Ovarian Stimulation and the Endometrium

New Approaches and Insights

Ovarian stimulation and the Endometrium: *new approaches and insights.*

Thesis, Erasmus University, Rotterdam, The Netherlands

The work presented in this thesis was performed at the Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands.

ISBN: 978-90-8559-524-3

Cover design by: Guus E. van der Gaast

Printed by: Optima Grafische Communicatie, Rotterdam

Parts of this PhD thesis have been published previously and have been reproduced with permission of the publishers:

© Reproductive Sciences (Reprod Sci)

© Reproductive Biomedicine Online (Reprod BioMed Online)

© British Journal of Obsterics and Gynaecology (BJOG)

The following companies are gratefully acknowledged for their financial contribution in the publication of this thesis:

Ferring BV, Raadgevers Kuijkhoven, Bayer Schering Pharma, BARD Benelux NV., Covedien Nederland BV.

Ovarian Stimulation and the Endometrium

New Approaches and Insights

Ovariële stimulatie en het Endometrium

nieuwe benaderingen en inzichten

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

dinsdag 12 mei 2009 om 16:00 uur

door

MARK HINKO van der GAAST

geboren te Wolvega

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. B.C.J.M. Fauser	(Erasmus MC & UMC Utrecht)
	Prof.dr. N.S. Macklon	(Erasmus MC & UMC Utrecht)
Overige leden:	Prof.dr. C.W. Burger	(Erasmus MC)
	Prof.dr. J.A. Land	(UMC Groningen)
	Prof.dr. J. Lindemans	(Erasmus MC)

- "You've got to be very careful if you don't know where you're going,
because you might not get there." -

Yogi Berra (1925–)
Baseball player New York Yankees

Voor Chantal, Max, Janneke, Luuk & Klara

CONTENTS

1.

INTRODUCTION

1.1 THE ENDOMETRIUM AS A DETERMINANT FACTOR FOR IVF OUTCOME

When one considers the recognized 'causes' of infertility, problems with gamete quality and availability rank highly, as does the means of their transport, whether it be through the fallopian tubes in the female or vas deferens in the male. Conception is seen as the primary goal, and fertility therapies are currently focused on helping couples achieve this. In around 25% of couples with fertility problems, medical investigation reveals no such clear cause. It is now understood however, that while a couple may succeed in conception, implantation failure may be the underlying reason for their difficulties in achieving a pregnancy (Macklon *et al.,* 2002). Implantations depend on the succesful interaction between the embryo and the endometrium of the uterus. Since the earliest days of *in vitro* fertilization (IVF) the focus of attention has been the embryo. In order to obtain multiple embryos to allow selection for transfer to the uterus, complex ovarian stimulation regimens have been devised which result in many oocytes being harvested for fertilization. In most women, these stimulation regimens succefully achieve this aim. Yet despite the ability to produce large numbers of embryos for selection for transfer, pregnancy rates from IVF remain around 25% per started cycle (ESHRE 2008). Once the embryo is transferred into the uterus, it must interact with the endometrium, and in the majority of treatment cycles, this is the moment in which failure occurs.

The role played by the endometrium as a determinant factor of fertility treatment, and in particular IVF outcomes remains unclear. Moreover, the impact of ovarian stimulation on endometrial quality is only now beginning to be elucidated. In this introductory chapter, the hormonal control of endometrial development is reviewed, and current approaches for assessing the receptivity of the endometrium to the embryo are critically assessed (Chapter **1.1.1**). The concept of the 'implantation window' is introduced (Chapter **1.1.2**) and the molecular regulation of endometrial maturation and implantation is reviewed (Chapter **1.2**), with emphasis on the most important of the described markers of receptivity (Chapter **1.3**). The challenges of studying the impact of ovarian stimulation on endometrium are described (Chapter **1.4**) as a prelude to introducing a novel means of addressing these by studying endometrial secretion fluid (Chapter **1.5**). These considerations lead to the defining of the objectives of the research described in this thesis (Chapter **1.6**).

1.1.1 Hormonal regulation of the endometrium during the menstrual cycle

During the normal human menstrual cycle (Figure **1.1**), ovarian steroid hormones estradiol (E) and progesterone (P) induce endometrial proliferation, differentiation and menstruation. Following menstruation, only the vascularized endometrial basal layer (*lamina basalis*) remains, containing the blind ending remnants of the endometrial glands with cells which will proliferate into new uterine cylindrical luminal and glandular epithelium. During the *follicular phase* of the menstrual cycle, estradiol production in the granulosa cells of the dominant follicle increases rapidly once a diameter of 10 mm has been reached (Fauser *et*

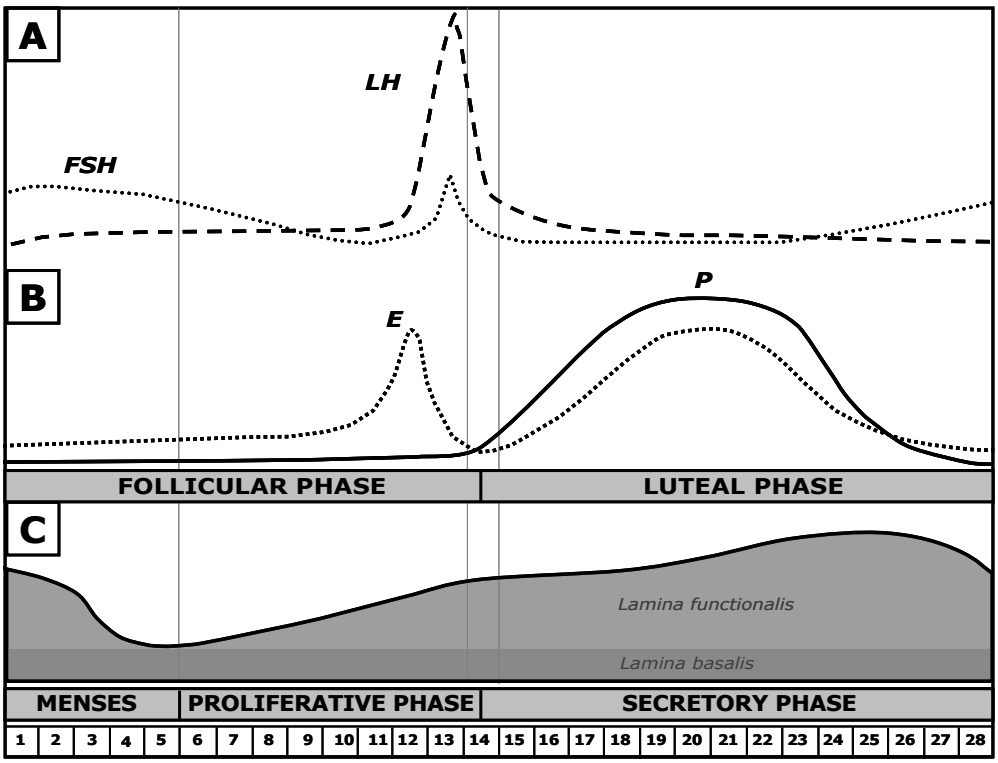


Figure 1.1 *Cyclic changes of the pituitary hormones (**A**): Follicle Stimulating Hormone (FSH; stimulates follicle growth), and Luteinizing Hormone (LH: induces follicular ovulation 36 hours after the surge); ovarian steroids (**B**): estradiol (E) and progesterone (P); and endometrial changes (**C**) in a normalized 28 day cycle. The endocrinological follicular and luteal phase are related to the endometrial menses plus proliferative, and secretory phases, respectively. (Copyright 2009, MH van der Gaast. All rights reserved).*

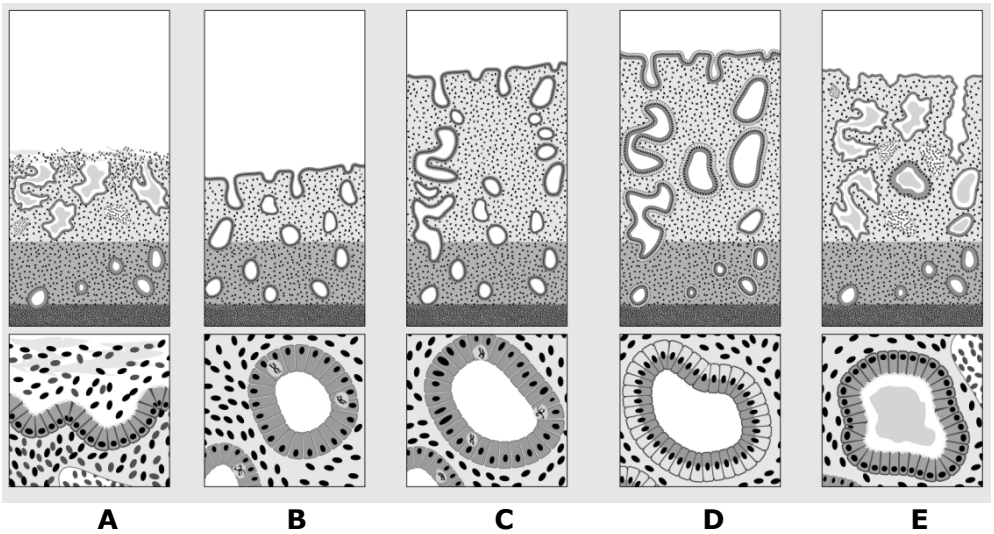


Figure 1.2 *Endometrial histology changes during the menstrual cycle. (**A**) Early proliferative phase; (**B**) Late proliferative phase; (**C**) Early secretory phase; (**D**) Midsecretory phase; (**E**) Late secretory phase. Highly magnified endometrial glandular cells corresponding with the low magnification above. (Copyright 2009, GE van der Gaast. All rights reserved).*

al., 1997). This rise in serum estradiol results in proliferation of endometrial luminal and glandular epithelium, and stroma by increased oedema and mitotic activity in fibroblasts (Figure **1.2A & B**). This is the functional layer (*lamina functionalis*), necessary for embryo implantation. At the end of the endometrial proliferation phase, just before follicular ovulation, the glands are now narrow straight tubes, with epithelial cells with minimal basal glycogen accumulation, and stromal mitotic activity is reaching a peak (Johannisson *et al.,* 1987). After ovulation (on cycle day 14 in a normalized cycle of 28 days) granulosa cells of the ruptured follicle becomes luteinized by incorporation of lipid-rich vacuoles within the its cytoplasm. This *corpus luteum* starts with the production of progesterone and as result endometrial glands secrete the intracellular accumulated glycogen together with polysaccharide protein complexes into the glandular lumen (Figure **1.2C** & **D**). Glandular ducts are now coiled and the lumen is distended as result of secretion accumulation. The thickness of the stromal layer increases as result of oedema, and the arteries become tortuous. Besides the oedema, a stromal leukocytic infiltration of lymfocytes, neutrophilic granulocytes and macrophages occurs.

At the end of the *luteal phase* of the cycle, if the corpus luteum is not rescued by human choriongonadotropin (hCG) produced by trophoblast cells of the early pregnancy, progesterone production declines and eventually stops. The endometrial functional layer disintegrates and consequently a new cycle starts with a next menstruation.

Endometrial tissue dating

In the first article of the first issue of *Fertility and Sterility* Noyes, Hertig and Rock established the dating criteria of endometrial tissue in 300 endometrial biopsy specimens (Noyes *et al.,* 1950). They studied histological changes of endometrial tissue during the menstrual cycle using 8 criteria (Figure **1.3**): (a) mitosis in the endometrial glands; (b) pseudostratification of nuclei; (c) basal vacuolation; (d) secretion in glandular lumen; (e) stromal edema; (f) pseudodecidual reaction; (g) stromal mitosis; and (h) leucocytic infiltration of polymorphonuclear cells (neutrophilic granulocytes & macrophages).

The principle aim of these criteria was to relate endometrial response to the cycle day and matching ovarian hormone levels, and detect abnormal endometrial changes. In different endometrial compartments (e.g. epithelium, glands and stroma). Since the endometrial dating criteria have been described and established, they have been used as the 'gold standard' for endometrial evaluation in fertility work up and research in women of subfertile couples. However, the original study of Noyes and co-workers contained a number of what we would now consider to be methodological 'errors'. First, the 300 biopsies were taken from women who were part of a 'sterile couple', not proven fertile females with normal menstrual cycles. Second, the specimens were selected in retrospect from the files in the pathology department. Third, the luteal day on which the specimen was obtained, was calculated by subtracting 14 days from the start of the next menses. Here, the assumption of a fixed luteal phase length was used. Finally, endometrial dating may have an acceptable variation of plus-minus 2 days to be 'in–phase', and was assumed to be normal

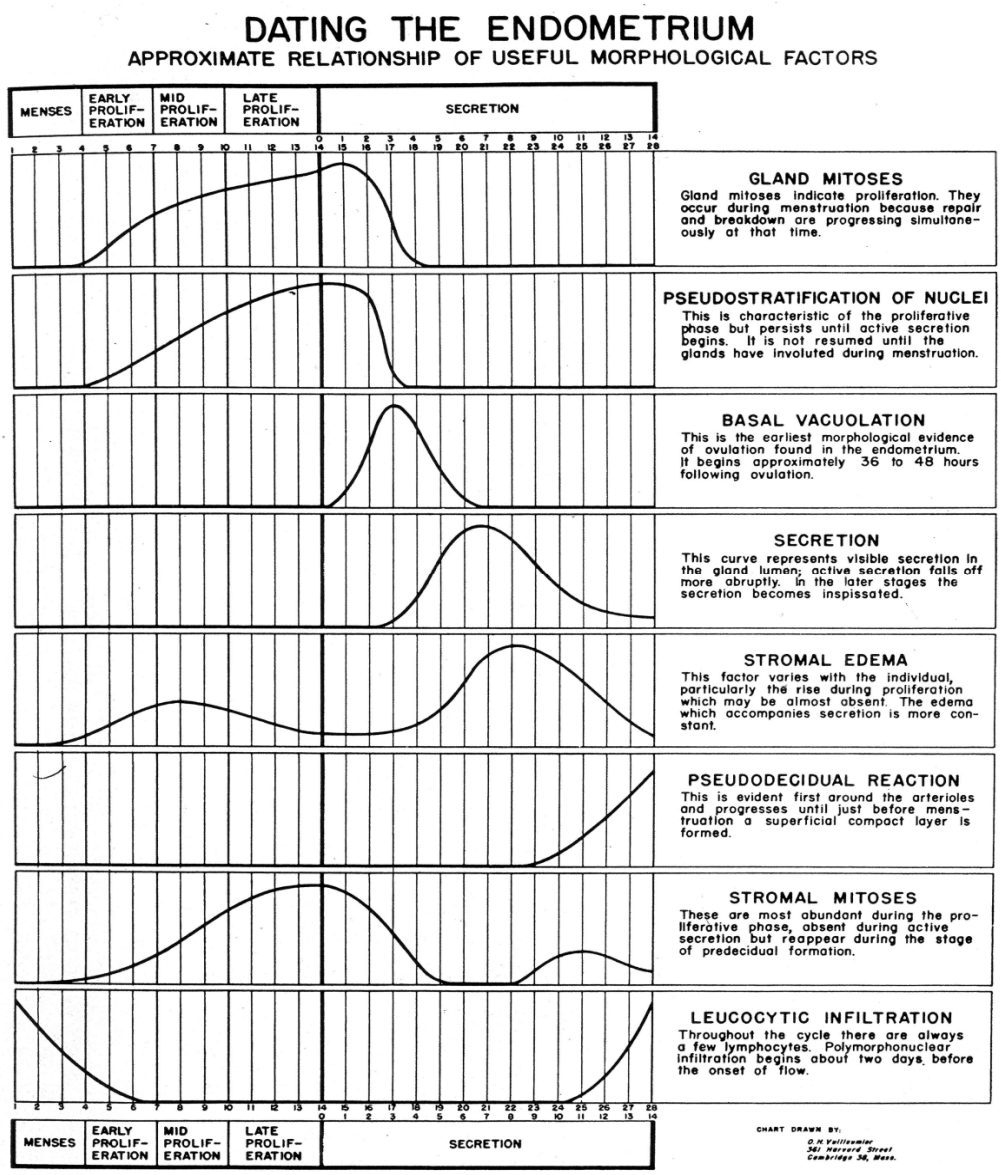


Figure 1.3 The original figure with the 8 criteria for endometrial dating described by Noyes *et al.*, 1950 (reproduced with permission).

(see subtitle of Figure **1.3**: 'approximate relationship of useful morphological factors'). This may be due to individual interpretation of the histological slides. Moreover, no inter- and intraobserver, and intra-patient variability were calculated.

More than 50 years elapsed before the value of the 'Noyes criteria' were analyzed for accuracy and reproducibility in fertile women (Murray *et al.,* 2004), the value of the criteria in discriminating subfertile from fertile women (Coutifaris *et al.,* 2004), and the interobserver and intraobserver variability in endometrial

dating (Myers *et al.,* 2004). All three studies showed that the 'Noyes criteria' did not meet the accuracy, reproducibility and variability to use these criteria for clinical practice or for research to evaluate receptivity and fertility in patients.

More advanced tools have been developed in molecular biology, reproductive endocrinology and ultrasound, and as result more increasing insight has gained in menstrual cyle and the process of embryo implantation after the morphological 'Noyes criteria' were defined. During the implantation process endometrium and the embryo interact with each other during the time the embryo is in the uterine cavity. This endometrial-embryonic dialogue in the luteal phase of the cycle leads to endometrial and embryological changes and a (higher) degree of endometrial receptivity and ultimately to embryo implantation and pregnancy. This receptive period in the luteal phase is short and known as the 'implantation window'.

1.1.2 The 'implantation window' concept

Human embryo implantation in endometrium remains a poorly understood complex phenomenon. This process can be divided in three arbitrary phases: (1) the *apposition phase*, in which the embryo is next to the endometrial lining with embryo-endometrial communication; (2) the *attachment phase*, embryonal blastocyst is now being hatched to the endometrial luminal epithelium by adhesion molecules; and finally (3) the *invasion phase*, embryonal trophoblast invades the endometrial lamina functionalis.

Successful embryo implantation occurs in a limited period in the luteal phase of the human menstrual cycle, the 'implantation window' or 'nidation window' (Psychoyos, 1973). There is no general consensus on the exact time frame of this putative window. In a normalized cycle of 28 days (Figure **1.1**) implantation occurs in approximately 24 hours between the 19th (5 days after ovulation) and 24th cycle day, and may continue for 5 to 7 days (Hertig *et al.,* 1956; Psychoyos, 1993). The endometrium becomes non-receptive again after this window has been closed, and until the next menstruation. This window concept was confirmed with urine hCG, which showed that ongoing pregnancy rates drop dramatically if embryo implantation occurs later than the 10th post-ovulatory day (Wilcox *et al.,* 1999). During this period in the luteal phase of the human menstrual cycle a number of temporally specific morphological and molecular events (Table **1.1**) have been demonstrated in the endometrial tissue and its secretion fluid. These will be discussed in detail in chapter **1.3**. Much of these changes occur in the absence of the embryo and are controlled by ovarian steroid hormone changes. However, increasing insight in endometrial-embryonic dialogue has revealed that the endometrium is a paracrine target for further development of attachment and invasion of the embryo.

After disruption of the luminal epithelium and the basal membrane, trophoblast penetrates the endometrial stroma by degradation and lysis of the extracellular matrix (ECM) by enzymes. These enzymes, matrix metalloproteinases (MMP), are a family of endopeptidases which can be divided in mainly 3 groups: (a) gelatinases (MMP-2 & -9), for degrading gelatins and collagen; (b) collagenases (MMP-1, -8, -13, and -18), for degrading collagen; (c) stromelysins (MMP-3, -7, -10, -11, and -12), degrading collagen, fibronectin,

Table 1.1. *Overview of most important implantation markers.*

Type of Change	Marker of implantation
Structural change	Pinopodes (uterodomes)
Molecular change	Adhesion molecules: -Mucins (MUC-1, MUC-4) -Carbohydrates (Selectins & Galectins) -Integrins -Cadherins -Trophinin-tastin-bystin complex -Heparin-binding Epidermal Growth Factor (HB-EGF) Glycodelin A (GdA; Placental Protein 14, PP14)
Regulation of implantation	Estrogens (E), Progesterone (P) and receptors (ER & PR), Cytokines: -Interleukin family -Leukaemia Inhibitory Factor (LIF) -Colony Stimulating Factor 1 (CSF-1) -Epidermal Growth Factor (EGF) -Transforming Growth Factor-β (TGF-β) -Activins and Inhibins -Chemokines Other: -Insulin Growth Factor (IGF) system -Calcitonin -Leptin
Vascular factors	Vascular Endothelial Growth Factor (VEGF) Nitric Oxide (NO)

laminin, gelastin, elastin, and proteoglycans. For balancing the degradation for cytotrophoblast invasion, and decidual regeneration, a subtle regulation of enzyme activity is necessary. This regulation mechanism contains tissue inhibitors of metalloproteinases (TIMPs; 3 types with a different affinity pattern for MMPs), produced by endometrium (of and limits invasion. Other regulators of MMP activity are IL-1, -6, -10, -15, LIF, EGF, TGF, IGF-II, IGFBP-1 and hCG (Chaouat *et al.,* 2005; Licht *et al.,* 2007). To reduce cohesion between decidual cells it is necessary to downregulate integrin expression may be caused by cytokines (Grosskinsky *et al.,* 1996; Simon *et al.,* 1997; Tabibzadeh *et al.,* 1995b).

Despite extensive research, the process of human embryo implantation process remains to be fully elucidated. However, in the next section, our current understanding of the main regulators is briefly reviewed.

1.2 ENDOCRINE & PARACRINE REGULATORS OF IMPLANTATION

1.2.1 Progesterone (P) and estrogen (E), and their receptors

Ovarian steroids E and P are the primary messengers that induce endometrial

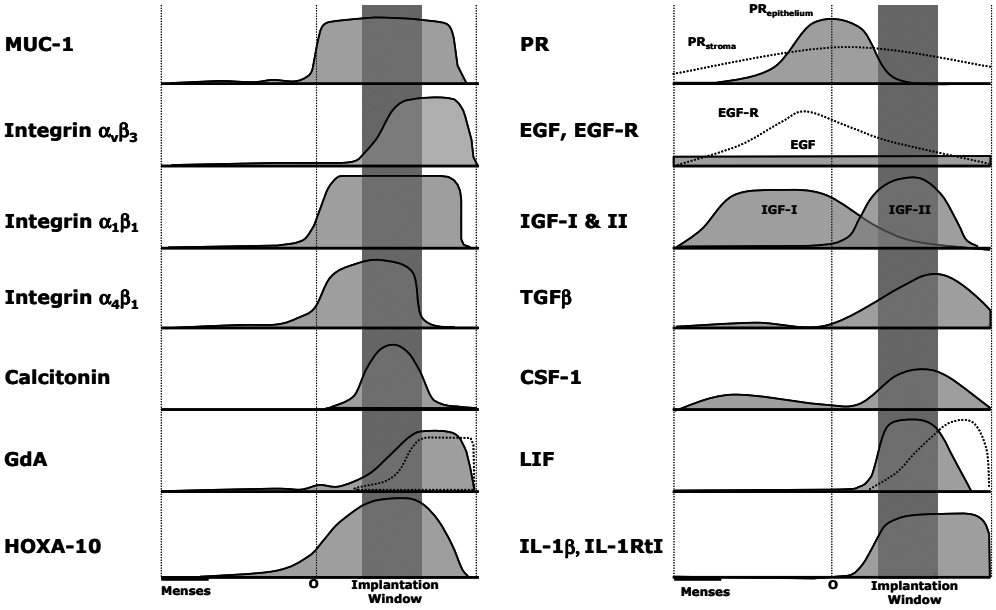


Figure 1.4 *Temporal expression profiles of various endometrial markers during the human menstrual cycle, in relation with the menses, ovulation (O) and implantation window (cycle day 20 – 24). The dotted line in the LIF and GdA expression profiles are levels in flushings from the uterine cavity. For abbreviations see page 169.*

differentiation and maturation. Serum levels are cycle dependent (Figure **1.1**).

Estrogen receptors (ER) and progesterone receptors (PR) are nuclear receptors. Both receptors have two isoforms: ERα and for ERβ is observed throughout the menstrual ERβ; PR_A and PR_B. In the human endometrium a relatively low expression level of ERβ is observed throughout the menstrual cycle (Rey *et al.,* 1998). In healthy women with a normal menstrual cycles, the total PR levels, and PR_A - PR_B ratio vary during the menstrual cycle (Mangal *et al.,*

Table 1.2. *Steroid regelatory effects on various endometrial factors of in vitro and in vivo studies. For abbreviations see page 169.*

hCG		Estrogen		Progesterone	
Decreased expresion	**Increased expression**	**Decreased expresion**	**Increased expression**	**Decreased expresion**	**Increased expression**
Prolactin IGFBP-1 M-CSF TIMP1-3	COX-2 LIF VEGF MMP-9	COX-1	ER, PR L-selectin HB-EGF IGF-I TGFβ VEGF HOXA10	ER, PR β_3 integrin LIF COX-1	pinopodes MUC-1 L-selectin HB-EGF IL-1 CSF-1 TNFα TGFβ IGF-II Calcitonin VEGF(-R) NOS GdA HOXA10

1997). In the peri-implantation period a marked PR_B rise with an increase of total PR level is observed. In the secretory phase PR levels fall sharply, with decreasing expression of PR_A preceded by PR_B. In contrast with the cyclic variation of PR protein, the cellular mRNA content appears to be unchanged during the menstrual cycle (Ingamells *et al.,* 1996). This suggests a post-transcriptional PR expression regulation. Both steroid receptors are present in endometrial epithelial and stromal cells (Lessey *et al.,* 1988). PR synthesis is mediated by E through ER. ER and PR levels increase during the endometrial proliferative phase, and are maximal at the pre-ovulatory and immediate post-ovulatory period (Lessey *et al.,* 1988). After ovulation the serum concentrations of E and P decrease and increase, respectively (Figure **1.1**). P inhibits the ER synthesis and as result, PR is no longer upregulated. Both receptor levels fall sharply in epithelial cells after ovulation (Lessey *et al.,* 1988). In contrast with ER, PR staining shows continue expression in stromal cells (Figure **1.4**) (Bergeron *et al.,* 1988; Garcia *et al.,* 1988; Lessey *et al.,* 1988). E and P modulate the expression of many putative markers of endometrial maturation / receptivity (Table **1.2**).

1.2.2 Human chorionic gonadotropin (hCG)

hCG is member of the glycoprotein hormone family which also contains FSH, LH and TSH. The α-subunit is the same in all members, the β-subunit is different for each member and is coded by chromosome 19 (hCG, LH, TSH) or chromosome 11 (FSH). The β-subunit of LH and hCG hormones have a 96% identical peptide sequence, which allows these hormones to share the same hCG/LH receptor. A recent study showed that this receptor expressed cycle-dependent in endometrium (Licht *et al.,* 2003b). Besides the gonads, this receptor is present in many other tissues (Licht *et al.,* 2003b). hCG is produced in larger quantities by the embryonic synctiotrophoblast after fusion with cytotrophoblast. But before this trophoblast differentiation hCG transcribed from the genes in the 8-cell embryo's (Bonduelle *et al.,* 1988) and production detected in blastocysts (Lopata *et al.,* 1989). This hormone may considered as one of the first early regulatory factor in the implantation process of the embryo into the endometrium (Table **1.2**).

Intracavitary administered hCG resulted in a significant decrease of intrauterine production of Prolactin, IGFBP-1 (Licht *et al.,* 1998; Licht *et al.,* 2002), and M-CSF (Licht *et al.,* 2002). A significant increase of COX-2 enzyme (Zhou *et al.,* 1999), LIF (Licht *et al.,* 2002; Perrier d'Hauterive S. *et al.,* 2004), VEGF (Licht *et al.,* 2002; Licht *et al.,* 2003a), MMP-9 (Licht *et al.,* 2001b), and trophinin (Sugihara *et al.,* 2008) was observed.

1.2.3 Cytokines, chemokines and growth factors

These factors belong to an autocrine and paracrine acting family of (glyco) proteins with an activating, proliferative and differentiating effect on various tissues.

The *IL-1 family* consists of 3 related polypeptides: two agonists (IL-1α and IL-1β) and a competitive receptor antagonist (IL-1ra). IL-1β appears in luteal human endometrium (Kauma *et al.,* 1990) and is localized in the stromal

compartment (Simon *et al.,* 1993). Furthermore, IL-1β is produced by the blastocyst probably as response on receptive endometrium, before and after embryo implantation (De los Santos *et al.,* 1996).

IL-6 is up-regulated in the secretory period of human endometrium, predominantly in glandular and luminal epithelial cells (Tabibzadeh *et al.,* 1995a; Vandermolen *et al.,* 1996; Von Wolff *et al.,* 2000). Furthermore, the IL-6 protein levels in the endometrial secretion fluid (Table **1.4**) were also increased in the secretory phase of the menstrual cycle (Von Wolff *et al.,* 2002). Endometrial IL-6 expression drops in the late secretory phase (Tabibzadeh *et al.,* 1995a). High serum E levels have detrimental effects on epithelial IL-6 expression but not on endometrial secretions during IVF treatment (Makkar *et al.,* 2006).

IL-11 protein, which is a member of the IL-6 family, is present on glandular and luminal epithelial cells wit a menstrual cycle variation, and weak but more constant in the stroma (Cork *et al.,* 2001; Dimitriadis *et al.,* 2000; Linjawi *et al.,* 2004). IL-11 also appeared in endometrial secretion (Table **1.4**) retrieved by flushing (Makkar *et al.,* 2006).

The chemo-attractant cytokines (chemokines) include *IL-8*, *MCP* (monocyte chemo-attractant protein)-1 and 3. IL-8 and MCP-1 have been localized in human glandular and luminal epithelium as well as to the endothelial cells and are up-regulated by the administration of progesterone (Luk *et al.,* 2008). IL-12 showed decreased mRNA content in secretory endometrium compared to recurrent spontaneous abortion and idiopathic subfertile women (Ledee-Bataille *et al.,* 2004a; Lim *et al.,* 2000).

IL-13 and *IL-15* protein and mRNA expression occurs throughout the menstrual cycle, with a peak in the peri-ovulatory period (Chegini *et al.,* 2002). Both cytokines have been reported to be more highly expressed in women with recurrent abortion (Chegini *et al.,* 2002).

IL-18 is expressed in endometrial tissue (Ledee-Bataille *et al.,* 2004a) and endometrial secretion fluid (Table **1.4**) (Ledee-Bataille *et al.,* 2004b). In normal fertile women a 100% moderate IL-18 staining was demonstrated, but idiopathic subfertile women expressed 37% weak, 17% moderate and in 46% strong tissue staining. IL-18 may affect activity of uNK cells which are involved in invasion of the trophoblast (Croy *et al.,* 2003).

CSF-1 (colony stimulating factor-1) (Kauma *et al.,* 1991; Pampfer *et al.,* 1992; Von Wolff *et al.,* 2000), *EGF* (epidermal growth factor), *TGFβ* (transforming growth factorβ) superfamily, *IGF* (Insulin like growth factor) system and HB-EGF are expressed in endometrial tissue during the whole menstrual cycle (Figure **1.4**) and are increased by ovarian steroids (Table **1.2**). TGFβ-1 (Polli *et al.,* 1996) and activin A (Florio *et al.,* 2003) are also present in endometrial secretions (Table **1.4**).

LIF (leukemia inhibitory factor) is an highly glycosylated 40-50 kDa glycoprotein which is a member of the IL-6 family. LIF mRNA and protein expression in human endometrial luminal and glandular epithelium is observed during the whole menstrual cycle (Figure **1.4**), but is increased in the mid- and late secretory phase with a maximum on cycle day 21 to 26 (Arici *et al.,* 1995; Charnock-Jones *et al.,* 1994; Chen *et al.,* 1995; Vogiagis *et al.,* 1996). LIF is also found in uterine flushings from 6th day with a peak on the 12th day from

the LH surge (Table **1.4**). In normal cycling fertile women a higher LIF level was observed in flushings compared to idiopathic infertile women (Laird *et al.,* 1997). Moreover, endometrial cells *in vitro* from fertile women produced more LIF in culture medium than from infertile women (Hambartsoumian, 1998), and subfertile women with hydrosalpinges and initial decreased LIF expression showed an increased LIF expression following a salpingectomy (Seli *et al.,* 2005). Endometrial LIF expression was not directly altered by ovarian steroids (Table **1.2**), but by paracrine and/or autocrine pathways with leptin, TNFα, IGF, TGFβ, and hCG (Gonzalez *et al.,* 2004; Kimber, 2005; Perrier d'Hauterive S. *et al.,* 2004). hCG produced in blastocysts may increase local LIF production in endometrial cells and lumen, and may induce directly and locally increased endometrial receptivity. LIF receptors contain two chains: an α-chain (LIF-Rα) and a glycoprotein 130 (gp130). The α-chain induces gp130 dimerization after the cytokine binds the α-chain, and gp130 will be utilized for further signal transduction. This glycoprotein is also used in signal transduction after binding IL-6 and -11 to their own α-chain (IL-6Rα and IL-11Rα) (Lass *et al.,* 2001). In contrast with LIF mRNA, LIF-R mRNA is expressed in human blastocysts (Charnock-Jones *et al.,* 1994). LIF affects endometrial cells and blastocysts, which also produces LIF itself (Aghajanova, 2004). LIF-R expression may be induced by LIF levels, which are increased in the midluteal phase (Charnock-Jones *et al.,* 1994). The levels of gp130 are increased between 6th and 13th day after the LH surge. Both receptors are observed in endometrial luminal and glandular epithelium (Aghajanova *et al.,* 2003; Cullinan *et al.,* 1996). Subfertile patients have reduced soluble gp130 during the implantation window compared to fertile controls (Sherwin *et al.,* 2002). LIF and LIF-R are increased coexpressed with the pinopodes in glandular and luminal epithelial cells respectively during the mid-secretory period (Aghajanova *et al.,* 2003). In infertile women LIF expression in glandular epithelium was decreased in postovulatory endometrium compared to the fertile women (Dimitriadis *et al.,* 2006). Furthermore, luteal LIF expression is 6.4 fold higher in women who became pregnant in a consecutive IVF treatment cycle than who did not conceive (Serafini *et al.,* 2008).

1.2.4 Glycodelin A (GdA)

GdA is a 28 kDa glycoprotein that contains 17% carbohydrates. It is detected in serum, and male and female reproductive tissues (Seppala *et al.,* 1998). As result many names have been used for this protein, such as placental protein 14 (PP14), endometrial protein 15, pregnancy associated endometrial α_2-globulin (α_2PEG), chorionic α_2-microglobulin, α-uterine protein (AUP), progestagen associated endometrial protein (PAEP), and progesterone-dependent protein (PEP).

It is clear that GdA is produced in secretory and decidualized endometrium, and then secreted in the glands and uterine cavity (Figure **1.4**). Serum and endometrial GdA levels are affected by cyclic changing serum concentrations of E and P (Borri *et al.,* 1998; Westergaard *et al.,* 1998). However, in contrast with serum E concentrations, GdA is not entirely correlated with the serum P concentrations. This suggests that GdA is probably also influenced by other

factors. In endometrial tissue GdA can be detected from the 4th postovulatory/retrieval day and increases until the end of the secretory phase (Brown *et al.,* 2000; Klentzeris *et al.,* 1994). Retarded endometrium histology shows a lower GdA expression (Klentzeris *et al.,* 1994). Furthermore, ovarian stimulation for IVF showed increased cellular expression (Brown *et al.,* 2000). GdA is also observed in flushings obtained from the uterine cavity (Table **1.4**) on 5th day after LH surge until the end of the cycle (Li *et al.,* 1993a).

Despite numerous studies focused on GdA, little information is available concerning its precise function, but it is associated with cell recognition and differentiation (Seppala, 2004). GdA has suppressing activity on natural killer cells (Clark *et al.,* 1996; Okamoto *et al.,* 1991), which may offer an immunosuppressive milieu in the uterine cavity, allowing implantation.

1.2.5 Vascular factors

A primary and unique feature of the endometrium is the cyclical increase in vascularization necessary for maturation and receptivity, and the vascular constriction which results in endometrial shedding.

Angiogenic factors

Angiogenesis is the formation of new blood vessels from pre-existing mature vessels. At the present time, many factors have shown to influence direct or indirect the angiogenesis process *in vitro*, such as TNFα (tumor necrosis factor α), a-FGF (acidic fibroblast growth factor), b-FGF (basic fibroblast growth factor), TGF (transforming growth factor) α and β, IL-8, RGF (epidermal growth factor), angiogenin, angiopoetin, HGF (hepatocyte growth factor), PlGF (placental growth factor), $\alpha_v\beta_3$ integrin, leptin and VEGF (vascular endothelial growth factor) (Risau, 1997).

Nitric Oxide (NO)

NO relaxes vascular smooth muscles via the cGMP mediated pathway and as result vasodilatation. Endometrial expression of endothelial nitric oxide synthase (eNOS), one of three enzymes which converts L-Arginine to nitric oxide (NO), is increased in the secretory phase of the menstrual cycle (Khorram *et al.,* 1999), and mainly expressed in endometrial glands (Tseng *et al.,* 1996).

1.2.6 Calcitonin

Calcitonin is a peptide hormone which regulates the calcium metabolism. It is expressed in endometrial glandular epithelium (Ding *et al.,* 1994). After endometrial expression from cycle day 17 until 25 (Figure **1.4**) with a maximum between 19 and 21 (Kumar *et al.,* 1998). Calcitonin is observed in endometrial secretions in animals (Zhu *et al.,* 1998) and the precise role in embryo implantation remains uncertain. Probably the changes in the cellular calcium metabolism results in an alteration of expression of adhesion molecules.

1.2.7 Leptin

Expression of leptin and leptin receptor (OB-R) in endometrial glandular and luminal epithelium is increased in proliferative phase (cd 7 until 14) and

midluteal phase (cd 20 until 24) when compared with the early and late luteal phase (Alfer *et al.,* 2000). Infertile women do not express functional OB-R (Alfer *et al.,* 2000), suggesting a role in fertility, but it remains uncertain which pathway is being used. However, recently a study revealed a signaling role of leptin for lysophosphatidic acid (LPA_3), which is involved in COX-2 metabolism during the implantation process (Ye *et al.,* 2005).

1.2.8 HOXA10 genes

HOX genes are transcriptional factors which play an important role in the tissue identity during the embryonic Müllerian duct development and are expressed in adult life. In mice these genes have shown the importance in endometrial receptivity (Satokata *et al.,* 1995), probably alteration of HOX gene expression may be correlated with implantation failure in humans.

HOXA10 is expressed in the endometrial glands and stroma throughout the menstrual cycle, with increase in the mid-secretory phase until the end of the cycle (Figure **1.4**) (Taylor *et al.,* 1999). HOXA10 genes are up-regulated by E and P (Table **1.2**) (Taylor *et al.,* 1998). Perhaps the HOX genes regulate other genes downstream for an appropriate endometrial maturation and finally receptive endometrium for the embryo during the implantation window.

1.2.9 Prostaglandins (PG) and Cyclooxygenase (COX) system

Embryo implantation into endometrium requires the biosynthesis of prostaglandins (PG). In the PG synthesis pathway, cyclooxygenase (COX) is the rate-limiting enzyme which converts archidonic acid to endoperoxidase (prostaglandin H_2, PGH_2), the common substrate for various PGs (Smith *et al.,* 1996b). Two COX isoforms exists: COX-1, production is inhibited by estrogens and progesterone, and consequently the endometrial expression in glandular and luminal epithelium drops in the midluteal phase of the menstrual cycle; and COX-2, which production is not affected by ovarian steroids but by inflammatory stimuli (Masferrer *et al.,* 1995) and presence of a animal blastocysts (Charpigny *et al.,* 1997).

Little information is available concerning the role of PG in the implantation and placentation process. Local expression of COX-2, probably induced by embryo presence in humans, may result in local production of PG. PG showed increased vascular permeability in animals (Dey *et al.,* 1980) and facilitating local endometrial edema. Increased vascular permeability is in many species one of the first signs of implantation (Psychoyos ,1973).

In gestation PG concentration in decidual cells drop considerably compared to concentrations in the normal menstrual cycle (Maathuis *et al.,* 1978c). Probably pregnancy is maintained by PG synthesis inhibition, and defect in inhibition may result in pregnancy loss (Jaschevatzky *et al.,* 1983).

1.3 PUTATIVE MARKERS OF ENDOMETRIAL RECEPTIVITY

1.3.1 Pinopodes

Pinopodes are dome-shaped cytoplasmatic protrusions which appear on the apical side of endometrial luminal epithelial cells for approximately 48 hours between day 19 and 21 in a normalized menstrual cycle of 28 days (Nikas, 1999a; Nikas *et al.,* 1999b; Psychoyos *et al.,* 1971). Because the protrusions are co-expressed with the loss of steroid receptors and maximal expression of $\alpha_v\beta_3$ integrin, OPN, LIF (Aghajanova *et al.,* 2003; Nikas *et al.,* 2002) and HB-EGF (Stavreus-Evers *et al.,* 2002), and coincide with the 'window of implantation', pinopodes may play a role in embryo implantation.

