with an advanced stage of ovarian aging, who might benefit most from natural cycle IVF.

The shift towards a more physiological approach not only affects ovarian hyperstimulation. A more individualized approach to ovulation induction in anovulatory patients may improve success rates in terms of (singleton) pregnancies together with a reduction in complications. Gonadotrophin ovulation induction is associated with high ovulation and conception rates at the expense of an increase in multifollicular development, which may result in multiple pregnancies and OHSS. In order to reduce these risks several dose regimens have been tested. The gonadotrophin step-down protocol (van Santbrink *et al.*, 1995a) may more closely mimic FSH serum concentrations during the follicular phase of the normal menstrual cycle, subsequently restricting the time the FSH concentration is above the threshold. However, since WHO 2 anovulatory patients represent a heterogeneous group of patients, inter- and intra-individual variation in sensitivity to exogenous FSH is impressive. Disclosing the underlying pathophysiology of ovarian dysfunction within the different subgroups might result in more physiological ovarian stimulation in these patients. A normal response to stimulation may facilitate the most healthy follicle (with good quality oocyte) to develop. Moreover, monofollicular growth and hormone concentrations within the normal range might

reduce the negative impact on luteal phase characteristics, endometrial receptivity and consequently might increase chances for implantation and ongoing pregnancy.

LH participates, with FSH, in normal follicular development. In the initial stages of follicle development, LH contributes to cell proliferation and differentiation and activates and maintains androgen synthesis through the presence of LH receptors on theca cells. In the pre-ovulatory stage, the appearance of LH receptors on granulosa cells facilitates a direct intervention of LH on follicle maturation and the implication of LH in oocyte modification through paracrine mediation of somatic cells. Finally, the pre-ovulatory LH surge controls ovulation by the induction of the follicle rupture, secretion of progesterone and the formation of the corpus luteum (Hillier, 2001; Hillier, 2002). Apart from the pre-ovulatory LH surge, LH levels are low during the normal menstrual cycle. However, approximately 40% of women with normogonadotrophic anovulation present with LH levels above the normal range. Whether these elevated LH levels are involved in ovarian dysfunction by stimulating androgen synthesis or whether these elevated LH levels merely constitute an epi-phenomenon to an inherent ovarian defect remains controversial (Laven *et al.*, 2002). In a subgroup of PCOS patients with elevated LH serum concentrations, we showed that the newly available GnRH antagonists are capable of suppressing elevated LH levels to normal range values, in doses similar to the ones previously shown to prevent a premature LH rise during ovarian hyperstimulation. However, the suppression of elevated LH levels per se did not re-establish normal follicle development. This is consistent with the observation that treatment of PCOS patients with a combination of a GnRH antagonist and pulsatile GnRH did re-establish a normal LH secretion pattern, but did not induce ovulation (Dubourdieu *et al.*, 1993). Moreover, initial LH concentrations have low predictive value for ovarian response to ovulation induction or the chance to conceive (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2002). In summary, elevated LH levels should be regarded as an epiphenomenon associated with PCOS, rather than being the cause of PCOS. However, to some extent FSH levels were also suppressed following GnRH antagonist administration by which folliculogenesis and dominant follicle selection might not be sufficiently supported.

Insulin has a wide range of acute metabolic and anabolic actions, of which its effects on glucose metabolism are the most well known. Insulin acts through binding to its cell surface transmembrane receptor, followed by a complex intracellular pathway bringing out the different effects of insulin. Various molecular mechanisms can account for a reduced response to insulin. Insulin resistance initially results in lower levels of glucose uptake from the blood into the target tissues, followed by a compensatory hyperinsulinemia in order to overcome hyperglycaemia. Insulin resistance or hyperinsulinemia is one of the prominent features of normogonadotrophic anovulation, including PCOS. Sex steroids (androgens and oestrogens) and insulin interact in their actions on tissues (Livingstone and Collison, 2002). Hyperinsulinemia at the ovarian level is proposed to increase androgen production (Gilling-Smith *et al.*, 1994; Dunaif, 1997), and oestradiol synthesis (Erickson *et al.*, 1990; Coffler *et al.*, 2003) and it may cause arrested early antral follicle development. Insulin reduces circulating levels of SHBG by inhibiting its production in the liver, which further accounts for elevated free androgen levels, previously shown as the most prominent endocrine predictor of ovar-

ian response during CC therapy in PCOS patients (Imani *et al.*, 2000). Moreover, high levels of androgens or oestrogens may promote insulin resistance, further contributing to the vicious circle. Since it has been suggested that the degree of insulin resistance is the best indication of increased requirement for FSH (Homburg *et al.*, 1996), insulin may directly affect follicular development by increasing the FSH threshold. It is suggested that hyperinsulinemia directly stimulates cytochrome P450c17 in the theca cells (Nestler and Jakubowicz, 1996; Nestler and Jakubowicz, 1997) and aromatase in granulosa cells (la Marca *et al.*, 2002; Coffler *et al.*, 2003), independent of FSH, resulting in an enhanced production of androgens and oestrogens. This change in endocrine milieu may induce an overreacted negative feedback resulting in a reduced FSH release during early follicular growth followed by follicle arrest. Insulin sensitizing drugs, such as metformin, may improve the endocrine milieu by both reducing insulin resistance and hyperandrogenism, which might affect the FSH threshold and restore menstrual cyclicity (Velazquez *et al.*, 1997). We observed that metformin therapy normalises the endocrine profile, resulting in reduced androgen levels and increased endogenous gonadotrophin levels, hence an indication of a recovering negative feedback system. This normalisation of endocrine profile resulted in more physiologically reacting ovaries, facilitating monofollicular development during FSH induction of ovulation with a reduced amount of exogenous FSH needed. Although the numbers of patients in our study were small, it confirms findings in previous studies and a recent meta-analysis in normogonadotrophic anovulatory patients (Lord *et al.*, 2003). This study demonstrates that a more patient tailored approach in the treatment of this heterogeneous group of normogonadotrophic anovulatory patients might reduce risks for complications during ovulation induction with exogenous FSH.