1.3.2 Mucins

Mucine-1 (MUC-1) & 4 (MUC-4)

MUC-1 is 200 **–** 500 nm long epithelial polymorphic glycosylated transmembrane protein, which extends above the glycocalyx layer on the surface of endometrial luminal epithelium. In contrast with other species, in humans this layer is increased from the early until late endometrial secretory phase (Hey *et al.,* 1994). In flushings obtained from the uterine cavity in the natural menstrual cycle showed increased MUC-1 levels 7 **–** 13 days after LH surge (Figure **1.4**) (Hey *et al.,* 1995). *In vitro* studies showed increased expression with the presence of an embryo and P (Hey *et al.,* 1994; Hoffman *et al.,* 1998). Because the increased thickness of the MUC-1 layer on top of the epithelial surface in the endometrial receptive period, it is suggested that MUC-1 may be a selective barrier to prevent binding of adhesion molecules present on *suboptimal* endometrium and/or embryos. *In vitro* blastocysts induce shedding of the layer by paracrine alteration of the MUC-1 molecule (Meseguer *et al.,* 2001). This may allow embryonal attachment to the endometrium. Another explanation is that MUC-1 may attach embryos initially by binding the selectins on the embryonal trophectoderm, before the embryo finally binds the epithelial surface of the endometrium with other adhesion molecules, such as integrins (Genbacev *et al.,* 2003).

Expression of MUC-4, another mucine which is expressed on glandular and luminal epithelium of the endometrium, was not different in subfertile patients from the fertile patients in the luteal phase (Koscinski *et al.,* 2006). This study showed a focal expression of MUC-4 in certain areas of the endometrial epithelium.

Recently, MUC-16 another membrane associated mucin has been identified which may be associated with endometrial receptivity (Gipson *et al.,* 2007). MUC-16, was lost from uterodome surfaces in all samples taken LH+6 to LH+8. This suggests that MUC-16 prevents cell-adhesion to the endometrial epithelium.

Mouse Ascites Golgi (MAG)

MAG is a mucine molecule that is expressed on the endometrial luminal epithelium on cycle day 18 and 19 in a normalized menstrual cycle of 28 days (Kliman *et al.,* 1995). Abnormal endometrial MAG expression is found in women

with idiopathic subfertility and failed embryo transfer after IVF (Catalanotti *et al.,* 2006).

1.3.3 Adhesion molecules

Adhesion molecules are expressed on the cellular surface for intercellular contact. For these molecules it is possible to bind 2 identical adhesion molecules, such as cadherins, but frequently adhesion molecules interact with molecules with a complementary structure. The adhesion molecule family contains carbohydrate chains (selectins and lectins), integrins, cadherins, immunoglobins and trophinin-tastin-bystin complex.

Carbohydrates

Carbohydrates are probably the first adhesion molecules that are involved in the initial embryo hatching in the implantation cascade. After the embryo is attached to the endometrium through these binding sites, other cell adhesion molecules may contribute to a more stable adhesion to the endometrial epithelium.

P-Selectin, which is expressed on human embryos (Campbell *et al.,* 1995), has shown to interact with fucosylated carbohydrate chains on glycoproteins. These glycoproteins, sialyl-Lewis-x (sialyl-Le-x) (Hey *et al.,* 1996), H-type-1-antigen (H-type-1; Fucα1-2Galβ1-3GlcNAcβ1-) and Lewis-y-carbohydrate (Le-y; Fucα1-2Galβ1-4[Fucα1-3]GlcNAcβ1-) antigens (Ravn *et al.,* 1992), appear on animal blastocysts and on human and animal endometrial luminal and glandular epithelium (Ravn *et al.,* 1994). No embryo implantation has been observed after blocking Le-y antigen glycoprotein with monoclonal antibodies (Wang *et al.,* 1998).

Galectins are members of the lectins family, which contain one or more carbohydrate recognition domains with affinity for β-galactocides (Leffler *et al.,* 2004). Galectins have been shown to play a role in cell adhesion, cell migration and chemotaxis (Almkvist *et al.,* 2004). Galectin-1 and -3 are expressed in the menstrual cycle with increased expression in the secretory phase (von Wolff *et al.,* 2005), predominantly in endometrial stroma and endometrial epithelium respectively. This cyclicity of these galectins suggest involvement in embryo implantation, however, evidence is absent so far.

Recently, a new carbohydrate binding protein, L-Selectin, has been observed on the embryonal surface (Genbacev *et al.,* 2003). Increased L-selectin expression was observed in early and mid-secretory phase of the menstrual cycle (Lai *et al.,* 2005), and the ligand is localized in endometrial glandular and luminal epithelial cells (Wang *et al.,* 2008). Implantation and pregnancy rates were higher in women with higher levels of L-Selectin ligand in the early secretory phase (Foulk *et al.,* 2007; Wang *et al.,* 2008). Therefore, it has been suggested that this protein is also involved in implantation by hatching of the embryo on the epithelial cell of the endometrium before it interacts more firmly with integrins.

Integrins

Integrins are transmembrane heterodimeric glycoproteins, which consists of an α- and a β-chain. These adhesion molecules are involved in intercellular and cell-

ECM binding in the whole body, and can bind another adhesion molecules and ECM components.

Cell adhesion molecules have also been shown to be of importance in the development of endometrial receptivity. Three integrins $\alpha_1\beta_1$, $\alpha_4\beta_1$ and $\alpha_V\beta_3$ are expressed (Figure **1.4**) on the luminal endometrial epithelium during the secretory phase, framing the 'window of implantation' (Lessey *et al.,* 1992). Expression of $\alpha_V\beta_3$ starts from cycle day 19 – 20 in a 28 day menstrual cycle until pregnancy is established, or begin of a new cycle. The other 2 integrins, $\alpha_1\beta_1$ and $\alpha_4\beta_1$, are expressed from ovulation until both disappears on cycle day 24 and 28, respectively (Acosta *et al.,* 2000; Creus *et al.,* 1998; Lessey *et al.,* 1994). On the apical side of the endometrial epithelium $\alpha_V\beta_3$ is co-expressed with pinopodes (Nikas *et al.,* 2002) and with osteopontin (OPN) (Apparao *et al.,* 2001) in the secretory period. OPN is a 70 kDa adhesion protein with a RGD sequence which is a binding site for $\alpha_V\beta_3$ (Lessey *et al.,* 1994; Lessey, 1998a) and several other integrins (Bayless *et al.,* 1998; Hu *et al.,* 1995; Liaw *et al.,* 1995; Smith *et al.,* 1996a) and has been detected on glandular epithelial cells (Apparao *et al.,* 2001; Von Wolff *et al.,* 2001a, 2001b) and in uterine secretions (Von Wolff *et al.,* 2001a,2001b) during the secretory phase of the menstrual cycle. Furthermore, $\alpha_V\beta_3$ and OPN are both present on endometrial, trophoblast, and pre-implantation embryonal cells (Campbell *et al.,* 1995).

Down regulation of $\alpha_V\beta_3$ by E and P (Lessey *et al.,* 1998b) indicates that implantation and receptivity may arise as a result of a downregulation of ERs and PRs during the mid-secretory phase (Creus *et al.,* 2002; Garcia *et al.,* 1988; Lessey *et al.,* 1988). Furthermore, endometrial epithelium showed increased β_3 containing integrins during co-culture experiments in presence of a blastocyst. Probably, this increase of β_3 containing integrins was mediated by embryo produced IL-1 (Simon *et al.,* 1997). Integrins expression regulation by ovarian hormones may be mediated by cytokines and growth factors, as has been shown in *in vitro* studies (Lessey *et al.,* 1998b). Furthermore, disappearance of PR is related to the increasing expression of β_3 containing integrins (Lessey *et al.,* 1996).

OPN is an ECM component but also a cytokine, produced by leucocytes (Johnson *et al.,* 2003), and endometrial cells during the window of implantation (Apparao *et al.,* 2001). OPN expression is under control of steroid hormones, IL-1, TGFβ, TNFα, and IFNγ (Johnson *et al.,* 2003).

Trophinin-tastin-bystin complex

Trophinin is a transmembrane glycoprotein on trophoblast and epithelial endometrium cells (Fukuda *et al.,* 1995). Tastin (trophinin assisting protein) is a cytoplasmatic protein, necessary for trophinin to act as an adhesion molecule. Tastin connects the intracytoplasmatic part of trophinin with the cytoskeleton (Fukuda *et al.,* 1995). The expression of trophinin on the endometrial cells shows some dense spots ('plaques'), which may act as binding sites for the embryo (Fukuda *et al.,* 1995). Intercellular protein bystin is necessary to connect two trophinin molecules of two different cells (Suzuki *et al.,* 1998).

Trophinin is expressed on human endometrial cells on cycle day 16 and 17 in a normalized cycle of 28 days (Fukuda *et al.,* 1995). Then it disappears and is

only present in the mucus glycocalyx layer, to appear again in trophoblast at the uteroplacental interface (Suzuki *et al.,* 1999). *In vitro* studies showed trophinin expression in endometrial cells is induced by hCG and IL-1β (Sugihara *et al.,* 2008).

1.4 THE PROBLEM OF STUDYING RECEPTIVITY DURING IVF TREATMENT CYCLES

To study markers of endometrial receptivity and/or maturation (see Chapter **1.3**) invasive retrieval of endometrial tissue is required. So far, only 4 studies (Table **1.3**) studied the effects of transcervical biopsy of the endometrium in a conception or treatment cycle (Abate *et al.,* 1987; Kolibianakis *et al.,* 2002a; Papanikolaou *et al.,* 2005; Ubaldi *et al.,* 1997). These studies showed no adverse effect on pregnancy rates (34%) after invasive retrieval of endometrial tissue and ET into the biopsied uterus. However, in all four studies the endometrial biopsy was performed before the putative endometrial 'implantation window' (i.e. before 5 days postovulation or post OPU), and in 3 of the four studies biopsy and ET was not on the same day.

Table 1.3. *Literature overview of transcervical endometrial biopsy in conception/IVF cycles with the number of ongoing pregnancies and early pregnancy loss (EPL) or spontaneous abortion (%).*

	Pts	Ovarian stimulation	Biopsy	ET	Pregnancy (%)	
					term	EPL
Abate *et al.,* 1987	24	??	OPU+1.5d	OPU+1.5d	6 (25)	9 (38)
Ubaldi *et al.,* 1997	60	hMG+agonist hCG	OPU	OPU+2d	16 (27)	1 (2)
Kolibianakis *et al.,* 2002a	55	FSH+antagonist hCG	OPU	OPU+3/5d	8 (15)	6 (11)
Papanikolaou *et al.,* 2005	8	FSH+antagonist hCG	OPU-2d	OPU+3/5d	3 (38)	1 (12)
Van der Gaast *et al*, 2000 (not published)	10	FSH+agonist hCG	OPU+5d	OPU+5d	0 (0)	2 (20)
Total	**157**				**33 (21)**	**19 (12)**

Pts = patients; OPU = oocyte pick up; d = day; ET = embryo transfer; EPL = early pregnancy loss; hMG= human menopausal gonadotropin; FSH = follicle stimulating hormone; agonist = GnRH agonist; antagonist = GnRH antagonist; hCG = human chorionic gonadotropin

1.5 ANALYSIS OF ENDOMETRIAL SECRETION FLUID: A POTENTIAL NON-INVASIVE TECHNIQUE FOR ASSESSING ENDOMETRIAL MATURATION AND RECEPTIVITY.

A non-invasive technique was developed in the late sixties to obtain endometrial secretion fluids *in vivo* by flushing, washing or aspiration of the uterine cavity with a transcervically inserted flexible catheter.

1.5.1 Composition of endometrial fluid

Fluid in the uterine cavity is produced by glandular secretion and transudation from stromal blood vessels in the endometrium. Composition of endometrial fluid are proteins, amino acids, electrolytes, glucose, urea, cytokines and growth factors, metalloproteinases and their inhibitors, and cells. The composition varies during the menstrual cycle as result of changing ovarian steroids, and may provide a nourishing environment for ascending sperm cells and descending blastocyst, and defense mechanism against bacteria and viruses.

Proteins

These intracavitary fluid proteins comprises immunoglobins, protease inhibitors, albumin, transferin, lactoferin, lysosome, β-amylase and other glycosides, and are secretional products of the endometrial glands, transudate from the vessels, and cellular content of the exfoliated endometrial cells from the lining (Bernstein *et al.,* 1971; Wolf *et al.,* 1975). Table **1.4** is an overview of studies using transcervical aspirations and flushings in humans. The protein composition, expressed as an electropheretic profile, comprises proteins with molecular weight between 6.5 and 68 kDa (Beier *et al.,* 1998; Beier-Hellwig *et al.,* 1988; Beier-Hellwig *et al.,* 1989; Daniel, Jr. 1973; Maathuis *et al.,* 1978b; Roberts *et al.,* 1976; Shirai *et al.,* 1972; Wolf *et al.,* 1975). This protein profile obtained in the aspirations or flushings changed during the menstrual cycle (Figure **1.5**). Postovulatory up-regulation of expression of the 12 – 18 kDa region reflects an adequate endometrial maturation, and a receptive uterine micro-environment may be considered for embryos to implant (Beier-Hellwig *et al.,* 1989; Beier-Hellwig *et al.,* 1994). This region contain α-globulin, β-globulin, histones (H2A, H2B, H3, H4), haptoglobin and cyclophilin A (Beier *et al.,* 1998). Human endometrial tissue cultures secrete about 20 proteins into the culture medium. Besides the non-plasma and uterine specific proteins, endometrial fluid also contains serum proteins which may represent blood contamination. However, this was excluded in some studies (Maathuis *et al.,* 1978a; Maathuis *et al.,* 1978b; Shirai *et al.,* 1972), therefore these proteins may be exsudation process through the endometrial lining into the luminal cavity of the uterus. This process depends on ovarian steroids because uterine blood flow and endometrial growth are influenced by E and P.

Electrolytes

Potassium and calcium levels in endometrial fluid show cyclic variation (Casslen *et al.,* 1984), but sodium, chloride, glucose, fructose and urea did not.

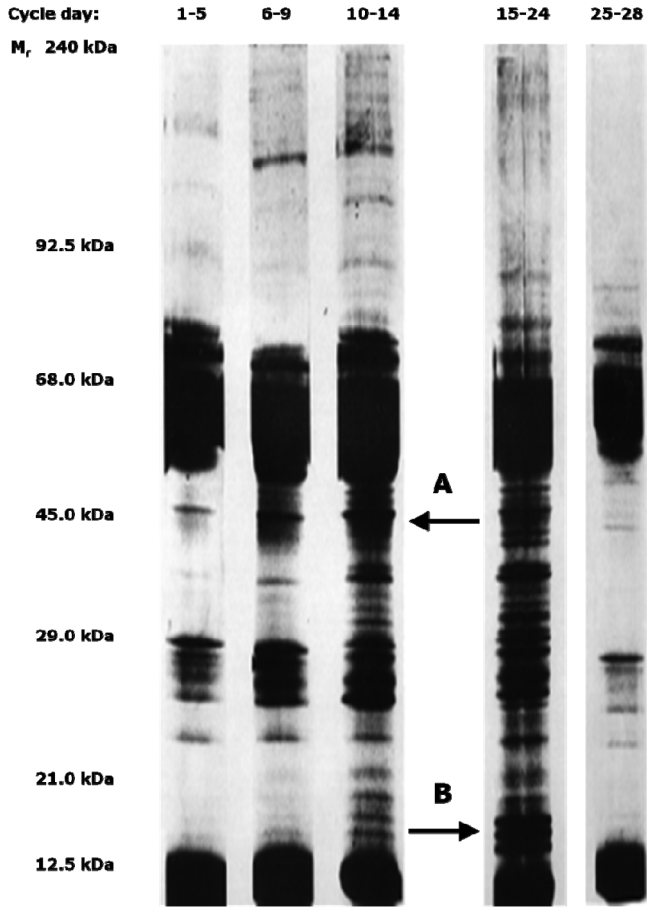


Figure 1.5. *Protein profiles in human endometrial fluid on different cycle days. In the proliferative phase an increased expression is shown in the 34 – 45 kDa region (**A**), while in the secretory phase an increased expression of 12.5 – 18 kDa region (**B**) was observed post-ovulatory.*

Decreased concentrations were observed peri-ovulatory, possibly for facilitation of sperm transport.

Steroids

Ovarian steroid content in endometrial flushings (Table **1.4**) showed minimal cyclic variations during natural menstrual cycles (Bischof *et al.,* 1984; Fazleabas *et al.,* 1987; Stone *et al.,* 1986). However, in these studies each patient was flushed once during the study cycle, and the timing of the flushing was determined with cycle history and endometrial biopsy dating without confirmation with TVS or LH testing.

Cytokines, growth factors and angiogenic factors

Cytokines and growth factors observed in uterine fluid are LIF, GdA, IL-1, IL-6, IL-11, IL-18, LIF, EGF, CSF, VEGF, IGFBP-1 in transcervical flushings (Table **1.4**), and washings in posthysterectomy specimens (Von Wolff *et al.,* 2002), intra-uterine continuous microdialysis system (Licht *et al.,* 2001b).

LIF showed an decreased level in secretions of normal fertile patients compared to subfertile patients (Laird *et al.,* 1997; Ledee-Bataille *et al.,* 2002).

However, two other studies could not confirm these results (Mikolajczyk *et al.,* 2003; Olivennes *et al.,* 2003).

GdA is only expressed in endometrial secretion fluid in the secretory phase (Bell *et al.,* 1987) from 5 days after the LH surge and onwards (Li *et al.,* 1993a). GdA levels were decreased in patients with subfertility and recurrent spontaneous abortions in the mid- and late secretory phase (Dalton *et al.,* 1995; Dalton *et al.,* 1998; MacKenna *et al.,* 1993). Ovarian stimulation for IVF had no effect on GdA levels (Ng *et al.,* 2004). Patients with intra-uterine pathological conditions, such as polyps and fibroids, showed increased GdA levels (Richlin *et al.,* 2002).

Interleukines *IL-1*, *IL-6*, *IL-11* and *IL-18* have been studied in endometrial secretion fluid after hormone treatment (Table **1.4**). Furthermore, other cytokines and growth factors have been studied with a intra-uterine microdialysis system (IUMD) (Licht *et al.,* 1998; Licht *et al.,* 2001b). This system, which was left *in utero* on 3 different periods in the menstrual cycle (late follicular phase and early and late luteal phase) each time for 8 hours, measured IL-1, IL-6, EGF, M-CSF, LIF, VEGF IGFBP-1, prolactin and hCG. Compared to the microdialysis system of Edwards *et al.* (1968), this novel system is able to measure these parameters dynamic *in vivo* after intra-uterine hCG perfusion. This system showed that hCG altered intra-uterine cytokines and growth factors, but some effects measured with this system may be artefacts as result of this system was kept *in vivo* for a prolonged time.

Cells

Endometrial secretion fluid contain mononuclear phagocytic cells, granulocytes, endometrial glandular cells and some other cells (Casslen *et al.,* 1982). The total amount of cells, as well all cell populations separately, were decreased in the uterine flushings during the luteal phase compared to other cycle periods. Low numbers of phagocytic cells may be important for embryo implantation.

1.5.2 Flushing of uterine cavity: technique and results

Before the discomfort of the flushing procedure was studied (Li *et al.,* 1993c), transcervical endometrial flushing in humans had been performed for approximately 25 years, since 1968 (Kar *et al.,* 1968). Flushing of the endometrial cavity was not more painful than endometrial tissue sampling with a Pipelle, but the discomfort was more than undergoing a cervical PAP smear or antecubital blood sampling. No major complications occurred in the 90 flushing procedures.

Flushing showed no effect on pregnancy rate when performed in IVF treatment cycles (Ledee-Bataille *et al.,* 2004b; Olivennes *et al.,* 2003). However, in these two studies flushing was carried out on the day of oocyte retrieval, not in the luteal phase during the 'implantation window'.

Flushing during ovarian stimulation for IVF has been studied in three studies (Ledee-Bataille *et al.,* 2004b; Ng *et al.,* 2004; Olivennes *et al.,* 2003), but in 2 studies an ovarian stimulation treatment was compared with patients without treatment. In these studies no effect of the stimulation was shown on LIF and GdA expression (Ng *et al.,* 2004; Olivennes *et al.,* 2003). In 2 of the 3 studies

an embryo was transferred in the same cycle as the flushing was performed (Ledee-Bataille *et al.,* 2004b; Olivennes *et al.,* 2003).

1.6 STUDY OBJECTIVES

The main focus of this thesis is the impact of ovarian stimulation on endometrial maturation, and particularly receptivity for implantation in the 'implantation window'.

In **Section I** the following questions are addressed:

- what is the current knowledge of the effect of ovarian stimulation on the corpus luteum function, endometrium and implantation process.
- is it possible to define an optimal number of retrieved oocytes which has a minimal impact on endometrial maturation and receptivity and enough choice to transfer the best quality embryo, and which leads to a maximal pregnancy rate.
- what are the endometrial gene expression changes during the putative 'window of implantation' :
  - comparing natural and IVF stimulated cycles.
  - comparing with and without progesterone luteal support.

In **Section II**, a novel approach to obtaining endometrial tissue for analysis is investigated:

- to study in safety and feasibility of obtaining endometrial secretion fluid just prior to ET in a conception-treatment IVF cycle. Determining safety by comparing the pregnancy rates with a matched controlled cohort group. The amount of obtained fluid was determined.
- to compare endometrial secretion fluid markers with conventional endometrial tissue markers of maturation.
- assessment of cellular and functional endometrial secretion response to ovarian stimulation in IVF cycles, compared to natural cycles in the same patients. Furthermore, the impact of luteal P supplementation was determined.

Table 1.4. *Literature overview of transcervical flushing/washing (¶) or aspiration (†) of the uterine cavity for determining protein content/profile, leukemia inhibitory factor (LIF), Interleukin (IL), and Glycodelin-A (GdA), with ovulation determined with menstruation or cycle history (history M/C), basal body temperature (BBT), LH surge (LH), biopsy endometrium (biopsy).*

Marker	Publication	Patients	Aspiration/Flushing #	Aspiration/Flushing time	Results
Protein	Kar *et al.*, 1968†	36 normal fertile pts	1x	history M/C	.↑protein content in pts with IUD.
		144 normal fertile pts+IUD	1x	history M/C	.protein content not different in follicular & luteal phase.
	Shirai *et al.*, 1972¶†	152 normal fertile pts			.protein profile:
		28 group I (es)	1x	BBT + 0-5d	20/28 same protein pattern
		36 group II (ms)	1x	BBT + 6-10d	24/36 same protein pattern
		26 group III (ls)	1x	BBT +11d-M	21/26 same protein pattern
		62 excluded (blood flush)			.flushing & serum different protein pattern.
					.in group II blastokinin (uteroglobin).
	Daniel, Jr. 1973¶	2 normal fertile pts	2x	cd 1 & 8 cd 15 & 23	blastokinin (uteroglobin) on cd23
	Wolf *et al.*, 1975¶	36 subfertile pts (natural cycle) 11 29	1x	historyM/&BBT: preovulatory postovulatory	.protein band at relative mobility 0.64 expressed in luteal phase (highest in midsecretory period). .no blastokinin (uteroglobin) expression.
	Roberts *et al.*, 1976¶	32 normal fertile pts 4 12 9	1x	history M/C: cd 4-14 cd 15-22 cd 23-28	.1-21 mg protein, dip in luteal phase. .10 uterine specific protein bands: 1 protein band is overexpressed late luteal period; others no cycle specificity. .no blastokinin (uteroglobin) expression.
	Voss *et al.*, 1977¶	?? normal fertile pts 7-12 7-12	1x	whole cycle, biopsy: proliferative (p) secretory (s)	.no different protein profile in proliferative & secretory phase. .no uteroglobin expression. .↑binding of P in to proteins in flushing, compared to serum; not different in pre-post-ovulatory flushing. .proteins in uterine fluid originate from selective filtration through endometrium.

pts = patients; RSA = recurrent spontaneous abortion; ; (c)d = (cycle)day; OPU = ovum pick up; OR = under anaesthesia in operation theatre; PR = pregnancy rate; ep / mp / lp = early / mid / late proliferative; es / ms / ls = early / mid / late secretory

Table 1.4. *Continued.*

Marker	Publication	Patients	Aspiration/Flushing #	Aspiration/Flushing time	Results
Protein	Maathuis *et al.*, 1978a¶	55 normal fertile pts 3 ep; 12 mp; 9 lp ; 4 es; 9 ms; 4 ls; 4 excluded	1x	history M/C: preovulatory postovulatory	.protein content same in 3 stages of follicular phase, and same 3 stages of luteal phase. .protein content proliferative > luteal phase.
	Maathuis *et al.*, 1978b¶	52 normal fertile pts 5 ep; 13 mp; 11 lp ; 6 es; 11 ms; 6 ls	1x	history M/C: preovulatory postovulatory	.11 uterine specific protein bands: all without cycle specificity. .no blastokinin (uteroglobin) expression.
	Sylvan *et al.*, 1981†	19 normal fertile pts in OR 8 11		whole cycle, biopsy: proliferative (p) secretory (s)	.protein content not different in both groups. .'peak III' proteins (69kDa), profound present in secretory phase (10/11 pts), almost not in proliferative phase (1/8 pts).
	MacLaughlin *et al.*, 1983¶	18 normal fertile pts in OR 9 9	1x	whole cycle, biopsy: proliferative (p) secretory (s)	.protein content not different in both groups. .↓protein content compared to serum .2D proteomics: .spots flushing < serum .cluster of proteins only expressed in secretory phase
	Sullivan *et al.*, 1984¶	21 normal fertile pts in OR 41 38 5	1x	whole cycle, biopsy: proliferative (p) secretory (s) menstrual (m)	.protein content in flush: p < s < m. .secretory component (SC): s > p > m. (incl IgA) .SC/total protein ratio: no difference p/s/m.
	Cowan *et al.*, 1986¶	18 normal fertile pts	1x	at random in whole cycle	.↑expression uteroglobin like antigen in early & midluteal phase. .not clear whether induced by P or not.

pts = patients; RSA = recurrent spontaneous abortion; ; (c)d = (cycle)day; OPU = ovum pick up; OR = under anaesthesia in operation theatre; PR = pregnancy rate; ep / mp / lp = early / mid / late proliferative; es / ms / ls = early / mid / late secretory

Table 1.4. *Continued.*

Marker	Publication	Patients	Aspiration/Flushing #	Aspiration/Flushing time	Results
Protein	MacLaughlin *et al.,* 1986¶†	56 normal fertile pts in OR	1x	whole cycle, biopsy:	.protein content in flushing/aspiration: .es **<** p < ms < ls .flushing not different from aspiration.
		57		proliferative (p)	.2D proteomics: .aspiration had greater spot resolution.
		15, 12, 15		secretory (es, ms, ls)	.different pattern for serum & flushing. .cyclic changes in protein patterns better observed in aspirations compared to flushings, but no significant changes detectable with available techniques. .27 uterine specific proteins, from which 8 expressed in all uterine fluid samples.
	Cowan *et al.,* 1986¶	18 normal fertile pts	1x	at random in whole cycle	.↑expression uteroglobin like antigen in early & midluteal phase. .not clear whether induced by P or not.
	Singh *et al.,* 1993¶	30 normal fertile pts 30 subfertile pts	??x ??x	history M/C: cd 5-8 (ep) cd 9-12 (lp) cd 13-16 (ov) cd 17-20 (es) cd 21-24 (ms) cd 25-28 (ls)	.↑protein content in infertile pts, in all periods of the cycle. .largest difference in protein content in ov. .both pts groups ep,lp,ov > es,ms,ls.
	Beier-Hellwig *et al.,* 1989† Beier-Hellwig *et al.,* 1994†	>300 subfertile pts	1x	at random in whole cycle	protein profile in menstrual cycle.
	Müller-Schöttle *et al.,* 1999†	115 normal fertile pts	1x	whole cycle, biopsy:	ELISA uteroglobin analysis: .secretion fluid: p < es < ms > ls (ms significant the highest level compared with other 3 periods) .secretion fluid > tissue.
		5		cd 3-14 (p)	
		32		cd 15-19 (es)	
		29		cd 20-23 (ms)	
		32		cd 34-28 (ls)	

pts = patients; RSA = recurrent spontaneous abortion; ; (c)d = (cycle)day; OPU = ovum pick up; OR = under anaesthesia in operation theatre; PR = pregnancy rate; ep / mp / lp = early / mid / late proliferative; es / ms / ls = early / mid / late secretory

Table 1.4. *Continued.*

Marker	Publication	Patients	Aspiration/Flushing #	Aspiration/Flushing time	Results
GdA	Bell *et al.*, 1987¶	31 normal fertile pts	1x	history M/C	.follicular phase: 0/16 GdA +ve flushing. .luteal phase: 7/15 GdA +ve flushing.
	MacKenna *et al.*, 1993¶	12 normal fertile pts 16 subfertile pts	1-3x 1-3x	LH+7/10/12d LH+7/10/12d	.GdA content not different in both groups LH+7d. .↓GdA content in subfertile pts on LH+10 & 12d. .content in flushing volume, not related to protein.
	Li *et al.*, 1993a¶	29 subfertile/RSA pts	1x	LH+2.5-9d (& biopsy)	.GdA +ve flushing from LH+5d. .+ve flushing is related with 'in phase' endometrium histology. .content in flushing volume, not related to protein.
	Li *et al.*, 1993b¶	23 normal fertile pts: 7 16	1x 2-7x	LH+10-14d in whole cycle	.↑GdA content in secretory phase, from LH+6d; maximal in LH+10-14d. .content in flushing volume, not related to protein. .significant positive correlation flushing and serum GdA .
	Dalton *et al.*, 1995¶	8 normal fertile pts 35 RSA pts	1-7x 1x	luteal phase LH+7	.GdA +ve flushing from LH+6d. .↓GdA content in RSA pts.
	Dalton *et al.*, 1998¶	15 normal fertile pts	1x 2x	LH+10/12d LH+10 & 12d	.↓GdA content in RSA pts on LH+10 & 12d. .↓GdA content on LH+10/12 in RSA pts conceived in subsequent cycle.
		49 RSA pts	1x	LH+7/10/12d	.correlation GdA serum-secretion (r=0.569; P<0.05). .content in flushing volume, not related to protein.
	Hamilton *et al.*, 1998¶	392 subfertile pts	1x	cd 1-16	.↓GdA content in flushing in follicular/proliferative period. .no difference in infertility type. .GdA content related to protein content; better correlation GdA with protein content than with recovered volume.

pts = patients; RSA = recurrent spontaneous abortion; ; (c)d = (cycle)day; OPU = ovum pick up; OR = under anaesthesia in operation theatre; PR = pregnancy rate; ep / mp / lp = early / mid / late proliferative; es / ms / ls = early / mid / late secretory

Table 1.4. *Continued.*

Marker	Publication	Patients	Aspiration/Flushing #	Aspiration/Flushing time	Results
GdA	Richlin *et al.*, 2002	44 subfertile pts: 20 normal cavity 12 myoma 12 polyp	1x	cd 5-14	.↑GdA pts with intra-uterine polyps. .no different GdA content in pts with myoma. .content in flushing volume, not related to protein.
	Ng *et al.*, 2004¶	107 subfertile pts: 36 natural cycles 71 IVF cycles	 1x 1x	 LH+7d hCG+7d	.GdA content in flushing not different in both groups (GdA per volume or per protein content). .GdA content relation with serum E.
	Salim *et al.*, 2007¶	16 normal fertile pts 20 RSA pts 8 pts with septate uterus	1x 1x 1x	LH+7d LH+7d LH+7d	.↑GdA content in normal fertile pts. .no GdA content difference in septate uterus pts and RSA pts.
LIF	Laird *et al.*, 1997¶	21 normal fertile pts 22 subfertile pts;33 RSA pts	1-6x 1x	luteal LH+7/10/12d	.normal pts LIF +ve flushing from LH +7d, max. on LH +12d. .↓LIF content in normal fertile pts compared to subfertile pts (LH+10d).
	Ledee-Bataille *et al.*, 2002¶	33 subfertile pts	1x	cd 26 in 2 natural cycles	↓LIF content in pts who became pregnant in subsequent cycles.
	Olivennes *et al.*, 2003¶	148 subfertile pts +IVF 12 normal fertile pts	1x 0x	OPU (+ET) no flush	.flushing has no effect on PR. .no relation LIF and PR.
	Mikolajczyk *et al.*, 2003¶	16 normal fertile pts 49 subfertile pts; 30 RSA pts	1x 1x	LH+9-11d LH+9-11d	.↓LIF content in subfertile pts. .correlation LIF in serum & flushing.
	Mikolajczyk *et al.*, 2007¶	16 normal fertile pts 54 subfertile pts	1x 1x	O+7-9d O+7-9d	.↓LIF content in subfertile pts. .lowest LIF content in idiopatic subfertile pts. .LIF level at 2.31 pg/ml a 95.7% sensitivity and 81.8% specificity for prediction pregnancy in future.

pts = patients; RSA = recurrent spontaneous abortion; ; (c)d = (cycle)day; OPU = ovum pick up; OR = under anaesthesia in operation theatre; PR = pregnancy rate; ep / mp / lp = early / mid / late proliferative; es / ms / ls = early / mid / late secretory

Table 1.4. *Continued.*

Marker	Publication	Patients	Aspiration/Flushing #	Aspiration/Flushing time	Results
IL-1	Simon *et al.,* 1996¶	20 donor oocyte acceptors +HRT (mock cycle)	3x	cd 9/15/20	.↑IL-1(α &) β expression on cd20. .IL-1ra expression remain the same.
IL-2	Makkar *et al.,* 2008¶	17 subfertile pts 32 subfertile pts +IVF (E on hCG <20000pmol/L) 32 subfertile pts +IVF (E on hCG >20000pmol/L)	1x 1x 1x	LH+7d LH+7d LH+7d	.IL-2 mainly not detectable. .IL-2 in high serum E concentration group.
IL-6 **IL-11**	Makkar *et al.,* 2006¶	17 subfertile pts 31 subfertile pts +IVF (E on hCG <20000pmol/L) 32 subfertile pts +IVF (E on hCG >20000pmol/L)	1x 1x 1x	LH+7d LH+7d LH+7d	.IL-6 no difference in 3 groups. .↓IL-11 in high serum E concentration group.
IL-18	Ledee-Bataille *et al.,* 2004b¶	133 subfertile pts +IVF	1x	OPU (+ET)	.IL-18 -ve flushing: PR 0.38. .IL-18 +ve flushing: PR 0.16.
TGFβ-1	Polli *et al.,* 1996	?? patients IVF	??x	??	.secretion is modulated during the menstrual cycle. .may be controlled by steroids.
Steroids **PAPP-A**	Bischof *et al.,* 1984¶	23 pts with abnormal cycles	1x	whole cycle, biopsy: ep-lp-es-ls	.PAPP-A content significant ep **>** lp & es **<** ls. .E & P content doesn't vary with histological cycle dating, however for P: es > ep = lp = ls.
Steroids **Protein**	Stone *et al.,* 1986¶	73 normal fertile pts in OR	1x	history M/C: cd 6-10 (ep) cd 11-14 (lp) cd 15-20 (es) cd 21-28 (ls)	.no significant variation of steroid hormone concentration & protein content during the menstrual cycle. .significant ↓E,DHEAS & Prolactin content in es, compared to ep+lp+ls. .comparing es with ep+lp+ls.

pts = patients; RSA = recurrent spontaneous abortion; ; (c)d = (cycle)day; OPU = ovum pick up; OR = under anaesthesia in operation theatre; PR = pregnancy rate; ep / mp / lp = early / mid / late proliferative; es / ms / ls = early / mid / late secretory

Table 1.4. *Continued.*

Marker	Publication	Patients	Aspiration/Flushing #	Aspiration/Flushing time	Results
Steroids	Fazleabas *et al.*, 1987¶	29 normal fertile pts in OR 16 endometriosis pts in OR	1x 1x	history M/C: cd 0-8 (ep) cd 9-14 (lp) cd 15-22 (es) cd 23-28 (ls)	.no statistical protein content variation during menstrual cycle; no difference in normal & endometriosis pts. .no statistical P content variation during menstrual cycle; ↑P serum in es & ls. .P content in ls in normal **>** endometriosis pts. .protease inhibitor activity ep **<** lp, & es **>** ls.
MUC	Hey *et al.*, 1995¶	16 normal fertile pts 41 RSA pts	3x 3x	LH+7,10,13d LH+7,10,13d	.47/57 +ve flushing in all pts. .↑MUC content after LH+6d. .↓MUC content in RSA pts, significant in LH+10d.
Amylase	Singh, 1995¶	30 normal fertile pts 30 subfertile pts	??x ??x	history M/C: cd 5-8 (ep) cd 9-12 (lp) cd 13-16 (ov) cd 17-20 (es) cd 21-24 (ms) cd 25-28 (ls)	.amylase vary during menstrual cycle. .↓amylase content in subfertile pts.
Activin A	Florio *et al.*, 2003¶	25 normal fertile pts	1x	whole cycle, biopsy: proliferative (p) secretory (s)	.Activin A content significant p **<** s. .positive correlation Activin A with cd and endometrial thickness assessed by US.

pts = patients; RSA = recurrent spontaneous abortion; ; (c)d = (cycle)day; OPU = ovum pick up; OR = under anaesthesia in operation theatre; PR = pregnancy rate; ep / mp / lp = early / mid / late proliferative; es / ms / ls = early / mid / late secretory

2.

OVARIAN STIMULATION FOR IVF AND ENDOMETRIAL RECEPTIVITY – THE MISSING LINK.

Mark H. van der Gaast, Nicole G. Beckers, Karin Beier-Hellwig, Henning M. Beier, Nick S. Macklon, Bart C.J.M. Fauser.

Ovarian stimulation for IVF and endometrial receptivity - the missing link.

Reprod BioMed Online ***2002****;5:36-43.*

2.1 INTRODUCTION

During the normal menstrual cycle, the spontaneous LH surge results in luteinization of theca and granulosa cells, a switch from the predominant production of E to P, the induction of final stages of oocyte maturation, and subsequent follicle rupture and release of the oocyte. Modified granulosa cells represent the most important P producing cells of the corpus luteum. LH is the principle trophic hormone for the corpus luteum (Filicori *et al.,* 1984; Hutchison *et al.,* 1984) and diminished of LH support induces luteolysis (Casper *et al.,* 1979). It was confirmed that continued support of the corpus luteum by LH is critical and that luteal regression becomes non-reversible in case endogenous support is lacking beyond 72 hours (Dubourdieu *et al.,* 1991; Hutchinson-Williams *et al.,* 1990; Polson *et al.,* 1987; Weissman *et al.,* 1996). The pulsatile release of LH is closely controlled by E and P feedback actions (Nippoldt *et al.,* 1989). Adequate corpus luteum function is required for conception to occur. Luteal regression during the normal menstrual cycle is caused by reduced responsiveness of the ageing corpus luteum to LH (Zeleznik, 1998), which can be overcome by increasing doses of LH or human chorion gonadotropin (hCG) (Duffy *et al.,* 1999). At present most centers carrying out in vitro fertilization (IVF) employ high dose gonadotropin ovarian stimulation regimens in order to harvest as many oocytes as possible. This approach allows the generation of numerous embryos, enabling selection of high quality embryos for transfer into the uterine cavity (Templeton *et al.,* 1998). The non-physiological hormonal milieu thus created may have detrimental consequences for oocyte and embryo quality as well as endometrial receptivity, either directly or indirectly.

Since IVF treatment first became available, significant progress has been made in improving stimulation protocols, preventing premature luteinization, fertilizing oocytes and optimizing embryo culture conditions. Despite these improvements, pregnancy rates remain around 25% per cycle. The effects of multiple follicle development on oocyte quality and fertilization rates *in vitro* have not been clearly established. In this article, the impact of currently applied ovarian stimulation protocols and compounds on corpus luteum function and the conditions required to enable implantation of the embryo are reviewed.

2.2 OVARIAN STIMULATION FOR IVF AND CORPUS LUTEUM DYSFUNCTION

From the very beginning of IVF it became established that ovarian stimulation induces abnormal luteal function (Edwards *et al.,* 1980; Messinis *et al.,* 1987a; Jones Jr., 1996; VandeVoort *et al.,* 1989). Further confirmation of the phenomenon was provided in a recent study of normo-ovulatory volunteers, in whom the induction of development of multiple dominant follicles per sé elicited a shortening of the luteal phase (Hohmann *et al.,* 2001). However the study of the impact of ovarian stimulation on the luteal phase has been complicated by the widespread use of GnRH agonist co-treatment for preventing a premature LH rise. GnRH agonist co-treatment itself has an impact on the luteal phase.

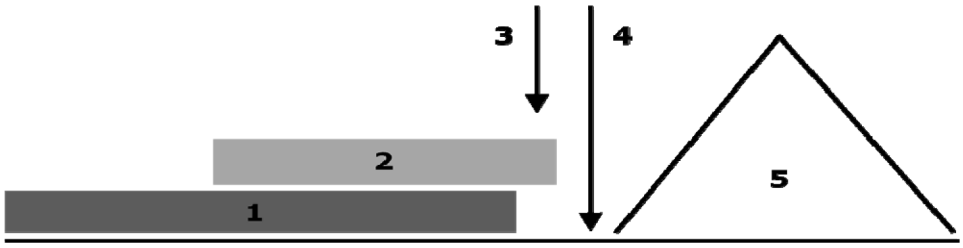


Figure 2.1. *Hypothetical causes of luteal and endometrial dysfunction following ovarian stimulation protocols for IVF.*
(1) GnRH analogue: GnRH agonists / GnRH antagonist co-treatment for the prevention of a premature LH rise.
(2) ovarian stimulation: stimulation of growth of multiple dominant follicles will give rise to multiple corpora lutea and therefore induce abnormal luteal phase steroid secretion.
(3) hCG bolus injection for triggering final stages of oocyte maturation. This can now be replaced by either recombinant LH or a GnRH agonist bolus to induce an endogenous gonadotropin surge.
(4) oocyte retrieval: removal of granulosa cells may impair P production in the luteal phase.
(5) high early luteal phase steroid concentration: supra-physiologic steroid levels may diminish pituitary LH release through negative feedback action, which may induce early luteolysis.

Due to retarded pituitary recovery from down-regulation after late follicular phase cessation of the GnRH agonist (Donderwinkel *et al.,* 1993; Smitz *et al.,* 1992), exogenous luteal support is necessary. Under current ovarian stimulation regimens which include not only exogenous gonadotropins and GnRH agonists but also the administration of a bolus of hCG to induce oocyte maturation, several mechanisms of action underlaying abnormal luteal function following ovarian stimulation for IVF can be proposed (Figure **2.1**). For a review see also Tavaniotou *et al.* (2001b).

2.2.1 Oocyte retrieval and removal of granulosa cells

It may be hypothesized that follicle puncture and removal of the oocyte along with large amounts of cumulus / granulosa cells could interfere with subsequent corpus luteum function, since luteinized granulosa cells form the most important component of P producing cells. Several early studies reported conflicting results (Feichtinger *et al.,* 1982; Garcia *et al.,* 1981; Kerin *et al.,* 1981; Vargyas *et al.,* 1986), and all in all this did not appear to be an important factor. It remains uncertain therefore whether luteal support should be provided in 'natural cycle' IVF where the only intervention is oocyte pick-up (Pelinck *et al.,* 2002).

2.2.2 GnRH analogue co-treatment

Several studies established the profound suppression of pituitary gonadotropin release after cessation of GnRH agonist (Smitz *et al.,* 1988; Sungurtekin *et al.,* 1995).

Earlier cessation of the GnRH agonist co-medication during follicular phase stimulation aimed at enabling recovery of pituitary desensitization around the beginning of the luteal phase was not shown to be successful (Beckers *et al.,* 2000; Pantos *et al.,* 1994). Because of the known rapid recovery of pituitary

gonadotropin release following cessation of the GnRH antagonist, it is tempting to speculate that GnRH antagonist co-treatment can be applied for IVF without disrupting luteal phase endocrinology. Two initial pilot studies regarding the use of GnRH antagonist for IVF without luteal support were inconclusive (Albano *et al.,* 1999; De Jong *et al.,* 2000). However, a recent study focusing on the non-supplemented luteal phase endocrinology after GnRH antagonist use, clearly showed abnormal steroid profiles along with extremely low LH levels and a profoundly reduced luteal phase lengths (Beckers *et al.,* 2003). Moreover, a clear drop in serum LH concentrations was shown during the early luteal phase following HMG stimulation alone for IVF or in association with GnRH antagonist (Tavaniotou *et al.,* 2001a).

2.2.3 Approaches for the induction of final oocyte maturation

A late follicular phase bolus dose of hCG represents the standard of care as substitute for the endogenous LH surge for the induction of oocyte maturation in IVF. Due to the extended circulating half live of hCG compared to LH, exogenous hCG is also implicated in the development of multiple corpora lutea, sustained luteotrophic effects and ovarian stimulation syndrome. Initial protocols for ovarian stimulation without GnRH analogue co-treatment allowed for the administration of a GnRH agonist bolus inducing an endogenous LH (and FSH) surge due to the initial flare-effect. Its use as an alternative to hCG for the induction of oocyte maturation in IVF has been established (Gonen *et al.,* 1990) as well as luteal phase steroid levels closer to normo-ovulatory cycles (Lanzone *et al.,* 1994). A recent study confirmed the use of GnRH agonist for triggering final oocyte maturation after GnRH antagonist co-treatment during ovarian stimulation for IVF (Fauser *et al.,* 2002). Again, luteal phase steroid levels were closer to the physiological range presumably due to the absence of extended corpus luteum support by hCG. However, detailed endocrine studies of the luteal phase without luteal support after alternative approaches for inducing oocyte maturation after GnRH antagonist co-treatment clearly showed advanced luteolysis resulting in decreasing steroid levels, reduced luteal phase length and compromised pregnancy rates (Beckers *et al.,* 2003). Hence, replacing hCG as trigger to induce final stages of oocyte maturation by the short acting recombinant LH or by inducing an endogenous gonadotropin surge does not circumvent the problem of subsequent luteal dysfunction.

2.2.4 Stimulation per se and high early luteal phase steroid levels

During the luteal phase of the normal menstrual cycle, the pulsatile release of LH is tightly controlled by changes in steroid feedback resulting in a decrease in both pulse frequency and amplitude (Filicori *et al.,* 1984; Soules *et al.,* 1984). Normal corpus luteum function is dependent on sustained pituitary gonadotropin secretion throughout the luteal phase (Hutchison *et al.,* 1984). Indeed, corpus luteum function can be sustained by exogenous LH/hCG (Duffy *et al.,* 1999; Zeleznik, 1998) and luteolysis can be induced by extended early suppression of gonadotropin (LH) release in hypogonadotropic hypogonadism (Weissman *et al.,* 1996) or by the administration of GnRH agonist (Casper *et al.,* 1979), GnRH antagonist (Dubourdieu *et al.,* 1991) or estrogens (Choudary *et al.,* 1969;

Hutchison *et al.,* 1987; Johansson *et al.,* 1971; Messinis *et al.,* 1987b; Schoonmaker *et al.,* 1982). It could be confirmed in normo-ovulatory volunteers that even minor elevations in P are capable of reducing gonadotropin secretion (Gibson *et al.,* 1991).

A similar early luteolysis has been observed during the non-supplemented luteal phase after IVF. This condition gives rise to the following paradoxical situation; distinctly increased early luteal phase steroid levels coinciding with an early decrease in steroids and a reduced luteal phase length. This phenomenon was already described during early studies in IVF (for review see Jones Jr., (1996) and has recently been confirmed in detail (Beckers *et al.,* 2000; Beckers *et al.,* 2003). A correlation between luteal phase length and early luteal phase serum E levels could also be confirmed. Indeed, recent studies from our group also established that only women where growth of multiple dominant follicles could be induced present with a reduced luteal phase length emphasizing a direct correlation between follicular and luteal phase events (Hohmann *et al.,* 2001).

Based on the studies discussed above the following mechanism of action may be proposed for luteal dysfunction following ovarian stimulation for IVF: The induction of growth of multiple dominant follicles results in the development of multiple corpora lutea, together producing large amounts of estrogens and progestagen during the early luteal phase. Due to negative feedback actions, pituitary LH release is suppressed inducing advanced luteal regression unless support is provided by means of hCG or LH. Indeed, luteal phase defects are less obvious following a large pre-ovulatory bolus dose of hCG due to the extended half life. In case 10,000 IU is administered, circulating hCG will remain for 7 to 10 days providing continued support of the corpus luteum (Beckers *et al.,* 2000). Further studies should be developed along these lines, to enhance our understanding of luteal defects in IVF patients.

2.3 OVARIAN STIMULATION AND COMPROMISED ENDOMETRIAL RECEPTIVITY

The endometrial morphology changes under direct and indirect control of ovarian sex steroids during the menstrual cycle. After ovulation has initiated the transition of the human endometrium, implantation of the embryo occur between 5th and 10th day thereafter. During ovarian stimulation in the proliferative phase of the treatment cycle, the endometrium is exposed to supraphysiological concentrations of ovarian steroids. In addition, current regimens mean that, like the corpus luteum, the endometrium is also subjected to the impact of GnRH agonists or antagonists. These agents may alter endometrial morphology and cellular adhesion molecule expression in the secretory phase, and may play a role in consequent impaired implantation.

2.3.1 The role of sex steroids in endometrial maturation

For normal endometrial morphology to occur, all that is needed is an appropriate E priming phase followed by a sequence of endometrial changes induced by P.

Exposure to E is a requirement for endometrial priming and proliferation, resulting in the necessary induction of estradiol alpha receptors (ER or ERα) and progesterone receptors (PR). In order to bring about sufficient priming of ER and PR in the nuclei of the endometrial glands and stroma, exposure to E should exceed a concentration and duration threshold. The concept of threshold exposure to E was supported by studies in which the duration of exposure to E without P was varied from 5 days to 6 weeks without altering the quality of endometrial receptivity to the embryo induced by subsequent P exposure (Navot *et al.,* 1991). However, exposure to P in the proliferative phase of endometrial development may have detrimental effects. P antagonizes the proliferative effects of E on the endometrial glands by down regulating ERs, followed by a subsequent disappearance of the PRs (Garcia *et al.,* 1988). This antagonistic relationship with E has led to the suggestion that E/P ratio may be of importance in the secretory transformation of the endometrium.

2.3.2 Impact of ovarian stimulation on endometrial maturation

The consequences of exposing the endometrium to a non-physiological hormonal environment has been the subject of several studies (Bourgain *et al.,* 2002; Cohen *et al.,* 1984; Frydman *et al.,* 1982; Garcia *et al.,* 1984; Graf *et al.,* 1988; Meyer *et al.,* 1999; Sterzik *et al.,* 1988; Ubaldi *et al.,* 1997). These studies indicate that ovarian stimulation, particularly with clomiphene citrate has an adverse affect on the process of endometrial maturation. While approximately 50% of endometrial biopsies performed around the day of embryo transfer showed a delay in endometrial maturation, other studies have indicated that ovarian stimulation with gonadotropins results in advancement of endometrial maturation (Bourgain *et al.,* 2002; Garcia *et al.,* 1984; Meyer *et al.,* 1999; Ubaldi *et al.,* 1997). Moreover, no clinical pregnancies were observed in endometrial advancement of more than 3 days (Ubaldi *et al.,* 1997). Consequently, these studies imply that the embryo will meet poorly receptive endometrium on the day of transfer, and as result the implantation may be impaired. While it has been suggested that one reason for the low implantation rate in IVF treatment maybe a corpus luteum insufficiency following the removal of granulosa cells by follicular aspiration (Kreitmann *et al.,* 1981), one of the studies suggested this not to be the case. More than 50% of the women studied by Sterzik *et al.* (1988) in whom a follicular aspiration was performed showed a normal endometrial morphology. However, almost all the patients with no follicular aspiration had endometrial deficiency or atrophy.

Clinical studies using the oocyte donation model have confirmed the detrimental effect of ovarian stimulation on endometrial receptivity and embryo implantation (Paulson *et al.,* 1990) and the negative influence of supraphysiological E on the day of hCG administration and during the peri-implantation period has been clearly demonstrated (Simon *et al.,* 1995). Recently, the deleterious effects of increasing estradiol levels on *in vitro* embryo development and thus implantation were reported (Valbuena *et al.,* 2001).

The effect of elevated P levels on implantation is less clear. While some studies indicate that premature luteinization, as defined by high P on the day of hCG, is detrimental for IVF outcome (Fanchin *et al.,* 1993; Silverberg *et al.,*

1991) others have shown P elevation to be non-predictive of IVF outcome (Check *et al.,* 1994; Ubaldi *et al.,* 1997). Pellicer *et al.* (1996) found that while increased serum E levels negatively correlated with pregnancy and implantation, serum P showed no correlation. The effect of exposure to ovarian stimulation on endometrial morphology is extensively reviewed in this series of articles by Kolibianakis *et al.* (2002b). The difficulties inherent in studying the endometrium in this clinical context is highlighted by the same authors. The invasive nature of endometrial biopsy limits its application in possible conception cycles, and questions remain over the inter- and intra- observer variation in interpretation of biopsy specimens. *In vitro* models for studying the molecular processes involved in human implantation and the effect of ovarian stimulation on both the endometrial and embryonic components of implantation have revealed much new information and led to the identification of many molecular markers of endometrial receptivity (Pellicer *et al.,* 2002). However, novel, non-invasive and integrative techniques are required to analyze the effect of therapeutic interventions on the endometrium *in vivo*.

2.3.3 Interventions to reduce E levels

Given the evidence for a negative impact of supraphysiological steroid levels on the endometrium, interventions aimed at reducing these levels may prove of benefit. In order to test this, Simon *et al.* (1998a) applied the step down principle for ovarian stimulation (Fauser *et al.,* 1993) in women undergoing IVF who were known to be high responders with the aim of reducing the number of functionally active mature follicles. After the FSH 'threshold' for follicle development (Brown, 1978) was reached, the dose of exogenous FSH was reduced, dropping the FSH concentration below this threshold. The 'FSH window' (Fauser *et al.,* 1997) was thus shortened, leading to a reduction in the number of recruited and mature follicles and a lower serum E level. The implantation rate and pregnancy rate were 29% and 64% respectively in the women treated with the 'step-down' protocol following an unsuccessful hyper-responsive cycle as compared with 9% and 24% respectively in high responders who received the standard protocol in their following cycle. Another approach to reduce E serum levels, that of providing mild ovarian stimulation for IVF has been studied by our group (de Jong *et al.,* 2000; Hohmann *et al.,* 2003). By commencing gonadotropin in the mid follicular phase, the 'FSH window' is extended with a moderate FSH dose. This mild stimulation regimen yields fewer embryos that standard stimulation regimens but these embryos obtained demonstrate improved implantation rates. This may reflect selection at the oocyte level or improved endometrial and embryo quality due to lower E levels (Valbuena *et al.,* 2001).

2.3.4 GnRH antagonists and endometrial receptivity

Initial dose finding studies clearly showed a GnRH antagonist dose dependent decrease in implantation, with the complete absence of pregnancies with doses above 1 mg/day in the follicular phase (The ganirelix dose-finding study group, 1998). A recent meta-analysis of 5 randomized studies comparing GnRH antagonist vs. agonist co-treatment demonstrated certain advantages with GnRH

antagonist use, but also revealed the presence of decreased implantation rates in a clinical setting (Al Inany *et al.,* 2002). The underlying mechanism of decreased implantation remains unclear, but current data suggest that this involves compromised endometrial receptivity rather than embryo quality. Possible mechanisms underlying this observation have been the subject of considerable debate (Diedrich *et al.,* 2001; Hernandez, 2000; Mannaerts *et al.,* 2000). It remains unclear for instance whether this concerns a direct or indirect effect mediated for instance through LH suppression, or whether or not adjustments in treatment regimens may reduce the observed effect of GnRH antagonists on implantation rates.

Evidence for a direct effect of the GnRH antagonist on the endometrium remains sparse. GnRH receptor has been shown to be present in human endometrium with increased expression in the mid-secretory phase compared to the proliferative phase in the epithelial and stromal cells (Casan *et al.,* 1998; Raga *et al.,* 1998). *In vitro* and *in vivo* studies have shown that GnRH controls hCG production and secretion by the placenta, resulting in a parallel expression of GnRH in the placenta and hCG serum concentrations. Studies have been carried out with continuous administration of GnRH agonist during the secretory phase attempting to prevent implantation and induce abortion. Instead, it seemed to enhance implantation compared to the routine 'long protocol' IVF cycles (Fujii *et al.,* 2001), possibly by enhancing trophoblastic hCG release.

2.4 CONCLUSION

The follicle(s) and corpus luteum produce E and P, which are required for the proliferation and subsequent secretory activities in order to render the endometrium receptive to embryo implantation. Due to ovarian stimulation, and GnRH analogue co-treatment, the corpus luteum and subsequently the endometrium function may be compromised, and as a result pregnancy rates may be reduced. Interpreting in vivo data relating to the effects of ovarian stimulation for IVF on the luteal endocrinology and endometrium is complicated by the possible effects of pituitary down regulation with GnRH agonists, the use of hCG to trigger oocyte maturation, and the luteal phase supplementation with hCG or progestins. In order to assess the impact of altered follicular and luteal phase endocrinology on the outcome of stimulated cycles, it is important to consider the rate of early pregnancy loss following spontaneous normo-ovulatory conception (Wilcox *et al.,* 1999; for review see Macklon *et al.,* 2002).

Corpus luteum function may be impaired as a result of pituitary LH suppression due to negative feedback by the supra-physiologic E and P levels during the early luteal phase of IVF cycles. A lack of sufficient support by endogenous LH induces premature luteolysis and a short luteal phase. Excess of E concentrations in the luteal phase appear to reduce endometrial receptivity by altering morphology, endometrial modulators and adhesion molecules, while inadequate corpus luteum function reflected by an abnormal pattern of luteal P concentrations reduce the chance of successful implantation of the embryo.

As demonstrated by new data from our group, GnRH antagonists provide the

opportunity to minimize the degree of ovarian stimulation, potentially remove the need for hormonal intervention in the luteal phase, and make the use of alternatives to hCG such as recombinant human LH possible. Alternative approaches to ovarian stimulation may mitigate against the non-physiological consequences of current standard stimulation protocols. Our group recently carried out studies to assess the concept of extending the duration for which FSH concentrations exceed the 'threshold' level required for dominant growth follicle (Fauser *et al.,* 1997) as a mean of providing moderate ovarian stimulation for IVF (De Jong *et al.,* 2000; Hohmann *et al.,* 2003). Moreover, we have shown preliminary results of endometrial secretory function after either GnRH analogue or GnRH antagonist co-treatment with ovarian stimulation (Van der Gaast *et al.*, unpublished observations, Table **1.3**). These data may imply reduced endometrial receptivity after ovarian stimulation treatment with GnRH antagonist, as recently shown in a meta-analysis by Al Inany *et al.* (2002).

The subtle and complex ways in which the endometrium and corpus luteum responds to a non-physiological endocrine environment remain to be fully elucidated and will continue to challenge those working towards optimizing stimulation regimens for IVF.

3.

OPTIMUM NUMBER OOCYTES FOR A SUCCESFUL FIRST IVF TREATMENT CYCLE.

Mark H. van der Gaast MH, Marinus J.C. Eijkemans, Jeroen B. van der Net, Curt W. Burger, F.E. van Leeuwen, Bart C.J.M. Fauser, Nick S. Macklon.

Optimum number oocytes for a successful first IVF treatment cycle.

Reprod BioMed Online ***2006****;13:476-80.*

3.1 INTRODUCTION

In order to increase pregnancy rates in IVF, ovarian stimulation protocols are employed to stimulate multi-follicular growth and to allow the retrieval of multiple oocytes (Fauser *et al.,* 2005). This strategy allows for the selection of one or more embryos for transfer. When a 'poor response' to a conventional ovarian stimulation results in few oocytes being obtained, the chance of conceiving is low (Tarlatzis *et al.,* 2003). Within the margins of safety required to prevent the complication of ovarian hyperstimulation syndrome, this paradigm suggests that the more oocytes obtained, the higher the chance of conception (Edwards *et al.,* 1996; Fauser *et al.,* 1999). There is, however, increasing evidence that ovarian stimulation and excessive response may have detrimental effects on oocyte and embryo quality (Greb *et al.,* 1997; Pena *et al.,* 2002; Simon *et al.,* 1995; Valbuena *et al.,* 1999), and endometrial receptivity (Bourgain *et al.,* 2003; Devroey *et al.,* 2004; Kodaman *et al.,* 2004; Macklon *et al.,* 2000a). It has been proposed that there may be an optimum range of oocytes for achieving embryo transfer and pregnancy (Fauser *et al.,* 2004; Popovic-Todorovic *et al.,* 2003). There are however, few reliable data available defining this optimal range.

The aim of this study was to test in a large cohort of patients the hypothesis that the relationship between the chance of undergoing ET and achieving a pregnancy versus the number of oocytes obtained at retrieval is represented by a hyperbolic distribution. In addition, we wished to identify the 'optimal' response to ovarian stimulation in terms of number of oocytes for achieving pregnancy. We used a large national database and analyzed it with the multivariate approach to correct the pregnancy rates for various factors which are known to affect ovarian response to hormonal ovarian stimulation (Templeton *et al.,* 1996), and treatment variables, such as gonadotropin dosage end luteal support.

3.2 MATERIAL AND METHODS

3.2.1 Study Population

The study population, study procedures and data collection methods have been described elsewhere (De Boer *et al.,* 2004; Klip *et al.,* 2001). In brief, the OMEGA study was initiated in 1995 to examine the late effects of hormone stimulation. This nationwide retrospective cohort study comprised 19,840 women treated with IVF. Women with subfertility of >1 year duration were included if they had completed at least one IVF treatment cycle between January 1983 (the start of IVF treatment in the Netherlands) and January 1995.

Inclusion criteria for entry into the analysis were: (a) first IVF cycles in which ovarian stimulation was carried out with exogenous gonadotropins along with gonadotropin releasing hormone (GnRH) agonist co-treatment, (b) those in whom oocytes were retrieved, and (c) age between 18 and 45 years. Data from donor oocyte cycles were excluded. In the Netherlands, widespread availability of treatment and standardization of IVF stimulation protocols (gonadotropins

with GnRH agonist co-treatment, final oocyte maturation with human chorionic gonadotropin (hCG), and luteal phase support) developed after 1990 (De Boer *et al.,* 2004). Prior to this date, practice was highly variable, and data relating to cycles carried out earlier than this were excluded. The remaining 7,422 first IVF treatment cycles were used for multivariate analysis.

3.2.2 Statistical Analysis

For each oocyte number the mean and 95% confidence interval (95% CI) was calculated for the pregnancy rate (PR) per ET (PR/ET) and PR per started IVF cycle (PR/C) in the overall cohort using multivariate analysis. In this analysis the relation was assessed for each possible confounding factor. Then the relationship between number of oocytes and IVF outcome was assessed after correction for age, fecundity, cause of subfertility, gonadotropin dosage, whether or not luteal support was provided and type of luteal support, and number of transferred embryos all together, resulting in distribution curves. Cycles in which more than 24 oocytes were obtained were excluded from analysis due to their relative rarity. Subgroup analysis was carried out to evaluate the impact of age over 38 years on the optimum number of oocytes obtained to achieve pregnancy.

In all statistical analyses a *P* value <0.05 was considered statistically significant. The analysis was performed with a commercially available software package (Statistical Package for Social Sciences (SPSS, Chicago, IL, USA)).

3.3 RESULTS

Of the selected 7,422 women who achieved oocyte retrieval in the first IVF cycle, an ET was performed in 6,310 patients (85%). In these women a mean of 2.1 (SD $\pm$ 1.2) embryos were transferred, and 1,776 patients (24%) conceived. The effect of number of retrieved oocytes on the pregnancy rate is shown in Figure **3.1**. In this cohort, the mean number of oocytes at retrieval associated with the highest chance of conceiving per ET (PR/ET) and per started cycle (PR/C) was 13.1 (SD ± 1.2). The fall in pregnancy rates observed when more than this number of oocytes was obtained was not due to decreased ET rate as this remained stable at 93–95% when 4 or more oocytes were obtained.

Age over 38 years was associated with a non-significant increase in the optimal number of oocytes at retrieval for achieving pregnancy following ET (Table **3.1**).

3.4 DISCUSSION

This large cohort study confirms that there is an optimal range of oocytes obtained in response to a given degree of ovarian stimulation for IVF, below and above which outcomes are compromised. The number of oocytes associated with the optimum chance of conceiving after ET was 13. Although this rose to 17 in women above 38 years, this difference did not reach statistical significance. Previous studies addressing the effect of oocyte number on IVF outcome have

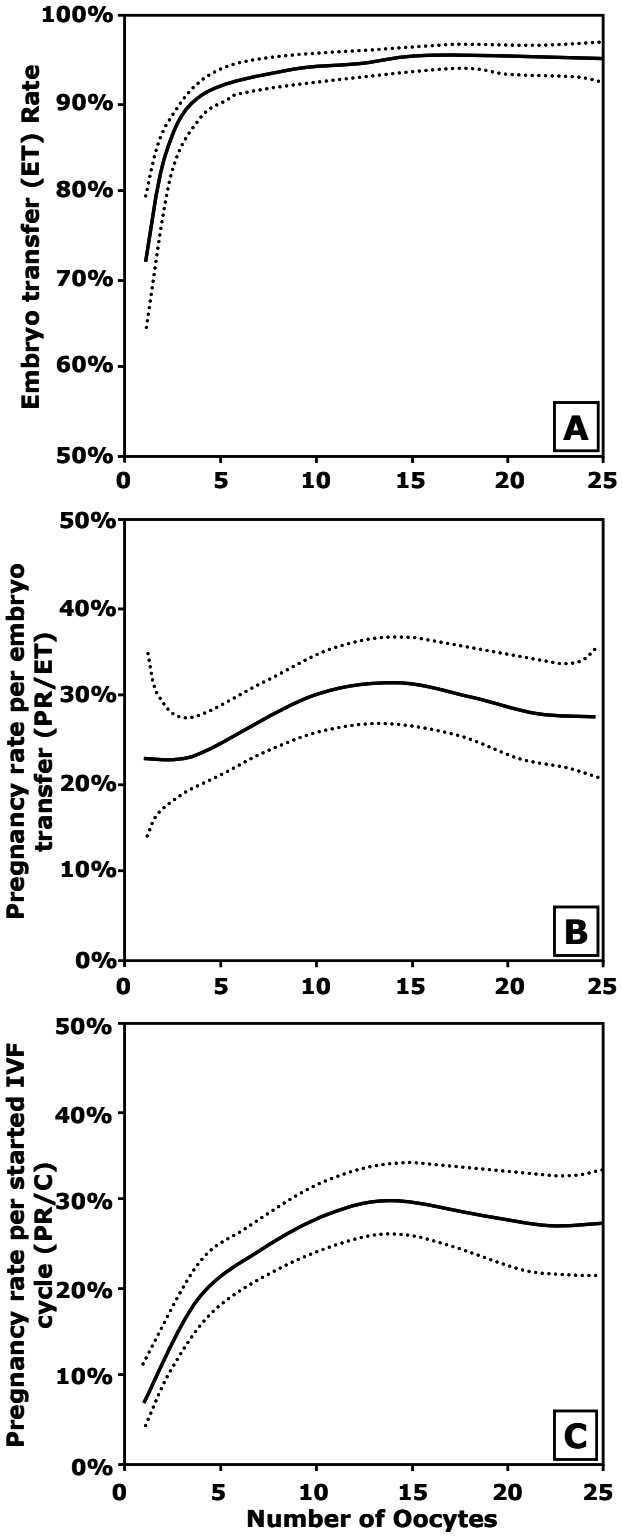


Figure 3.1 *The number of retrieved oocytes (mean with 95% CI) in relation to embryo transfer (ET;* ***A)****, pregnancy per embryo transfer (PR/ET;* ***B****) and pregnancy per started IVF cycle (PR/C;* ***C****). The optimal number of obtained oocytes is 13 to conceive.*

Table 3.1 *Number (mean ± SD) of oocytes, embryos in transfer, and optimal number of oocytes to retrieve for an optimal pregnancy rate per transfer (PR/ET) in the overall group, in the patients of 38 years and older, and patients who had previously delivered.*

	Overall	Age ≥ 38 yrs	Previous live birth
Number of patients	7422	767	1620
Number of oocytes	9.9 ± 6.8	6.9 ± 4.7	10.0 ± 6.8
Number of embryos in transfer	2.1 ± 1.2	2.2 ± 1.2	2.2 ± 1.1
Optimum number of oocytes for PR/ET	13.1 ± 2.7	17.6 ± 2.6	14.4 ± 3.4

shown a clear improvement in outcome in association with increased response to stimulation (Sharma *et al.,* 2002; Templeton *et al.,* 1996; Yih *et al.,* 2005). However, most studies have focused on the impact of poor ovarian response in relation to age and ovarian reserve, and have not addressed this relationship between number of oocytes obtained and outcome across the complete range of ovarian response. In the present study, the fall in pregnancy rates observed when more than 13 oocytes were obtained indicates that ovarian stimulation aimed simply at achieving the maximum harvest of oocytes is ill founded. Indeed, these findings are consistent with the concept that within the cohort of follicles sensitive to stimulation, only those most sensitive to stimulation are likely to yield high quality embryos, and that the additional oocytes obtained in response to maximal stimulation are unlikely to be of sufficient quality to result in conception. However, alternative explanations for the reduction in pregnancy rates when higher number of oocytes are obtained include a direct effect of excessive estradiol on oocyte quality (Fauser *et al.,* 2005; Hohmann *et al.,* 2003), or reduced endometrial receptivity resulting from excessive estradiol levels, as has been previously suggested (Fauser *et al.,* 2005; Macklon *et al.,* 2000a; Simon *et al.,* 1998a; Van der Gaast *et al.,* 2002).

A potential weakness of this study relates to the age of the data, which is derived from IVF cycles carried out 10 – 15 years ago. Since the period in which the data were collected, many improvements have occurred in IVF treatment. These include laboratory performance, stimulation protocols, and embryo transfer techniques. We therefore selected for analysis only those cycles in which the 'long' down-regulation protocol was employed. For many clinics this protocol is still the current standard approach to ovarian stimulation in IVF treatments. Moreover, our analysis aimed to test the hypothesis that the relationship between the chance of ET and pregnancy versus number of oocytes is represented by a hyperbolic distribution. Therefore although modern protocols and laboratory techniques may have improved outcomes generally, the effect on the analyzed relationship is likely to be minor.

The introduction of GnRH antagonist and the development of mild IVF stimulation protocols have encouraged a paradigm shift in relation to ovarian stimulation for IVF (Fauser *et al.,* 1999; Hohmann *et al.,* 2003; Macklon *et al.,* 2000b; Macklon *et al.,* 2006). 'Poor' ovarian response has been shown to be a relative concept, dependent on the degree of ovarian stimulation. In a recent study more than half of the pregnancies obtained following mild stimulation,

occured in women who had produced 4 or less oocytes. However, when similar oocyte numbers were obtained following conventional stimulation no pregnancies were obtained (Hohmann *et al.,* 2003). Moreover, in a recent study in which pre-implantation genetic screening was used to assess embryo quality, aneuploidy rates were reduced in embryos obtained after a mild stimulation protocol compared with a conventional long protocol (Baart *et al.,* 2006). Although fewer oocytes and embryo were obtained following mild stimulation, a median of 2 euploid embryos were obtained following both stimulation protocols. In another study, a high ovarian response was associated with increased number of embryos with chromosome abnormalities compared to controls (Munne, 2006). These studies suggest that excessive ovarian stimulation removes a 'selection pressure' which in the spontaneous cycle, increases the chance that the ovulated oocyte is chromosomally normal. This concept has been previously formulated by Edwards (2003) in which it was suggested that IVF may be interfering with evolutionary pressures, by interfering with 'natural selection' of the 'fittest' oocyte.

It may also therefore be the case that oprimal outcomes following mild stimulation for IVF are associated with a smaller number of obtained oocytes than those observed in the present study. In a randomized study comparing mild to conventional ovarian stimulation, half of all pregnancies after the former regimens occured in women in whom 4 or less oocytes were obtained. No pregnancies occured when 4 or less oocytes were obtained following conventional stimulation. It is therefore likely that 5 – 10 oocytes represent the optimum after mild stimulation, rather than 10 – 15 as seen in the present study. The increasing and welcome trend of transferring fewer embryos reduces the need for multiple embryos in IVF, and may encourage wider implementation of mild ovarian stimulation.

In conclusion, the results of this study show that following correction for other important confounding factors, an optimal number of retrieved oocytes to achieve pregnancy following long stimulation protocols can be identified. Aiming for even moderately high numbers of oocytes might impair IVF outcomes. These data are consistent with the concept argued by Fauser *et al.* (1999) that ovarian stimulation should aim at obtaining the optimal rather than maximal number of oocytes for IVF. The findings of this study support the case for milder ovarian stimulation regimens producing fewer but higher quality oocytes without compromising endometrial receptivity and implantation.

4.

THE IMPACT OF OVARIAN STIMULATION WITH FSH IN COMBINATION WITH GNRH ANTAGONIST ON ENDOMETRIAL GENE EXPRESSION IN THE WINDOW OF IMPLANTATION.

Nick S. Macklon, Mark H. van der Gaast, Amy Hamilton, Bart C.J.M. Fauser, Linda C. Guidice.

The impact of ovarian stimulation with recombinant FSH in combination with GnRH antagonist on the endometrial transcriptome in the window of implantation.

Reprod Sci ***2008****;15:357-65.*

4.1 INTRODUCTION

Ovarian stimulation in the context of IVF treatment aims to generate multiple oocytes which, when fertilized, allow the selection of one or more embryos for transfer into the uterus (Macklon *et al.,* 2006). Current stimulation protocols result in supraphysiological oestradiol concentrations, and there is considerable evidence from rodent (Ma *et al.,* 2003) and human studies (Devroey *et al.,* 2004) that the resultant abnormal steroid milieu is detrimental to endometrial receptivity and therefore to implantation.

The availability of genome-wide microarray techniques provides the opportunity to study the impact of ovarian stimulation on the endometrium at the level of gene expression. The high degree of concordance in gene expression between subjects has facilitated progress in this field (Kao *et al.,* 2002; Talbi *et al.,* 2006), and a number of studies have now been published which demonstrate the relationship between cyclic hormonal changes and gene expression throughout the menstrual cycle. Differences in gene expression have been identified between proliferative and secretory endometrium (Borthwick *et al.,* 2003; Kao *et al.,* 2002) and early-secretory and midsecretory endometrium (Horcajadas *et al.,* 2004; Martin *et al.,* 2002; Riesewijk *et al.,* 2003). The effect of ovarian stimulation on endometrial gene expression has also been subject to microarray gene expression studies. In a previous study, endometrium from oocyte donors was obtained in each donor's natural cycle on day LH+7 and on day hCG+7 in the same donor following stimulation with human menopausal gonadotropin (hMG) and highly purified FSH (FSH HP) in combination with GnRH agonist and no exogenous luteal support (Horcajadas *et al.,* 2005). More than 200 genes were differentially expressed with > 3-fold change in hCG +7 vs. LH +7 (Horcajadas *et al.,* 2005).

In recent years, the introduction of GnRH antagonists into clinical practice has enabled the development of shorter stimulation protocols, since their mode of action allows administration to be limited to a short period at the end of the follicular phase (Tarlatzis *et al.,* 2006). This is in contrast to GnRH agonists, which act by downregulating the pituitary gland; a process which takes around 2 weeks (Macklon *et al.,* 2006). A previous study by Mirkin *et al.* (2004) assessed the impact of recombinant FSH (recFSH) and GnRH antagonist treatment on the endometrial gene expression profile. Data from subjects in natural cycles (on day LH +8) and in others undergoing stimulated cycles (day hCG+9) were compared, with and without micronized progesterone luteal support. A small variation in gene expression between the non-stimulated and stimulated cycles was observed, with 18 genes showing 1.55 – 3.0 fold changes in expression. In a further study, the impact of different doses of GnRH antagonists in combination with recFSH ovarian stimulation on the endometrial transcriptome was addressed (Simon *et al.,* 2005). However, as in the study of Mirkin *et al.* (2004), the subjects undergoing ovarian stimulation did not serve as their own controls in the unstimulated cycle. Moreover, the majority of subjects previously studied received progesterone supplementation in the luteal phase which may have masked the effects of the stimulation protocol itself.

In order to determine the effects of ovarian stimulation with recFSH and co-treatment with GnRH antagonist on endometrial gene expression during the putative 'window of implantation', in the current study, endometrial biopsies were obtained for microarray analysis in the natural and stimulated cycle of 4 oocyte donor volunteers. No progesterone luteal support was administered.

4.2 MATERIAL AND METHODS

4.2.1 Subjects

This study was approved by the ethics review board of the Erasmus Medical Center, Rotterdam, The Netherlands and the Committee on Human Research, the University of California, San Francisco, and a signed written informed consent was obtained. Subjects were oocyte donors for IVF of less than 40 years of age, with regular menstrual cycles of 25 – 35 days duration, no anatomical uterine abnormalities, and proven previous fertility. In order to participate, subjects had to abstain from oral contraception exposure for at least 1 month prior to the study.

4.2.2 Assessments

All participants were initially studied in their spontaneous cycle prior to commencing a stimulation cycle. The subjects were monitored using transvaginal ultrasound on cycle day 2 (to exclude ovarian cysts) and daily from cycle day 8 to detect ovulation in the natural cycle or to time oocyte retrieval in the stimulated IVF cycle.

Ovarian stimulation was initiated on cycle day 2 using a fixed daily dose of 150 IU sc recFSH (Puregon®, Organon, Oss, The Netherlands). To prevent premature luteinisation, GnRH antagonist (Orgalutran®, Organon) 0.25 mg daily sc was initiated on the day that the largest follicle was at least 14 mm in diameter and was continued until at least one follicle was at least 18 mm in diameter. 10000 IU sc hCG (Pregnyl®, Organon) was then administered to trigger final oocyte maturation. Oocyte retrieval was performed 35 hours later. Luteal phase support was not provided.

Endometrial tissue sampling from the uterine fundus was performed with a Pipelle de Cornier® (CDD Laboratoire, France) 5 days after the spontaneous ovulation in the natural cycle, and the ovum pick up in the hyperstimulated cycle. Immediately after the tissue was obtained, it was frozen in a –70°C freezer for later analysis.

4.2.3 Total RNA isolation and microarray preparation

Total RNA was isolated from individual tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The RNA preparations were then Dnase treated and purified using the RNeasy kit Mini Kit (QIAGEN, Valencia, CA.) Some samples with a lower RNA yield were concentrated by adding 10% sodium acetate, 1% glycogen, and 2.5 vol 100% cold ethanol. They were then incubated overnight at –20°C, centrifuged at 4°C for 30 min at 20,000 × g, and washed once with cold 80% ethanol, with air

drying of the RNA pellet. Samples were stored in RNase-free H2O, and the purity was analyzed by both the 260/280 absorbance ratio as well as gel electrophoresis. First and second-strand cDNAs were prepared according to the Affymetrix microarray preparation protocol (Affymetrix, Santa Clara,CA). Samples were then cleaned using the Affymetrix sample clean-up module (Affymetrix). The Enzo BioArray High Yield T7 Transcript labeling kit (Enzo) was used to generate biotinylated cRNA for hybridization to Affymetrix U133 Plus 2.0 chips. Samples were fragmented and hybridized overnight and subsequently scanned using an HR3000 Scanner.

4.2.4 Microarray gene expression data analysis

Samples were processed in GeneSpring data analysis software (Agilent Technologies). The raw data files (.CEL files) which contain the probe level intensities were imported into GeneSpring using the robust multi-array analysis (RMA) which normalizes the data and performs background adjustments. The normalized data were then subjected to statistical analysis and fold change filtration.

In order to identify significant changes in expression levels between the natural and stimulated cycle, a Student's *t*-test was performed to identify genes that were statistically different with a *P*-value <0.05 using a Benjamini-Hochberg multiple testing correction. Statistically significant genes were then subjected to a fold change analysis and genes up or down regulated by 1.5 fold or higher in the Stimulated vs. Natural cycle were retained. This analysis generated fold change values in each gene and measured their statistical significance (*P*-values).

4.2.5 Hierarchical clustering

Hierarchical clustering clusters samples by their expression level similarities. We used a combined gene list of differentially regulated genes throughout the menstrual cycle (Talbi *et al.,* 2006) to cluster the samples according to cycle phase. The output is displayed as a hierarchical tree with branches indicating a mathematical distance between individual samples.

4.2.6 Gene ontologies (GO) clustering

Differentially expressed genes between stimulated cycles and natural cycles were queried for their gene ontology classifications using the GOTree machine web-based analysis from the Oak Ridge National Laboratory (Zhang *et al.,* 2004), maintained by Vanderbilt University, Tennessee, USA (http://bioinfo.vanderbilt.edu/webgestalt). Only genes unique to particular gene clusters were analysed. Gene Ontology categories significantly present in our data set in the major categories of biological processes, molecular functions and cellular components were determined by methods previously described (Talbi *et al.,* 2006).

4.2.7 Microarray validation by real-time PCR

In order to technically validate the data derived from microarray analysis, real-time PCR studies were carried out. 1 µg of total RNA was used to generate cDNA

by reverse transcription using the iScript cDNA synthesis kit (Bio-Rad). Serial dilutions of the template were made and a standard curve for each gene to be analyzed was performed to determine primer efficiency and the optimal dilution range for the template. Samples were run using RPL19 as a normalizer because this stable transcript demonstrates low standard deviation of Ct values in repeated real-time analyses (Talbi *et al.,* 2006). Primers were designed to be intron-spanning in order to correct for genomic contamination. Real-time PCR was performed in triplicate 25 µl reactions using the Stratagene Brilliant SYBR Green Master Mix and the Stratagene MX3005P QPCR machine. Fold change increases and/or decreases in the stimulated samples relative to the normal samples were calculated as previously described (Talbi *et al.,* 2006) and statistical significance was determined by a paired Student's *t*-test on log transformed normalized values.