In the current approach to treatment of normogonadotrophic anovulatory patients, all patients start with CC treatment, followed by gonadotrophin ovulation induction and IVF respectively, if no pregnancy occurred. However, as stated before, the heterogeneous group of WHO 2 anovulation shows marked variation in ovarian response to ovulation induction. Treatment modalities in normogonadotrophic anovulation should become more patient tailored and therefore it seems reasonable to implicate new approaches to the conventional WHO 2 anovulation treatment protocol. The use of prediction models will help to choose an individualized treatment pathway per patient. Since exercise and weight reduction improve insulin sensitivity and hyperandrogenism and hence ovarian response to ovulation induction (Clark *et al.*, 1998; Norman *et al.*, 2004), life style modifications should be strongly advocated as first line treatment, especially in obese, insulin resistant anovulatory patients (Norman *et al.*, 2002). Ovulation induction can be started with CC or tamoxifen, which show comparable effectivity (Messinis and Nillius, 1982), or with metformin alone in insulin resistant patients. In case of CC resistance, a combination of CC and insulin sensitizing drugs should be the second choice of treatment. The use of aromatase inhibitors in ovulation induction is promising and should be further investigated (Mitwally and Casper, 2001; Mitwally and Casper, 2004). Depending on individual prognosis of success and risks, ovulation induction with gonadotrophins can be started using the step-up or step-down protocol, eventually combined with insulin sensitizing drugs (this thesis). Since initial LH levels merely constitute an epiphenomenon associated with PCOS, there is

no place for GnRH analogues (co)treatment in ovulation induction (Dubourdieu *et al.*, 1993). Recently, laparoscopic electrocautery has been introduced as an alternative treatment to CC-resistant PCOS patients. Laparoscopic electrocautery alone and followed by CC treatment or exogenous gonadotrophin treatment if no pregnancy occurred, showed pregnancy rates comparable to those obtained after directly commencing gonadotrophin treatment in CC-resistant patients, but showed a marked reduction in multiple pregnancies (Bayram *et al.*, 2004a). Although further research is needed concerning the efficacy of this therapy, first studies on health-economics are promising (Van Wely *et al.*, 2004a; Van Wely *et al.*, 2004b). IVF represents the final treatment for WHO 2 anovulatory infertility, although depending on prognosis some patients will skip ovulation induction starting directly with IVF. Currently, the most commonly applied IVF stimulation protocol in these patients is the long protocol, using GnRH agonists for pituitary down regulation and the administration of high doses of exogenous gonadotrophins. Ovarian response to hyperstimulation is reported to be increased in these patients, compared to normo-ovulatory women (MacDougall *et al.*, 1992; MacDougall *et al.*, 1993). However, since inhibin B serum levels in WHO II patients (reflecting the number of healthy non-atretic follicles) were similar to those in normo-ovulatory controls (Laven *et al.*, 2001), this enhanced ovarian response might only reflect stimulation of atretic follicles. Therefore it is reasonable to believe that PCOS patients may benefit more from a minimal stimulation protocol, which only selects the healthiest oocytes of the cohort. Ovarian stimulation can be started after spontaneous menses or a progesterone withdrawal bleeding similar to gonadotrophin ovulation induction. As soon as dominant follicles are selected, a GnRH antagonist should be administered in order to prevent premature luteinisation or a premature LH surge. Eventually co-treatment with insulin sensitizers may normalise ovarian response in IVF, as demonstrated in this thesis for gonadotrophin ovulation induction in insulin resistant anovulatory patients. Further investigations for minimal ovarian stimulation protocols in PCOS patients are needed. The accompanying benefits of minimal stimulation have been discussed in extent previously.

In conclusion, this thesis provides further insight in the manipulation of follicle growth in normo-ovulatory cycles and disturbed follicle development. These findings are useful for the development of a milder, more individualized approach to ovarian stimulation for the induction of multifollicular growth or ovulation induction in anovulatory cycles. This approach may improve the balance between success and overall costs and risks. The findings in this thesis indicate that success should not be measured as number of follicles or oocytes retrieved, since quantity does not equal quality. The knowledge that a milder, more physiological approach to ovarian stimulation for the induction of multiple follicle development will select the healthiest oocytes of the cohort, leaves no longer approval to profound hyperstimulation. Moreover, this knowledge further stimulates the introduction of more individualized protocols to ovulation induction in anovulatory patients. A more physiological approach to ovulation induction will facilitate monofollicular growth closer mimicking normal ovarian physiology. The selection of the healthiest follicle in a monofollicular cycle will result in a better quality of oocyte and endometrium, subsequently improving success rates in terms of singleton pregnancy.

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Summary / Samenvatting

Summary

Chapter 1

The introduction of this thesis starts with general information on reproduction, subfertility and reproductive medicine. It continues with a brief overview of current knowledge regarding the function of the human ovary, describing ovarian development and early and advanced follicle development. The importance of the threshold/window concept for the selection of a single dominant follicle is stressed in more detail. Furthermore, a description of disturbed follicle development and the classification of anovulatory disorders is offered. After a discussion on management of subfertility and the differences between ovulation induction and ovarian (hyper)stimulation, the study objectives are provided.

Chapter 2

Section 2.1

To test whether the administration of low-dose exogenous FSH initiated during the early, mid or late follicular phase can induce multiple dominant follicle development, a prospective, randomized trial was performed in normo-ovulatory women. Forty normal weight women participated. Administration of a fixed dose (75 IU) of recombinant FSH was started on either cycle day 3, 5 or 7 until the induction of ovulation with human chorionic gonadotrophin. Frequent transvaginal ultrasound scans and blood sampling were performed. Multifollicular growth occurred in all groups (overall in 60%), although day 7 starters showed less multifollicular growth. Age, cycle length and initial FSH and inhibin B concentrations were similar between subjects with single or multiple follicle development. However, for all women, the lower the BMI, the more follicles emerged. If multifollicular growth occurred, the length of the luteal phase was reduced and midluteal serum concentrations of LH and FSH were decreased while oestradiol and inhibin A were increased. In conclusion, interference with decremental serum FSH concentrations by administration of low dose FSH starting on cycle day 3, 5 or as late as day 7, is capable of disrupting single dominant follicle selection. The role of BMI in determining ovarian response suggest that differences in pharmacokinetics of exogenous FSH are involved. Multifollicular growth *per se* has a distinct effect on luteal phase characteristics.