4.3 RESULTS

The overall median age of the study subjects was 32 (range, 31 – 38) year. The median duration of the stimulated cycle was significantly shorter than the spontaneous cycle (P <0.05). Further data relating to the characteristics of the cycles are given in Table **4.1**.

Good quality RNA was obtained from all 8 samples. Ovarian stimulation was shown to have a profound impact on the expression of a large number of genes and of GO categories, compared to the natural cycle in the same patient. Table **4.2** provides an overview of all genes with statistically significant differences greater than a 2 fold up or down regulation. 142 genes were significantly upregulated according to the applied criteria, and 98 significantly downregulated. The principal observations were the marked upregulation of SCYB 13, which encodes the chemokine ligand CXCL 13, involved in regulating cytosolic calcium ion concentration, and of DKK1 which codes for the Dickkopf homolog which regulates growth factor and Wnt signal transducer activity. Other notably upregulated genes include that coding for steroidogenic acute regulatory protein, insulin-like growth factor binding proteins 4 and 5, and Homeobox C6.

Hierarchical cluster analysis of control spontaneous cycle samples demonstrated cluster patterns similar to those of early-secretory phase of the cycle in terms of gene expression profile. However, the samples taken following ovarian stimulation clustered in a pattern close to that observed in late-secretory phase endometrium, indicating a profound change in gene expression between the spontaneous and stimulated cycle independent of biological variation between patients (Figure **4.1**).

The GO categories with relatively enriched gene number involving upregulated genes are given in Table **4.3**, and those involving downregulated genes are given in Table **4.4**. While many groups were affected, prominent categories of upregulated genes included those involved in cell adhesion, T cell receptor signaling, regulation of signal transduction, cell growth, proliferation and programmed cell death. GO categories of down regulated genes indicated

Table 4.1 *Subject and cycle characteristics.*

	median	(range)
Age (years)	32	(31– 38)
Spontaneous cycle duration (days)*	27	(26 – 29)
Stimulated cycle duration (days) *	24.5	(21 – 26)
Number of oocytes in stimulated cycle	6	(5 – 12)
Estradiol levels on day of LH peak (pmol/l) **	397	(256 – 462)
Estradiol levels on day of hCG injection (pmol/l)**	1790	(1710 – 1884)

Significantly shorter stimulated cycles in duration than spontaneous cycle (P <0.05)*; significantly higher estradiol levels in stimulated cycles than spontaneous cycle (P <0.01)**

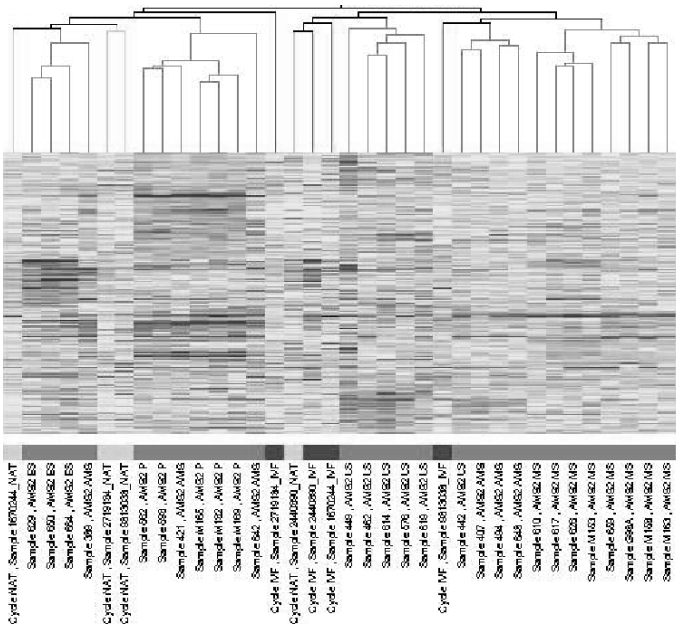


Figure 4.1.
Heirarchical clustering of natural and IVF stimulated cycle samples (taken at 5 postovulatory days) with samples of known histology and phase specificity in the proliferative, early-secretory, mid-secretory, and late secretory phases of the menstrual cycle. Natural cycle samples (highlighted yellow) cluster predominately with the early-secretory phase, while samples from IVF stimulated cycles highlighted red) cluster with the late-secretory phase.

reduced transmembrane receptor protein kinase activity following ovarian stimulation.

4.3.1 Validation of the microarray data by real-time PCR

In order to validate the data generated by microarray analysis, real-time PCR was performed on natural and stimulated cycle samples. Figure **4.2** shows data obtained from PCR on samples from 4 genes selected on the basis of the extent of their dysregulation following ovarian hyperstimulation. CXCL 13, DKK-1, STAR and TIMP 3, all of which were significantly upregulated on microarray analysis were found to be upregulated by PCR (fold changes: 15, 12, 4 and 3 respectively).

4.4 DISCUSSION

As far as we are aware this is the first study to report the impact of ovarian stimulation by recFSH in association with GnRH antagonist on endometrial gene expression in non-progesterone supplemented cycles, compared to natural cycles in the same subject.

This stimulation protocol is being increasingly adopted into clinical practice. Recombinant human FSH offers improved purity and consistency compared with urinary derived preparations (Talbi *et al.,* 2006), and the use of GnRH antagonist to prevent a premature LH surge enables a shorter treatment period, since suppression of pituitary LH production is immediate. However, the widespread adoption of GnRH antagonists into daily practice in IVF has been hindered by concern over results from clinical studies suggesting a slightly lower pregnancy rate compared to GnRH agonist protocols (Al Inany *et al.,* 2002). Although a recent meta-analysis did not confirm this difference in terms of live birth rates (Kolibianakis *et al.,* 2006), it has been speculated that endometrial quality may be more detrimentally effected in GnRH antagonist-, compared to GnRH agonist-based stimulation protocols (Tarlatzis *et al.,* 2006).

In a previous study in 8 subjects addressing the differential effect of GnRH agonist and antagonist exposure on endometrial gene expression. 13 genes were found to be relatively upregulated in the GnRH agonist group (Mirkin *et al.,* 2004). These included major histocompatibility important in endometrial development and implantation such as LIF, IGFBP-1, GdA and CXCL14, that were highly up-regulated in the window of implantation in the study by Talbi *et al* (Talbi *et al.,* 2006), were down-regulated in treatment cycles. These data provided further evidence of abnormal endometrial development in gonadotropin stimulated cycles. However, in a recent study comparing endometrial gene expression in the progesterone-supplemented luteal phase of cycles in which GnRH agonists versus GnRH antagonists were employed, no significant differences in gene expression profiles were observed (Simon *et al.,* 2005). Although this would appear to suggest that the GnRH antagonists in clinical use have no additional detrimental effect on endometrial receptivity at the gene level, possible subtle effects may have been masked by the effect of exogenous progesterone on gene expression.

The presented data demonstrate that when normal fertile women are exposed to ovarian stimulation using recFSH in combination with GnRH antagonist, extensive changes occur in endometrial gene expression. Since no luteal hormonal supplementation was given, the impact of ovarian stimulation on endometrial gene expression is revealed, without confounding by exogenous progesterone administration. In a previous study in which serum progesterone levels were measured daily throughout the cycle, exogenous progesterone administration was shown to significantly increase serum levels in the early to mid-luteal phase compared with both the stimulated, nonsupplemented cycle and the spontaneous cycle (Beckers *et al.,* 2000). In another study, this difference was no longer evident 9 days after hCG administration or an LH peak (Mirkin *et al.,* 2004).

Table 4.2 *Selected genes demonstrating statistically significant differences greater than a 2 fold up or down regulation in the stimulated versus spontaneous cycle are given.*

Upregulation		Downregulation	
Gene Symbol	**Fold**	**Gene Symbol**	**Fold**
CXCL13	7.83	CD36	0.18
DKK1	6.69	SFRP4	0.18
SLC16A7	5.28	IDH1	0.29
Clusterin	4.09	ANGPT1	0.42
SCYA8	4.03	Syndecan 2	0.43
STAR	4.01	VLDLR	0.44
PAPSS2	3.61	ESR1	0.46
S100A4	3.54	SLC12A6	0.48
IGFBP5	3.30	FGF13	0.49
HOXC6	3.27		
IGFBP4	3.02		
CSPG2	3.01		
S100A6	2.94		
APOE	2.85		
EMP1	2.78		
CTSW	2.77		
CEBPG	2.75		
COL15A1	2.66		
ISG15	2.65		
CD14	2.63		
ALDH1A3	2.60		
PTGDS	2.55		
IGFBP6	2.53		
TIMP1	2.53		
ANXA4	2.51		
ANGPTL1	2.39		
IFI35	2.36		
SERPINA3	2.35		
CYP4B1	2.34		
Transcription factor 8	2.34		
IFITM2	2.31		
IER3	2.30		
TIMP3	2.30		
IL15	2.29		
IFITM3	2.20		
ID4	2.19		
IGSF4	2.19		
ECGF1	2.13		
COX7A1	2.12		
IFITM1	2.12		
BCL3	2.02		
IGF2	2.02		
TGFBR2	2.02		
THY1	2.00		

The genes most highly upregulated following stimulation reflect a wide variety of processes important to endometrial maturation and implantation. Genes coding for chemokines (SCYB 13) and modulators of growth factor activity and Wnt signaling (DKK1) were highly upregulated following ovarian stimulation. These and other genes observed in the present study to be upregulated in the stimulated cycle have been previously shown to be upregulated in the luteal phase versus the proliferative phase (Kao *et al.,* 2002; Talbi *et al.,* 2006), and the mid-luteal versus the early luteal phase (Carson *et al.,* 2002), suggesting

their progesterone dependence. Moreover, DKK1 mRNA and protein expression have been found to be upregulated in human endometrial stromal cells within 3 hours of treatment with progesterone (Tulac *et al.,* 2006). DKK1 inhibits Wnt signaling by binding LRP 4/6 (Mao *et al.,* 2001) and probably has an important role in modulating endometrial glandular development (Lane *et al.,* 1997). This suggests that supraphysiological levels of progesterone and estrogen present in the early luteal phase of the non-progesterone supplemented stimulated cycle (Beckers *et al.,* 2000; Beckers *et al.,* 2003) may be responsible for additional expression of progesterone modulated genes. The observation in the present and previous studies of a shorter luteal phase in the stimulated, non-progesterone supplemented cycle provides a clinical manifestation of this dysregulation in gene expression.

Upregulation of genes involved in immunomodulation at the endometrial-embryo interface, such as IL-15, was observed. Excessive production of certain cytokines as a result of ovarian stimulation may alter the populations of immunoactive cells in the endometrium, resulting in an antiimplantation (Th1 as opposed to Th2 dominant) environment. Studies in rodents have suggested that such a shift towards Th1 dominance may be detrimental to successful implantation (Blois *et al.,* 2004).

Since previous studies addressing the effect of ovarian stimulation on endometrial gene expression have employed different stimulation regimens, it has been suggested that the large number of genes apparently dysregulated may reflect a general advancement in endometrial maturation caused by ovarian stimulation, rather than selected gene dysregulation. Although there is evidence that the early luteal phase endometrium shows histological evidence of advancement, recent data suggest that by 7 days following hCG administration, the endometrium is back 'in phase' (Kolibianakis *et al.,* 2003). In the present study, the endometrial biopsies were taken at this later stage of the luteal phase. Thus, the observed alterations in gene expression following ovarian stimulation likely reflect selected gene dysregulation.

Previous studies have been notable for the high level of consistency in individual endometrial gene expression profiles. This phenomenon has been observed in studies of cyclical variation in endometrial gene expression (Kao *et al.,* 2002) and in studies of gene expression in endometriosis (Giudice *et al.,* 1999). However, there is little overlap in the individual genes reported to be upregulated or downregulated. Of four studies addressing cyclic changes in endometrial gene expression, only three (osteopontin, apolipoprotein D and DKK1) were consistently reported to be significantly upregulated.

Previous studies addressing the impact of ovarian stimulation similarly reveal discordant profiles of gene expression. For instance, other studies demonstrate considerable dysregulation of genes coding for factors known to be important regulators of endometrial receptivity, such as glycodelin and LIF (Horcajadas *et al.,* 2007). In contrast, we have observed significant up-regulation in other factors associated with implantation such as TIMPs, annexins, interleukins and IGFBPs, and no effect on expression of LIF or GdA. However a previous study comparing endometrial gene expression in progesterone supplemented cycles stimulated with recFSH and GnRH antagonist with natural cycles in other

Table 4.3 *The gene ontogeny catagories with relatively enriched gene number involving upregulated genes are given in ratio of observed to expected.*

In biological process			In Molecular function		
Category	**Ratio**	***P***	**Category**	**Ratio**	***P***
fibroblast proliferation	20	0.004	PDGF receptor binding	25	0.002
fibroblast proliferation regulation	20	0.004	hematopoietin/IFN-class cytokine receptor signal transducer activity	25	0.002
rRNA transcription	20	0.004	amine oxidase activity	20	0.003
Tcell receptor signaling pathway	16.67	0.006	phospholipase activity	18.75	<0.001
RNA elongation from RNA Polymerase II promoter	16.67	0.006	metalleopeptidase inhibitor activity	18.75	<0.001
negative regulation of angiogenesis	13.04	0.001	insulin-like growth factor binding	11.63	<0.001
regulation of neurogenesis	10.34	0.002	transmembrane receptor protein ser/thr kinase activity	11.11	0.002
phosphate transport	5.66	<0.001	TGFβ activity	11.11	0.002
angiogenesis	5.56	<0.001	calcium-dependent phospholipid binding	7.32	0.007
blood vessel morphogenesis	5.30	<0.001	growth factor binding	5.61	<0.001
vasculature development	5.30	<0.001	antioxidant activity	5.56	0.006
blood vessel development	5.30	<0.001	glycosaminoglycan binding	5.00	<0.001
transmembrane receptor protein ser/thr kinase signaling pathway	4.82	0.009	heparin binding	4.96	0.001
			polysaccharide binding	4.82	<0.001
			pattern binding	4.81	<0.001
			ECM structural constituent	4.12	0.002
			phospholipid binding	3.38	0.005
innate immune response	3.85	0.009	endopeptidase inhibitor activity	3.25	0.003
skeletal development	3.75	<0.001	protease inhibitor activity	3.24	0.003
cell growth	3.56	<0.001	enzyme inhibitor activity	3.03	<0.001
regulation of cell size	3.56	<0.001	growth factor activity	2.98	0.003
inorganic anion transport	3.32	0.002	carbohydrate binding	2.92	<0.001
cell growth regulation	3.20	0.006	receptor signaling protein activity	2.89	0.007
cellular morphogenesis	3.12	<0.001	cytoskeletal protein binding	2.28	0.004
anion transport	3.08	0.002			
growth	2.88	0.003			
inflammatory response	2.64	0.003			
organ morphogenesis	2.44	0.008			
cell proliferation regulation	2.41	0.002			
cell proliferation	2.40	<0.001			
signal transduction regulation	2.37	0.01			
organ development	2.33	<0.001			
morphogenesis	2.29	<0.001			
immune response	2.21	<0.001			
cell death	2.10	<0.001			
death	2.09	<0.001			
cell adhesion	2.05	<0.001			
apoptosis	2.03	<0.001			
programmed cell death	2.02	0.002			

subjects also demonstrated significant upregulation of IGFBP-5. Up-regulation of this gene in progesterone-supplemented cycles is unanticipated, as this IGFBP is the only IGFBP whose expression is suppressed in the secretory, compared to the proliferative, phase (Giudice *et al.,* 1991). The observation of upregulation of

Table 4.4 *The gene ontogeny catagories with relatively enriched gene number involving downregulated genes are given in ratio of observed to expected.*

In Molecular function		
Category	**Ratio**	***P***
low-density lipoprotein binding	25	0.002
cation chloride symporter activity	25	0.002
ephrin receptor activity	18.75	<0.001
transmembrane receptor protein tyrosine kinase activity	6.25	0.004
transmembrane receptor protein kinase activity	5.13	0.008

IGFBP-5 in the present study, where no progesterone supplementation was given, provides further evidence that endometrial gene dysregulation following ovarian stimulation is not fully attributable to the resultant supraphysiological concentrations of sex steroids. IGFBPs have been shown to have an important role in the regulation of endometrial cellular mitosis and differentiation and embryo-endometrial communication (Frost *et al.,* 1993). Up-regulation of IGFBP-5 may inhibit IGF action within endometrium at this stage of the cycle, as IGFBP-1 levels begin to increase in microenvironments in the tissue.

There are several possible reasons underlying the differences in results between studies. Differences may be related to differing sequences being represented in different chips, the use of different types of chips, altered hybridization techniques and differences in the software used for analyzing data. Previous studies have employed arrays and analysis systems which generate considerable number of uninterpretable negative intensity values (Kao *et al.,* 2002), or a restricted number of transcripts (Mirkin *et al.,* 2004). Moreover differences in the timing in the cycle, different complements of cells present in

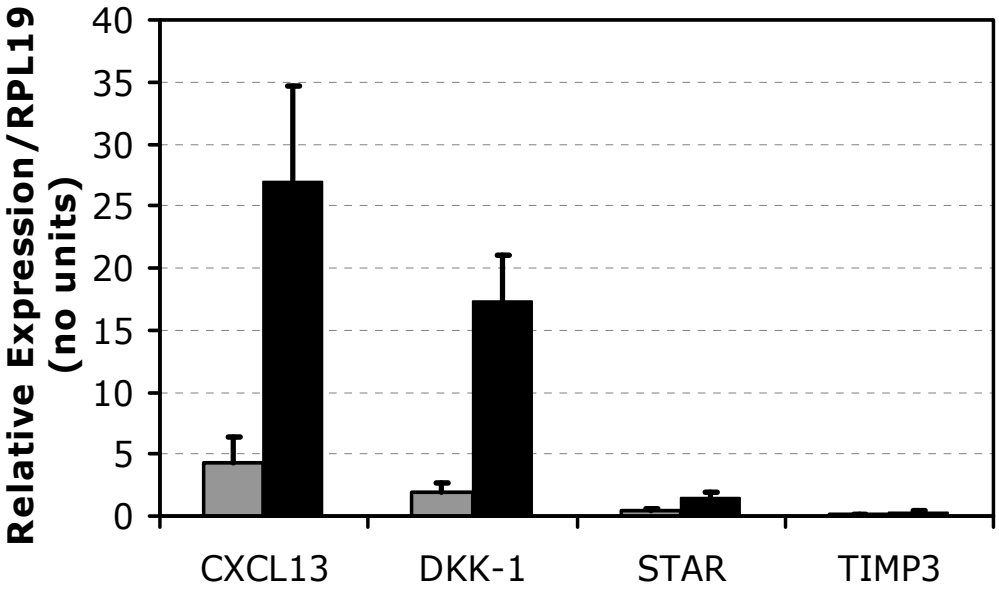


Figure 4.2. *Data from real time PCR analysis) for 4 genes observed to be significantly upregulated (±SD) following ovarian stimulation compared to the spontaneous cycle. Relative expression data (normalized to RPL19) of natural (grey bars) vs. stimulated IVF (black bars) cycles from 4 patients is shown.*

the biopsy, and even the position in the uterus from which the biopsy is obtained may explain variation in results (Talbi *et al.,* 2006). However, a further explanation for differences between the present findings and those reported in a previous study may be the different stimulation protocols.

Although the present study reveals a different gene different gene expression profile to that reported in studies in which GnRH agonist was employed, current knowledge relating to the importance of individual gene expression does not allow conclusions to be drawn as to whether one or other dysregulated profile indicates a more receptive endometrium. While studies such as that presented provide information on the impact of hormonal stimulation of the ovary on endometrial receptivity and demonstrate both the complexity and hormone sensitivity of genetic regulation of endometrial receptivity, it remains unclear whether the altered gene expression profile may underlie the clinically observed reduction in receptivity observed following ovarian stimulation. However, our study demonstrates that genes involved in cell adhesion, T-cell receptor signaling, and regulation of signal transduction are particularly susceptible to dysregulation by ovarian stimulation. The next challenge is to identify the downstream protein gene products expressed by the endometrium in the molecular cross talk with the implanting embryo. Data from this and other gene expression studies provide an indication as to the proteins likely to be the key determinants of a receptive or nonreceptive endometrium.

5.

ENDOMETRIAL FLUID ASPIRATION: A NON-INVASIVE MEAN OF OBTAINING MATERIAL FOR ANALYSIS OF MARKERS OF RECEPTIVITY.

Mark H. van der Gaast, Karin Beier-Hellwig, Bart C.J.M. Fauser, Henning Beier, Nick S. Macklon.

Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates.

Reprod BioMed Online ***2003****;7:105-9.*

5.1 INTRODUCTION

Successful implantation depends on a complex process of interaction between the embryo and the endometrium. Only if the endometrium is receptive to the embryo will apposition, implantation and trophoblast invasion occur. The period for which the endometrium is receptive to embryo implantation appears to be limited and is often referred to as the 'window of implantation'. This widely used term emerged following investigations of the role of estrogens in the control of the receptivity of endometrium to apposition and implantation of the blastocyst (Finn *et al.,* 1974; McLaren, 1973; Psychoyos, 1963). The terms 'receptivity' and 'window of implantation' are currently used synonymously to describe the physiological and structural stage of the endometrium during the luteal phase of the cycle in which attachment and implantation can be achieved. The time frame of the implantation window has been defined differently by various investigators according to different cell biological markers and levels of methodological resolution (Beier-Hellwig *et al.,* 1989; Bergh *et al.,* 1992; Lessey *et al.,* 1995; Nikas *et al.,* 1995).

For more than 50 years, the secretory transformation of the endometrium has been assessed by histological dating according to the criteria of Noyes (Noyes *et al.,* 1950). More recently, immunohistochemical molecular markers (including cytokines and adhesion molecules) have been used for cell biological assessment of receptivity (for review, see Giudice, 1999a). For such studies, endometrial tissue should ideally be biopsied during the window or receptivity. In order to assess the impact of biopsy immediately prior to embryo transfer, we previously performed endometrial biopsy with a Pipelle endometrial aspirator (Laboratoire CCD, Paris, France) one hour prior to embryo transfer in a pilot study of 13 patients. Only two biochemical and no ongoing pregnancies resulted (Van der Gaast *et al.*, unpublished data, Table **1.3**). In view of the poor observed outcome, the study was not extended.

The analysis of endometrial secretion may constitute an alternative, less invasive technique. The 'cross-talk' which occurs between the embryo and endometrium prior to and during the process of implantation results in production and release of molecules into endometrial secretion. The expression of these molecules is temporally related to the phase of endometrial development (Lessey *et al.,* 2000). Previous studies have reported detectable expression of leukaemia inhibitory factor (LIF) (Cullinan *et al.,* 1996; Laird *et al.,* 1997; Ledee-Bataille *et al.,* 2002), Glycodelin A (GdA, PP14) (Li *et al.,* 1993a), interleukines (Simon *et al.,* 1998b), macrophage colony-stimulating factor (M-CSF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) (Classen-Linke *et al.,* 2000), insulin-like growth factor binding protein-1 (IGFBP-1), prolactin, and human chorionic gonadotropin (hCG) (Licht *et al.,* 1998) in the endometrial secreted fluid, obtained during the luteal phase. In recent years an approach has been described whereby endometrial secretion directly aspirated from uterine cavity can be analyzed for the expression of these and additional proteins by electropheretic techniques (Beier-Hellwig *et al.,* 1988). It has been shown that the protein profile expressed in endometrial secretions alters with the

phase of the cycle, and that characteristic patterns are predictive of receptivity, and subsequent implantation and pregnancy (Beier *et al.,* 1998; Beier, 2000).

The aim of this study was to assess whether aspiration of endometrial secretions prior to embryo transfer for analysis of receptivity in a treatment cycle might be carried out without disrupting the process of implantation.

5.2 MATERIAL AND METHODS

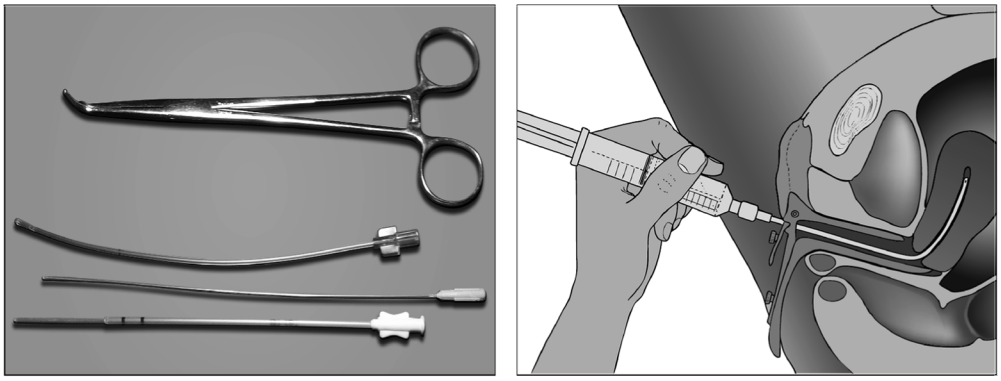
5.2.1 Subjects and treatment

The study was approved by the local ethics committee of the Erasmus Medical Center. After informed consent, 66 women undergoing IVF were recruited to the study regardless of the individual indications for IVF. The patients were treated with recombinant FSH (Puregon®; Organon Nederland B.V., Oss, The Netherlands) and GnRH agonist (triptorelin (Decapeptyl®); Ferring BV., Hoofddorp, The Netherlands) or GnRH antagonist (cetrorelix (Cetrotide®); Serono Benelux BV, The Hague, The Netherlands). Oocytes were retrieved and fertilized *in vitro*. After 3 to 5 days of culture no more than 2 embryos, which were selected according to a previously described classification (Huisman *et al.,* 2000), were transferred. Immediately prior to the embryo transfer, aspiration of the endometrial secretion was performed as described below. Discomfort and side effects were assessed. A pregnancy test was performed 18 days after oocyte retrieval, and if positive, an ultrasound was performed 5 weeks later to assess the presence of a gestational sac and fetal heart activity.

The control patients were matched for stimulation treatment protocol, age, number of oocytes retrieved and number and quality of embryos available for transfer. They underwent embryo transfer over the same period as the study patients, and all embryo transfers and endometrial secretion aspirations were performed by the same investigator (M.vdG.).

5.2.2 Aspiration procedure

In all 66 patients who underwent the transcervical uterine aspiration, endometrial secretion was obtained. Two catheter systems were tested. Initially, an embryo transfer catheter (Repromed®, International Medical Products B.V., Zutphen, The Netherlands) with only one outlet was tested. In order to increase the volume of secretion available for analysis, a double outlet soft insemination catheter (ASSA med GmbH, Bexbach, Germany), stiffened with the stylette of the guide catheter of the embryo transfer set, was substituted after 20 patients.
With the patient lying in the lithotomy position, the cervix was cleansed with a swab after insertion of a speculum. The catheter was gently introduced transcervically into the uterine cavity. When the catheter was correctly positioned, the stylette was removed and a 10 ml syringe was connected to the insemination catheter. Suction was gradually applied. Following the application of suction, the catheter was clamped just distal to the external os of the cervix with an artery clamp, and removed (Figure **5.1**). The tip was cut off and placed in an Eppendorf cup with 1 ml of distilled water and frozen to -20°C. Immediately thereafter, the normal embryo transfer procedure was carried out.



A **B**

Figure 5.1. *Material necessary for endometrial secretion aspiration (**A**) and the position in the uterine cavity (**B**). (Copyright 2009, MH & GE van der Gaast. All rights reserved).*

5.2.3 Statistical Analysis

In order to exclude a major reduction in implantation as a result of fluid aspiration, a power calculation was carried out which indicated that at least 60 subjects were required in each arm to demonstrate a reduction in pregnancy rates per embryo transfer from 30% to 10% with a *P* value <0.05 and 80% power.

Possible differences between the study and control group in age, number of collected oocytes, number and quality of obtained embryos, and day of the embryo transfer after oocyte retrieval were analyzed by the Student's paired *t* - test or the Wilcoxon matched pairs signed rank sum test, after the study patients were matched with the control group. For analysis of the impact of aspiration on outcome, data from both catheter systems were combined. Implantation rate differences were tested by the χ^2 test. Differences in positive pregnancy test rate and the ongoing pregnancy rate were analyzed using the McNemar test. For analysis of the impact of aspiration on outcome, data from both catheter systems were combined.

5.3 RESULTS

No significant differences in age, number of collected oocytes, number of embryos or median embryo score were observed between the study group and control group (Table **5.1**).

Biochemical and ongoing pregnancy rates in the study group patients were not significantly different to those observed in the control group (Table **5.1**). No discomfort or side effects of the aspiration were reported by any of the patients.

Table 5.1. *Patient characteristics and clinical IVF outcome (mean ± SD).*

	Study Group (n = 66)	Control Group (n = 66)	*p*
Age*	34 ± 3.9	34 ± 3.7	0.86[a]
No. retrieved oocytes*	9.1 ± 5.4	9.1 ± 4.8	0.96[a]
No. obtained embryo's*	5.2 ± 3.6	5.1 ± 3.3	0.84[a]
Embryo score[#]	2 (1-4)	2 (1-4)	0.59[b]
No. of embryo transfers3 / 4 / 5 days after OPU[#]	30 / 16 / 20	30 / 16 / 20	
Implantation rate (%)	29/125 (23)	23/127 (18)	0.32[c]
Positive pregnancy test (%)	24 (36)	22 (33)	0.84[d]
Ongoing pregnancy (%)	22 (33)	20 (30)	0.85[d]

OPU = ovum pick-up; * mean ± SD; [#] median (range); [a] Student's paired *t* test; [b] Wilcoxon matched pairs signed ranksum test; [c] χ^2 test; [d] McNemar test.

5.4 DISCUSSION

The findings of this steady indicate that the removal of endometrial secretions immediately prior to embryo transfer provides sufficient material for analysis of markers of receptivity without disrupting embryo implantation. This approach may overcome one of the barriers to the *in vivo* investigation of endometrial receptivity in conception cycles: disruption of the process of implantation (Van der Gaast *et al.,* 2002). While endometrial biopsy performed in IVF patients shortly after oocyte retrieval (Abate *et al.,* 1987; Kolibianakis *et al.,* 2002a; Papanikolaou *et al.,* 2005; Ubaldi *et al.,* 1997) does not seem to have any adverse effects on implantation, we observed a negative on implantation when the biopsy was taken immediately prior to the embryo transfer.

Uterine flushing and uterine dialysis offer alternative techniques for obtaining endometrial material for analysis. Endometrial secretions can be analyzed for functional markers of endometrial receptivity (Li *et al.,* 1993a; Li *et al.,* 1993b; Li *et al.,* 1993c; Laird *et al.,* 1997; Ledee-Bataille *et al.,* 2002; Li *et al.,* 1998). However, animal studies suggest that uterine flushing may disrupt the endometrial epithelium (Milligan *et al.,* 1984). To date, no case control studies have been carried out showing the impact of these techniques on pregnancy rates.

Beier and coworkers have demonstrated quantitative and qualitative changes of the protein patterns of endometrial secretion during the menstrual cycle (Beier-Hellwig *et al.,* 1989; Beier, 1998). Analysis of the protein pattern is capable of indicating an adequate microenvironment in the uterine cavity, which in turn could facilitate the embryos attachment and implantation. This technique may therefore offer a useful alternative to histological evaluation of endometrial biopsies (Beier *et al.,* 1994).

Our study shows that aspiration of endometrial secretion, a simple and non-invasive technique, may be safely carried out immediately prior to embryo transfer without disrupting implantation. This study was powered to exclude a reduction of pregnancy rates from 30% to 10% per embryo transfer. However in order to exclude a more subtle impact, a far larger study would have been required. Since a trend towards increased pregnancy rates after endometrial secretion removal was observed, it is unlikely that a larger sample size would have led to different conclusions. No adverse effects or side effects were reported. Indeed, This positive effect may be due to the removal of excessive cervical mucus prior to embryo transfer. However, the technique employed, whereby the catheter is clamped prior to removal from the cavity to prevent unintended aspiration of cervical mucus, renders this explanation unlikely. Recently, a pilot study showed that removal of ultrasonic visible fluid accumulation in the uterine cavity before the embryo transfer may have a beneficial effect on the implantation process (Griffiths *et al.,* 2002). By reducing the volume of fluid in the uterine cavity aspiration of secretions prior to ET may facilitate the process of implantation, namely adhesion of the embryo to the endometrial surface.

In conclusion, endometrial secretion aspiration may provide a tool to assess endometrial receptivity in treatment and conception cycles (Beier-Hellwig *et al.,* 1994), without compromising embryo implantation and establishment of clinical pregnancy. Prospective studies designed to further validate this approach to determining endometrial receptivity are now ongoing.

6.

COMPARISON OF ENDOMETRIAL SECRETION ANALYSIS WITH ENDOMETRIAL TISSUE MATURATION IN THE WINDOW OF IMPLANTATION.

Mark H. van der Gaast, Nick S. Macklon, Karin Beier-Hellwig, Claudia A. Krusche, Bart C. J. M. Fauser, Henning M. Beier, and Irmgard Classen-Linke.

The feasibility of a less invasive method to assess endometrial maturation - comparison of simultaneously obtained uterine secretion and tissue biopsy.

*BJOG **2009**;314:304-12.*

6.1 INTRODUCTION

Human implantation is a complex process which is dependent on multiple, successive interactions between the embryo and the endometrium. It is only successful when it occurs during a brief period of the secretory phase of the menstrual cycle (Hertig *et al.,* 1956; Wilcox *et al.,* 1999), usually referred to as the 'implantation window' or 'window of receptivity' (Psychoyos, 1993). The traditional 'gold standard' technique for assessing endometrial differentiation and maturation was described by Noyes *et al.* (1950), and these histological criteria are widely applied. However, the clinical value of only these criteria in terms of predicting endometrial receptivity and consequently fertility are limited (Coutifaris *et al.,* 2004; Murray *et al.,* 2004; Myers *et al.,* 2004). Moreover, the histological approach to monitor endometrial maturation requires an invasive biopsy which excludes its use during the luteal phase of cycles in which implantation is the endpoint objective as in *in vitro* fertilization (IVF) (Van der Gaast *et al.,* 2003).

In recent years, less invasive techniques have become available to study endometrial maturation. Aspiration and flushing of the endometrial cavity in the peri-implantation period of menstrual cycles has been performed without detrimental effect on pregnancy rates (Li *et al.,* 1993c; Olivennes *et al.,* 2003; Van der Gaast *et al.,* 2003). Analysis of endometrial secretions showed proteins originating from transudate of serum, leakage products of apoptotic epithelial cells, and glandular secretion (Beier, 1974). The composition of the secretions varies during the menstrual cycle as result of changing ovarian steroid levels. estradiol (E) regulates transudation by blood vessel dilatation and permeability, and progesterone (P) controls secretory activity of the endometrial glands. Protein composition analysis has been performed throughout the spontaneous menstrual cycle, and revealed significant protein pattern changes for normal endometrium transition from proliferative to secretory (Beier *et al.,* 1998). Endometrial gene expression studies have shown Glycodelin A (GdA) to be upregulated during the implantation window (Kao *et al.,* 2002), and both GdA and another putative marker of endometrial receptivity, Leukemia Inhibitory Factor (LIF), have been identified in endometrial secretions. These markers vary during the menstrual cycle (Bell *et al.,* 1987; Laird *et al.,* 1997; Li *et al.,* 1993b; Tuckerman *et al.,* 2004) and appear to be differentially expressed between fertile and subfertile women (Dalton *et al.,* 1995; Laird *et al.,* 1997; MacKenna *et al.,* 1993; Mikolajczyk *et al.,* 2003). However, the degree of correlation between the endometrial secretion levels of P, LIF, and GdA with the endometrial tissue markers of maturation PR and Ki-67, and Noyes criteria, is not known.

The principle aim of this study is to examine whether putative markers of endometrial maturation obtained in aspirated endometrial secretion fluids correlated with endometrial maturation monitored by the traditional histological Noyes criteria combined with immunohistochemical detection of endometrial PR and Ki-67 expression. In addition, markers of endometrial maturation were correlated with serum E and P levels.

6.2 METHODS

6.2.1 Subjects

This prospective study was approved by the local ethics committee of the Erasmus Medical Centre. Women attending the out-patient department of the fertility clinic were asked to participate in the study after exclusion of major uterine pathology and cervical Chlamyida infection. Approval and written informed consent was obtained after the first visit from 34 women. In order to limit the heterogeneity of the study population, inclusion criteria were: age (ranging between 18–40 years), normal regular menstrual cycles, (ranging between 25–35 days), no uterus anomaly which could impair embryo implantation or pregnancy evolution, no hormonal contraception for at least 2 months prior to the study, no endometriosis, and no medical history of disease which may impair implantation or pregnancy.

6.2.2 Assessments

Ovarian follicle development was monitored by transvaginal ultrasound (TVS) by a single operator (M.vdG.) on cycle day 2 and 8, and then daily until the diameter of the leading follicle measured 18 mm. Since changes in endometrial markers of maturation and receptivity were to be related to the day of ovulation, daily TVS was continued until spontaneous follicle rupture had been identified by the disappearance of the leading follicle and the presence of free fluid in the pouch of Douglas (Ecochard *et al.,* 2000a). This validated method has been shown to be a reliable means of detecting ovulation for the purposes of endometrial dating (Guermandi *et al.,* 2001; Shoupe *et al.,* 1989). On the 5th day after ovulation, the endometrial cavity and cervix length were measured by TVS, blood sampling was performed and transcervical aspiration of endometrial secretion fluid was carried out, as described previously (see Chapter **5.2.2**; Van der Gaast *et al.,* 2003). In short, after removal of excessive cervical mucus an insemination catheter (ASSA med GmbH, Bexbach, Germany) was cautiously inserted in the uterine cavity at a depth of the measured cervix length and 1 cm beyond. Suction was gently applied while simultaneously rotating the catheter. Immediately after the aspiration, the outside of the tip of the double outlet soft insemination catheter was cleaned with a sterile cloth. The tip containing the secretion fluid was cut off into an Eppendorf cup and stored at –20°C until final assessment. Immediately following aspiration, an endometrial biopsy was obtained using a suction curette (Pipelle de Cornier®, C.C.D. Laboratoire, France).

Each specimen was labelled using a coding system for which the observers in the laboratory were blinded.

6.2.3 Serum hormone assay

Blood was centrifuged for 10 minutes at 1500 × g immediately after obtaining the samples, and the aliquots of serum were extracted and stored at –20°C until assessment. Serum E and P were measured by an immunofluorometric assay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA) performed in the

same laboratory. Respective intra- and interassay coefficients of variation for P were less than 10% for both, and less than 5% and 7% for E.

6.2.4 Endometrial tissue analysis

After fixation in neutral buffered 3.7% formalin, the biopsied tissue samples were dehydrated with increasing ethanol concentration before they were embedded in paraffin. A portion of endometrial tissue from each specimen was routinely cut, mounted and stained with hematoxylin-eosin for histological dating according to the criteria of Noyes *et al.* (1950).

For immunohistochemistry, 5 µm thick paraffin sections were cut, deparaffinized and rehydrated in PBS. The slides were heated in citrate buffer by microwave (4 × 5 min; 600 W) for epitope retrieval and the immunohistochemical staining was performed by a streptavidin-biotin-peroxidase method (Histostain-SP Kit; Zymed Laboratories Inc, Berlin, Germany).

The primary monoclonal antibody MIB-1 (M 7240, DakoCytomation, Hamburg, Germany) which was used to detect the nuclear proliferation marker Ki-67 in endometrial tissue was diluted 1:100 in PBS/1.5% BSA and applied overnight at 4°C. Visualising of the Ki-67 antigen was performed by peroxidase catalyzing the substrate and converting the chromogen aminoethylcarbazole (Zymed Laboratories Inc, Berlin, Germany) to a red deposit.

For the detection of estrogen and progesterone receptor (ER and PR) the same immunhistochemical procedure was used as described for Ki-67. The primary monoclonal anti-PR antibody (clone PgR 636, M 3569, DakoCytomation, Hamburg, Germany), which recognizes the A and B isoform, was diluted 1:50. The anti ER-α antibody (clone ER 6 F11, Novocastra, Newcastle upon Tyne, UK) was diluted 1: 40.

As a negative control, the primary antibodies were replaced with non-immune mouse IgG at the same concentration. None of the negative controls revealed a positive staining. As a positive control we used archival emdometrial paraffin blocks with known positive reactivity for the studied antibodies.

All endometrial tissue assessments were performed by two observers (I.C.L., C.A.K.), who were blinded for the results of endometrial secretion fluid assessment and E and P serum levels. Since all samples were taken exactly on the 5th day after ovulation we looked for the typical criteria according to Noyes *et al.* (1950), i.e. the occurrence of glycogen vacuoles which first appear subnuclear and then shift from a subnuclear to a supranuclear location (Hendrickson *et al.,* 1997). This has also been taken in account by a recent study by Tuckerman *et al.* (2004). In addition to these data, we evaluated immunohistochemical staining of Ki-67 to obtain the proliferation status of epithelial versus stromal cells as well as the PR and ER expression in the glands. Endometrial epithelial expression of PR and ER was graded on a scale of 0–2+, based on the intensity of cells with positive nuclear staining: 0 if none stained, 1+ if weak staining, 2+ if strong staining. The expression of Ki67 in the glandular epithelium was graded on a scale of 0–3+ based on the percentage of cells with positive nuclear staining: 0 if none stained, 1+ if < 10% stained, 2+ if 10–50% stained and 3+ if >50% stained.

6.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and laser densitometry

Secretion fluids were removed from the tip of the catheter, incubated for 10 minutes in an ultrasound bath at 4°C and then centrifuged at 20,800 × g. Protein concentration of the supernatant was determined (Lowry *et al.,* 1951) and the protein composition was analyzed by SDS-PAGE using a 8.3–16.6% acrylamide separating gel with 5% acrylamide containing stacking gel (Laemmli, 1970). Each lane was loaded with 50 µg protein diluted with sample buffer. The gels were stained with Coomassie brilliant blue. Molecular weight was estimated by comparison to protein standards electrophoresed in adjacent lanes of the gel. Many protein bands (Figure **1.5**) appear among the lower-molecular weight fractions (68 kDa and 6.5 kDa area) in electrophoresed endometrial secretions (Beier *et al.,* 1998). The most pronounced and heavily staining of which are albumin at 68 kDa and the two of α- and β-chains of haemoglobin, close to the position of 12.5 kDa. Bands below 68 kDa which form 3 groups of similarly sized, partly faintly staining bands are the focus of our assessments. Group A is represented by bands between 45 and 34 kDa, group B from 29 to 25 kDa, and group C from 18 to 12 kDa. Group C awaits its completion at the time of about 2 days after ovulation. Particular attention is paid to the 3 intensely staining bands in the range of 15–18 kDa. The 12.5 kDa-protein fraction decreases in width during the periovulatory period and remains less prominent during the luteal phase. Such pattern of the luteal phase is designated as 'optimal luteal phase pattern'. Suboptimal or impaired luteal phase patterns showed some or significant changes in group B and C fractions.

The relative density of each band was measured using a scanning laser densitometer, He-Ne-Laser 633 nm (LKB Ultrascan XL, Pharmacia-LKB, Freiburg, Germany) and the GelScan XL software package. Protein pattern analyses were scored semi-quantitatively as an optimal, suboptimal, or non-luteal protein pattern, according to previously published criteria (Beier-Hellwig *et al.,* 1989). All samples were analyzed independently by two observers (K.H.B. and H.M.B), who were blinded to the endometrial tissue assessment results.

6.2.6 LIF, GdA, and P assessment in endometrial secretions by ELISA

The intra- and interassay variations of the LIF ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany) were 2.4% and 6.1%, respectively. The detection limit was 8 pg/ml. The intra- and interassay variation of the GdA ELISA (BIOSERV, Rostock, Germany) was 8.3% and 4.58%, respectively.

6.2.7 Statistical analysis

Due to the novel nature of this study, it was not possible to carry out an appropriate power calculation. However, it was considered that the study of 30 subjects would be sufficient to reveal important correlations.

Pearson's correlation coefficients were used for relation analysis of endometrial dating (histological Noyes criteria and cellular maturation markers), serum steroid levels, and endometrial secretion P, LIF and GdA levels. In all statistical analyses a $P < 0.05$ was considered statistically significant. The

analysis was performed with a commercially available software package (GraphPad Prism 3.00).

6.3 RESULTS

Of the 34 patients recruited to the study, 3 yielded insufficient endometrial tissue and these were excluded from further analysis. The median age was 32 (range 21–38) years and the median luteal cycle length was 14 (range 11–16) days (Table **6.1**).

Although the endometrial biopsy and the endometrial secretion samples had been taken on the 5th day after ovulation as checked by TVS, the stage of endometrial maturation differed substantially between subjects with respect to both Noyes criteria combined with immunohistochemical markers. Glycogen vacuoles in the endometrial glandular epithelium, were observed in all but one subject (patient nr. 23 showed already a nonvacuolated secretory appearance). Since glycogen vacuoles are the hallmark of the early secretory phase (post-ovulatory days 2–5) and disappear after post-ovulatory days 5, all biopsies could be dated between post-ovulatory days 2 and 5. Analysis of tissue Ki-67 and PR expression enabled more precise assessment of maturation. When Ki-67 staining cells within the glandular epithelium were few or absent, the endometrium was dated as postovulatory days 4–5½, reflecting 'adequate' endometrial maturation. When 10–50% of glands showed Ki-67 positive cells, the endometrium was dated as postovulatory days 3–4, characterizing slightly retarded maturation since proliferation was continuing. When more than 50% of glands showed Ki-67 positive cells the endometrium was dated as postovulatory days 2–3 referring to retarded maturation (Figure **6.1**).

Strong staining of PR in the glands was dated as postovulatory days 2–4. Less intense staining of the PR in the glandular epithelial cells in contrast to the stromal cells, indicating the down-regulation of PR, was dated as postovulatory day 4–5½ (Figure **6.1**).

All results combined resulted in the final dating of the endometrial biopsies as given in Table **6.1**.

6.3.1 Endometrial tissue dating relation with serum hormone levels

Serum E and P levels showed a significant positive correlation with endometrial dating by Noyes criteria in combination with the immunohistochemical assessment of markers Ki-67 and PR ($R = 0.568$, $P = 0.006$ for serum E; and $R = 0.408$, $P = 0.023$ for serum P; Figure **6.2A** and **6.2B**).

6.3.2 Endometrial tissue dating relation with proteins and endometrial secretion fluid marker levels

Endometrial dating using the histological Noyes criteria combined with PR and Ki-67 immunostaining showed no correlation to the protein content in endometrial secretion fluids ($R = 0.013$, $P = 0.945$; Figure **6.3C**). In the ten subjects who demonstrated adequate or advanced endometrial maturation at the

Table 6.1. *Characteristics of the 31 women. Each serum, endometrial and secretion sample was obtained on the 5th day after spontaneous ovulation. Endometrial tissue dating (postovulatory days, POD), assessed with histological Noyes criteria and immunohistochemical markers, is expressed in postovulatory days. SDS-PAGE scored profile of the secretions means: 1, impaired; 2, suboptimal; 3, optimal luteal phase pattern.*

#	Age (yr)	Parity	Cycle length (days)		Serum		Endometrial tissue	Endometrial secretion				
			Follicular	Luteal	E (pmol/ml)	P (nmol/ml)	Dating (POD)	SDS-PAGE protein profile scores	P (ng/ml)	Protein (mg/ml)	LIF (pg/ml)	GdA (ng/ml)
1	28	0	14	16	315	50.4	3.0	2	0.61	0.19	-	-
2	28	1	16	15	264	40.5	2.5	3	0.74	1.06	5.23	14.26
3	38	0	11	14	295	29.4	3.5	3	0.37	0.36	19.25	18.58
4	28	0	17	13	305	16.1	2.0	1	0.79	0.77	10.35	8.39
5	34	1	13	16	293	50.1	3.0	3	0.60	0.62	7.27	5.87
6	29	0	12	15	555	52.1	4.5	1	0.34	0.32	19.5	14.26
7	30	0	16	14	307	20.5	2.5	2	0.51	0.39	11.56	1.15
8	24	0	16	15	314	34.5	2.0	2	0.48	0.46	7.54	0.13
9	37	2	10	16	269	22.7	2.5	3	0.55	0.86	5.64	2.17
10	21	1	19	15	594	29.0	3.5	3	0.30	0.5	7.62	1.92
11	32	0	18	14	464	28.4	2.5	1	0.31	0.27	16.7	5.61
12	34	1	9	13	314	34.9	2.5	3	0.47	0.44	6.29	-
13	34	0	13	14	547	30.8	3.5	3	0.33	0.32	8.66	0.38
14	26	1	16	15	250	21.9	2.5	2	0.45	0.4	16.47	0.60
15	33	3	14	16	295	28.5	3.0	3	0.48	0.65	7.46	1.66
16	31	0	16	14	404	15.5	2.5	3	0.59	0.65	8.54	3.06
17	33	0	10	16	264	28.6	2.5	3	0.35	0.41	13.54	3.19
18	35	0	12	13	590	191	4.5	2	0.96	0.67	10.86	4.21
19	26	0	17	15	402	53.8	4.5	3	0.60	0.59	12.93	-
20	27	0	19	13	602	100	5.0	3	0.71	0.47	8.85	2.04
21	31	1	21	12	459	33.9	3.0	1	0.48	0.78	9.78	4.09
22	27	2	13	14	324	33.4	2.5	3	0.92	1.01	2.74	4.73
23	24	1	22	11	576	43.1	5.5	2	0.31	0.51	6.78	4.98
24	36	2	17	14	362	21.1	3.5	2	0.31	0.54	13.48	1.40
25	38	2	10	16	324	22.8	3.5	3	0.63	0.63	10.46	14.35
26	38	7	14	11	358	20.5	3.5	1	0.44	0.45	7.71	14.35
27	32	2	15	13	462	42.2	4.5	2	0.21	0.34	15.55	3.2
28	32	2	14	13	1177	53.0	5.0	2	0.40	0.64	6.50	25.76
29	38	1	16	13	256	20.9	5.0	3	0.33	1.09	8.25	4.13
30	32	2	13	13	402	27.6	4.5	2	0.61	0.75	-	28.76
31	32	2	15	11	392	36.5	4.5	3	0.30	0.52	-	40.18

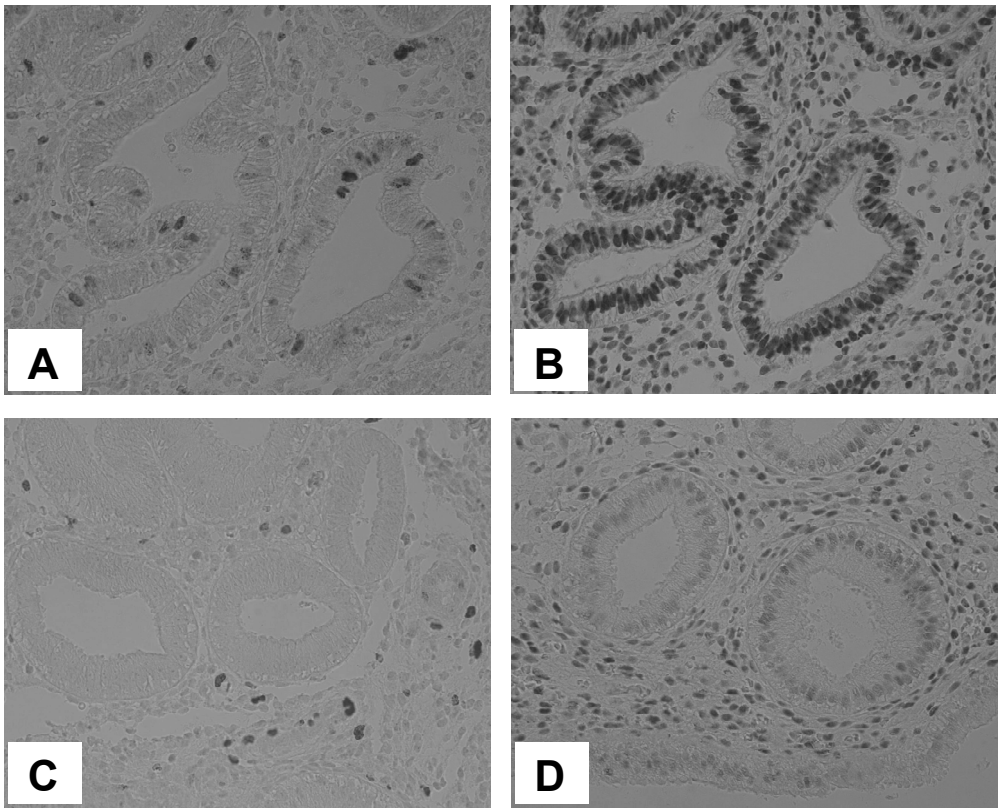


Figure 6.1. *Immunostaining for the proliferation marker Ki-67 (MIB-1) (**A**, **C**) and the progesterone receptor (PR) (**B**, **D**). Representative staining of two differently dated endometrial biopsies obtained on the 5th day after ovulation (postovulatory days). Sample **A**, **B** was dated as postovulatory days 2-3 (retarded maturation) according to strong staining of PR and Ki-67 in the glands. Sample **C**, **D** was dated as postovulatory days 4-5 (adequate maturation) according to weak staining of PR and no staining of Ki-67 in the glands. Magnification: 400×.*

tissue level, analysis of USE revealed an optimal pattern in four (40%), a suboptimal pattern in five (50%) and an impaired luteal phase pattern in only one subject (10%) (Figure **6.4**).

Of the 21 subjects with slightly delayed endometrial maturation according to the dating of endometrial biopsies, 12 (57%) subjects showed an optimal luteal phase pattern, five subjects (24%) a suboptimal and four subjects (19%) an impaired luteal phase pattern according to the assessments of the endometrial secretion samples (Figure **6.4**).

Endometrial secretion levels of GdA revealed a positive correlation with endometrial dating (R = 0.376, *P* = 0.048, Figure **6.3B**). In contrast to GdA, secretory LIF (R = 0.105, *P* = 0.594 in Figure **6.3A**) was not correlated with endometrial dating.

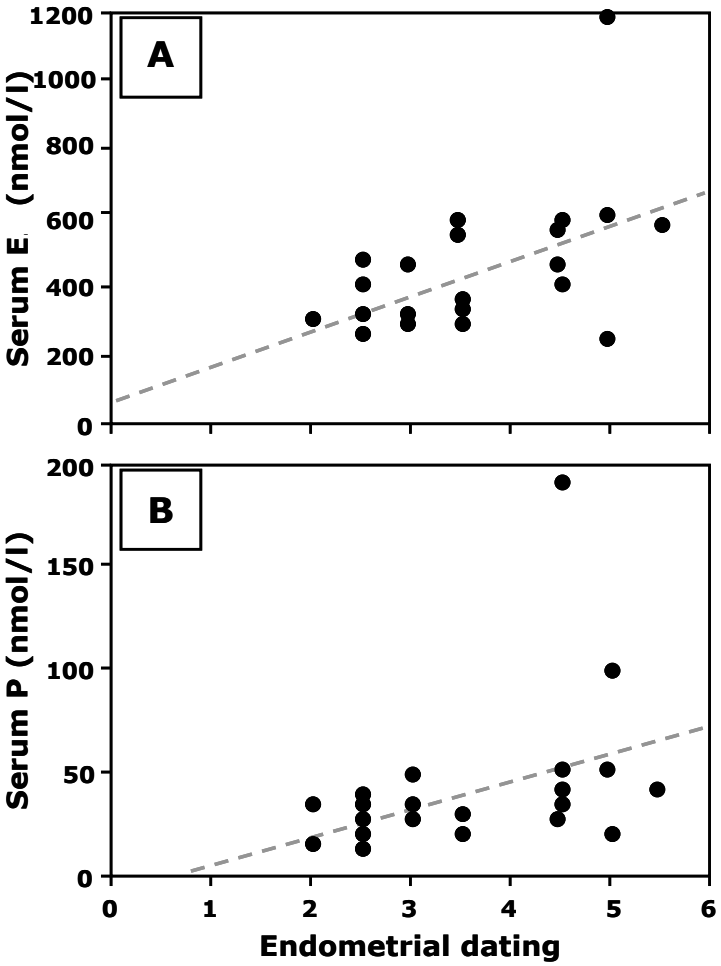


Figure 6.2. *Correlations between the endometrial tissue dating (postovulatory days) with the serum concentration of estradiol (E) (**A**, R = 0.568, P = 0.006) and with the serum concentration of progesterone (P) (**B**, R = 0.408, P = 0.023).*

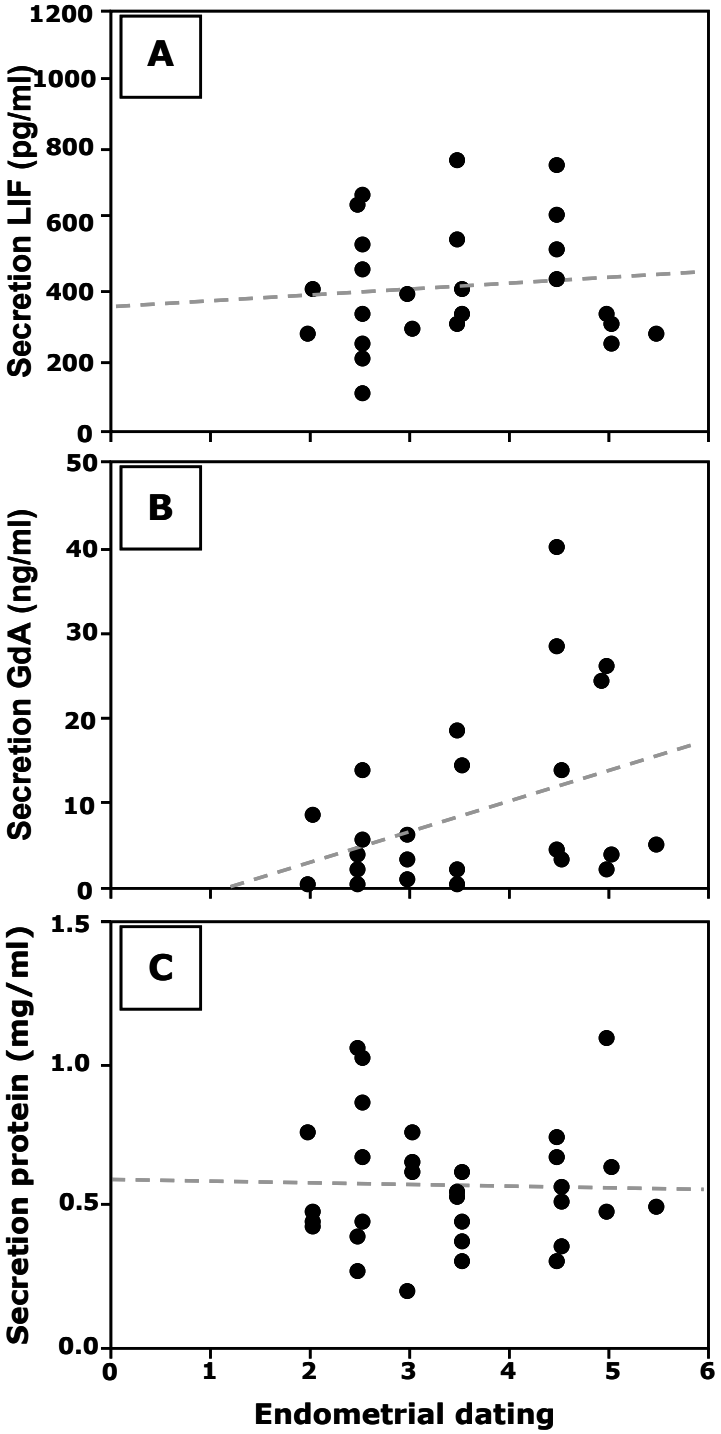


Figure 6.3. *Correlations between the endometrial tissue dating (postovulatory days) and LIF in endometrial secretions (**A**, R = 0.105, P = 0.594), GdA (**B**, R = 0.376, P = 0.048) in endometrial secretions and protein content in endometrial secretions (**C**, R = 0.013, P = 0.945).*

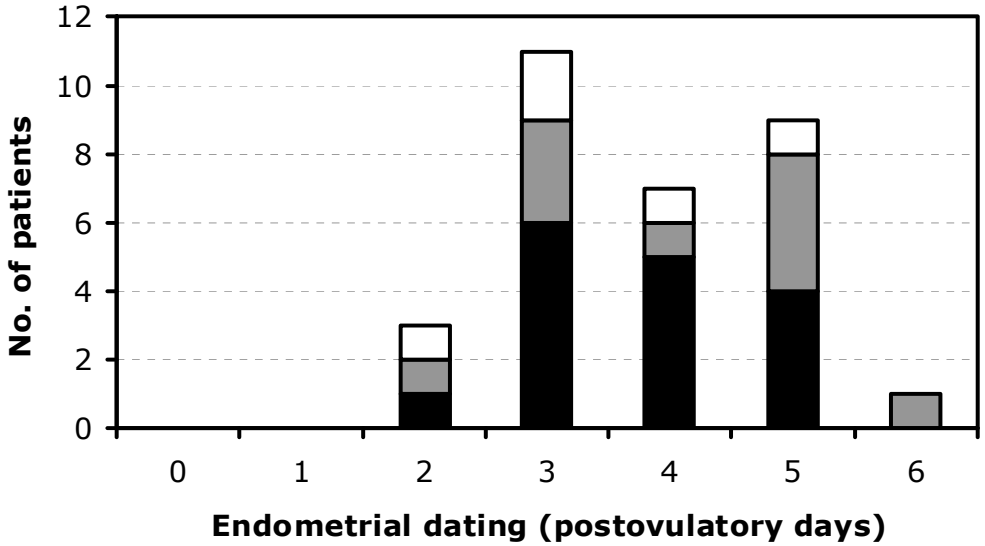


Figure 6.4. *Number of patients with optimal luteal phase pattern (black bars), suboptimal (grey bars), and impaired luteal phase pattern (white bars) according to scored protein profiles in endometrial secretions compared with endometrial dating (postovulatory days) determined in endometrial tissue obtained on the 5th day after ovulation. Only 5 women showed an impaired luteal phase pattern but independent of the endometrial dating.*

6.4 DISCUSSION

The principle aim of this study was to compare the assessment of endometrial maturation parameters that can be measured in endometrial secretion samples obtained by minimally-invasive techniques to those assessed in tissue biopsies. The results of endometrial dating assessed by the combination of the Noyes criteria and the immunohistochemical staining patterns of endometrial PR and Ki-67 expression were compared with the overall protein content, the 1D gel electophoresis protein pattern as well as LIF and GdA content of endometrial secretion fluids. As far as we are aware, these factors have never been studied simultaneously in endometrial secretions (obtained by aspiration or flushing) and conventional endometrial maturation parameters in tissue from the same subject.

Endometrial biopsy prior to embryo transfer in IVF cycles negatively affects implantation rates (Ledee-Bataille *et al.,* 2004a; Olivennes *et al.,* 2003; Van der Gaast *et al.,* 2003). In contrast, no decrease in pregnancy rates have been observed following transcervical aspiration of endometrial secretion fluids immediately prior to embryo transfer (Ledee-Bataille *et al.,* 2004a; Olivennes *et al.,* 2003; Van der Gaast *et al.,* 2003). This technique can be applied during the window of implantation without disrupting the implantation process (Ledee-Bataille *et al.,* 2004a; Olivennes *et al.,* 2003; Van der Gaast *et al.,* 2003), and is associated with less discomfort than endometrial suction microbiopsy (Li *et al.,* 1993c). No major complications following this approach have been reported.

Endometrial dating by assessment of the protein patterns of endometrial secretion fluids did not significantly correlate to that based on the combination

of Noyes criteria and immunohistochemical PR and Ki-67 staining patterns. Previous studies have indicated that protein profile expression in endometrial secretions undergo cyclical changes, demonstrating significant differences between the proliferative, periovulatory and late secretory phases (Beier-Hellwig *et al.,* 1989; Beier-Hellwig *et al.,* 1994). However, the resolution of 1D SDS-PAGE may be insufficient to demonstrate changes in protein profile expression within only few days between the early to mid-luteal phase.

E and P are the key modulators of endometrial maturation. Consistent with this, a significant correlation was observed between endometrial maturation determined by the Noyes criteria combined with immunohistochemical assessments, and serum E and P levels.

The total protein content of endometrial secretion samples did not vary according to endometrial dating at the tissue level. This finding is consistent with several other studies which did not find a significant cycle dependent variation of the total protein content of endometrial flushings (MacLaughlin *et al.,* 1983; Sylvan *et al.,* 1981; Voss *et al.,* 1977). In contrast, two studies reported a higher protein content in endometrial secretion samples retrieved from secretory phase (MacLaughlin *et al.,* 1986; Sullivan *et al.,* 1984). This discrepancy may be due to differences in analyzing techniques. Moreover, different proteins may subtly vary during the menstrual cycle without measurable variations in the total protein content. It is likely that changes are too small and the present method of analysis too crude to discriminate cyclic variation.

Two specific proteins reported to have key roles in implantation, GdA and LIF (Diedrich *et al.,* 2007), were analysed in the endometrial secretion samples. LIF has been shown to be essential for implantation in mice (Stewart *et al.,* 1992) and is upregulated in the human endometrium at the time of implantation (Charnock-Jones *et al.,* 1994; Classen-Linke *et al.,* 1998). LIF modulates the invasiveness of trophoblast cells (Bischof *et al.,* 1995) and affects immune tolerance by controlling HLA-G expression of invasive cytotrophoblast cells (Bamberger *et al.,* 2000) during implantation. It has been suggested that impaired LIF production may underlie some cases of recurrent miscarriage (Piccinni *et al.,* 1998; Steck *et al.,* 2004). Furthermore, the amount of LIF in endometrial flushings was recently described to be highly predictive for pregnancy in the future menstrual cycles (Mikolajczyk *et al.,* 2007). However, this study was based on returned voluntary questionnaires.

We detected LIF in aspirated endometrial secretions in all patients with endometrial tissue dating of 2 days or later after ovulation. This is consistent with previous studies which have shown that LIF appears in endometrial flushings in the early luteal phase from postovulatory day 2 and onwards (Laird *et al.,* 1997; Mikolajczyk *et al.,* 2003). However, the present study showed no correlation between LIF levels in endometrial secretion samples with the P serum concentration in the early luteal phase. Furthermore, we found no significant correlation between LIF and endometrial maturation based on the combined assessments at the tissue level. This may be due to the large range of the LIF concentrations observed in endometrial secretions (Laird *et al.,* 1997; Ledee-Bataille *et al.,* 2002; Mikolajczyk *et al.,* 2003; Olivennes *et al.,* 2003). Furthermore, there is evidence that LIF expression is not primarily modulated by

steroid hormones,(Ng *et al.,* 2004) but by TGF-β, TNF-α and IL-1β (Arici *et al.,* 1995). Although LIF has been shown in mice to be crucial for implantation (Stewart *et al.,* 1992) its importance and regulation pathways in human implantation remains unclear. In conclusion, the LIF content of endometrial secretion fluids cannot be used as marker for endometrial maturity.

The second candidate marker for endometrial maturation was GdA. Global gene profiling studies revealed a significant increase of GdA expression during the window of implantation (Kao *et al.,* 2002). Furthermore, endometrial gene expression studies suggest that sex steroids play an important role in regulating endometrial GdA expression (Horcajadas *et al.,* 2007) Like LIF, GdA may also suppress the maternal immune response possibly through suppression of the NK cells (Okamoto *et al.,* 1991), and an immunoprotective role for GdA during implantation and placentation has been proposed (Seppala *et al.,* 2007)

In this study, GdA was detected in endometrial secretions when the endometrium was dated as post-ovulatory day 2 and onwards. Additionally, GdA expression increased together with the endometrial maturation detected at the tissue level. This is consistent with a previous study showing a positive correlation between GdA levels in endometrial flushings and endometrial maturation (Tuckerman *et al.,* 2004). Other studies have identified GdA in uterine flushings 3–4 days after ovulation (Dalton *et al.,* 1995; Li *et al.,* 1993a). Moreover, fertile patients showed higher levels of GdA in uterine flushings compared to the subfertile controls (Dalton *et al.,* 1998), suggesting that an increase of GdA might facilitate implantation. In the present study, GdA protein levels in the endometrial fluid clearly correlated with the combined endometrial dating using the Noyes criteria and immunohistochemical marker. These findings support a possible role for endometrial secretion GdA level as a marker of endometrial maturation. Further studies are required to ascertain whether endometrial secretion GdA concentrations provide a more specific and sensitive predictor of implantation.

Although the study subjects were similar in terms of cycle characteristics, they differed in cause of subfertility. However, since both endometrial secretion and biopsy material were compared within each subject and each subject was its own control, the effect of heterogeneous fertility on the presented analysis is likely to be limited.

In conclusion, we showed that putative secretory receptivity markers can be simultaneously analyzed in endometrial secretions, which can be retrieved without disrupting endometrial receptivity. Only the GdA level was significantly correlated with endometrial dating. Therefore, the measurement of GdA in non-invasively obtained endometrial secretion samples may represent a novel diagnostic tool to monitor endometrial maturation in a way that does not affect implantation. This study also demonstrated that it is possible to assess a small array of putative receptivity markers simultaneously in a single endometrial secretion sample. This opens the possibility for studying the complex intrauterine regulatory networks prior to implantation and the identification of further important regulators of endometrial maturation and receptivity. In contrast to more invasive techniques, endometrial fluid aspiration makes it possible to correlate such markers directly with successful implantation.

7.

THE IMPACT OF OVARIAN STIMULATION WITH FSH IN COMBINATION WITH GNRH ANTAGONIST ON ENDOMETRIAL SECRETION FLUID AND TISSUE IN THE WINDOW OF IMPLANTATION.

Mark H. van der Gaast, Irmgard Classen-Linke, Claudia A. Krusche, Karin Beier-Hellwig, Bart C. J. M. Fauser, Henning M. Beier and Nick S. Macklon.

Impact of ovarian stimulation on midluteal endometrial tissue and secretion markers of receptivity.

Reprod BioMed Online ***2008****;17:553-63.*

7.1 INTRODUCTION

Despite increasingly efficient oocyte recovery and fertilization techniques, pregnancy rates in IVF remain 20 – 30% per embryo transfer procedure. Failure of implantation is now one of the most important limitations on IVF results (Fauser *et al.,* 2005). Although, abnormal embryos probably account for a large proportion of implantation failures, an unfavorable intra-uterine environment may also contribute to implantation failure (Beier, 1974; Edwards, 1994). There is also evidence that supraphysiological elevated E serum concentrations, as result of ovarian stimulation regimes employed in IVF treatment cycles, disrupt the endocrinology of the luteal phase (Beckers *et al.,* 2003), peri-ovulatory endometrial histology (Devroey *et al.,* 2004) and implantation (Simon *et al.,* 1995).

Co-treatment with GnRH antagonist instead of GnRH agonist, to prevent premature luteinization, has enabled the development of milder ovarian stimulation protocols which may have a reduced impact on endometrial receptivity (Boomsma *et al.,* 2006). However, concerns remain regarding a possible detrimental effect of GnRH antagonists on endometrial receptivity (Martinez-Conejero *et al.,* 2007; Tarlatzis *et al.,* 2006). Moreover, the luteal phase following ovarian stimulation for IVF co-treated with GnRH antagonist has been shown to be abnormal (Beckers *et al.,* 2003). We have recently demonstrated that ovarian stimulation with recFSH and GnRH antagonist co-treatment without P supplementation results in dysregulation of 192 genes within the endometrium (Chapter **4**; Macklon *et al.,* 2008). Those gene array studies are performed using total tissue samples of endometrial biopsies for intracellular gene activity assessment. However, little information exists regarding the effect of ovarian stimulation on protein expression at the endometrial embryo interface.

In vivo study of endometrial receptivity in the oocyte donation model permits the use of endometrial biopsy material for analysis during the 'window of implantation' without disrupting implantation. The 'classical' technique used to assess endometrial maturation is the histological dating of biopsy material using the criteria described by Noyes (Noyes *et al.,* 1950). However, Noyes criteria have been shown to be poorly correlated with endometrial receptivity and implantation (Bergh *et al.,* 1992). Moreover, dating by Noyes is subject to variable interpretation due to a lack of distinct sequential endometrial changes (Murray *et al.,* 2004), and large intra- and intersubject and inter-observer variability (Myers *et al.,* 2004). To improve the assessment of endometrial maturation, expression of functional markers such as the proliferation marker Ki-67 and of ER and PR can be analyzed by immunohistochemistry (Classen-Linke *et al.,* 1998).

Furthermore, the viscous fluid secreted by the endometrium, which reflects additional aspects of endometrial function as well as the embryo-endometrial dialogue prior to implantation, is another important compartment in the assessment of endometrial maturation and differentiation (Beier-Hellwig *et al.,* 1989; Giudice, 1999a; Herrler *et al.,* 2003; Lindhard *et al.,* 2002). Endometrial secretion has been shown to contain (a) proteins originating from transudation

of serum, (b) leakage products of apoptotic epithelial cells and (c) proteins secreted from glandular epithelium, and to undergo significant changes in protein content in the transition from the proliferative into the secretory phase (Beier, 1974; Beier *et al.,* 1998; Maathuis *et al.,* 1978b). Endometrial secretion composition varies during the menstrual cycle as a result of changes in ovarian steroid serum concentration (Maathuis *et al.,* 1978b). Furthermore, endometrial secretion contains cytokines such as leukemia inhibitory factor (LIF) (Laird *et al.,* 1997) and interleukins (Makkar *et al.,* 2006; Simon *et al.,* 1996) as well as steroid hormones (E, P and their precursors) (Stone *et al.,* 1986). We have previously shown that endometrial secretion can be withdrawn for analysis immediately prior to embryo transfer in IVF cycles without disrupting implantation (Van der Gaast *et al.,* 2003). This approach circumvents one of the major challenges of endometrial research: investigating endometrial performance during the 'window of implantation' without disrupting endometrial function and the following process of implantation. With this technique, factors that are involved in endometrial differentiation and receptivity can be directly correlated with the outcomes of embryo transfer.

In order to gain a greater insight into the impact of ovarian stimulation with recFSH and GnRH antagonist cotreatment on endometrial receptivity at the time of embryo transfer, we employed this approach in combination with analysis of tissue markers of endometrial maturation. In this prospective cohort study using a multi-marker approach, we compared the natural and the stimulated cycle in the same patient, and in addition we studied the effect of ovarian stimulation with and without luteal progesterone supplementation on endometrial maturation. Further, the relationship between endometrial secretion levels of LIF, GdA and P and serum P and E levels was studied.

7.2 MATERIAL AND METHODS

7.2.1 Subjects

This prospective study was approved by the local ethics review board. Women of proven fertility, attending the out-patient fertility department to donate oocytes on a voluntary basis, were asked to participate in this study. Approval and written informed consent was obtained from all participating oocyte donor patients.

Inclusion criteria were: (a) age under 40 years; (b) regular menstrual cycles, ranging between 25–35 days; (c) no uterine abnormalities detectable by ultrasound; (d) completed family; (e) no hormonal contraception for 2 months prior to inclusion; (f) no history of endometriosis; and (g) no concurrent medical condition.

7.2.2 Assessments

Ovarian follicle development was monitored by transvaginal ultrasound (TVS) in all patients by the same operator (M.vdG.); initially in a spontaneous menstrual cycle and subsequently in a stimulated IVF cycle (Figure **7.1**). In the spontaneous cycle, TVS was performed on cycle day 2 and daily from day 8 until

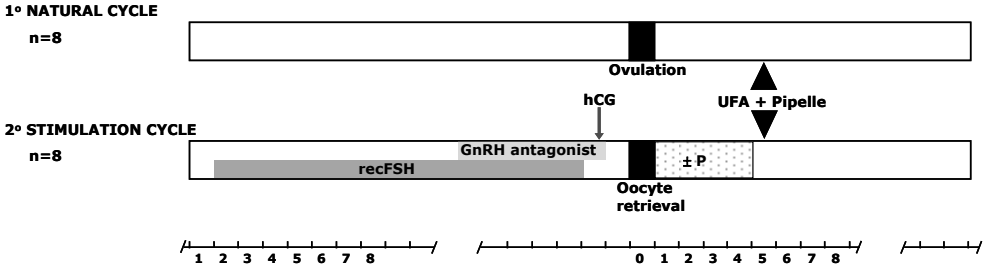


Figure 7.1. *Schematic representation of the assessments (ESA, endometrial secretion aspiration; Pipelle biopsy) in the spontaneous natural cycle and stimulated IVF cycles. Following a spontaneous natural cycle, all 8 patients underwent an IVF cycle to donate oocytes using exogenous recombinant FSH (recFSH) stimulation from cycle day 2, and GnRH antagonist co-treatment commenced when the leading follicle had a diameter of ≥ 14 mm until hCG was administered for final oocyte maturation. After oocyte retrieval 4 oocyte donors received vaginal progesterone (P) for luteal phase support until day 5, and the other 4 donors did not.*

spontaneous follicle rupture, identified by disappearance of the leading follicle and the presence of free fluid in the pouch of Douglas (Ecochard *et al.,* 2000b).

In the stimulation IVF cycle, patients received a fixed daily dose of recombinant FSH (Puregon®, Organon, Oss, The Netherlands) 150 IU sc from cycle day 2 onwards. GnRH antagonist (Orgalutran®, Organon) 1 mg sc daily was commenced on the day on which the leading follicle reached a diameter of ≥ 14 mm, and both medications were continued until at least one follicle reached a diameter of at least 18 mm. On that day hCG (Pregnyl®, Organon) 10,000 IU sc was administered for the induction of final oocyte maturation. Oocytes were retrieved 35 hours later. In the first 4 patients P supplementation (Progestan®, Organon) 200 mg tid was provided for 5 days after oocyte retrieval for IVF.

On the 5th day after ovulation or oocyte retrieval in the spontaneous and stimulation IVF cycle respectively, the uterine cavity and cervix length were measured by TVS. Blood sampling was performed and transcervical aspiration of endometrial secretion fluid was then performed, as described previously (Van der Gaast *et al.,* 2003). Briefly, an insemination catheter (ASSA med GmbH, Bexbach, Germany) was cautiously inserted in the uterine cavity at a depth of the measured cervix length and 1 cm beyond. Then suction was gently applied while simultaneously rotating the catheter. Immediately after the aspiration, the outside of the tip of the double outlet soft insemination catheter was cleaned with a sterile cloth. The tip containing the secretion fluid was cut off into an Eppendorf cup and stored at –20°C until final assessment. An endometrial biopsy was then obtained transcervically using a suction curette (Pipelle de Cornier®, C.C.D. Laboratoire, France). Biopsied tissue was fixed in neutral buffered 3.7% formalin.

Each specimen was labeled using a coding system for which the observers in the laboratory were blinded.

7.2.3 Serum hormone assays

Blood was centrifuged for 10 minutes at 1500 ×g immediately after obtaining the samples, and the aliquots of serum were extracted and stored at –20°C until assessment. Serum E and P were measured by an immunofluorometric assay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA) performed in the same laboratory. Respective intra- and interassay coefficients of variation for P were less than 10% for both, and less than 5% and 7% for E.

7.2.4 Endometrial tissue analysis

Biopsied tissue was fixed in neutral buffered 3.7% formalin, and dehydrated with increasing ethanol concentration before it was embedded in paraffin. A portion of endometrial tissue from each specimen was routinely cut and mounted and stained with hematoxylin-eosin for histological dating according to the criteria of Noyes (Noyes *et al.,* 1950). Paraffin-embedded sections were deparaffinized and rehydrated in PBS. The slides were heated in a citrate buffer by microwave (4×5 min; 600 W) for epitope retrieval and the immunhistochemical staining was performed by a steptavidin-biotin-peroxidase method (Histostain-SP Kit; Zymed Laboratories Inc, Berlin, Germany).

The primary monoclonal antibody MIB-1 (M 7240, DakoCytomation, Hamburg, Germany) which was used to detect the nuclear proliferation marker Ki-67 in endometrial tissue was diluted 1:100 in PBS/1.5% BSA and applied overnight at 4°C. Visualising of the Ki-67 antigen was performed by peroxidase catalyzing the substrate and converting the chromogen AEC (Zymed Laboratories Inc, Berlin, Germany) to a red deposit.

For the detection of estrogen and progesterone receptor (ER and PR) the same immunhistochemical procedure was used as described for MIB-1. The primary monoclonal antibody PR (clone PgR 636, M 3569, DakoCytomation, Hamburg, Germany) which recognizes the A and B forms of the receptor was diluted 1:50, and the primary monoclonal antibody ER (clone ER 6 F11, Novocastra, Newcastle upon Tyne, UK) which recognizes ER-α was diluted 1:40.

As negative control the primary antibodies were replaced with non-immune mouse IgG at the same concentration. None of the controls revealed a positive staining.

Endometrial maturity in biopsied tissue was assessed by Noyes histological criteria together with immunohistochemical Ki-67, ER and PR which were scored semi-quantitatively (Hendrickson *et al.,* 1997; Lessey *et al.,* 1988; Tuckerman *et al.,* 2004). Glycogen vacuoles in the endometrial glandular epithelium which are the hallmark of the early secretory phase (post-ovulatory days (POD) 2-5), were observed in all subjects. Analysis of tissue Ki-67 and PR expression enabled more precise assessment of maturation. When Ki-67 staining cells within the glandular epithelium were few or absent, the endometrium was dated as 3+ reflecting 'adequate' endometrial maturation. When 10-50% of glands showed Ki-67 positive cells, the endometrium was dated as 2+, characterizing slightly retarded maturation (1 day retarded) since proliferation was continuing. When more than 50% of glands showed Ki-67 positive cells the endometrium was dated as 1+ , indicating retarded maturation (2 days retarded). Strong staining of PR in the glands was dated as 2+ or 1+ (slightly or retarded

maturation). Less intense staining of the PR in the glandular epithelial cells in contrast to the stromal cells, indicating the down-regulation of PR, was dated as adequate maturation (3+). All results combined resulted in the final dating of the endometrial biopsies as given in Table **7.3**. All endometrial tissue assessments were performed by two observers (I.C.-L. and C.A.K.), who were blinded to the endometrial secretion fluid assessment results. The correlation between the scorings of the two different assessors was 87.5%.