Section 2.2

This study was performed to investigate the relationship between serum concentrations of inhibin A, B, and E_2 and the number of developing follicles during the administration of FSH in various regimens in normo-ovulatory volunteers and to evaluate if inhibins act as suitable markers for the number of developing follicles during ovarian stimulation. To address this issue, serial hormone determinations and assessment of follicle numbers were carried out during unstimulated cycles and during various interventions

with exogenous FSH. Subjects were randomized for FSH administration in a single high dose (375 IU: Group A) during the early follicular phase, 5 consecutive low doses starting in the mid follicular phase (75 IU: Group B) or daily low doses (75 IU) during the early to late follicular phase (starting on cycle days 3, 5 or 7: Groups C, D and E, respectively).

This study showed that extending the FSH window increases the number of small antral follicles and hence inhibin B serum concentrations. If such an intervention resulted in multifollicular growth, mid follicular phase inhibin B ($P = 0.001$) as well as late follicular phase inhibin B and A levels were significantly ($P < 0.05$ and $P < 0.01$, respectively) increased compared to monofollicular cycles or the natural cycle. Although mid follicular inhibin B levels correlated well with the number of small antral ($P < 0.05$) and pre-ovulatory ($P < 0.001$) follicles in the late follicular phase, mid follicular inhibin A and E_2 serum concentrations only correlated with the number of pre-ovulatory follicles ($P < 0.001$ and $P < 0.01$, respectively). In conclusion, the present data extend our understanding of the relationship between follicle dynamics, serum inhibins and FSH during ovarian hyperstimulation. However, although mid follicular inhibin B does correlate with the number of developing follicles, it does not facilitate the identification of women at risk for multiple follicle development.

Chapter 3

Section 3.1

This section is meant as an introduction to section 3.2. It describes the enormous expansion IVF treatment has undergone since its first successful report in the natural cycle. In order to improve success rates of IVF treatment, GnRH agonists were introduced to prevent premature luteinisation and ovulation during ovarian hyperstimulation. The mechanism of action of GnRH agonists is discussed. Over the years IVF treatment has become extremely complex, expensive, time consuming and not without risks. Recently, serious concerns related to the management of assisted reproductive therapies, like IVF, have been expressed. The clinical availability of GnRH antagonists offers the opportunity to develop alternative approaches to ovarian stimulation, since these compounds are characterized by an immediate suppression of pituitary gonadotrophin release and a rapid recovery after cessation.

Section 3.2

Extending the FSH window for multifollicular development by administering FSH from the midfollicular phase onward constitutes a novel, mild protocol for ovarian stimulation for IVF based on the physiology of single dominant follicle selection in normoovulatory women. To test the outcomes from this protocol, 142 IVF patients were randomized to either a GnRH agonist long protocol or one of two GnRH antagonist protocols commencing recombinant FSH on cycle day 2 or cycle day 5. A fixed dose (150 IU/day) of exogenous FSH was used for ovarian stimulation, and GnRH antagonist co-treatment was initiated on the day when the leading follicle had reached 14 mm diameter. Frequent transvaginal ultrasound scans and blood sampling were performed. This study showed that application of the described mild ovarian stimulation protocol

resulted in pregnancy rates per started IVF cycle similar to those observed after profound stimulation with GnRH agonist co-treatment despite a shorter stimulation and a 27% reduction in exogenous FSH. A higher cancellation rate before oocyte retrieval was compensated by improved embryo quality concomitant with a higher chance of undergoing embryo transfer. A relatively low number of oocytes retrieved after mild ovarian stimulation distinctly differs from the pathological reduction in the number of oocytes retrieved after profound ovarian stimulation (poor response) associated with poor IVF outcome. The relatively small number of oocytes obtained after mild ovarian stimulation may represent the best of the cohort in a given cycle.

Chapter 4

Section 4.1

This section provides an introduction to alternative approaches to the management of anovulatory patients and ovulation induction. WHO 2 group patients represent a very heterogeneous group of patients, which shows a marked variation in ovarian response during ovulation induction. The importance of a more patient tailored approach to this category of patients is stressed.

Section 4.2

Elevated LH concentration is a common feature in PCOS. This study was designed to investigate whether elevated LH levels in PCOS might be suppressed to normal range values by the administration of low doses of GnRH antagonist, which subsequently might reverse the anovulatory status of these patients. In order to address this issue, 24 PCOS patients with elevated endogenous LH concentrations were randomized into three different dose groups, receiving either 0.125 mg (group A), 0.250 mg (Group B) or 0.500 mg (Group C) ganirelix sc daily for 7 subsequent days. During the first day of treatment, LH and FSH levels were assessed at 20 minute intervals, during 8 hours. Thereafter LH, FSH, androgens, E_2 and inhibins were assessed daily and frequent ultrasound scans were performed for 7 days to record follicle development.

The study showed that repeated GnRH antagonist administration induced a significant suppression of LH (and to a lesser extent of FSH) serum levels, which was comparable between the different doses. Six hours after ganirelix administration, endogenous LH was suppressed by 49%, 69% and 75%, and endogenous FSH was suppressed by 23%, 19% and 25%, respectively. The decrease in serum LH and FSH levels was transient and lasted for 12 hours, whereafter serum levels returned to baseline levels at 24 hours after drug administration. Only in the highest dose group a suppression of androgen levels after prolonged treatment was observed. E_2 levels decreased significantly ($P < 0.001$) and suppression was most pronounced in group C. Inhibin B levels did not change during the treatment period. Spontaneous follicle development or ovulations were not recorded during the course of treatment. In conclusion, the present study demonstrated that the GnRH antagonist ganirelix is capable of normalising elevated LH in PCOS patients, in doses similar to the ones previously shown to prevent a premature LH rise during ovarian hyperstimulation for IVF. In addition, the transient suppression of elevated endogenous LH levels per se does not

re-establish normal follicle development in PCOS. However, follicle development may be insufficiently supported by the accompanied subtle suppression of endogenous FSH. Similarly, a transient decline in E_2 levels is not effectively restoring normal pituitary ovarian feedback. Moreover, these results support the contention of a limited role of LH in the pathogenesis of PCOS.