7.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and laser densitometry

Secretion fluids were removed from the tip of the catheter, incubated for 10 minutes in an ultrasound bath at 4°C and then centrifuged at 20,800 × g. Protein concentration of the supernatant was determined (Lowry *et al.,* 1951), and the protein composition was analyzed by SDS-PAGE using a 8.3–16.6% acrylamide separating gel with 5% acrylamide containing stacking gel. Each lane was loaded with 50 µg protein diluted with sample buffer. The gels were stained with Coomassie brilliant blue. Molecular weight was estimated by comparison to protein standards electrophoresed in adjacent lanes of the gel. The relative density of each band was measured using a scanning laser densitometer, He-Ne-Laser 633 nm (LKB Ultrascan XL, Pharmacia-LKB, Freiburg, Germany) and the GelScan XL software package (Figure **7.2**).

Figure **7.2** illustrates the data produced by this technique. A considerable number of protein bands appear among the lower-molecular weight fractions. This area is represented by the proteins between 68 kDa and 6.5 kDa. The totally expressed pattern under these conditions comprises some 60–70 bands, the most pronounced and heavily staining of which are albumin at 68 kDa and the two of α- and ß-chains of haemoglobin, close to the position of 12.5 kDa. Bands below 68 kDa which form 3 groups of similarly sized, partly faintly staining bands are the focus of our assessments. Group A is represented by bands between 55 and 34 kDa, group B from 29 to 25 kDa, and group C from 18 to 12 kDa. Group C awaits its completion at the time of about 48 hrs after ovulation. Particular attention is paid to the 3 intensely staining bands in the range of 15–18 kDa. The 12.5kDa-protein fraction decreases in width during the periovulatory period and remains less prominent during the luteal phase. Such pattern of the luteal phase is designated 'adequate luteal phase pattern' or 'optimal luteal phase pattern'. We have published 4 such patterns of patients, who experienced normal clinical pregnancies which began in such cycles and delivered healthy babies (Beier-Hellwig *et al.,* 1989, see Fig. 7). Impaired luteal phase patterns showing significant changes in Group B and Group C fractions where obtained after various treatments with progesterone antagonists (RU486 and Onapristone), as reported in the same publication (Beier-Hellwig *et al.,* 1989, see Figs. 8 and 9).

Protein pattern analyses were scored semi-quantitatively according to previously published criteria (Beier-Hellwig *et al.,* 1989). All samples were analyzed independently by two observers (K.B.H. and H.M.B), who were blinded

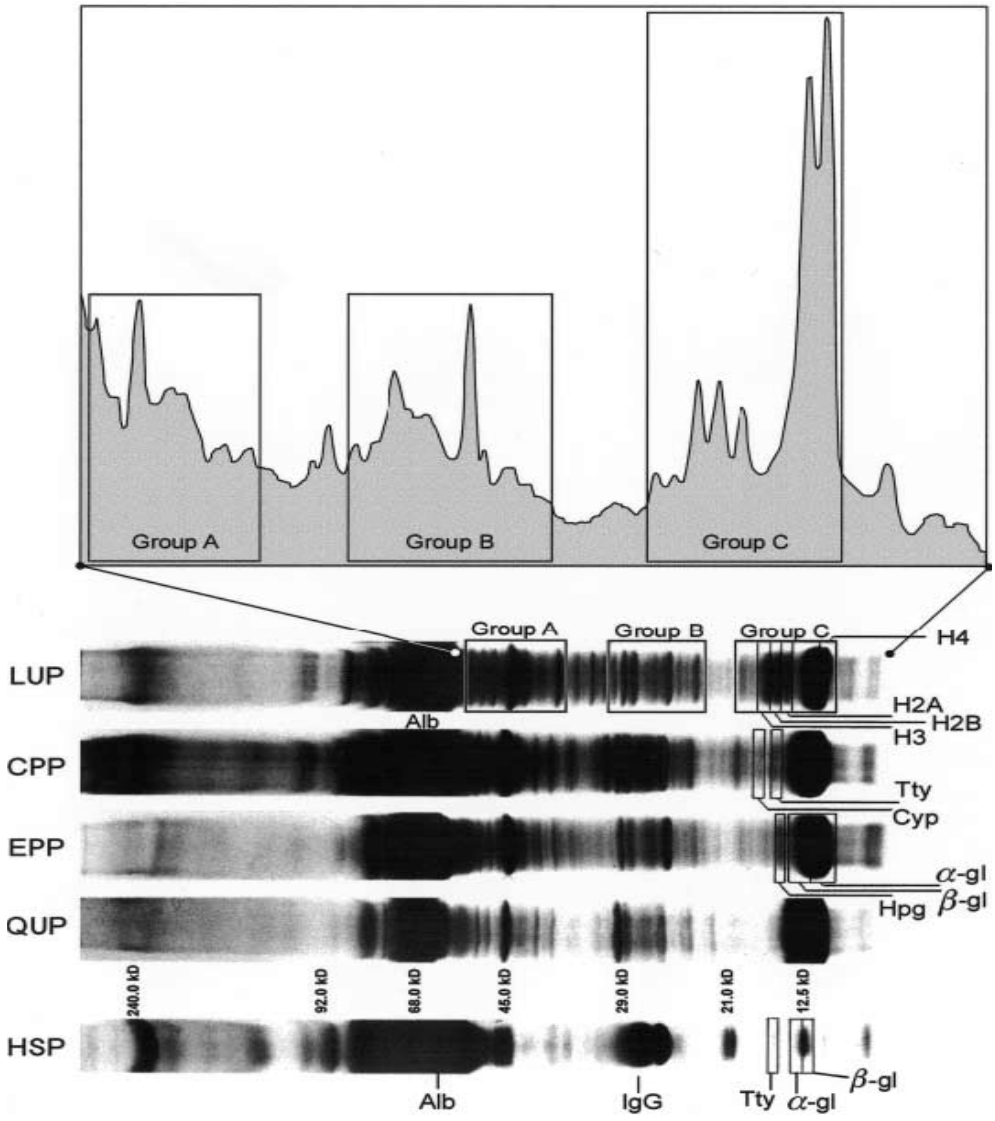


Figure 7.2. *Protein patterns of human uterine secretion at various stages of the menstrual cycle, in comparison to the protein pattern of human blood serum (HSP). The patterns demonstrated are from the quiescent uterine phase (QUP), early proliferative phase (EPP), late proliferative phase (CPP), and the luteal uterine phase (LUP).The molecular weight ranges of the proteins are depicted between the HSP and QUP patterns. Albumin (Alb), immunoglobin (IgG), histones (H2A, H2B, H3, H4), cyclophilin (Cyp), transthyretin (Tty), haptoglobin (Hpg) and haemoglobin chains of α-globin and β-globin (α-gl, β-gl) are indicated. Changes in particular protein bands are devided in groups, which are shown in boxes (groups A, B and C). The upper part is the laser densitometric representation of the LUP protein profile showing changed expression of these families of proteins, in particular appearing when compared to the proliferative phase of the menstrual cycle (kindly provided by Oxford University Press from Beier and Beier-Hellwig 1998).*

to the endometrial tissue assessment results. The correlation between the scorings of the two different assessors was 93.8%.

7.2.6 LIF, GdA, and P assessment in endometrial secretions by ELISA

The intra- and interassay variations of the LIF ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany) were 2.4% and 6.1%, respectively. The detection limit was 8 pg/ml. The intra- and interassay variation of the GdA ELISA (BIOSERV, Rostock, Germany) was 8.3% and 4.58%, respectively. The P ELISA was purchased from IBL-Hamburg (Hamburg, Germany). The intra- and interassay variations were 6.4% and 6.63%, and the detection limit was 0.045 ng/ml.

7.2.7 Data analysis

The Wilcoxon matched pairs signed rank sum and Mann-Whitney test were used for comparing natural to stimulated IVF cycles, and IVF cycles with to without luteal support, respectively. Pearson's correlation coefficients were used for relation analysis of serum and secretion fluid levels of P, LIF and GdA. In all statistical analyses a *P* value <0.05 was considered statistically significant. The analysis was performed with a commercially available software package (GraphPad Prism 3.00).

7.3 RESULTS

The mean age of subjects was 34.3 year (range, 31.5–38.3) and median parity was 2 (range, 1–7). In two of the 10 patients recruited to the study, detection of the spontaneous ovulation by ultrasound in the natural cycle failed and therefore time of ovulation could not be determined, and they were withdrawn from the study. Of the remaining 8 patients no significant difference in age and parity was

Table 7.1. *Ovarian steroid serum concentrations on postovulatory day 5 (median and range): comparison between the natural and stimulated IVF cycle, and comparison between the stimulated IVF cycles with and without luteal progesterone supplementation.*

	Natural vs. Stimulation cycle		***P*** †	**Stimulation cycle with vs. without luteal support**		***P****
	Natural cycle (n = 8)	**IVF cycle** (n = 8)		**IVF + P** (n = 4)	**IVF - P** (n = 4)	
E (pmol/L)	377 (256-180)	1860 (1220-2800)	0.01	2130 (1220-12800)	1790 (1710-1880)	0.34
P (nmol/L)	25.2 (20.5-53.0)	209 (91.1-822)	0.01	227 (104-822)	193 (91.1-223)	0.49

† Wilcoxon matched pairs signed ranksum test
***** Mann-Whitney test

observed between the 4 patients with, and the 4 patients without luteal P supplementation in the stimulated cycles.

7.3.1 Serum steroid concentrations

Serum E and P serum levels measured 5 days after ovulation and oocyte retrieval were significantly higher in all stimulated IVF cycles compared to the spontaneous natural cycles (Table **7.1**). No differences in E and P serum levels between stimulated IVF cycles with and without luteal P supplementation were detected (Table **7.1**).

7.3.2 Endometrial tissue analysis

There were no obvious differences in the intensity and localization of Ki-67, ER and PR immunostaining 5 days after spontaneous ovulation in the natural cycle or after oocyte retrieval in the stimulated IVF cycle (Figure **7.3**). However, significant differences were observed between stimulated IVF cycles supplemented with P versus non-supplemented cycles for staining for Ki-67 (Figure **7.3A**, median total staining scores, 1.5 vs. 3.25 respectively; $P = 0.03$ and Figure **7.4**) and ER (Figure **7.3C**, median total staining scores, 2.5 vs. 3.5 respectively; $P = 0.04$). No differences were found in PR expression between natural and stimulated cycles, whether or not luteal phase support was provided (Figure **7.3B**).

Endometrial dating by Noyes criteria combined with immunohistochemical assessment, showed no differences between natural and stimulated cycles, nor between P supplemented and non-supplemented stimulation cycles (respectively; postovulatory day 4.25 ± 0.66 vs. 4.19 ± 0.60 ($P = 1.0$); 4.38 ± 0.63 vs. 4.0 ± 0.58 ($P = 0.49$)).

7.3.3 Endometrial secretion analysis

The total protein content of endometrial secretion samples did not significantly differ between stimulated and spontaneous cycles, or between stimulated cycles with or without P supplementation. Uterine secretion levels of LIF, GdA and P did not significantly differ between natural and stimulated cycles independent of the application of luteal phase support (Table **7.2**).

Figure **7.5** shows the relationships of concentration differences between the stimulated and the natural cycle in each patient for serum P, as well as P, LIF and GdA in endometrial secretions. A strong correlation between P changes in uterine fluids with those of serum P (Figure **7.5A**; $R_P = 0.71$, $P = 0.04$) and E (data not shown; $R_E = 0.74$, $P = 0.04$) was observed. In uterine secretion samples, changes in GdA concentration, but not in LIF concentration, were significantly correlated with changes in P endometrial secretion levels (respectively Figure **7.5C**; $R_{GdA} = 0.81$, $P = 0.01$; Figure **7.5B**; $R_{LIF} = 0.69$, $P = 0.06$).

Table **7.3** demonstrates the assessments of luteal phase patterns during natural and during stimulation cycles from 8 subjects, respectively. The so-called 'secretory protein pattern score' is used within 3 assessment categories, optimal luteal phase pattern, suboptimal luteal phase pattern, and non-luteal phase pattern. In both the natural and stimulation cycle group 3 optimal scores were

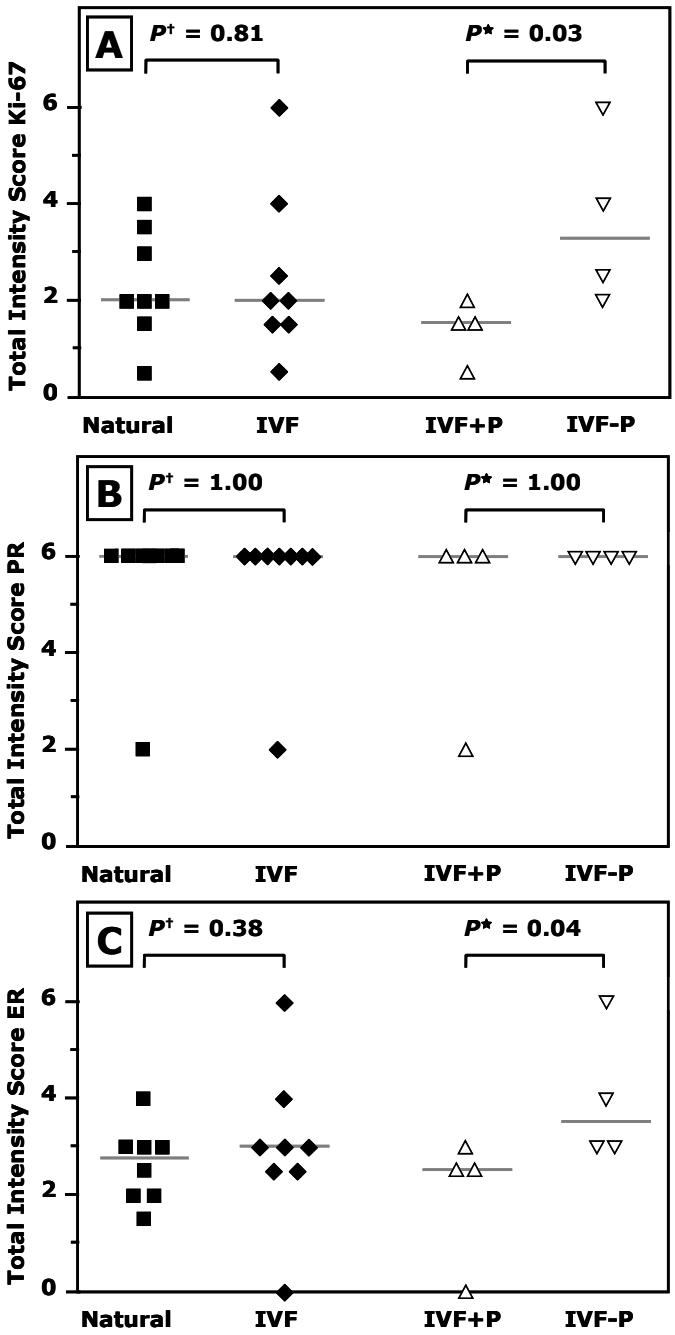


Figure 7.3. *Endometrial maturation assessment during the secretory phase as indicated after immunohistochemical analysis using a scatter plot with the median of the staining intensity scores (0, no; 0.5, weak; 1, moderate; 2, strong staining; scores in the luminal epithelium, glandular epithelium and stroma were added resulting in a score between 0 and 6) of proliferation marker Ki-67 (**A**), progesterone receptor (PR; **B**) and estrogen receptor (ER; **C**). Comparison of natural (squares) vs. IVF (diamonds) cycle, and IVF cycle with (up triangles) vs. without (down triangles) luteal phase support using Wilcoxon matched pairs signed ranksum† and Mann-Whitney* test respectively.*

observed. In natural cycles there are 4 suboptimal scores, and in stimulation cycles, only two. The 'non-luteal phase' score was found in just one natural cycle sample, but in 3 stimulation cycles. The question whether the luteal phases were identical in the same subjects or whether ovarian stimulation altered the score appeared to be answered by data from three patients: two optimal luteal phases stayed constant, and the only non-luteal phase appearing in natural circumstances stayed unchanged after stimulation. From the four 'suboptimal luteal' phases, one was unchanged (number 1), 2 dropped in score (number 5 and 7), and one improved (number 4) following ovarian stimulation. Finally, one optimal luteal phase dropped down to a suboptimal score. Patient 3 and patient 6 showed unchanging scores in both protein pattern assessments and endometrial morphological maturation assessment.

7.3.4 Comparison of endometrial cellular and secretion markers

The combined histological and immunohistochemical assessments were scored as adequate or slightly retarded but conducive to implantation in 7 respectively 8 of 8 women in the natural and in the stimulated cycle (Table **7.3**). No marked differences in cellular maturation score were observed between the natural and stimulated cycle. Protein pattern analyses were also scored as 'optimal' or 'suboptimal' but conducive to implantation in 7 out of 8 women in the spontaneous natural cycle, and in 5 out of 8 subjects in the stimulated IVF cycle (Table **7.3**). Again, no significant detrimental effect of ovarian stimulation on secretory protein pattern score was observed.

7.4 DISCUSSION

This preliminary study is the first in which the impact of ovarian stimulation on endometrial maturation during the luteal phase has been studied in both endometrial biopsies and in endometrial secretions. The principal observation was that the majority of the assessed markers, which reflect the differentiation and maturation status of the endometrium, were not profoundly disrupted by ovarian stimulation with recombinant FSH and GnRH antagonist co-treatment, when compared with the natural spontaneous cycle in the same subject.

Previous studies employing Noyes criteria to date endometrium at the time of oocyte retrieval have shown ovarian stimulation to be associated with advancement of endometrial maturation (Develioglu *et al.,* 1999; Kolibianakis *et al.,* 2002a; Papanikolaou *et al.,* 2005; Saadat *et al.,* 2004). However, it is uncertain to what extent data derived from the peri-ovulatory period can be extrapolated to the peri-implantation phase. In other words, it is unclear whether endometrial advancement observed during the peri-ovulatory phase reflects the status of endometrial differentiation in the peri-implantation phase. In a previous study, the observed advanced endometrial maturation in tissue retrieved on day of oocyte retrieval after ovarian stimulation, disappeared when assessed in the same subjects later in the luteal phase (Kolibianakis *et al.,* 2003). Others found advanced endometrial maturation on day of oocyte retrieval to have an effect on the endometrial status during the implantation window

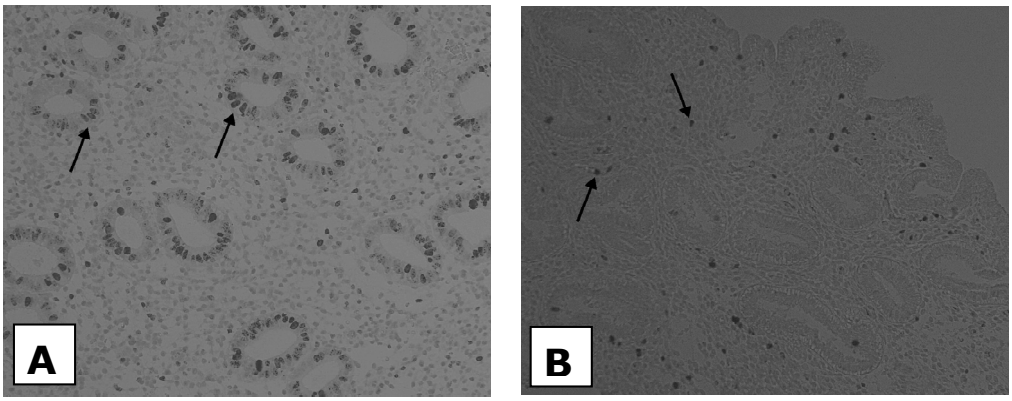


Figure 7.4. *Immunohistochemical staining of the proliferation marker Ki-67 (MIB-1). Representative strong staining of Ki-67 in the glandular epithelial cells (red deposit), dated as two days retarded maturation (**A**); representative no staining in the glandular epithelium (**B**) was dated as adequate endometrial maturation. Arrows indicate positive cells. Magnification: 200x.*

(Develioglu *et al.,* 1999; Saadat *et al.,* 2004). However, all these studies employed histological endometrial dating, which is discussed to lack precision and accuracy (Murray *et al.,* 2004; Myers *et al.,* 2004).

The proliferative phase endometrium shows strong Ki-67 staining in the nuclei of epithelial and stromal cells. During the secretory phase Ki-67 staining of glandular and luminal epithelial cells decreases to nearly zero around day 20 or 21 (Shiozawa *et al.,* 1996). Therefore, in addition to histological

Table 7.2. *Total protein content of endometrial secretion and concentrations of leukemia inhibitory factor (LIF), glycodelin (GdA) and progesterone on postovulatory day 5 (median and range), compared between the spontaneous natural and stimulated IVF cycle, and compared between the stimulated IVF cycles with and without luteal progesterone supplementation.*

	Natural vs. Stimulation cycle		***P*** †	**Stimulation cycle with vs. without luteal support**		***P****
	Natural cycle (n = 8)	**IVF cycle** (n = 8)		**IVF + P** (n = 4)	**IVF - P** (n = 4)	
protein content (mg/mL)	0.60 (0.34-1.09)	1.03 (0.42-2.31)	0.38	1.03 (0.45-2.31)	0.81 (0.42-1.54)	0.86
LIF (ng/mg prot.)	8.25 (0.0-15.6)	5.57 (1.72-19.3)	0.63	4.52 (2.55-19.3)	6.61 (1.72-15.1)	1.00
GdA (ng/mg prot.)	27.3 (2.60-77.3)	9.67 (0.75-60.3)	0.44	6.87 (6.84-12.5)	23.3 (0.75-60.3)	0.70
P (ng/mg prot.)	0.625 (0.30-1.0)	0.96 (0.42-2.35)	0.22	1.03 (0.62-2.35)	0.88 (0.42-1.30)	0.70

† Wilcoxon matched pairs signed ranksum test
***** Mann-Whitney test

advancement, ovarian stimulation might be expected to cause a further decrease in Ki-67 expression in the early luteal phase. However, we observed no difference in Ki-67 expression at day 19 between the natural and stimulated cycle. On the other hand, when IVF treatment cycles with and without luteal P supplementation were compared, a significantly higher level of Ki-67 expression was observed in the latter group, suggesting that endometrial advancement in stimulated cycles might be related to progesterone exposure (Develioglu *et al.,* 1999; Saadat *et al.,* 2004). A similar pattern was detected in endometrial ER expression, which showed no difference between the natural and stimulated cycle. However, those receiving progesterone supplementation demonstrated significantly reduced ER expression, consistent with the known down-regulatory effect of progesterone on ER expression in the early luteal phase (Lessey *et al.,* 1988). Although previous studies of the effect of ovarian stimulation on Ki-67, ER and PR expression during the peri-ovulatory period have shown inconsistent findings (Bourgain *et al.,* 2002; Papanikolaou *et al.,* 2005), these preliminary data suggest that in addition to estrogen levels (Ma *et al.,* 2003) luteal phase progesterone levels may determine the rate of endometrial maturation.

Studies of the impact of ovarian stimulation on endometrial gene expression during the luteal phase have shown a number of genes regulated by progesterone to be markedly dysregulated following ovarian stimulation (Horcajadas *et al.,* 2005; Macklon *et al.,* 2008; Mirkin *et al.,* 2004; Simon *et al.,* 2005). This would appear at odds with our findings. However, protein products are only partially correlated to upstream gene expression and many factors including rate of protein breakdown can influence the concentration of proteins expressed.

Ultimately, not the endometrial genes but their protein products characterize the intra-uterine environment during the periconceptional phase. Although many secretory endometrial proteins have been identified, their impact on endometrial function and maturation, and their role in embryo implantation remains unclear. However, proteins have been postulated as important regulators of endometrial receptivity (Ledee-Bataille *et al.,* 2005) and embryo-endometrial cross-talk (Simon *et al.,* 1997). Particular attention has been paid to LIF, which has been shown to be crucial for successful implantation in the mouse (Stewart *et al.,* 1992). Like LIF, another putative marker of implantation GdA has also been found in human endometrial secretions from day 3–4 in the luteal phase (Li *et al.,* 1993a; Seppala *et al.,* 2002). In this study, we observed no statistically significant changes in uterine secretion GdA levels after ovarian stimulation compared to the natural cycle. This finding was consistent with the findings of a previous cross-sectional study of endometrial flushing in subfertile patients (Ng *et al.,* 2004). In contrast, analysis of endometrial tissue revealed higher GdA expression after ovarian stimulation (Brown *et al.,* 2000). This discrepancy may relate to the difference in stimulation protocols and the compartment being analysed, as these data were derived from tissue rather than secretion analysis. Another study showed no difference in LIF levels in endometrial secretions after IVF stimulation compared to the group without stimulation (Olivennes *et al.,* 2003). These results were also in accordance with our findings, but in contrast with our study, endometrial secretion was obtained from subfertile patients on

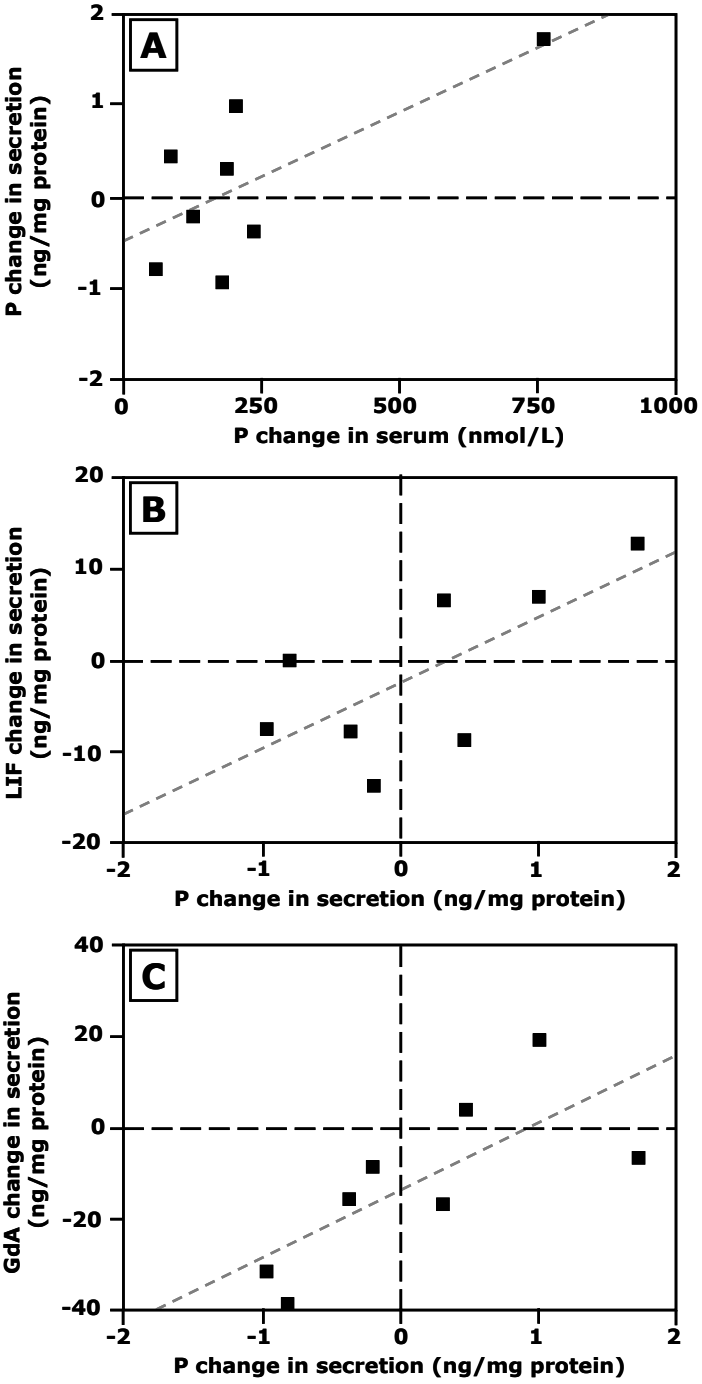


Figure 7.5. *Scatter plots relating changes of serum P in serum, and P, LIF and GdA in uterine secretion samples. Changes in levels were calculated by substracting the level measured in natural cycle from the levels measured in the stimulated cycles in each subject. Statistical analysis revealed a strong correlation of P changes in secretions with P changes in serum (**A**; R_P = 0.71, P = 0.04), and with GdA changes in secretions (**C**; R_{GdA} = 0.81, P = 0.01), but not with LIF changes in secretions (**B**; R_{LIF} = 0.69, P = 0.06).*

Table 7.3. *Comparing endometrial tissue morphology with protein pattern of the endometrial secretion on postovulatory day 5. Endometrial maturation assessment was performed using combined histological and immunohistochemical (MIB/ Ki-67, PR, ER) analysis: +++ = adequate maturation, ++ = slightly retarded maturation, + = retarded maturation. Assessment of luteal phase secretion fluid as indicated by uterine fluid protein pattern after SDS-PAGE and laser densitometry: +++ = optimal, ++ = suboptimal, + = non-luteal phase pattern.*

Subject	Natural cycle		Stimulation cycle	
	Cellular endometrial maturation score	Secretory protein pattern score	Cellular endometrial maturation score	Secretory protein pattern score
1	++	++	+++	++
2	+	+++	+++	+++
3	+++	+	+++	+
4	+++	++	++	+++
5	+++	++	+++	+
6	+++	+++	+++	+++
7	+++	++	++	+
8	+++	+++	++	++

day of oocyte retrieval, rather than on the day of embryo transfer (ET), and the LIF levels were compared with matched control subjects.

Protein expression profiles in uterine secretions have been shown to change during the spontaneous natural cycles (Beier *et al.,* 1973; Beier-Hellwig *et al.,* 1989; Beier-Hellwig *et al.,* 1994; Maathuis *et al.,* 1978b). The significance of endometrial secretion and its protein patterns for implantation in animal models have been demonstrated by the identification of luteal phase patterns which give rise to successful implantation after embryo transfer (Beier, 1974). Endometrial tissue from a normal uterus releases luteal phase specific proteins in its secretion when it has transformed adequately from proliferative into the secretory state. Consistent with the lack of advancement observed on the endometrial tissue level, the protein profiles of the endometrial secretions revealed evidence of a slight endometrial retardation. A non-significant trend towards weakening of secretory capacity was observed in 3 out of 8 subjects within their stimulated IVF cycles (Table **7.3**), whereas 4 of the 8 subjects showed constant performance, regardless whether it was a optimal luteal phase pattern or a non-luteal phase pattern. One patient showed an improved pattern shift from 'suboptimal' to an 'optimal' score.

The combined analysis of endometrial tissue and endometrial secretions after ovarian stimulation compared to the natural cycle in the same subject at the beginning of the window of implantation provides novel data on the impact of IVF treatment on endometrial differentiation. Despite the small number of subjects included in this preliminary study, the paired design of this study allows cautious conclusions to be drawn. The present study demonstrates that ovarian stimulation with GnRH antagonist co-treatment has little impact on tissue-derived markers of endometrial maturation, or on LIF, GdA, and P levels in the uterine cavity during the 'window of implantation'. Our data are consistent with the concept that markers of endometrial receptivity alter between the time of

oocyte retrieval and ET 5 days after oocyte retrieval, thus limiting the accuracy of predicting endometrial maturation during the peri-implantation period.

New techniques, genome and protein microarrays, are required which enable the study of endometrial differentiation in the peri-implantation phase in IVF treatment cycles. Aspiration of endometrial secretions offers a non-invasive technique to study the 'black box' of implantation without disrupting the implantation process. Further development and application of such techniques will enable elucidation of what constitutes the optimal periconceptional uterine environment for implantation, and the impact of ovarian stimulation on endometrial receptivity.

8.

DISCUSSION, CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Over the past two and a half decades IVF has evolved from a rarely performed surgical procedure to a routine outpatient based treatment for infertility. Although pregnancy rates have increased as result of development in technology and clinical protocols, side effects are still common, and efficacy remains relatively low, with ongoing pregnancy rates per started cycle around 25% (ESHRE 2008) (Fauser *et al.,* 2005; Macklon *et al.,* 2006). In recent years much attention has been given to ovarian function, follicular growth, and clinical aspects of ovarian stimulation and management of the luteal phase. The aim of ovarian stimulation for IVF became the retrieval of as many oocytes as possible in each stimulation cycle, to allow the production of multiple embryos for selection and transfer into the uterus. This remains the basis of practice in most centres (de Boer *et al.,* 2004; Van der Gaast *et al.,* 2006). However, as we have shown in this thesis, pregnancy rates are not linearly correlated to the number of oocytes obtained; indeed following conventional stimulation with the long GnRH protocol, rates were observed to fall when more than 12 oocytes were obtained. Possible reasons for this include a possible effect on the quality of the occytes obtained, and/or an impact of ovarian stimulation on the endometrium.

Hormonal ovarian stimulation affects the profiles of the ovarian steroids E and P in the luteal phase (Beckers *et al.,* 2000), and in combination with GnRH antagonist without P supplementation a decrease of pregnancy rates occur (Beckers *et al.,* 2003). Furthermore, with increasing retrieved oocytes from by oocyte pickup after vigorous ovarian stimulation, decreasing pregnancy rates occur after a optimum of 10-14 oocytes (Van der Gaast *et al.,* 2006). Contradictory data is available of endometrial maturation in the luteal phase after ovarian stimulation. Most studies show advanced endometrial maturation with asynchrony of epithelium and stroma (Basir *et al.,* 2001; Meyer *et al.,* 1999; Noci *et al.,* 1997). However, according to our study (Van der Gaast *et al.,* 2008) another study showed no advancement of endometrial maturation between natural and IVF cycles (Lukassen *et al.,* 2004). Minimal stimulation may inflict a moderate ovarian response with minimal endometrial alteration of maturation (Basir *et al.,* 2001). Furthermore, irrespective of the ovarian stimulation, endometrial tissue advancement in biopsies retrieved in the early luteal phase has less pronounced advancement if retrieved later in the luteal phase (Kolibianakis *et al.,* 2003). This shows that early luteal assessment of endometrial maturation from receptivity point of view is not very useful. P supplementation is very importantant for establishing pregnancy when recFSH and GnRH(ant)agonist is used (Beckers *et al.,* 2003). Endometrium shows early changes whether or not P supplementation was used. However, luteal support, only provided in the early secretory phase for a short time, resulted in significant differences in cellular maturation markers. Significant lower Ki-67 and ER but unchanged PR expression when luteal support was provided, indicating advanced/retarded endometrial maturation (Van der Gaast *et al.,* 2008).

The role of the oocyte is still important for implantation and pregnancy, but this role appears to be limited. Only 36% of the retrieved oocytes stimulated with mild ovarian stimulation appeared chromosomally normal in young (Baart *et al.,* 2006) and older (Staessen *et al.,* 2004) IVF patients. In a historical cohort a clinical pregnancy rate per transfer and pregnancy rate per started cycle rose to a maximum of 28% and 24% respectively when 10–14 oocytes were retrieved (Van der Gaast *et al.,* 2006). Obtaining more than 14 oocytes was associated with declining pregnancy rates per embryo transfer, but the optimal range of retrieved oocytes for IVF outcomes was higher in women with ovulatory subfertility. Recently, a greater awareness of the possible impact of hard ovarian stimulation on IVF outcomes has grown, more milder IVF treatment cycles have been developed (Hohmann *et al.,* 2003) which impose a reduced burden on patients. Recent studies have shown that mild stimulation in combination with single embryo transfer can result in similar live brith rates over a period of 12 months as more traditional strategies, while reducing risks, costs and patient burden (Heijnen *et al.,* 2007). But the final pregnancy rate remain 43 to 45% after multiple IVF treatment cycles. To increase the pregnancy rates further, a novel approach of the IVF treament is necessary.

When endometrial gene expression is compared between natural and stimulated IVF cycle with recFSH and GnRH antagonist in fertile patients 142 genes were significantly upregulated and 98 significantly downregulated in the not P supplemented midluteal phase (Macklon *et al.,* 2008). The principal observations were the marked upregulation of SCYB 13, DKK1, IGFBP-4 and -5, and Homeobox C6. Gene ontogeny (GO) categories of upregulated genes included those involved in cell adhesion, T-cell receptor signaling, regulation of signal transduction, cell growth, proliferation and programmed cell death. GO catagories of down regulated genes indicated reduced transmembrane receptor protein kinase activity following ovarian stimulation. Several gene microarray studies have been performed to assess gen expression changes during the 'implantation window' comparing proliferative and secretory endometrium in the natural cycles (Borthwick *et al.,* 2003; Carson *et al.,* 2002; Kao *et al.,* 2002; Riesewijk *et al.,* 2003). The significant up-regulation of OPN in all five studies is remarkable, since it mediates adhesion and migration during the implantation. Ovarian stimulation for IVF has a large effect on gene expression in secretory endometrium (Horcajadas *et al.,* 2005). They identified 281 up-regulated and 277 down-regulated genes in IVF stimulated cycles. Another study did not confirm these results (Mirkin *et al.,* 2005). No effect in GdA and LIF gene expression was found. Furthermore, no effect on gene expression was shown whether or not P suplementation was provided. Despite techniques to standardize the results, the results of endometrial gene expression during the implantation window comparing natural and stimulated IVF cycles assessments are different. These differences are most likely due to the heterogeneity of the cell types in endometrial tissue samples. Gene expression in epithelial and stromal cells, devided by microdissection techniques, are different (Yanaihara *et al.,* 2004; Yanaihara *et al.,* 2005).

The implantation of the embryo in the endometrium is limited in a tight time frame, the 'implantation window'. This window occurs between 6 to 10 days after the ovulation. Histological markers (Noyes *et al.,* 1950) and molecular endometrial markers (Hoozemans *et al.,* 2004; Lindhard *et al.,* 2002) have been used to assess endometrial receptivity. The morphological 'Noyes criteria' are not suitable for assess maturation of endometrium tissue (Coutifaris ,2004; Coutifaris *et al.,* 2004; Garcia, 2004; Kazer, 2004; Lamb, 2004; McDonough, 2004; Murray *et al.,* 2004; Myers *et al.,* 2004), and until now no key marker has been identified which is mandatory for the implantation process or limits the implantation window in the luteal phase of the menstrual cycle. Moreover, endometrial tissue retrieval in the peri-implantation period during the menstrual cycle may disrupt the implantation process and decrease pregnancy rates in the same cycle (Van der Gaast *et al.,* 2002). By influencing this process it is unlikely that endometrial biopsy prior to embryo transfer in IVF cycles or in the luteal phase of the cycle can be used in conception cycles. A less invasive assessment is required to study embryo implantation in the same natural or IVF stimulated cycle with embryo implantation or pregnancy as ultimate endpoint. These methods can be used to study the effects of ovarian stimulation on endometrial maturation and implantation. Therefore we studied the safety of endometrial secretion retrieval, and possibility to study more than one putative endometrial receptivity and/or maturation markers.

Endometrial fluid creates an environment for embryos to develop further towards a blastocyst and finally to implant into the endometrial tissue. To allow this the endometrial secretions contain nourishing and signalling compounds, such as: proteins, electrolytes, cytokines, growth factors, and steroid hormones. These contents may play a role in the dialogue between the embryo and endometrium during the implantation process. This fluid may reflect the endometrial condition during the retrieval and consequently the endometrial receptivity at that moment. We first studied the effect of fluid aspiration just prior to an embryo transfer on the implantation (Van der Gaast *et al.,* 2003). This technique showed no decrease of pregnancy rates when performed on the day of embryo transfer during an IVF cycle according to te other studies in wich the fluid retrieval was performed on day of oocyte pick-up (Ledee-Bataille *et al.,* 2004a; Olivennes *et al.,* 2003). Consistent with an other study (Li *et al.,* 1993c), our patients showed no major discomfort or pain during this procedure, nor infections were recorded. We conclude that fluid aspiration is a safe method for obtaining enough material for uterine secretion analysis. This allows analysis of fluid serving as receptivity markers during conception cycles just before the 'implantation window' without affecting the implantation process due to disruption of the endometrial lining.