Section 4.3

A placebo controlled double blind assessment was performed to assess whether the addition of metformin to gonadotrophin ovulation induction in insulin-resistant, normogonadotrophic, anovulatory women alters ovarian responsiveness to exogenous FSH. After a progestagen withdrawal bleeding patients were randomized for either metformin (n = 11) or placebo (n = 9). In case of absent ovulation, exogenous FSH was subsequently administered to induce ovulation. Only during metformin treatment body mass index and androgen (androstenedione and testosterone) levels decreased, whereas FSH and luteinizing hormone levels increased significantly. In the metformin group a single patient ovulated before the initiation of exogenous FSH. Significantly more monofollicular cycles and lower pre-ovulatory oestradiol concentrations were observed in women receiving metformin next to FSH, compared to FSH alone. In conclusion, metformin co-treatment in a group of insulin-resistant, normogonadotrophic, anovulatory patients resulted in normalisation of the endocrine profile and facilitated monofollicular development during FSH induction of ovulation.

Chapter 5

In this chapter the reader is provided an overview of results and conclusions from the preceding studies. These findings are discussed in view of existing knowledge and future perspectives.

Samenvatting

Hoofdstuk 1

De introductie van dit proefschrift start met algemene informatie over voortplanting, subfertiliteit en voortplantingsgeneeskunde. Er wordt vervolgd met een kort overzicht van de huidige kennis over de functie van het humane ovarium door middel van een beschrijving van de ontwikkeling van het ovarium en de vroege- en late follikel ontwikkeling. Het belang van het zogenaamde "threshold/window concept" voor de selectie van een enkele dominante follikel wordt benadrukt en meer gedetailleerd beschreven. Vervolgens wordt er een beschrijving gegeven van gestoorde follikel ontwikkeling en de classificatie van anovulatoire stoornissen. Na een discussie over de behandeling van subfertiliteit en de verschillen tussen ovulatie inductie en ovariële (hyper)stimulatie, worden de studiedoelen gepresenteerd.

Hoofdstuk 2

Paragraaf 2.1

Om te testen of de toediening van een lage dosis exogeen FSH geïnitieerd tijdens de vroege-, mid- of late folliculaire fase multifolliculaire groei kan induceren, werd een prospectief, gerandomiseerd onderzoek verricht onder normo-ovulatoire vrouwen. Veertig vrouwen met een normaal gewicht participeerden. Toediening van een vaste dosis (75 IU/dag) recombinant FSH werd gestart op cyclusdag 3, 5 of 7 tot ovulatie inductie met hCG. Frequente transvaginale echografieën en bloedafnames werden verricht. Multifolliculaire groei vond plaats in alle groepen (in totaal 60%), hoewel dag 7 starters minder multifolliculaire groei lieten zien. Leeftijd, cycluslengte en initiële FSH and inhibine B spiegels waren vergelijkbaar tussen de personen met mono- of multifolliculaire follikel groei. Echter, voor alle vrouwen gold dat hoe lager de BMI, des te meer follikels werden geselecteerd. Als multifolliculaire groei plaats vond, was de lengte van de luteale fase verkort en waren de midluteale serum spiegels van LH en FSH verlaagd en van oestradiol en inhibine A verhoogd. Concluderend, interferentie met dalende serum FSH concentraties door toediening van een lage dosering FSH beginnend op cyclusdag 3, 5 of zelfs zo laat als dag 7, is in staat tot het verstoren van het zich ontwikkelen van een enkele dominante follikel. De rol van BMI in het bepalen van ovariële respons suggereert de betrokkenheid van verschillen in farmacokinetiek van exogeen FSH. Multifolliculaire groei op zich zelf heeft een bepalend effect op de karakteristieken van de luteale fase.

Paragraaf 2.2

Deze studie werd verricht om de relatie tussen serum concentraties van inhibine A, B en oestradiol en het aantal zich ontwikkelende follikels te onderzoeken tijdens de toediening van FSH in verschillende regimes in normo-ovulatoire vrijwilligers en om te evalueren of inhibines gebruikt kunnen worden als voorspellers voor het aantal zich

ontwikkelende follikels tijdens stimulatie. Om deze onderzoeksvraag te beantwoorden, werden series bepalingen van hormoon spiegels en follikel aantallen verricht tijdens ongestimuleerde cycli en tijdens verschillende interventies met exogeen FSH. Personen werden gerandomiseerd voor FSH toediening in een enkele hoge dosis (375 IU: groep A) tijdens de vroeg folliculaire fase, in 5 opeenvolgende lage doses beginnend in de midfolliculaire fase (75 IU: groep B) of in dagelijkse lage doses (75 IU) tijdens de vroege, mid en laat folliculaire fase (beginnend op cyclusdag 3, 5 of 7: groep C, D en E, respectievelijk).

Deze studie liet zien dat bij verlenging van het FSH window het aantal kleine antrale follikels stijgt en dus ook de inhibine B concentratie. Indien een dergelijke interventie resulteerde in multifolliculaire groei, dan stegen de mid-folliculaire fase inhibine B ($P = 0.001$) als ook laat-folliculaire fase inhibine B en A spiegels ($P < 0.05$ en $P < 0.01$) significant in vergelijking met monofolliculaire cycli en de natuurlijke cyclus. Hoewel de mid-folliculaire inhibine B spiegels goed correleerden met het aantal kleine antrale ($P < 0.05$) en pre-ovulatoire ($P < 0.001$) follikels in de laat-folliculaire fase, correleerden de mid-folliculaire inhibine A en E_2 serum concentraties slechts met het aantal pre-ovulatoire follikels ($P < 0.001$ en $P < 0.01$, respectievelijk). Concluderend: de gepresenteerde data verbeteren ons begrip van de relatie tussen follikel dynamiek, serum inhibines en FSH tijdens ovariële hyperstimulatie. Echter, hoewel mid-folliculair inhibine B correleert met het aantal zich ontwikkelende follikels, vergemakkelijkt het de identificatie van vrouwen at risk voor multi folliculaire ontwikkeling niet.

Hoofdstuk 3

Paragraaf 3.1

Deze paragraaf is bedoeld als introductie voor paragraaf 3.2. Het beschrijft de enorme vlucht die de IVF behandeling heeft ondergaan sinds de eerste succesvolle vermelding in de natuurlijke cyclus. Om het succes van de IVF behandeling te bevorderen, werden GnRH agonisten geïntroduceerd ter voorkoming van premature luteïnisatie en ovulatie tijdens ovariële hyperstimulatie. Het werkingsmechanisme van GnRH agonisten wordt beschreven. Over de jaren is de IVF behandeling steeds complexer, duurder en langer geworden en niet zonder risico's. Recent zijn er serieuze bedenkingen geuit gerelateerd aan het beleid rondom geassisteerde voortplanting, zoals IVF. De klinische beschikbaarheid van GnRH antagonisten geeft de mogelijkheid om alternatieve benaderingen voor ovarium stimulatie te ontwikkelen, doordat deze stoffen gekarakteriseerd worden door een directie suppressie van de hypofysaire gonadotrofine secretie en een snel herstel na onttrekking.

Paragraaf 3.2

Verlenging van het FSH "window" voor multifolliculaire groei door toediening van FSH vanaf de mid folliculaire fase, vormt een nieuw, mild protocol voor ovariële stimulatie voor IVF gebaseerd op de fysiologie van monofolliculaire groei in normo-ovulatoire vrouwen. Om de uitkomsten van dit protocol te testen werden er 142 IVF patiënten gerandomiseerd voor een lang protocol met toediening van een GnRH agonist of voor één van twee GnRH antagonist protocollen met start van recombinant FSH

op cyclusdag 2 of 5. Een vaste dosis (150 IU/dag) exogeen FSH werd gebruikt voor ovarium stimulatie, en GnRH antagonist co-behandeling werd begonnen op de dag dat de grootste follikel een diameter van 14 mm had bereikt. Frequente transvaginale echografieën en bloedafnames werden verricht. Deze studie liet zien dat applicatie van het beschreven milde protocol resulteerde in zwangerschapscijfers per gestarte cyclus die vergelijkbaar waren met de cijfers na sterke stimulatie met GnRH agonist co-behandeling ondanks een kortere stimulatie en 27% reductie in exogeen FSH. Een hoger percentage afgelastingen voor de eicel punctie werd gecompenseerd dor een verbeterde embryo kwaliteit, gepaard gaand met een grotere kans op embryo terugplaatsing. Een relatief laag aantal eicellen verkregen na milde stimulatie verschilt duidelijk van een pathologische reductie van het aantal eicellen verkregen na sterke stimulatie (slechte respons), dat geassocieerd wordt met een slechte IVF uitkomst. Het relatief kleine aantal eicellen verkregen na milde ovariële stimulatie vertegenwoordigt mogelijk het beste deel van het cohort in een bepaalde cyclus.

Hoofdstuk 4

Paragraaf 4.1

Deze paragraaf geeft een introductie over alternatieve benaderingen in het beleid van anovulatoire patiënten en ovulatie inductie. Patiënten in de WHO 2 groep vertegenwoordigen een erg heterogene groep patiënten, die een sterke variatie laten zien in ovariële respons tijdens ovulatie inductie. Het belang van een meer individuelere benadering bij deze patiënten categorie wordt benadrukt.

Paragraaf 4.2

Een verhoogde LH concentratie is een veel voorkomende bevinding in PCOS. Deze studie is ontworpen om te onderzoeken of de verhoogde LH spiegels in PCOS onderdrukt kunnen worden naar waarden binnen de normale range door toediening van een lage dosering GnRH antagonist, dat vervolgens wellicht de anovulatoire status van deze patiënten kan herstellen. Om dit te onderzoeken werden 24 PCOS patiënten met verhoogde endogene LH concentraties gerandomiseerd voor 3 verschillende dosering groepen, waarbij dagelijks 0.125 mg (groep A), 0.250 mg (groep B) of 0.500 mg (groep C) ganirelix sc werd toegediend op 7 opeenvolgende dagen. Tijdens de eerste behandelingsdag werden LH en FSH spiegels bepaald met tussenpozen van 20 minuten, gedurende 8 uur. Daarna werden gedurende 7 dagen dagelijks de LH, FSH, androgenen, E_2 en inhibine concentraties bepaald gecombineerd met frequente echografieën ter controle van follikel ontwikkeling.

De studie liet zien dat een herhaalde toediening van GnRH antagonist een significante suppressie veroorzaakte van LH (en in mindere mate van FSH) serum spiegels, die vergelijkbaar was tussen de verschillende doseringen. Zes uur na ganirelix toediening was het endogene LH onderdrukt met 49%, 69% en 75%, en het endogene FSH met 23%, 19% en 25%, respectievelijk. De daling in serum concentraties van LH en FSH was tijdelijk en duurde 12 uur, waarna de serumconcentraties 24 uur na toediening van het medicament terug gingen naar de uitgangswaarden. Alleen in de hoogste dosis groep werd een daling van de androgeen spiegels gezien na langere behandeling.

E_2 spiegels werden significant ($P < 0.001$) lager; deze suppressie was het meest duidelijk in groep C. Inhibine B spiegels veranderden niet tijdens de behandelingsperiode. Spontane follikel ontwikkeling of ovulaties werden niet gezien tijdens de behandeling. Concluderend: de huidige studie laat zien dat de GnRH antagonist ganirelix in staat is om de verhoogde LH spiegels in PCOS patiënten te normaliseren, in doseringen vergelijkbaar met de doseringen voorheen gebruikt ter preventie van een premature LH stijging tijdens ovariële hyperstimulatie voor IVF. Daarbij wordt de normale folliculaire groei in PCOS niet hersteld door tijdelijke onderdrukking van de verhoogde endogene LH spiegel op zich zelf. Echter, follikel ontwikkeling kan onvoldoende gestimuleerd zijn door de bijgaande subtiele daling van endogeen FSH. Hiermee vergelijkbaar veroorzaakt een tijdelijke daling in E_2 spiegels geen effectief herstel van de normale hypofysaire-ovariële terugkoppeling. Bovendien ondersteunen de bevindingen de opvatting dat LH een beperkte rol heeft in de pathogenese van PCOS.

Paragraaf 4.3

Een placebo gecontroleerd dubbel blind onderzoek werd verricht om te beoordelen of de toevoeging van metformine aan gonadotrofine ovulatie inductie in insuline resistente, normogonadotrope, anovulatoire vrouwen de ovariële gevoeligheid voor FSH beïnvloedt. Na een progestagene onttrekkingsbloeding werden patiënten gerandomiseerd voor of metformine (n = 11) of placebo (n = 9). Bij uitblijven van ovulatie werd vervolgens exogeen FSH toegediend voor ovulatie inductie. Alleen tijdens metformine behandeling daalden de BMI en de androgeen spiegels (androsteendion en testosteron), terwijl FSH en LH spiegels significant stegen. In de metformine groep ovuleerde één enkele patiënt voor de start van exogeen FSH. Significant meer monofolliculaire cycli en lagere pre-ovulatoire oestradiol spiegels werden gezien in de vrouwen die metformine ontvingen naast FSH, vergeleken met alleen FSH behandeling. Concluderend: metformine co-behandeling in een groep insuline resistente, normogonadotrope, anovulatoire patiënten resulteerde in normalisatie van het endocriene profiel en vergemakkelijkte monofolliculaire ontwikkeling tijdens ovulatie inductie met FSH.