Before the effects of ovarian stimulation on endometrial tissue and secretions was studied, we first evaluated the relation between endometrial maturation, assessed by the combination of histological Noyes criteria and immunohistochemical PR and Ki-67 staining patterns, and endometrial fluid samples with the protein content and pattern, and the LIF, GdA, P levels (Van der Gaast *et al.,* 2009). This is the first study which studies multiple contents

simultaneously in endometrial secretions and relate it to the endometrial maturation retrieved from the same patient in the same cycle. We showed a positive correlation between maturation and GdA levels in secretions, but not with the P and LIF levels. Because of the low number of patients and consequently the low power of the study, we assume that GdA levels are more related to the endometrial maturation than LIF levels. Recently a study showed that the LIF levels in endometrial flushings is highly predictive for pregnancy in the future menstrual cycles (Mikolajczyk *et al.,* 2007). However, this study was relying on patient response by returning voluntary questionnaires. This study may be a biased, because patients may not return questionnaires due to disappointment when a patient did not got pregnant. We also showed in our patient population the the difference in LIF levels in patients who conceived in the past and who did not (Van der Gaast, not published data). This suggests that LIF levels may have an important role in the embryo implantation process, but may also promote embryonic development (Dimitriadis *et al.,* 2005). Although the exact role of GdA in endometrial receptivity remains unclear, fertile patients showed higher levels of GdA in uterine flushings compared to the subfertile controls (Dalton *et al.,* 1998), suggesting that an increase of GdA might facilitate implantation. Furthermore, GdA levels in endometrial secretion samples may provide a method for assessing endometrial maturation in potential conception cycles without disrupting implantation (Li *et al.,* 1993b; Van der Gaast *et al.,* 2009). GdA expression in endometrial cells is directly regulated by P and not E (Mueller *et al.,* 2000). Our study also showed no relation of GdA levels in secretion with serum E levels nor by serum P levels, but only with P levels in endometrial secretions (Van der Gaast *et al.,* 2009). These results may reflect a stronger relation between P and GdA in secretions due to low patient number as mentoined earlier. According to other studies P levels in endometrial secretions do not show relation with endometrial maturation (Bischof *et al.,* 1984; Fazleabas *et al.,* 1987; Stone *et al.,* 1986), P levels in secretions and serum were significantly correlated. This suggests that P serum levels reflect levels of the hormone present in the endometrial lumen to act as a endometrial maturation modulator. Consistent with this, a significant correlation was observed between endometrial maturation and serum E and P levels. The exact role of this during endometrial preparation of embryo implantation is unknown.

Although the results of this study are preliminary, this is the first in which the impact of ovarian stimulation on endometrial maturation during the luteal phase has been studied in both endometrial biopsies and in endometrial secretions, in the same patient in a natural and stimulated IVF cycle. We showed no direct effect of the stimulation on LIF and GdA levels in secretions retrieved from the uterine cavity during the 'implantation window'. (Van der Gaast *et al.,* 2008) according to other studies (Ng *et al.,* 2004; Olivennes *et al.,* 2003). Ovarian stimulation has little impact on tissue markers of endometrial maturation, and on changes of LIF, GdA, and P levels in secretions. Endometrial maturation assessed with the histological endometrial dating, which is discussed to lack precision and accuracy (Murray *et al.,* 2004; Myers *et al.,* 2004), showed advanced maturation in previous studies (Develioglu *et al.,* 1999; Kolibianakis *et al.,* 2002a; Papanikolaou *et al.,* 2005; Saadat *et al.,* 2004). However, one study

showed advanced maturation in endometrial tissue retrieved, on day of oocyte retrieval after ovarian stimulation, disappeared when assessed in the same subjects later in the luteal phase (Kolibianakis *et al.,* 2003). This shows that it is unclear whether endometrial advancement observed during the peri-ovulatory phase reflects the status of endometrial differentiation in the peri-implantation phase. Analysis of endometrial tissue and endometrial secretions retriedved from the beginning of the 'window of implantation', comparing the natural and ovarian stimulation cycle in the same subject, provides novel data on the impact of IVF treatment on endometrial differentiation. Although, small number of subjects included in this study, the paired design of this study allows cautious conclusions to be drawn. The present study demonstrates that ovarian stimulation with GnRH antagonist co-treatment has little impact on tissue-derived markers of endometrial maturation, or on LIF, GdA, and P levels in the uterine cavity during the 'window of implantation'. Our data are consistent with the concept that markers of endometrial receptivity alter between the time of oocyte retrieval and ET 5 days after oocyte retrieval, thus limiting the accuracy of predicting endometrial maturation during the peri-implantation period.

Implantation is a complex process with a network of players influencing oocyte, endometrium, but also each other. To understand embryo implantation in endometrium it is important to study multiple factors instead to study only one factor and and show the role in implantation. In this thesis we showed a novel technique which may be used in conception cycles without effect on pregnancy rates and assess 5 parameters (protein content and profile, GdA level, LIF level, P level) simultaneously in one endometrial secretion sample. Recently new techniques became available during progress of the studies described in this thesis, to study an array of compounds in little sample volumes, such as proteomics (mass spectrometry or by antibody-based assays) and cytometric bead array (CBA) analysis of soluble cytokines. Application of these techniques makes it possible to make a gene, protein and/or cytokine 'fingerprint' of endometrial tissue and/or secretions retrieved from the uterus in the 'implantation window' is for near future the challenge and promise in embryo implantation research. A cohort study of 160 patients in whom uterine cavity aspiration has been performed in a natural and IVF stimulation cycle in the same subject which meets these criteria has been recently finished and submitted for publication. This study assess cytokine profiles produced by embryos and endometrium on only one day of the menstrual cycle. To understand implantation and to influence it to improve or hamper the process it is necessary to study embryo and endometrium and their interaction on more days of the cycle. But this is very expensive and needs many patients, and therefore cooperation of many reproductive medical centres are required to elucidate the last major enigma of achieving pregnancy in reproductive science.

REFERENCES

Abate V, De Corato R, Cali A, and Stinchi A (1987) Endometrial biopsy at the time of embryo transfer: correlation of histological diagnosis with therapy and pregnancy rate. *J In Vitro Fert Embryo Transf* 4, 173-176.

Acosta AA, Elberger L, Borghi M, Calamera JC, Chemes H, Doncel GF, Kliman H, Lema B, Lustig L, and Papier S (2000) Endometrial dating and determination of the window of implantation in healthy fertile women. *Fertil Steril* 73, 788-798.

Aghajanova L, Stavreus-Evers A, Nikas Y, Hovatta O, and Landgren BM (2003) Coexpression of pinopodes and leukemia inhibitory factor, as well as its receptor, in human endometrium. *Fertil Steril* 79, 808-814.

Aghajanova L (2004) Leukemia inhibitory factor and human embryo implantation. *Ann NY Acad Sci* 1034, 176-183.

Al Inany H and Aboulghar M (2002) GnRH antagonist in assisted reproduction: a Cochrane review. *Hum Reprod* 17, 874-885.

Albano C, Smitz J, Tournaye H, Riethmuller-Winzen H, Van Steirteghem A, and Devroey P (1999) Luteal phase and clinical outcome after human menopausal gonadotrophin/gonadotrophin releasing hormone antagonist treatment for ovarian stimulation in in-vitro fertilization/intracytoplasmic sperm injection cycles. *Hum Reprod* 14, 1426-1430.

Alfer J, Müller-Schöttle F, Classen-Linke I, von Rango U, Happel L, Beier-Hellwig K, Rath W, and Beier HM (2000) The endometrium as a novel target for leptin: differences in fertility and subfertility. *Mol Hum Reprod* 6, 595-601.

Almkvist J and Karlsson A (2004) Galectins as inflammatory mediators. *Glycoconj J* 19, 575-581.

Apparao KB, Murray MJ, Fritz MA, Meyer WR, Chambers AF, Truong PR, and Lessey BA (2001) Osteopontin and its receptor $\alpha v\beta 3$ integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. *J Clin Endocrinol Metab* 86, 4991-5000.

Arici A, Engin O, Attar E, and Olive DL (1995) Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. *J Clin Endocrinol Metab* 80, 1908-1915.

Baart EB, Martini E, Van den Berg I, Macklon NS, Galjaard RJ, Fauser BC, and Van Opstal D (2006) Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. *Hum Reprod* 21, 223-233.

Bamberger AM, Jenatschke S, Schulte HM, Loning T, and Bamberger MC (2000) Leukemia inhibitory factor (LIF) stimulates the human HLA-G promoter in JEG3 choriocarcinoma cells. *J Clin Endocrinol Metab* 85, 3932-3936.

Basir GS, WS O, Ng EH, and Ho PC (2001) Morphometric analysis of peri-implantation endometrium in patients having excessively high oestradiol concentrations after ovarian stimulation. *Hum Reprod* 16, 435-440.

Bayless KJ, Meininger GA, Scholtz JM, and Davis GE (1998) Osteopontin is a ligand for the $\alpha 4\beta 1$ integrin. *J Cell Sci* 111, 1165-1174.

Beckers NG, Laven JS, Eijkemans MJ, and Fauser BC (2000) Follicular and luteal phase characteristics following early cessation of gonadotrophin-releasing hormone agonist during ovarian stimulation for in-vitro fertilization. *Hum Reprod* 15, 43-49.

Beckers NG, Macklon NS, Eijkemans MJ, Ludwig M, Felberbaum RE, Diedrich K, Bustion S, Loumaye E, and Fauser BC (2003) Nonsupplemented luteal phase characteristics after the administration of recombinant human chorionic gonadotropin, recombinant luteinizing hormone, or gonadotropin-releasing hormone (GnRH) agonist to induce final oocyte maturation in *in vitro* fertilization patients after ovarian stimulation with recombinant follicle-stimulating hormone and GnRH antagonist cotreatment. *J Clin Endocrinol Metab* 88, 4186-4192.

Beier HM and Beier-Hellwig K (1973) Specific secretory protein of the female genital tract. *Acta Endocrinol Suppl (Copenh)* 180, 404-425.

Beier HM (1974) Oviducal and uterine fluids. *J Reprod Fertil* 37, 221-237.

Beier HM, Hegele-Hartung C, Mootz U, and Beier-Hellwig K (1994) Modification of endometrial cell biology using progesterone antagonists to manipulate the implantation window. *Hum Reprod* 9 (Suppl 1), 98-115.

Beier HM and Beier-Hellwig K (1998) Molecular and cellular aspects of endometrial receptivity. *Hum Reprod Update* 4, 448-458.

Beier HM (2000) The discovery of uteroglobin and its significance for reproductive biology and endocrinology. *Ann N Y Acad Sci* 923, 9-24.

Beier-Hellwig K, Sterzik K, and Beier HM (1988) Zur Rezeptivität des endometriums: Die diagnostik der proteinmuster des menslichen uterussekretes. *Fertilität* 4, 128-134.

Beier-Hellwig K, Sterzik K, Bonn B, and Beier HM (1989) Contribution to the physiology and pathology of endometrial receptivity: the determination of protein patterns in human uterine secretions. *Hum Reprod* 4, 115-120.

Beier-Hellwig K, Sterzik K, Bonn B, Hilmes U, Bygdeman M, Gemzell-Danielsson K, and Beier HM (1994) Hormone regulation and hormone antagonist effects on protein patterns of human endometrial secretion during receptivity. *Ann N Y Acad Sci* 734, 143-156.

Bell SC and Dore-Green F (1987) Detection and characterization of human secretory "pregnancy-associated endometrial alpha 2-globulin" in uterine luminal fluid. *J Reprod Immunol* 11, 13-29.

Bergeron C, Ferenczy A, Toft DO, Schneider W, and Shyamala G (1988) Immunocytochemical study of progesterone receptors in the human endometrium during the menstrual cycle. *Lab Invest* 59, 862-869.

Bergh PA and Navot D (1992) The impact of embryonic development and endometrial maturity on the timing of implantation. *Fertil Steril* 58, 537-542.

Bernstein GS, Aladjem F, and Chen S (1971) Proteins in human endometrial washings--a preliminary report. *Fertil Steril* 22, 722-726.

Bischof P, Schindler AM, Urner F, Mensi N, Herrmann WL, and Sizonenko PC (1984) Pregnancy-associated plasma protein-A (PAPP-A): concentration in uterine fluid and immunohistochemical localization in the endometrium. *Br J Obstet Gynaecol* 91, 863-869.

Bischof P, Haenggeli L, and Campana A (1995) Effect of leukemia inhibitory factor on human cytotrophoblast differentiation along the invasive pathway. *Am J Reprod Immunol* 34, 225-230.

Blois SM, Joachim R, Kandil J, Margni R, Tometten M, Klapp BF, and Arck PC (2004) Depletion of CD8+ cells abolishes the pregnancy protective effect of progesterone substitution with dydrogesterone in mice by altering the Th1/Th2 cytokine profile. *J Immunol* 172, 5893-5899.

Bonduelle ML, Dodd R, Liebaers I, Van Steirteghem A, Williamson R, and Akhurst R (1988) Chorionic gonadotrophin-β mRNA, a trophoblast marker, is expressed in human 8-cell embryos derived from tripronucleate zygotes. *Hum Reprod* 3, 909-914.

Boomsma CM and Macklon NS (2006) What can the clinician do to improve implantation? *Reprod BioMed Online* 13, 845-855.

Borri P, Noci I, Fuzzi B, Rice A, and Chard T (1998) The ovary is not a major source of placental protein 14 (glycodelin). *Hum Reprod* 13, 3418-3420.

Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC, and Smith SK (2003) Determination of the transcript profile of human endometrium. *Mol Hum Reprod* 9, 19-33.

Bourgain C, Ubaldi F, Tavaniotou A, Smitz J, Van Steirteghem AC, and Devroey P (2002) Endometrial hormone receptors and proliferation index in the periovulatory phase of stimulated embryo transfer cycles in comparison with natural cycles and relation to clinical pregnancy outcome. *Fertil Steril* 78, 237-244.

Bourgain C and Devroey P (2003) The endometrium in stimulated cycles for IVF. *Hum Reprod Update* 9, 515-522.

Brown JB (1978) Pituitary control of ovarian function - concepts derived from gonadotrophin therapy. *Aust N Z J Obstet Gynaecol* 18, 46-54.

Brown SE, Mandelin E, Oehninger S, Toner JP, Seppala M, and Jones HW, Jr. (2000) Endometrial glycodelin-A expression in the luteal phase of stimulated ovarian cycles. *Fertil Steril* 74, 130-133.

Campbell S, Swann HR, Seif MW, Kimber SJ, and Aplin JD (1995) Cell adhesion molecules on the oocyte and preimplantation human embryo. *Mol Hum Reprod* 10, 1571-1578.

Carson DD, Lagow E, Thathiah A, Al Shami R, Farach-Carson MC, Vernon M, Yuan L, Fritz MA, and Lessey B (2002) Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Mol Hum Reprod* 8, 871-879.

Casan EM, Raga F, Krüssel JS, Wen Y, Nezhat C, and Polan ML (1998) Immunoreactive gonadotropin-releasing hormone expression in cycling human endometrium of fertile patients. *Fertil Steril* 70, 102-106.

Casper RF and Yen SS (1979) Induction of luteolysis in the human with a long-acting analog of luteinizing hormone-releasing factor. *Science* 205, 408-410.

Casslen B, Kobayashi TK, and Stormby N (1982) Cyclic variation of the cellular components in human uterine fluid. *J Reprod Fertil* 66, 213-218.

Casslen B and Nilsson B (1984) Human uterine fluid, examined in undiluted samples for osmolarity and the concentrations of inorganic ions, albumin, glucose, and urea. *Am J Obstet Gynecol* 150, 877-881.

Catalanotti JS, Spandorfer SD, Barmat LI, Rosenwaks Z, McSweet JC, and Kliman HJ (2006) Mouse ascites Golgi mucin expression abnormalities in natural cycle endometrial biopsies predict subsequent in vitro fertilization-embryo transfer (IVF-ET) failure in patients with previous IVF-ET failures. *Fertil Steril* 85, 255-258.

Chaouat G, Ledee-Bataille N, Chea KB, and Dubanchet S (2005) Cytokines and implantation. *Chem Immunol Allergy* 88, 34-63.

Charnock-Jones DS, Sharkey AM, Fenwick P, and Smith SK (1994) Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J Reprod Fertil* 101, 421-426.

Charpigny G, Reinaud P, Tamby JP, Creminon C, and Guillomot M (1997) Cyclooxygenase-2 unlike cyclooxygenase-1 is highly expressed in ovine embryos during the implantation period. *Biol Reprod* 57, 1032-1040.

Check JH, Hourani C, Choe JK, Callan C, and Adelson HG (1994) Pregnancy rates in donors versus recipients according to the serum progesterone level at the time of human chorionic gonadotropin in a shared oocyte program. *Fertil Steril* 61, 262-264.

Chegini N, Ma C, Roberts M, Williams RS, and Ripps BA (2002) Differential expression of interleukins (IL) IL-13 and IL-15 throughout the menstrual cycle in endometrium of normal fertile women and women with recurrent spontaneous abortion. *J Reprod Immunol* 56, 93-110.

Chen DB, Hilsenrath R, Yang ZM, Le SP, Kim SR, Chuong CJ, Poindexter AN, and Harper MJ (1995) Leukaemia inhibitory factor in human endometrium during the menstrual cycle: cellular origin and action on production of glandular epithelial cell prostaglandin in vitro. *Hum Reprod* 10, 911-918.

Choudary JB and Greenwald GS (1969) Reversal by gonadotrophins of the luteolytic effect of oestrogen in the cyclic guinea-pig. *J Reprod Fertil* 19, 503-510.

Clark GF, Oehninger S, Patankar MS, Koistinen R, Dell A, Morris HR, Koistinen H, and Seppala M (1996) A role for glycoconjugates in human development: the human feto-embryonic defence system hypothesis. *Hum Reprod* 11, 467-473.

Classen-Linke I, Alfer J, Hey S, Krusche CA, Kusche M, and Beier HM (1998) Marker molecules of human endometrial differentiation can be hormonally regulated under in-vitro conditions as in-vivo. *Hum Reprod Update* 4, 539-549.

Classen-Linke I, Alfer J, Krusche CA, Chwalisz K, Rath W, and Beier HM (2000) Progestins, progesterone receptor modulators, and progesterone antagonists change VEGF release of endometrial cells in culture. *Steroids* 65, 763-771.

Cohen JJ, Debache C, Pigeau F, Mandelbaum J, Plachot M, and De Brux J (1984) Sequential use of clomiphene citrate, human menopausal gonadotropin, and human chorionic gonadotropin in human in vitro fertilization. II. Study of luteal phase adequacy following aspiration of the preovulatory follicles. *Fertil Steril* 42, 360-365.

Cork BA, Li TC, Warren MA, and Laird SM (2001) Interleukin-11 (IL-11) in human endometrium: expression throughout the menstrual cycle and the effects of cytokines on endometrial IL-11 production in vitro. *J Reprod Immunol* 50, 3-17.

Coutifaris C (2004) Histological dating of secretory endometrium: What controversy? *Fertil Steril* 82, 1301-1302.

Coutifaris C, Myers ER, Guzick DS, Diamond MP, Carson SA, Legro RS, McGovern PG, Schlaff WD, Carr BR, Steinkampf MP, Silva S, Vogel DL, and Leppert PC (2004) Histological dating of timed endometrial biopsy tissue is not related to fertility status. *Fertil Steril* 82, 1264-1272.

Cowan BD, North DH, Whitworth NS, Fujita R, Shumacher EK, and Mukherjee AB (1986) Identification of a uteroglobin-like antigen in human uterine washings. *Fertil Steril* 45, 820-823.

Creus M, Balasch J, Ordi J, Fabregues F, Casamitjana R, Quinto L, Coutifaris C, and Vanrell JA (1998) Integrin expression in normal and out-of-phase endometria. *Hum Reprod* 13, 3460-3468.

Creus M, Ordi J, Fabregues F, Casamitjana R, Ferrer B, Coll E, Vanrell JA, and Balasch J (2002) αvβ3 integrin expression and pinopod formation in normal and out- of-phase endometria of fertile and infertile women. *Hum Reprod* 17, 2279-2286.

Croy BA, Esadeg S, Chantakru S, Van den Heuvel M, Paffaro VA, He H, Black GP, Ashkar AA, Kiso Y, and Zhang J (2003) Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. *J Reprod Immunol* 59, 175-191.

Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA, and Stewart CL (1996) Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proc Natl Acad Sci USA* 93, 3115-3120.

Dalton CF, Laird SM, Estdale SE, Saravelos HG, and Li TC (1998) Endometrial protein PP14 and CA-125 in recurrent miscarriage patients; correlation with pregnancy outcome. *Hum Reprod* 13, 3197-3202.

Dalton CF, Laird SM, Serle E, Saravelos H, Warren MA, Li TC, and Bolton AE (1995) The measurement of CA 125 and placental protein 14 in uterine flushings in women with recurrent miscarriage; relation to endometrial morphology. *Hum Reprod* 10, 2680-2684.

Daniel JC, Jr. (1973) A blastokinin-like component from the human uterus. *Fertil Steril* 24, 326-328.

De Boer EJ, Van Leeuwen FE, Den Tonkelaar I, Jansen CA, Braat DD, and Burger CW (2004) [Methods and results of in-vitro fertilisation in the Netherlands in the years 1983-1994]. *Ned Tijdschr Geneeskd* 148, 1448-1455.

De Jong D, Macklon NS, and Fauser BC (2000) A pilot study involving minimal ovarian stimulation for in vitro fertilization: extending the "follicle-stimulating hormone window" combined with the gonadotropin-releasing hormone antagonist cetrorelix. *Fertil Steril* 73, 1051-1054.

De los Santos, Mercader A, Frances A, Portoles E, Remohi J, Pellicer A, and Simon C (1996) Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. *Biol Reprod* 54, 563-574.

Develioglu OH, Hsiu JG, Nikas G, Toner JP, Oehninger S, and Jones HWJ (1999) Endometrial estrogen and progesterone receptor and pinopode expression in stimulated cycles of oocyte donors. *Fertil Steril* 71, 1040-1047.

Devroey P, Bourgain C, Macklon NS, and Fauser BC (2004) Reproductive biology and IVF: ovarian stimulation and endometrial receptivity. *Trends Endocrinol Metab* 15, 84-90.

Dey SK, Chien SM, Cox CL, and Crist RD (1980) Prostaglandin synthesis in the rabbit blastocyst. *Prostaglandins* 19, 449-453.

Diedrich K, Fauser BC, Devroey P, and Griesinger G (2007) The role of the endometrium and embryo in human implantation. *Hum Reprod Update* 13, 365-377.

Diedrich K, Frydman R, Devroey P, Felberbaum R, Schally AV, Reissmann T, and Engel J (2001) Comment on the debate article: Embryo implantation: the Rubicon for GnRH antagonists. *Hum Reprod* 16, 1305-1306.

Dimitriadis E, Salamonsen LA, and Robb L (2000) Expression of interleukin-11 during the human menstrual cycle: coincidence with stromal cell decidualization and relationship to leukaemia inhibitory factor and prolactin. *Mol Hum Reprod* 6, 907-914.

Dimitriadis E, White CA, Jones RL, and Salamonsen LA (2005) Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update*. 11, 613-630.

Dimitriadis E, Stoikos C, Stafford-Bell M, Clark I, Paiva P, Kovacs G, and Salamonsen LA (2006) Interleukin-11, IL-11 receptorα and leukemia inhibitory factor are dysregulated in endometrium of infertile women with endometriosis during the implantation window. *J Reprod Immunol* 69, 53-64.

Ding YQ, Zhu LJ, Bagchi MK, and Bagchi IC (1994) Progesterone stimulates calcitonin gene expression in the uterus during implantation. *Endocrinology* 135, 2265-2274.

Donderwinkel PF, Schoot DC, Pache TD, De Jong FH, Hop WC, and Fauser BC (1993) Luteal function following ovulation induction in polycystic ovary syndrome patients using exogenous gonadotrophins in combination with a gonadotrophin-releasing hormone agonist. *Hum Reprod* 8, 2027-2032.

Dubourdieu S, Charbonnel B, Massai MR, Marraoui J, Spitz I, and Bouchard P (1991) Suppression of corpus luteum function by the gonadotropin-releasing hormone antagonist Nal-Glu: effect of the dose and timing of human chorionic gonadotropin administration. *Fertil Steril* 56, 440-445.

Duffy DM, Stewart DR, and Stouffer RL (1999) Titrating luteinizing hormone replacement to sustain the structure and function of the corpus luteum after gonadotropin-releasing hormone antagonist treatment in rhesus monkeys. *J Clin Endocrinol Metab* 84, 342-349.

Ecochard R and Gougeon A (2000a) Side of ovulation and cycle characteristics in normally fertile women. *Hum Reprod* 15, 752-755.

Ecochard R, Marret H, Rabilloud M, Bradai R, Boehringer H, Girotto S, and Barbato M (2000b) Sensitivity and specificity of ultrasound indices of ovulation in spontaneous cycles. *Eur J Obstet Gynecol Reprod Biol* 91, 59-64.

Edwards RG, Talbert L, Israelstam D, Nino HV, and Johnson MH (1968) Diffusion chamber for exposing spermatozoa to human uterine secretions. *Am J Obstet Gynecol* 102, 388-396.

Edwards RG, Steptoe PC, and Purdy JM (1980) Establishing full-term human pregnancies using cleaving embryos grown in vitro. *Br J Obstet Gynaecol* 87, 737-756.

Edwards RG (1994) Implantation, interception and contraception. *Hum Reprod* 9, 985-995.

Edwards RG, Lobo R, and Bouchard P (1996) Time to revolutionize ovarian stimulation. *Hum Reprod* 11, 917-919.

Edwards RG (2003) Towards single births after assisted reproduction treatment. *Reprod BioMed Online* 7, 506-508.

Fanchin R, De Ziegler D, Taieb J, Hazout A, and Frydman R (1993) Premature elevation of plasma progesterone alters pregnancy rates of in vitro fertilization and embryo transfer. *Fertil Steril* 59, 1090-1094.

Fauser BC, Donderwinkel P, and Schoot DC (1993) The step-down principle in gonadotrophin treatment and the role of GnRH analogues. *Baillieres Clin Obstet Gynaecol* 7, 309-330.

Fauser BC, Devroey P, Yen SS, Gosden R, Crowley WF, Jr., Baird DT, and Bouchard P (1999) Minimal ovarian stimulation for IVF: appraisal of potential benefits and drawbacks. *Hum Reprod* 14, 2681-2686.

Fauser BC and Van Heusden AM (1997) Manipulation of human ovarian function: physiological concepts and clinical consequences. *Endocr Rev* 18, 71-106.

Fauser BC, De Jong D, Olivennes F, Wramsby H, Tay C, Itskovitz-Eldor J, and van Hooren HG (2002) Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. *J Clin Endocrinol Metab* 87, 709-715.

Fauser BCJM and Macklon NS (2004) Medical approaches to ovarian stimulation for infertility. In Strauss JF III and Mastroianni L, Jr. (eds), *Reproductive Endocrinology: Physiology, Pathophysiology and Clinical Management*. Elsevier - Saunders, Philadelphia, pp. 965-1012.

Fauser BCJM, Devroey P, and Macklon NS (2005) Multiple birth resulting from ovarian stimulation for subfertility treatment. *Lancet* 365, 1807-1816.

Fazleabas AT, Khan-Dawood FS, and Dawood MY (1987) Protein, progesterone, and protease inhibitors in uterine and peritoneal fluids of women with endometriosis. *Fertil Steril* 47, 218-224.

Feichtinger W, Kemeter P, Szalay S, Beck A, and Janisch H (1982) Could aspiration of the Graafian follicle cause luteal phase deficiency? *Fertil Steril* 37, 205-208.

Filicori M, Butler JP, and Crowley WF, Jr. (1984) Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion. *J Clin Invest* 73, 1638-1647.

Finn CA and Martin L (1974) The control of implantation. *J Reprod Fertil* 39, 195-206.

Florio P, Severi FM, Luisi S, Ciarmela P, Calonaci G, Cobellis L, and Petraglia F (2003) Endometrial expression and secretion of activin A, but not follistatin, increase in the secretory phase of the menstrual cycle. *J Soc Gynecol Investig* 10, 237-243.

Foulk RA, Zdravkovic T, Genbacev O, and Prakobphol A (2007) Expression of L-selectin ligand MECA-79 as a predictive marker of human uterine receptivity. *J Assist Reprod Genet* 24, 316-321.

Frost VJ, Macaulay VM, Wass JA, and Holly JM (1993) Proteolytic modification of insulin-like growth factor-binding proteins: comparison of conditioned media from human cell lines, circulating proteases and characterized enzymes. *J Endocrinol* 138, 545-554.

Frydman R, Testart J, Giacomini P, Imbert MC, Martin E, and Nahoul K (1982) Hormonal and histological study of the luteal phase in women following aspiration of the preovulatory follicle. *Fertil Steril* 38, 312-317.

Fujii S, Sato S, Fukui A, Kimura H, Kasai G, and Saito Y (2001) Continuous administration of gonadotrophin-releasing hormone agonist during the luteal phase in IVF. *Hum Reprod* 16, 1671-1675.

Fukuda MN, Sato T, Nakayama J, Klier G, Mikami M, Aoki D, and Nozawa S (1995) Trophinin and tastin, a novel cell adhesion molecule complex with potential involvement in embryo implantation. *Genes Dev* 9, 1199-1210.

Garcia J, Jones GS, Acosta AA, and Wright GL, Jr. (1981) Corpus luteum function after follicle aspiration for oocyte retrieval. *Fertil Steril* 36, 565-572.

Garcia JE, Acosta AA, Hsiu JG, and Jones HW, Jr. (1984) Advanced endometrial maturation after ovulation induction with human menopausal gonadotropin/human chorionic gonadotropin for in vitro fertilization. *Fertil Steril* 41, 31-35.

Garcia E, Bouchard P, De Brux J, Berdah J, Frydman R, Schaison G, Milgrom E, and Perrot-Applanat M (1988) Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. *J Clin Endocrinol Metab* 67, 80-87.

Garcia JE (2004) Endometrial biopsy: A test whose time has come. *Fertil Steril* 82, 1293-1294.

Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Ilic D, Singer MS, Yang ZQ, Kiessling LL, Rosen SD, and Fisher SJ (2003) Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. *Science* 299, 405-408.

Gibson M, Nakajima ST, and McAuliffe TL (1991) Short-term modulation of gonadotropin secretion by progesterone during the luteal phase. *Fertil Steril* 55, 522-528.

Gipson IK, Blalock T, Tisdale A, Spurr-Michaud S, Allcorn S, Stavreus-Evers A, and Gemzell K (2007) MUC16 is Lost from the Uterodome (Pinopode) Surface of the Receptive Human Endometrium: In Vitro Evidence That MUC16 Is a Barrier to Trophoblast Adherence. *Biol Reprod* 78, 134-142.

Giudice LC, Milkowski DA, Lamson G, Rosenfeld RG, and Irwin JC (1991) Insulin-like growth factor binding proteins in human endometrium: steroid-dependent messenger ribonucleic acid expression and protein synthesis. *J Clin Endocrinol Metab* 72, 779-787.

Giudice LC (1999a) Potential biochemical markers of uterine receptivity. *Hum Reprod* 14 (Suppl 2), 3-16.

Giudice LC and Irwin JC (1999b) Roles of the insulinlike growth factor family in nonpregnant human endometrium and at the decidual: trophoblast interface. *Semin Reprod Endocrinol* 17, 13-21.

Gonen Y, Balakier H, Powell W, and Casper RF (1990) Use of gonadotropin-releasing hormone agonist to trigger follicular maturation for in vitro fertilization. *J Clin Endocrinol Metab* 71, 918-922.

Gonzalez RR, Rueda BR, Ramos MP, Littell RD, Glasser S, and Leavis PC (2004) Leptin-induced increase in leukemia inhibitory factor and its receptor by human endometrium is partially mediated by interleukin 1 receptor signaling. *Endocrinology* 145, 3850-3857.

Graf MJ, Reyniak JV, Battle-Mutter P, and Laufer N (1988) Histologic evaluation of the luteal phase in women following follicle aspiration for oocyte retrieval. *Fertil Steril* 49, 616-619.

Greb RR, Heikinheimo O, Williams RF, Hodgen GD, and Goodman AL (1997) Vascular endothelial growth factor in primate endometrium is regulated by oestrogen-receptor and progesterone-receptor ligands in vivo. *Hum Reprod* 12, 1280-1292.

Griffiths AN, Watermeyer SR, and Klentzeris LD (2002) Fluid within the endometrial cavity in an IVF cycle--a novel approach to its management. *J Assist Reprod Genet* 19, 298-301.

Grosskinsky CM, Yowell CW, Sun J, Parise LV, and Lessey BA (1996) Modulation of integrin expression in endometrial stromal cells in vitro. *J Clin Endocrinol Metab* 81, 2047-2054.

Guermandi E, Vegetti W, Bianchi MM, Uglietti A, Ragni G, and Crosignani P (2001) Reliability of ovulation tests in infertile women. *Obstet Gynecol* 97, 92-96.

Hambartsoumian E (1998) Endometrial leukemia inhibitory factor (LIF) as a possible cause of unexplained infertility and multiple failures of implantation. *Am J Reprod Immunol* 39, 137-143.

Hamilton JA, Iles RK, Gunn LK, Wilson CM, Lower AM, Chard T, and Grudzinskas JG (1998) Concentrations of placental protein 14 in uterine flushings from infertile women: validation of the collection technique and method of expression of results. *Hum Reprod* 13, 3357-3362.

Heijnen EM, Eijkemans MJ, De Klerk C, Polinder S, Beckers NG, Klinkert ER, Broekmans FJ, Passchier J, te Velde ER, Macklon NS, and Fauser BC (2007) A mild treatment strategy for in-vitro fertilisation: a randomised non-inferiority trial. *Lancet* 369, 743-749.

Hendrickson MR and Kempson RL (1997) Histology of the uterus and fallopian tubes. In Sternberg SS (ed), *Histology for Pathologists*. Lippincott-Raven, Philadelphia, USA, pp. 879-927.

Hernandez ER (2000) Embryo implantation and GnRH antagonists: embryo implantation: the rubicon for GnRH antagonists. *Hum Reprod* 15, 1211-1216.

Herrler A, von Rango U, and Beier HM (2003) Embryo-maternal signalling: how the embryo starts talking to its mother to accomplish implantation. *Reprod BioMed Online* 6, 244-256.

Hertig AT, Rock J, and Adams J (1956) A description of 34 human ova within the first 17 days of development. *Am J Anat* 98, 435-494.

Hey NA, Graham RA, Seif MW, and Aplin JD (1994) The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. *J Clin Endocrinol Metab* 78, 337-342.

Hey NA, Li TC, Devine PL, Graham RA, Saravelos H, and Aplin JD (1995) MUC1 in secretory phase endometrium: expression in precisely dated biopsies and flushings from normal and recurrent miscarriage patients. *Hum Reprod* 10, 2655-2662.

Hey NA and Aplin JD (1996) Sialyl-Lewis x and Sialyl-Lewis a are associated with MUC1 in human endometrium. *Glycoconj J* 13, 769-779.

Hoffman LH, Olson GE, Carson DD, and Chilton BS (1998) Progesterone and implanting blastocysts regulate Muc1 expression in rabbit uterine epithelium. *Endocrinology* 139, 266-271.

Hohmann FP, Laven JS, De Jong FH, Eijkemans MJ, and Fauser BC (2001) Low-dose exogenous FSH initiated during the early, mid or late follicular phase can induce multiple dominant follicle development. *Hum Reprod* 16, 846-854.

Hohmann FP, Macklon NS, and Fauser BC (2003) A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing

recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *J Clin Endocrinol Metab* 88, 166-173.

Hoozemans DA, Schats R, Lambalk CB, Homburg R, and Hompes PG (2004) Human embryo implantation: current knowledge and clinical implications in assisted reproductive technology. *Reprod BioMed Online* 9, 692-715.

Horcajadas JA, Riesewijk A, Martin J, Cervero A, Mosselman S, Pellicer A, and Simon C (2004) Global gene expression profiling of human endometrial receptivity. *J Reprod Immunol* 63, 41-49.

Horcajadas JA, Riesewijk A, Polman J, Van Os R, Pellicer A, Mosselman S, and Simon C (2005) Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. *Mol Hum Reprod* 11, 195-205.

Horcajadas JA, Pellicer A, and Simon C (2007) Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Hum Reprod Update* 13, 77-86.

Hu DD, Lin EC, Kovach NL, Hoyer JR, and Smith JW (1995) A biochemical characterization of the binding of osteopontin to integrins $\alpha v\beta 1$ and $\alpha v\beta 5$. *J Biol Chem* 270, 26232-26238.

Huisman GJ, Fauser BC, Eijkemans MJ, and Pieters MH (2000) Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture. *Fertil Steril* 73, 117-122.

Hutchinson-Williams KA, DeCherney AH, Lavy G, Diamond MP, Naftolin F, and Lunenfeld B (1990) Luteal rescue in in vitro fertilization-embryo transfer. *Fertil Steril* 53, 495-501.

Hutchison JS and Zeleznik AJ (1984) The rhesus monkey corpus luteum is dependent on pituitary gonadotropin secretion throughout the luteal phase of the menstrual cycle. *Endocrinology* 115, 1780-1786.

Hutchison JS, Kubik CJ, Nelson PB, and Zeleznik AJ (1987) Estrogen induces premature luteal regression in rhesus monkeys during spontaneous menstrual cycles, but not in cycles driven by exogenous gonadotropin-releasing hormone. *Endocrinology* 121, 466-474.

Ingamells S, Campbell IG, Anthony FW, and Thomas EJ (1996) Endometrial progesterone receptor expression during the human menstrual cycle. *J Reprod Fertil* 106, 33-38.

Jaschevatzky OE, Shalit A, Grunstein S, Kaplanski J, and Danon A (1983) Increased decidual prostaglandin E concentration in human abortion. *Br J Obstet Gynaecol* 90, 958-960.

Johannisson E, Landgren BM, Rohr HP, and Diczfalusy E (1987) Endometrial morphology and peripheral hormone levels in women with regular menstrual cycles. *Fertil Steril* 48, 401-408.

Johansson ED and Gemzell C (1971) Plasma levels of progesterone during the luteal phase in normal women treated with synthetic oestrogens (RS 2874, F 6103 and ethinyloestradiol). *Acta Endocrinol (Copenh)* 68, 551-560.

Johnson GA, Burghardt RC, Bazer FW, and Spencer TE (2003) Osteopontin: roles in implantation and placentation. *Biol Reprod* 69, 1458-1471.

Jones HW, Jr. (1996) What has happened? Where are we? *Hum Reprod* 11 (Suppl 1), 7-24.

Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA, and Giudice LC (2002) Global Gene Profiling in Human Endometrium during the Window of Implantation. *Endocrinology* 143, 2119-2138.

Kar AB, Engineer AD, Goel R, Kamboj VP, Dasgupta PR, and Chowdhury SR (1968) Effect of an intrauterine contraceptive device on biochemical composition of uterine fluid. *Am J Obstet Gynecol* 101, 966-970.

Kauma S, Matt D, Strom S, Eierman D, and Turner T (1990) Interleukin-1β, human leukocyte antigen HLA-DRα, and transforming growth factor-β expression in endometrium, placenta, and placental membranes. *Am J Obstet Gynecol* 163, 1430-1437.

Kauma SW, Aukerman SL, Eierman D, and Turner T (1991) Colony-stimulating factor-1 and c-fms expression in human endometrial tissues and placenta during the menstrual cycle and early pregnancy. *J Clin Endocrinol Metab* 73, 746-751.

Kazer RR (2004) Endometrial biopsy should be abandoned as a routine component of the infertility evaluation. *Fertil Steril* 82, 1297-1298.

Kerin JF, Broom TJ, Ralph MM, Edmonds DK, Warnes GM, Jeffrey R, Crocker JM, Godfrey B, Cox LW, Seamark RF, and Matthews CD (1981) Human luteal phase function following oocyte aspiration from the immediately preovular graafian follicle of spontaneous ovular cycles. *Br J Obstet Gynaecol* 88, 1021-1028.

Khorram O, Garthwaite M, and Magness RR (1999) Endometrial and myometrial expression of nitric oxide synthase isoforms in pre- and postmenopausal women. *J Clin Endocrinol Metab* 84, 2226-2232.

Kimber SJ (2005) Leukaemia inhibitory factor in implantation and uterine biology. *Reproduction* 130, 131-145.

Klentzeris LD, Bulmer JN, Seppala M, Li TC, Warren MA, and Cooke ID (1994) Placental protein 14 in cycles with normal and retarded endometrial differentiation. *Hum Reprod* 9, 394-398.

Kliman HJ, Feinberg RF, Schwartz LB, Feinman MA, Lavi E, and Meaddough EL (1995) A mucin-like glycoprotein identified by MAG (mouse ascites Golgi)

antibodies. Menstrual cycle-dependent localization in human endometrium. *Am J Pathol* 146, 166-181.

Klip H, Burger CW, de Kraker J, and van Leeuwen FE (2001) Risk of cancer in the offspring of women who underwent ovarian stimulation for IVF. *Hum Reprod* 16, 2451-2458.

Kodaman PH and Taylor HS (2004) Hormonal regulation of implantation. *Obstet Gynecol Clin North Am* 31, 745-766.

Kolibianakis EM, Bourgain C, Albano C, Osmanagaoglu K, Smitz J, Van Steirteghem AC, and Devroey P (2002a) Effect of ovarian stimulation with recombinant follicle-stimulating hormone, gonadotropin releasing hormone antagonists, and human chorionic gonadotropin on endometrial maturation on the day of oocyte pick-up. *Fertil Steril* 78, 1025-1029.

Kolibianakis EM and Devroey P (2002b) The luteal phase after ovarian stimulation. *Reprod BioMed Online* 5, 26-35.

Kolibianakis EM, Bourgain C, Platteau P, Albano C, Van Steirteghem AC, and Devroey P (2003) Abnormal endometrial development occurs during the luteal phase of nonsupplemented donor cycles treated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonists. *Fertil Steril* 80, 464-466.