Hoofdstuk 5

In dit hoofdstuk wordt aan de lezer een overzicht gegeven van de resultaten en conclusies verkregen uit de voorafgaande studies. Deze bevindingen worden besproken en beschouwd tegen de achtergrond van de bestaande inzichten en geplaatst in het licht van toekomstig onderzoek.

Publications and presentations

Publications and presentations

Publications included in the present thesis:

Hohmann F.P., Laven J.S.E., de Jong F.H., Eijkemans M.J.C. and Fauser B.C.J.M. (2001) Low-dose exogenous FSH initiated during the early, mid or late follicular phase can induce multiple dominant follicle development. *Human Reproduction,* 16 (5): 846-854.

Hohmann F.P., Laven J.S.E., de Jong F.H. and Fauser B.C.J.M. (2005) Relationship between inhibin A and B, oestradiol and follicle growth dynamics during ovarian stimulation in normo-ovulatory women. *European Journal of Endocrinology,* 152 (3): 395-401.

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Van Santbrink E.J.P., Hohmann F.P., Eijkemans M.J.C., Laven J.S.E. and Fauser B.C.J.M. (2005) Does metformin modify ovarian responsiveness during exogenous follicle-stimulating hormone ovulation induction in normogonadotrophic anovulation? A placebo controlled double blind assessment. *European Journal of Endocrinology*, 152 (4): 611–617.

Abstracts and presentations related to this thesis:

Hohmann F.P., Laven J.S.E., Eijkemans M.J.C. and Fauser B.C.J.M. (2000) Low dose exogenous FSH initiated during the early-, mid-, or late follicular phase: Effects on dominant follicle development. *SGI Year 2000 Annual Meeting, March, 2000, Chicago, USA [J. Soc. Gynecol. Investig. (2000), 7, (suppl.no.1) 581].*

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Dankwoord

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Lieve Elena (Martini), onze charmante Italiaanse en het levende bewijs dat 1968 een goed bouwjaar was. Toen ik net zwanger was van Stijn, genoot ik van jouw één-tweetje met je dikke buik tijdens de "woensdagmiddagsessies". Niet iedereen dacht hier echter hetzelfde over... Af en toe even uithuilen lucht gelukkig op! Fijn dat je je draai nu gevonden lijkt te hebben. Enne, laten we binnenkort weer eens echt afspreken, i.p.v. die halve minuutjes op het schoolplein?

En nu mijn paranimfen Annemarie (Wassing-)de Vet en Annemarie Mulders. Met Annemarie als meest directe collega in de eerste fase en Anne(marie) in de tweede fase, is "Annemarie" de rode draad van mijn promotie.

Lieve Annemarie, al tijdens mijn eerste baan als Agnio gynaecologie in Amsterdam leerden wij elkaar kennen als directe collega's. Na mijn overstap naar de cluster Rotterdam (RdGG), volgde jij ook snel (SFG). Als vanzelfsprekend kwam jij spoedig na mij de follikelgroep versterken. In deze periode kregen wij een hechte vriendschap en deelden we lief en leed (Is het schadelijk als je meer dan 1x per week een echo van de zwangerschap maakt? – nagellak, denk je dat ze dat ruiken? - Hoezo getuige van een huwelijk? – Ikea???? – Het is nota bene DDD bij de Bijenkorf!). Naast onderzoek, maakten wij beiden dezelfde verandering door en kozen voor de huisartsgeneeskunde. Het kan geen toeval zijn dat we nu het laatste stukje van de opleiding in dezelfde Haio-groep zitten. Annemarie, dank je voor je dierbare vriendschap. Ik bewonder je om hoe jij in het leven staat, en hoe je in staat bent jouw weg te kiezen. Je weet niet hoeveel ik van jou geleerd heb! Ook al maakten wij niet dezelfde keuze in voortzetting van het promotietraject, het blijft voor mij vanzelfsprekend dat jij een van mijn paranimfen bent. Laten we samen werken en heel vaak shoppen en nog heel lang vriendinnen blijven…

Lieve Anne, dierbare onderzoekscollega en vriendin! Erg knap (en meer dan terecht) dat jij mij bent voorgegaan met promoveren en dat jij straks aan mij Dé Beker mag uitreiken. Jouw werkhouding en doorzettingsvermogen zijn voor mij dat duwtje in de rug geweest om ook door te gaan. Anne, dank voor alle momenten dat je voor mij bent ingesprongen en inderdaad voor het delen van "bazenleed". Maar nog meer dank dat je er altijd was en voor je kleine attenties op exact het juiste moment (ik heb je kaart met klavertje 4 nog steeds…). De gynaecologenwereld mag in de handen knijpen met jou: Volgens mij bestaan er weinig mensen zo collegiaal, zo hardwerkend, zo gezellig en zo lief als jij. Enne, 112 en 156 scheelt maar een paar huizen, dus snel borreltijd…?

Nu de "jus" van het promoveren: deelgenoot worden van "Het follikelteam":

Lieve follikelboys, toen ik aantrad als eerste follikelgirl was ik nog "one of the boys". Al was het even wennen om oude verworvenheden te bestrijden (onze kamer op 8 Noord "=" rookkamer V&V), het voelde bevoorrecht om bij het team te horen: gezelligheid en borreltijd stonden voorop en jullie waren mijn voorbeeld. En, goed voorbeeld doet volgen, zodat ik ook langer dan de voorgeschreven 4 jaar voor promoveren heb genomen…

Beste Thierry Pache (leuk om je recent beter te hebben leren kennen), Evert van Santbrink (dank voor je adviezen en lunchgezelschap, ik zal je alleen nooit als "mijn baas" zien…), Jits Schipper (onvergetelijk jouw gezicht bij: "Jits, de eerste follikelboy is zwanger!") en Arne van Heusden (voetje vrijen in Parijs?!), dank voor jullie gezelligheid.

Lieve Bernd, met veel plezier denk ik terug aan onze gezamenlijke ritjes Leiden-Delft en terug. Jij bent degene geweest die mij heeft aangemoedigd de stap van Agnio naar arts-onderzoeker te maken. Wellicht was dit boekje er wel nooit gekomen als jouw oude autootje het niet begeven had…

Lieve Diederick, vanaf het eerste moment klikte het tussen ons. Tuurlijk kan je af en toe wat heetgebakerd reageren, en tuurlijk ben je niet altijd de makkelijkste… maar bij mij kan je geen fout meer maken sinds die zondagochtend 23 augustus 1998 om 06.00 uur….Vanaf toen heb ik geweten wie de echte Diederick is…

Lieve Mark, de enige drs. onder de boys. Ik ben er van overtuigd dat je over niet al te lange tijd ook aan de afronding zult beginnen, ondanks alle drukte van de kliniek en je huis vol kinders: Succes!

Beste Babek, ik hoop dat het je goed gaat (waar ook ter wereld)…

Vanaf het eerste moment heb ik er altijd (meestal?) bijgehoord. Ik hoop nog lang één van de boys te kunnen zijn (wat was ook alweer de regel?).

En zonder boys geen girls…

Lieve Nicole (Beckers), toen ik begon als onderzoeker, was jij nog "gewoon" één van de IVF-artsen, die daarnaast nog onderzoek deed. Dank voor je hulp bij het zetten van mijn eerste schreden in "IVF-land" en je altijd kundige adviezen. Nu bij jou ook eindelijk het einde inzicht is van je promotietraject ("je had al 3 keer kunnen promoveren als je niet zo eigenwijs was, artikelen genoeg!"), wens ik je veel succes met de hete adem van de baas. Ik heb begrepen dat hij nu een ander slachtoffer heeft moeten vinden….

Lieve (B)Esther (Baart), (H)Esther (Heijnen) en Christien (Weenen), dank jullie voor alle gezelligheid die ik met jullie heb gehad op onze "flex-plekken" op de kopkamer van 5-Noord. Nog altijd voel ik me welkom als ik af en toe eens kom buurten. Succes met alle onderzoeksfrustraties en denk aan the ultimate goal: Dé Beker!

Lieve Anne (von Bergh), wat een korte, maar intense periode als directe collega's hebben we samen doorgemaakt. We hebben gelachen en gehuild en veel stoute dingen besproken. Door jou ontdekte ik MSN en hervond ik het geluk in mijn leven…. 1000 maal dank! Ik ben blij dat ook jij weer vol vertrouwen de toekomst in durft te kijken. De 7 vette jaren zijn aangebroken!

Zonder ondersteuning ben je nergens:

Lieve Lizka (Nekrui), wat een gezelligheid dat jij bij ons op de afdeling kwam. Dank voor al je hulp bij het uitzoeken van samples. Ook bij jou is er een hoop gebeurd de laatste jaren. Fijn dat het geluk ook jou weer toelacht.

Joke Kuijpers, Eveline Ikking, Marita Meeuwes en Anita de Voogt (soms heb je het nodig om met iemand iets meer op afstand over privé-zaken te praten..), allemaal hartelijk dank voor jullie hulp bij van alles en nog wat. Dank voor jullie luisterend oor als ik even stoom moest afblazen…

Ontzettend veel dank aan alle medewerkers van lab Interne III: wat een hoop samples hebben jullie voor mij moeten verwerken. Het was fijn dat ik altijd bij jullie binnen kon lopen en dat er over "dit ene speciale verzoekje van mij" niet moeilijk werd gedaan.

Voor de uitvoering van mijn IVF studie veel dank aan de IVF-artsen, de secretaresses van de IVF (Annemarie Slingerland en Beate Pawliszak) en de medewerkers van het IVF-lab voor jullie welwillendheid om de logistiek op de poli en in het lab ietsjes te veranderen omwille van mijn onderzoek. Dank voor het scoren van velen embryo's; het heeft waardevolle informatie opgeleverd. En verder, de koffie op het IVF-lab smaakte toch weer anders dan elders in het ziekenhuis, wellicht door jullie gezelligheid en de droppot?

Ook dank aan de medewerkers van de poli gynaecologie en de prikkamer voor het aanhoren van mijn verhalen, het zoeken naar statussen, het prikken en vooral de opslag van samples.

En verder, niet in de minste plaats, dank voor alle gezelligheid en hulp aan alle medewerkers van de CRU. Inmiddels is er veel veranderd en de meeste van jullie werken ergens anders. Lieve Karin (ter Haar) en Christi (Wagemakers), jullie waren als echte vriendinnen voor mij. Wat hebben we een hoop gelachen (tot ik er buikpijn van kreeg). Karin, je bent de enige die ik ken die ook ooit eens met de gasslang aan de auto is weggereden… Dank hiervoor: gedeelde klunzigheid is altijd minder pijnlijk… Het wordt hoog tijd dat we weer eens afspreken.

En nu mijn "nieuwe leven": de huisartsen-wereld. Dank aan mijn groepsbegeleiders (Betsy Fu, Anne Weiland en Gerrit Jan Vrielink) en de betrokkenen van het huisartseninstituut, die mij de ruimte en mogelijkheden hebben gegeven mijn proefschrift weer op de rails te zetten. Zonder jullie begrip zou het nooit gelukt zijn de eindstreep te bereiken.

Mijn Haio-groep (Jack, Lieselot, Rim, Eveline, Chantal, Annemieke, Rachel, Chris, Bettina, Ashna en Dewi), bedankt voor jullie interesse in mijn promotie, en dat, terwijl jullie er eigenlijk niet in geïnteresseerd zijn. We zijn allemaal bijna klaar, maar onze wegen zullen elkaar vast nog vaker kruisen in huisartsenland.

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Als laatste mijn vrienden en familie…

Zonder jullie zou ik hier niet staan en zou ik niet zijn wie ik nu ben. Dankzij jullie heb ik ook in ongelukkige tijden altijd de zon zien schijnen aan het eind van de tunnel. Zoveel dierbare, vrolijke, gezellige en ontroerende momenten, zodat mijn accu weer kon worden opgeladen om de kar voort te trekken. Het blijft onmogelijk om alle dierbaren om me heen persoonlijk te bedanken. Een enkeling wil ik toch echt met naam noemen:

Lieve Eel (Eliane Leijten), het was liefde op het eerste gezicht in augustus 1987 voor de deur van "Bree 50"! Teveel momenten om op te noemen: de "Bora Bora", gezamenlijk dronkenschap, slapen in een kale kamer onder jouw jas, alweer een bushalte gemist.., wie praat er eigenlijk sneller en meer? Je was er altijd "for better and worse": Peter Rabbit's zusje en Pooh-beer zijn voor mij de bewijzen van echte vriendschap!

Lieve Beer (Ingrid de Beer), wat ben je een bijzondere vriendin! Als ik aan jou denk (en vooral wat we samen gedaan hebben, of nog beter: samen bedacht hebben..), moet ik lachen. We hebben zoveel samen ondernomen en zoveel verdrietige, angstige (paspoort kwijt?), onzekere (zwanger? wat nu?), blije en bijzondere (de geboorten van David, Ida en Lukas) momenten samen doorgemaakt. Weet je, ik heb me zelden zo ontroerd gevoeld als toen ik jullie kerstkaart met kerstcadeau 2002 ontving…. Zullen we dan toch maar in die commune gaan wonen als je terug komt uit Ghana?

En natuurlijk mijn andere vriendinnen uit Pseudomien (Nicolet, Eiske, Nienke, Gerry-Anne, Majella, Ellen, Maaike, Michaela en Letty), dank dat ik altijd op jullie heb kunnen rekenen en voor alle gezelligheid. Wie is hier het meest gezien? Dat is…. Ik hoop dat we nog vele jaren samen "bier met koekjes voor ontbijt" eten!

Lieve pappa, alweer 14 jaar geleden dat jij afscheid van ons hebt genomen. 14 jaar waarin zoveel is gebeurd, zoveel belangrijke momenten, waar ik je zo graag bij had willen hebben. Wat vind ik het ook nu weer intens jammer dat je dit boekje niet meer kunt zien en dat je 1 juni niet bij de verdediging en de feestelijkheden na afloop kunt zijn. Had je ooit gedacht dat juist ik zou gaan promoveren? Ik voel me trots dat ik nu een beetje meer in jouw voetsporen kan treden: ik ben nu alleen nog het streepje van de R verwijderd van jouw naambordje!

Lieve mamma, heel erg bedankt voor alle goede zorgen die je altijd hebt gegeven, ook als de afstand ons in de weg zat. Het is fijn te weten dat je altijd achter me staat, dat ik voor jou niet hoef te presteren en dat je van me houdt om wie ik ben. Voor jou telt alleen dat wij gelukkig zijn. Ik weet dat je het zwaar hebt gehad en ik ben trots op je dat je je al die jaren alleen zo krachtig hebt gedragen. Jouw kracht is de afgelopen jaren een groot voorbeeld voor mij geweest. Wat ben ik blij dat je met Klaas het geluk weer hebt hervonden!

En lieve Klaas, wat ben ik blij met jou om wie je bent, maar vooral omdat je mamma weer hebt laten stralen. Je bent onbetaalbaar!

Lieve Karin, Martijn en Wendy, dank voor jullie interesse en het vertrouwen in mijn kunnen. Het voelt goed te weten dat ik altijd op jullie kan rekenen als de nood echt hoog is. Ik wil nog heel veel kerstmissen met verhitte discussies tot in de kleine uurtjes met jullie vieren.

Lieve Elly en Joost, Margreet en Jan, wat voel ik me bevoorrecht dat ik in zo'n warme schoonfamilie ben beland. Vanaf het eerste moment heb ik me samen met de mannetjes welkom gevoeld bij jullie. Elly, duizend maal dank voor je lieve kaartjes, "het mamma van Kees zijn", voor alle maandagen en die constante berg strijk… Je bent onbetaalbaar! Joost, hoe vaak mogen de mannetjes nog kwartetten met "de pappa van Kees"? Ik hoop op nog vele "paashuisjes", "botenhuisjes" en andere gezelligheid, en, zoals Stijn het wel vaker verwoordt: "Wat zijn we toch een bofkonten!"

Allerliefste Stijn en Wiebe. Het boekje is af! De afgelopen jaren hebben jullie het niet altijd makkelijk gehad: een mamma met te weinig tijd en een hele hoop turbulentie in jullie jonge levens. Zonder het te weten zijn juist jullie het geweest die mij altijd de zin om door te gaan hebben gegeven. Dank jullie honderdmiljoentriljoen keer (is dat meer dan honderduizend?) voor alle glimlachjes en blijdschap die jullie mij de afgelopen jaren hebben gegeven. Jullie liefde is onuitputtelijk en een bron van energie voor mij geweest. Ik hou van jullie! We gaan er nu samen een geweldige tijd van maken.

Mijn liefste Kees, er zijn geen woorden voor, geen woorden voor, voor jou…
Jouw liefde, stimulans en geduld zijn onbeschrijflijk. Ik houd het daarom bij de volgende woorden:

Door jou weet ik dat één en één drie is en geloof ik weer in sprookjes....

Curriculum vitae auctoris

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The author of this thesis, Femke Pauline Hohmann, was born on the 26th of July 1968 in Leiden, The Netherlands. In 1986 she graduated from secondary school (VWO-B) at the Jacobus College in Enschede. She attended Medical School at the University of Leiden from 1988 – 1995 from which she graduated cum laude. From January 1996 to May 1998 she worked as a resident in Obstetrics and Gynaecology at the Sint Lucas Andreas Hospital (head: Dr. J.Th.M. van der Schoot) in Amsterdam and the Reinier de Graaff Hospital (head: Dr. J.C. Kuijpers) in Delft, respectively. In May 1998 she started as a PhD student at the Division of Reproductive Medicine (head: Prof.dr. B.C.J.M. Fauser), department of Obstetrics and Gynaecology of the Erasmus MC in Rotterdam, where she worked on the studies described in this thesis. During this PhD period her professional interest changed, partly because of the births of her two sons Stijn and Wiebe in 2000 and 2001 respectively. In March 2003 she started the training for General Practise at the Erasmus University in Rotterdam, which she expects to finish at the end of 2005.