Kolibianakis EM, Collins J, Tarlatzis BC, Devroey P, Diedrich K, and Griesinger G (2006) Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. *Hum Reprod Update* 12, 651-671.

Koscinski I, Viville S, Porchet N, Bernigaud A, Escande F, Defossez A, and Buisine MP (2006) MUC4 gene polymorphism and expression in women with implantation failure. *Hum Reprod* 21, 2238-2245.

Kreitmann O, Nixon WE, and Hodgen GD (1981) Induced corpus luteum dysfunction after aspiration of the preovulatory follicle in monkeys. *Fertil Steril* 35, 671-675.

Kumar S, Zhu LJ, Polihronis M, Cameron ST, Baird DT, Schatz F, Dua A, Ying YK, Bagchi MK, and Bagchi IC (1998) Progesterone induces calcitonin gene expression in human endometrium within the putative window of implantation. *J Clin Endocrinol Metab* 83, 4443-4450.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lai TH, Shih I, Vlahos N, Ho CL, Wallach E, and Zhao Y (2005) Differential expression of L-selectin ligand in the endometrium during the menstrual cycle. *Fertil Steril* 83 (Suppl 4), 1297-1302.

Laird SM, Tuckerman EM, Dalton CF, Dunphy BC, Li TC, and Zhang X (1997) The production of leukaemia inhibitory factor by human endometrium:

presence in uterine flushings and production by cells in culture. *Hum Reprod* 12, 569-574.

Lamb EJ (2004) Looking at the endometrial biopsy with evidence-based medicine. *Fertil Steril* 82, 1283-1285.

Lane TF and Leder P (1997) Wnt-10b directs hypermorphic development and transformation in mammary glands of male and female mice. *Oncogene* 15, 2133-2144.

Lanzone A, Fulghesu AM, Villa P, Guida C, Guido M, Nicoletti MC, Caruso A, and Mancuso S (1994) Gonadotropin-releasing hormone agonist versus human chorionic gonadotropin as a trigger of ovulation in polycystic ovarian disease gonadotropin hyperstimulated cycles. *Fertil Steril* 62, 35-41.

Lass A, Weiser W, Munafo A, and Loumaye E (2001) Leukemia inhibitory factor in human reproduction. *Fertil Steril* 76, 1091-1096.

Ledee-Bataille N, Lapree-Delage G, Taupin JL, Dubanchet S, Frydman R, and Chaouat G (2002) Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. *Hum Reprod* 17, 213-218.

Ledee-Bataille N, Dubanchet S, Coulomb-L'hermine A, Durand-Gasselin I, Frydman R, and Chaouat G (2004a) A new role for natural killer cells, interleukin (IL)-12, and IL-18 in repeated implantation failure after in vitro fertilization. *Fertil Steril* 81, 59-65.

Ledee-Bataille N, Olivennes F, Kadoch J, Dubanchet S, Frydman N, Chaouat G, and Frydman R (2004b) Detectable levels of interleukin-18 in uterine luminal secretions at oocyte retrieval predict failure of the embryo transfer. *Hum Reprod* 19, 1968-1973.

Ledee-Bataille N, Bonnet-Chea K, Hosny G, Dubanchet S, Frydman R, and Chaouat G (2005) Role of the endometrial tripod interleukin-18, -15, and -12 in inadequate uterine receptivity in patients with a history of repeated in vitro fertilization-embryo transfer failure. *Fertil Steril* 83, 598-605.

Leffler H, Carlsson S, Hedlund M, Qian Y, and Poirier F (2004) Introduction to galectins. *Glycoconj J* 19, 433-440.

Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, and McCarty KS (1988) Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab* 67, 334-340.

Lessey BA, Damjanovich L, Coutifaris C, Castelbaum A, Albelda SM, and Buck CA (1992) Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. *J Clin Invest* 90, 188-195.

Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW, and Sun J (1994) Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil Steril* 62, 497-506.

Lessey BA, Castelbaum AJ, Sawin SW, and Sun J (1995) Integrins as markers of uterine receptivity in women with primary unexplained infertility. *Fertil Steril* 63, 535-542.

Lessey BA, Yeh I, Castelbaum AJ, Fritz MA, Ilesanmi AO, Korzeniowski P, Sun J, and Chwalisz K (1996) Endometrial progesterone receptors and markers of uterine receptivity in the window of implantation. *Fertil Steril* 65, 477-483.

Lessey BA (1998a) Endometrial integrins and the establishment of uterine receptivity. *Hum Reprod* 13 (Suppl 3), 247-258.

Lessey BA and Arnold JT (1998b) Paracrine signaling in the endometrium: integrins and the establishment of uterine receptivity. *J Reprod Immunol* 39, 105-116.

Lessey BA, Castelbaum AJ, Wolf L, Greene W, Paulson M, Meyer WR, and Fritz MA (2000) Use of integrins to date the endometrium. *Fertil Steril* 73, 779-787.

Li TC, Dalton C, Hunjan KS, Warren MA, and Bolton AE (1993a) The correlation of placental protein 14 concentrations in uterine flushing and endometrial morphology in the peri-implantation period. *Hum Reprod* 8, 1923-1927.

Li TC, Ling E, Dalton C, Bolton AE, and Cooke ID (1993b) Concentration of endometrial protein PP14 in uterine flushings throughout the menstrual cycle in normal, fertile women. *Br J Obstet Gynaecol* 100, 460-464.

Li TC, MacKenna A, and Roberts R (1993c) The techniques and complications of out-patient uterine washing in the assessment of endometrial function. *Hum Reprod* 8, 343-346.

Li TC, Okon MA, Dalton CF, Heatley M, and Laird SM (1998) Is the measurement of placental protein-14 and CA-125 in plasma and uterine flushings useful in the evaluation of peri-menopausal and post- menopausal bleeding? *Hum Reprod* 13, 2895-2901.

Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, and Giachelli CM (1995) The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of $\alpha v\beta 3$ in smooth muscle cell migration to osteopontin in vitro. *J Clin Invest* 95, 713-724.

Licht P, Losch A, Dittrich R, Neuwinger J, Siebzehnrubl E, and Wildt L (1998) Novel insights into human endometrial paracrinology and embryo-maternal communication by intrauterine microdialysis. *Hum Reprod Update* 4, 532-538.

Licht P, Russu V, Lehmeyer S, Moll J, Siebzehnrubl E, and Wildt L (2002) Intrauterine microdialysis reveals cycle-dependent regulation of

endometrial insulin-like growth factor binding protein-1 secretion by human chorionic gonadotropin. *Fertil Steril* 78, 252-258.

Licht P, Russu V, Lehmeyer S, and Wildt L (2001a) Molecular aspects of direct LH/hCG effects on human endometrium--lessons from intrauterine microdialysis in the human female in vivo. *Reprod Biol* 1, 10-19.

Licht P, Russu V, and Wildt L (2001b) On the role of human chorionic gonadotropin (hCG) in the embryo- endometrial microenvironment: implications for differentiation and implantation. *Semin Reprod Med* 19, 37-47.

Licht P, Russu V, Lehmeyer S, Wissentheit T, Siebzehnrubl E, and Wildt L (2003a) Cycle dependency of intrauterine vascular endothelial growth factor levels is correlated with decidualization and corpus luteum function. *Fertil Steril* 80, 1228-1233.

Licht P, Von Wolff M, Berkholz A, and Wildt L (2003b) Evidence for cycle-dependent expression of full-length human chorionic gonadotropin/luteinizing hormone receptor mRNA in human endometrium and decidua. *Fertil Steril* 79 (Suppl 1), 718-723.

Licht P, Fluhr H, Neuwinger J, Wallwiener D, and Wildt L (2007) Is human chorionic gonadotropin directly involved in the regulation of human implantation? *Mol Cell Endocrinol* 269, 85-92.

Lim KJ, Odukoya OA, Ajjan RA, Li TC, Weetman AP, and Cooke ID (2000) The role of T-helper cytokines in human reproduction. *Fertil Steril* 73, 136-142.

Lindhard A, Bentin-Ley U, Ravn V, Islin H, Hviid T, Rex S, Bangsboll S, and Sorensen S (2002) Biochemical evaluation of endometrial function at the time of implantation. *Fertil Steril* 78, 221-233.

Linjawi S, Li TC, Tuckerman EM, Blakemore AI, and Laird SM (2004) Expression of interleukin-11 receptor alpha and interleukin-11 protein in the endometrium of normal fertile women and women with recurrent miscarriage. *J Reprod Immunol* 64, 145-155.

Lopata A and Hay DL (1989) The potential of early human embryos to form blastocysts, hatch from their zona and secrete HCG in culture. *Hum Reprod* 4, 87-94.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193, 265-275.

Luk J, Seval Y, Kayisili A, and Arici A (2008) The effect of sex steriods on IL-8 and MCP-1 expression in human endometrial endothelial cells - The gatekeeper of human endometrium . *Fertil Steril* 82, S62.

Lukassen HG, Joosten I, Van Cranenbroek B, Van Lierop MJ, Bulten J, Braat DD, and Van der Ma (2004) Hormonal stimulation for IVF treatment positively

affects the CD56bright/CD56dim NK cell ratio of the endometrium during the window of implantation. *Mol Hum Reprod* 10, 513-520.

Ma WG, Song H, Das SK, Paria BC, and Dey SK (2003) Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *Proc Natl Acad Sci USA* 100, 2963-2968.

Maathuis JB and Aitken RJ (1978a) Cyclic variation in concentrations of protein and hexose in human uterine flushings collected by an improved technique. *J Reprod Fertil* 52, 289-295.

Maathuis JB and Aitken RJ (1978b) Protein patterns of human uterine flushings collected at various stages of the menstrual cycle. *J Reprod Fertil* 53, 343-348.

Maathuis JB and Kelly RW (1978c) Concentrations of prostaglandins F2alpha and E2 in the endometrium throughout the human menstrual cycle, after the administration of clomiphene or an oestrogen-progestogen pill and in early pregnancy. *J Endocrinol* 77, 361-371.

MacKenna A, Li TC, Dalton C, Bolton A, and Cooke I (1993) Placental protein 14 levels in uterine flushing and plasma of women with unexplained infertility. *Fertil Steril* 59, 577-582.

Macklon NS and Fauser BC (2000a) Impact of ovarian hyperstimulation on the luteal phase. *J Reprod Fertil* 55, 101-108.

Macklon NS and Fauser BC (2000b) Regulation of follicle development and novel approaches to ovarian stimulation for IVF. *Hum Reprod Update* 6, 307-312.

Macklon NS, Geraedts JP, and Fauser BC (2002) Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. *Hum Reprod Update* 8, 333-343.

Macklon NS, Stouffer RL, Giudice LC, and Fauser BC (2006) The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocr Rev* 27, 170-207.

Macklon NS, Van der Gaast MH, Hamilton A, Fauser BC, and Giudice LC (2008) The impact of ovarian stimulation with recombinant FSH in combination with GnRH antagonist on the endometrial transcriptome in the window of implantation. *Reprod Sci* 15, 357-365.

MacLaughlin DT and Richardson GS (1983) Analysis of human uterine luminal fluid proteins following radiolabeling by reductive methylation: comparison of proliferative and secretory phase samples. *Biol Reprod* 29, 733-742.

MacLaughlin DT, Santoro NF, Bauer HH, Lawrence D, and Richardson GS (1986) Two-dimensional gel electrophoresis of endometrial protein in human uterine fluids: qualitative and quantitative analysis. *Biol Reprod* 34, 579-585.

Makkar G, Ng EH, Yeung WS, and Ho PC (2006) Reduced expression of interleukin-11 and interleukin-6 in the peri-implantation endometrium of excessive ovarian responders during in-vitro fertilization treatment. *J Clin Endocrinol Metab* 91, 3181-3188.

Makkar G, Yu Ng EH, Biu Yeung WS, and Ho PC (2008) Excessive ovarian response is associated with increased expression of interleukin-2 in the periimplantation endometrium. *Fertil Steril* (Epub ahead of print).

Mangal RK, Wiehle RD, Poindexter AN, III, and Weigel NL (1997) Differential expression of uterine progesterone receptor forms A and B during the menstrual cycle. *J Steroid Biochem Mol Biol* 63, 195-202.

Mannaerts B and Gordon K (2000) Embryo implantation and GnRH antagonists: GnRH antagonists do not activate the GnRH receptor. *Hum Reprod* 15, 1882-1883.

Mao B, Wu W, Li Y, Hoppe D, Stannek P, Glinka A, and Niehrs C (2001) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* 411, 321-325.

Martin J, Dominguez F, Avila S, Castrillo JL, Remohi J, Pellicer A, and Simon C (2002) Human endometrial receptivity: gene regulation. *J Reprod Immunol* 55, 131-139.

Martinez-Conejero JA, Simon C, Pellicer A, and Horcajadas JA (2007) Is ovarian stimulation detrimental to the endometrium? *Reprod BioMed Online* 15, 45-50.

Masferrer JL, Zweifel BS, Colburn SM, Ornberg RL, Salvemini D, Isakson P, and Seibert K (1995) The Role of Cyclooxygenase-2 in Inflammation. *Am J Ther* 2, 607-610.

McDonough PG (2004) Grading a developmental continuum-elegy on the rise and fall of the endometrial biopsy. *Fertil Steril* 82, 1286-1292.

McLaren A (1973) Blastocyst activation. In Segal SJ, Crozier R, Corfman PA *et al.* (eds), *The regulation of mammalian reproduction.* Charles C. Thomas, Springfield, pp. 321-334.

Meseguer M, Aplin JD, Caballero-Campo P, O'Connor JE, Martin JC, Remohi J, Pellicer A, and Simon C (2001) Human endometrial mucin MUC1 is up-regulated by progesterone and down- regulated in vitro by the human blastocyst. *Biol Reprod* 64, 590-601.

Messinis IE, Templeton A, and Baird DT (1987a) Luteal phase after ovarian hyperstimulation. *Br J Obstet Gynaecol* 94, 345-350.

Messinis IE and Templeton AA (1987b) Disparate effects of endogenous and exogenous oestradiol on luteal phase function in women. *J Reprod Fertil* 79, 549-554.

Meyer WR, Novotny DB, Fritz MA, Beyler SA, Wolf LJ, and Lessey BA (1999) Effect of exogenous gonadotropins on endometrial maturation in oocyte donors. *Fertil Steril* 71, 109-114.

Mikolajczyk M, Skrzypczak J, Szymanowski K, and Wirstlein P (2003) The assessment of LIF in uterine flushing--a possible new diagnostic tool in states of impaired fertility. *Reprod Biol* 3, 259-270.

Mikolajczyk M, Wirstlein P, and Skrzypczak J (2007) The impact of leukemia inhibitory factor in uterine flushing on the reproductive potential of infertile women - a prospective study. *Am J Reprod Immunol* 58, 65-74.

Milligan SR and Martin L (1984) The resistance of the mouse uterine lumen to flushing and possible contamination of samples by plasma and interstitial fluid. *J Reprod Fertil* 71, 81-87.

Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S, and Oehninger S (2005) In search of candidate genes critically expressed in the human endometrium during the window of implantation. *Hum Reprod* 20, 2104-2117.

Mirkin S, Nikas G, Hsiu JG, Diaz J, and Oehninger S (2004) Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. *J Clin Endocrinol Metab* 89, 5742-5752.

Mueller MD, Vigne JL, Vaisse C, and Taylor RN (2000) Glycodelin: a pane in the implantation window. *Semin Reprod Med* 18, 289-298.

Müller-Schöttle F, Classen-Linke I, Alfer J, Krusche C, Beier-Hellwig K, Sterzik K, and Beier HM (1999) Expression of uteroglobin in the human endometrium. *Mol Hum Reprod* 5, 1155-1161.

Munne S (2006) Chromosome abnormalities and their relationship to morphology and development of human embryos. *Reprod BioMed Online* 12, 234-253.

Murray MJ, Meyer WR, Zaino RJ, Lessey BA, Novotny DB, Ireland K, Zeng D, and Fritz MA (2004) A critical analysis of the accuracy, reproducibility, and clinical utility of histologic endometrial dating in fertile women. *Fertil Steril* 81, 1333-1343.

Myers ER, Silva S, Barnhart K, Groben PA, Richardson MS, Robboy SJ, Leppert PY, and Coutifaris C (2004) Interobserver and intraobserver variability in the histological dating of the endometrium in fertile and infertile women. *Fertil Steril* 82, 1278-1282.

Navot D, Bergh PA, Williams M, Garrisi GJ, Guzman I, Sandler B, Fox J, Schreiner-Engel P, Hofmann GE, and Grunfeld L (1991) An insight into early reproductive processes through the in vivo model of ovum donation. *J Clin Endocrinol Metab* 72, 408-414.

Ng EH, Laird SM, Li TC, Yeung WS, and Ho PC (2004) Concentrations of endometrial protein PP 14 and CA-125 in uterine flushings performed in natural and stimulated cycles. *Hum Reprod* 19, 905-910.

Nikas G, Drakakis P, Loutradis D, Mara-Skoufari C, Koumantakis E, Michalas S, and Psychoyos A (1995) Uterine pinopodes as markers of the 'nidation window' in cycling women receiving exogenous oestradiol and progesterone. *Hum Reprod* 10, 1208-1213.

Nikas G (1999a) Cell-surface morphological events relevant to human implantation. *Hum Reprod* 14 (Suppl 2), 37-44.

Nikas G, Develioglu OH, Toner JP, and Jones HWJ (1999b) Endometrial pinopodes indicate a shift in the window of receptivity in IVF cycles. *Hum Reprod* 14, 787-792.

Nikas G and Aghajanova L (2002) Endometrial pinopodes: some more understanding on human implantation? *Reprod BioMed Online* 4 Suppl 3, 18-23.

Nippoldt TB, Reame NE, Kelch RP, and Marshall JC (1989) The roles of estradiol and progesterone in decreasing luteinizing hormone pulse frequency in the luteal phase of the menstrual cycle. *J Clin Endocrinol Metab* 69, 67-76.

Noci I, Borri P, Coccia ME, Criscuoli L, Scarselli G, Messeri G, Paglierani M, Moncini D, and Taddei G (1997) Hormonal patterns, steroid receptors and morphological pictures of endometrium in hyperstimulated IVF cycles. *Eur J Obstet Gynecol Reprod Biol* 75, 215-220.

Noyes N, Hertig AT, and Rock J (1950) Dating the endometrial biopsy. *Fertil Steril* 1, 3-25.

Okamoto N, Uchida A, Takakura K, Kariya Y, Kanzaki H, Riittinen L, Koistinen R, Seppala M, and Mori T (1991) Suppression by human placental protein 14 of natural killer cell activity. *Am J Reprod Immunol* 26, 137-142.

Olivennes F, Ledee-Bataille N, Samama M, Kadoch J, Taupin JL, Dubanchet S, Chaouat G, and Frydman R (2003) Assessment of leukemia inhibitory factor levels by uterine flushing at the time of egg retrieval does not adversely affect pregnancy rates with in vitro fertilization. *Fertil Steril* 79, 900-904.

Pampfer S, Daiter E, Barad D, and Pollard JW (1992) Expression of the colony-stimulating factor-1 receptor (c-fms proto-oncogene product) in the human uterus and placenta. *Biol Reprod* 46, 48-57.

Pantos K, Meimeth-Damianaki T, Vaxevanoglou T, and Kapetanakis E (1994) Prospective study of a modified gonadotropin-releasing hormone agonist long protocol in an in vitro fertilization program. *Fertil Steril* 61, 709-713.

Papanikolaou EG, Bourgain C, Kolibianakis E, Tournaye H, and Devroey P (2005) Steroid receptor expression in late follicular phase endometrium in GnRH

antagonist IVF cycles is already altered, indicating initiation of early luteal phase transformation in the absence of secretory changes. *Hum Reprod* 20, 1541-1547.

Paulson RJ, Sauer MV, and Lobo RA (1990) Embryo implantation after human in vitro fertilization: importance of endometrial receptivity. *Fertil Steril* 53, 870-874.

Pelinck MJ, Hoek A, Simons AH, and Heineman MJ (2002) Efficacy of natural cycle IVF: a review of the literature. *Hum Reprod Update* 8, 129-139.

Pellicer A, Valbuena D, Cano F, Remohi J, and Simon C (1996) Lower implantation rates in high responders: evidence for an altered endocrine milieu during the preimplantation period. *Fertil Steril* 65, 1190-1195.

Pellicer A, Dominguez F, Remohi J, and Simon C (2002) Molecular basis of implantation. *Reprod BioMed Online* 5 (Suppl 1), 44-51.

Pena JE, Chang PL, Chan LK, Zeitoun K, Thornton MH, and Sauer MV (2002) Supraphysiological estradiol levels do not affect oocyte and embryo quality in oocyte donation cycles. *Hum Reprod* 17, 83-87.

Perrier d'Hauterive S, Charlet-Renard C, Berndt S, Dubois M, Munaut C, Goffin F, Hagelstein MT, Noel A, Hazout A, Foidart JM, and Geenen V (2004) Human chorionic gonadotropin and growth factors at the embryonic-endometrial interface control leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) secretion by human endometrial epithelium. *Hum Reprod* 19, 2633-2643.

Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, and Romagnani S (1998) Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat Med* 4, 1020-1024.

Polli V, Bulletti C, Galassi A, Borini A, Ciotti PM, Seracchioli R, Alfieri S, and Flamigni C (1996) Transforming growth factor-beta 1 in the human endometrium. *Gynecol Endocrinol* 10, 297-302.

Polson DW, Sagle M, Mason HD, Kiddy D, and Franks S (1987) Recovery of luteal function after interruption of gonadotrophin secretion in the mid-luteal phase of the menstrual cycle. *Clin Endocrinol (Oxf)* 26, 597-600.

Popovic-Todorovic B, Loft A, Bredkjaeer HE, Bangsboll S, Nielsen IK, and Andersen AN (2003) A prospective randomized clinical trial comparing an individual dose of recombinant FSH based on predictive factors versus a 'standard' dose of 150 IU/day in 'standard' patients undergoing IVF/ICSI treatment. *Hum Reprod* 18, 2275-2282.

Psychoyos A (1963) Precision sur l'état de 'non-receptivité' de l'uterus. CR Academy Sciences, Paris, 1153-1156.

Psychoyos A and Mandon P (1971) [Study of the surface of the uterine epithelium by scanning electron microscope. Observations in the rat at

the 4th and 5th day of pregnancy]. *C R Acad Sci Hebd Seances Acad Sci D* 272, 2723-2725.

Psychoyos A (1973) Hormonal control of ovoimplantation. *Vitam Horm* 31, 201-256.

Psychoyos A (1993) The implantation window: basic and clinical aspects. In *Perspectives in Assisted Reproduction*.pp. 57-62.

Raga F, Casan EM, Kruessel JS, Wen Y, Huang HY, Nezhat C, and Polan ML (1998) Quantitative gonadotropin-releasing hormone gene expression and immunohistochemical localization in human endometrium throughout the menstrual cycle. *Biol Reprod* 59, 661-669.

Ravn V, Teglbjaerg CS, Mandel U, and Dabelsteen E (1992) The distribution of type-2 chain histo-blood group antigens in normal cycling human endometrium. *Cell Tissue Res* 270, 425-433.

Ravn V, Mandel U, Svenstrup B, and Dabelsteen E (1994) Expression of type-2 histo-blood group carbohydrate antigens (Le(x), Le(y), and H) in normal and malignant human endometrium. *Virchows Arch* 424, 411-419.

Rey JM, Pujol P, Dechaud H, Edouard E, Hedon B, and Maudelonde T (1998) Expression of oestrogen receptor-alpha splicing variants and oestrogen receptor-beta in endometrium of infertile patients. *Mol Hum Reprod* 4, 641-647.

Richlin SS, Ramachandran S, Shanti A, Murphy AA, and Parthasarathy S (2002) Glycodelin levels in uterine flushings and in plasma of patients with leiomyomas and polyps: implications for implantation. *Hum Reprod* 17, 2742-2747.

Riesewijk A, Martin J, Van Os R, Horcajadas JA, Polman J, Pellicer A, Mosselman S, and Simon C (2003) Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Mol Hum Reprod* 9, 253-264.

Risau W (1997) Mechanisms of angiogenesis. *Nature* 386, 671-674.

Roberts GP, Parker JM, and Henderson SR (1976) Proteins in human uterine fluid. *J Reprod Fertil* 48, 153-157.

Saadat P, Boostanfar R, Slater CC, Tourgeman DE, Stanczyk FZ, and Paulson RJ (2004) Accelerated endometrial maturation in the luteal phase of cycles utilizing controlled ovarian hyperstimulation: impact of gonadotropin-releasing hormone agonists versus antagonists. *Fertil Steril* 82, 167-171.

Salim R, Miel J, Savvas M, Lee C, and Jurkovic D (2007) A comparative study of glycodelin concentrations in uterine flushings in women with subseptate uteri, history of unexplained recurrent miscarriage and healthy controls. *Eur J Obstet Gynecol Reprod Biol* 133, 76-80.

Satokata I, Benson G, and Maas R (1995) Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. *Nature* 374, 460-463.

Schoonmaker JN, Bergman KS, Steiner RA, and Karsch FJ (1982) Estradiol-induced luteal regression in the rhesus monkey: evidence for an extraovarian site of action. *Endocrinology* 110, 1708-1715.

Seli E, Kayisli UA, Cakmak H, Bukulmez O, Bildirici I, Guzeloglu-Kayisli O, and Arici A (2005) Removal of hydrosalpinges increases endometrial leukaemia inhibitory factor (LIF) expression at the time of the implantation window. *Hum Reprod* 20, 3012-3017.

Seppala M, Bohn H, and Tatarinov Y (1998) Glycodelins. *Tumour Biol* 19, 213-220.

Seppala M, Taylor RN, Koistinen H, Koistinen R, and Milgrom E (2002) Glycodelin: a major lipocalin protein of the reproductive axis with diverse actions in cell recognition and differentiation. *Endocr Rev* 23, 401-430.

Seppala M (2004) Advances in uterine protein research: reproduction and cancer. *Int J Gynaecol Obstet* 85, 105-118.

Seppala M, Koistinen H, Koistinen R, Chiu PC, and Yeung WS (2007) Glycosylation related actions of glycodelin: gamete, cumulus cell, immune cell and clinical associations. *Hum Reprod Update* 13, 275-287.

Serafini P, Rocha AM, Osorio CT, da S, I, Motta EL, and Baracat EC (2008) Endometrial leukemia inhibitory factor as a predictor of pregnancy after in vitro fertilization. *Int J Gynaecol Obste* 102, 23-27.

Sharma V, Allgar V, and Rajkhowa M (2002) Factors influencing the cumulative conception rate and discontinuation of in vitro fertilization treatment for infertility. *Fertil Steril* 78, 40-46.

Sherwin JR, Smith SK, Wilson A, and Sharkey AM (2002) Soluble gp130 is up-regulated in the implantation window and shows altered secretion in patients with primary unexplained Infertility. *J Clin Endocrinol Metab* 87, 3953-3960.

Shiozawa T, Li SF, Nakayama K, Nikaido T, and Fujii S (1996) Relationship between the expression of cyclins/cyclin-dependent kinases and sex-steroid receptors/Ki67 in normal human endometrial glands and stroma during the menstrual cycle. *Mol Hum Reprod* 2, 745-752.

Shirai E, Iizuka R, and Notake Y (1972) Analysis of human uterine fluid protein. *Fertil Steril* 23, 522-528.

Shoupe D, Mishell DR, Jr., Lacarra M, Lobo RA, Horenstein J, d'Ablaing G, and Moyer D (1989) Correlation of endometrial maturation with four methods of estimating day of ovulation. *Obstet Gynecol* 73, 88-92.

Silverberg KM, Burns WN, Olive DL, Riehl RM, and Schenken RS (1991) Serum progesterone levels predict success of in vitro fertilization/embryo transfer

in patients stimulated with leuprolide acetate and human menopausal gonadotropins. *J Clin Endocrinol Metab* 73, 797-803.

Simon C, Piquette GN, Frances A, and Polan ML (1993) Localization of interleukin-1 type I receptor and interleukin-1β in human endometrium throughout the menstrual cycle. *J Clin Endocrinol Metab* 77, 549-555.

Simon C, Cano F, Valbuena D, Remohi J, and Pellicer A (1995) Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. *Hum Reprod* 10, 2432-2437.

Simon C, Mercader A, Frances A, Gimeno MJ, Polan ML, Remohi J, and Pellicer A (1996) Hormonal regulation of serum and endometrial IL-1α, IL-1β and IL-1ra: IL-1 endometrial microenvironment of the human embryo at the apposition phase under physiological and supraphysiological steroid level conditions. *J Reprod Immunol* 31, 165-184.

Simon C, Gimeno MJ, Mercader A, O'Connor JE, Remohi J, Polan ML, and Pellicer A (1997) Embryonic regulation of integrins β3, α4, and α1 in human endometrial epithelial cells in vitro. *J Clin Endocrinol Metab* 82, 2607-2616.

Simon C, Garcia VJ, Valbuena D, Peinado JA, Moreno C, Remohi J, and Pellicer A (1998a) Increasing uterine receptivity by decreasing estradiol levels during the preimplantation period in high responders with the use of a follicle-stimulating hormone step-down regimen. *Fertil Steril* 70, 234-239.

Simon C, Moreno C, Remohi J, and Pellicer A (1998b) Molecular interactions between embryo and uterus in the adhesion phase of human implantation. *Hum Reprod* 13 (Suppl 3), 219-232.

Simon C, Oberye J, Bellver J, Vidal C, Bosch E, Horcajadas JA, Murphy C, Adams S, Riesewijk A, Mannaerts B, and Pellicer A (2005) Similar endometrial development in oocyte donors treated with either high- or standard-dose GnRH antagonist compared to treatment with a GnRH agonist or in natural cycles. *Hum Reprod* 20, 3318-3327.

Singh VN, Quadros M, and Singh JN (1993) Human uterine protein in relation to infertility. *Horm Metab Res* 25, 231-233.

Singh VN (1995) Human uterine amylase in relation to infertility. *Horm Metab Res* 27, 35-36.

Smith LL, Cheung HK, Ling LE, Chen J, Sheppard D, Pytela R, and Giachelli CM (1996a) Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by alpha9beta1 integrin. *J Biol Chem* 271, 28485-28491.

Smith WL and Dewitt DL (1996b) Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* 62, 167-215.

Smitz J, Devroey P, Camus M, Deschacht J, Khan I, Staessen C, Van Waesberghe L, Wisanto A, and Van Steirteghem AC (1988) The luteal phase and early pregnancy after combined GnRH-agonist/HMG treatment for superovulation in IVF or GIFT. *Hum Reprod* 3, 585-590.

Smitz J, Erard P, Camus M, Devroey P, Tournaye H, Wisanto A, and Van Steirteghem AC (1992) Pituitary gonadotrophin secretory capacity during the luteal phase in superovulation using GnRH-agonists and HMG in a desensitization or flare-up protocol. *Hum Reprod* 7, 1225-1229.

Soules MR, Steiner RA, Clifton DK, Cohen NL, Aksel S, and Bremner WJ (1984) Progesterone modulation of pulsatile luteinizing hormone secretion in normal women. *J Clin Endocrinol Metab* 58, 378-383.

Staessen C, Platteau P, Van Assche E, Michiels A, Tournaye H, Camus M, Devroey P, Liebaers I, and Van Steirteghem A (2004) Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. *Hum Reprod* 19, 2849-2858.

Stavreus-Evers A, Aghajanova L, Brismar H, Eriksson H, Landgren BM, and Hovatta O (2002) Co-existence of heparin-binding epidermal growth factor-like growth factor and pinopodes in human endometrium at the time of implantation. *Mol Hum Reprod* 8, 765-769.

Steck T, Giess R, Suetterlin MW, Bolland M, Wiest S, Poehls UG, and Dietl J (2004) Leukaemia inhibitory factor (LIF) gene mutations in women with unexplained infertility and recurrent failure of implantation after IVF and embryo transfer. *Eur J Obstet Gynecol Reprod Biol* 112, 69-73.

Sterzik K, Dallenbach C, Schneider V, Sasse V, and Dallenbach-Hellweg G (1988) In vitro fertilization: the degree of endometrial insufficiency varies with the type of ovarian stimulation. *Fertil Steril* 50, 457-462.

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, and Abbondanzo SJ (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 359, 76-79.

Stone BA, Petrucco OM, Seamark RF, and Godfrey BM (1986) Concentrations of steroid hormones, and of prolactin, in washings of the human uterus during the menstrual cycle. *J Reprod Fertil* 78, 21-25.

Sugihara K, Kabir-Salmani M, Byrne J, Wolf DP, Lessey B, Iwashita M, Aoki D, Nakayama J, and Fukuda MN (2008) Induction of trophinin in human endometrial surface epithelia by CGbeta and IL-1beta. *FEBS Lett* 582, 197-202.

Sullivan DA, Richardson GS, MacLaughlin DT, and Wira CR (1984) Variations in the levels of secretory component in human uterine fluid during the menstrual cycle. *J Steroid Biochem* 20, 509-513.

Sungurtekin U and Jansen RP (1995) Profound luteinizing hormone suppression after stopping the gonadotropin-releasing hormone-agonist leuprolide acetate. *Fertil Steril* 63, 663-665.

Suzuki N, Zara J, Sato T, Ong E, Bakhiet N, Oshima RG, Watson KL, and Fukuda MN (1998) A cytoplasmic protein, bystin, interacts with trophinin, tastin, and cytokeratin and may be involved in trophinin-mediated cell adhesion between trophoblast and endometrial epithelial cells. *Proc Natl Acad Sci USA* 95, 5027-5032.

Suzuki N, Nakayama J, Shih IM, Aoki D, Nozawa S, and Fukuda MN (1999) Expression of trophinin, tastin, and bystin by trophoblast and endometrial cells in human placenta. *Biol Reprod* 60, 621-627.

Sylvan PE, MacLaughlin DT, Richardson GS, Scully RE, and Nikrui N (1981) Human uterine luminal fluid proteins associated with secretory phase endometrium: progesterone-induced products? *Biol Reprod* 24, 423-429.

Tabibzadeh S, Kong QF, Babaknia A, and May LT (1995a) Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. *Hum Reprod* 10, 2793-2799.

Tabibzadeh S, Kong QF, Kapur S, Satyaswaroop PG, and Aktories K (1995b) Tumour necrosis factor-alpha-mediated dyscohesion of epithelial cells is associated with disordered expression of cadherin/beta-catenin and disassembly of actin filaments. *Hum Reprod* 10, 994-1004.

Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, Le Shay N, Nezhat CN, Kempson R, Lessey BA, Nayak NR, and Giudice LC (2006) Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 147, 1097-1121.

Tarlatzis BC, Zepiridis L, Grimbizis G, and Bontis J (2003) Clinical management of low ovarian response to stimulation for IVF: a systematic review. *Hum Reprod Update* 9, 61-76.

Tarlatzis BC, Fauser BC, Kolibianakis EM, Diedrich K, and Devroey P (2006) GnRH antagonists in ovarian stimulation for IVF. *Hum Reprod Update* 12, 333-340.

Tavaniotou A, Albano C, Smitz J, and Devroey P (2001a) Comparison of LH concentrations in the early and mid-luteal phase in IVF cycles after treatment with HMG alone or in association with the GnRH antagonist Cetrorelix. *Hum Reprod* 16, 663-667.

Tavaniotou A, Smitz J, Bourgain C, and Devroey P (2001b) Ovulation induction disrupts luteal phase function. *Ann N Y Acad Sci* 943, 55-63.

Taylor HS, Arici A, Olive D, and Igarashi P (1998) HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium. *J Clin Invest* 101, 1379-1384.

Taylor HS, Igarashi P, Olive DL, and Arici A (1999) Sex steroids mediate HOXA11 expression in the human peri-implantation endometrium. *J Clin Endocrinol Metab* 84, 1129-1135.

Templeton A, Morris JK, and Parslow W (1996) Factors that affect outcome of in-vitro fertilisation treatment. *Lancet* 348, 1402-1406.

Templeton A and Morris JK (1998) Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization. *N Engl J Med* 339, 573-577.

The ganirelix dose-finding study group (1998) A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon). The ganirelix dose-finding study group. *Hum Reprod* 13, 3023-3031.

Tseng L, Zhang J, Peresleni TY, and Goligorsky MS (1996) Cyclic expression of endothelial nitric oxide synthase mRNA in the epithelial glands of human endometrium. *J Soc Gynecol Investig* 3, 33-38.

Tuckerman E, Laird SM, Stewart R, Wells M, and Li TC (2004) Markers of endometrial function in women with unexplained recurrent pregnancy loss: a comparison between morphologically normal and retarded endometrium. *Hum Reprod* 19, 196-205.

Tulac S, Overgaard MT, Hamilton AE, Jumbe NL, Suchanek E, and Giudice LC (2006) Dickkopf-1, an inhibitor of Wnt signaling, is regulated by progesterone in human endometrial stromal cells. *J Clin Endocrinol Metab* 91, 1453-1461.

Ubaldi F, Bourgain C, Tournaye H, Smitz J, Van Steirteghem A, and Devroey P (1997) Endometrial evaluation by aspiration biopsy on the day of oocyte retrieval in the embryo transfer cycles in patients with serum progesterone rise during the follicular phase. *Fertil Steril* 67, 521-526.

Valbuena D, Jasper M, Remohi J, Pellicer A, and Simon C (1999) Ovarian stimulation and endometrial receptivity. *Hum Reprod* 14 (Suppl 2), 107-111.

Valbuena D, Martin J, de Pablo JL, Remohi J, Pellicer A, and Simon C (2001) Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. *Fertil Steril* 76, 962-968.

Van der Gaast MH, Beckers NG, Beier-Hellwig K, Beier HM, Macklon NS, and Fauser BC (2002) Ovarian stimulation for IVF and endometrial receptivity - the missing link. *Reprod BioMed Online* 5 (Suppl 1), 36-43.

Van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM, and Macklon NS (2003) Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates. *Reprod BioMed Online* 7, 105-109.

Van der Gaast MH, Eijkemans MJ, Van der Net JB, de Boer EJ, Burger CW, van Leeuwen FE, Fauser BC, and Macklon NS (2006) Optimum number of oocytes for a successful first IVF treatment cycle. *Reprod BioMed Online* 13, 476-480.

Van der Gaast MH, Classen-Linke I, Krusche CA, Beier-Hellwig K, Fauser BC, Beier HM, and Macklon NS (2008) Impact of ovarian stimulation on mid-luteal endometrial tissue and secretion markers of receptivity. *Reprod BioMed Online* 17, 553-563.

Van der Gaast MH, Macklon NS, Beier-Hellwig K, Krusche CA, Fauser BC, Beier HM, and Classen-Linke I (2009) The feasibility of a less invasive method to assess endometrial maturation-comparison of simultaneously obtained uterine secretion and tissue biopsy. *BJOG* 116, 304-312.

Vandermolen DT and Gu Y (1996) Human endometrial interleukin-6 (IL-6): in vivo messenger ribonucleic acid expression, in vitro protein production, and stimulation thereof by IL-1 beta. *Fertil Steril* 66, 741-747.

VandeVoort CA, Baughman WL, and Stouffer RL (1989) Comparison of different regimens of human gonadotropins for superovulation of rhesus monkeys: ovulatory response and subsequent luteal function. *J In Vitro Fert Embryo Transf* 6, 85-91.

Vargyas J, Kletzky O, and Marrs RP (1986) The effect of laparoscopic follicular aspiration on ovarian steroidogenesis during the early preimplantation period. *Fertil Steril* 45, 221-225.

Vogiagis D, Marsh MM, Fry RC, and Salamonsen LA (1996) Leukaemia inhibitory factor in human endometrium throughout the menstrual cycle. *J Endocrinol* 148, 95-102.

Von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, and Tabibzadeh S (2000) Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. *Mol Hum Reprod* 6, 627-634.

Von Wolff M, Strowitzki T, Becker V, Zepf C, Tabibzadeh S, and Thaler CJ (2001a) Endometrial osteopontin, a ligand of β3-integrin, is maximally expressed around the time of the "implantation window". *Fertil Steril* 76, 775-781.

Von Wolff M, Thaler CJ, Lumpp K, and Strowitzki T (2001b) Endometrial osteopontin is maximally expressed in the secretory phase of the menstrual cycle in endometrial glands and leukocytes and is secreted into the uterine cavity. *Hum Reprod* 16 (Suppl 1), 29.

Von Wolff M, Thaler CJ, Zepf C, Becker V, Beier HM, and Strowitzki T (2002) Endometrial expression and secretion of interleukin-6 throughout the menstrual cycle. *Gynecol Endocrinol* 16, 121-129.

Von Wolff M, Wang X, Gabius HJ, and Strowitzki T (2005) Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation. *Mol Hum Reprod* 11, 189-194.

Voss HJ and Beato M (1977) Human uterine fluid proteins: gel electrophoretic pattern and progesterone-binding properties. *Fertil Steril* 28, 972-980.

Wang XQ, Zhu ZM, Fenderson BA, Zeng GQ, Cao YJ, and Jiang GT (1998) Effects of monoclonal antibody directed to LeY on implantation in the mouse. *Mol Hum Reprod* 4, 295-300.

Wang B, Sheng JZ, He RH, Qian YL, Jin F, and Huang HF (2008) High expression of L-selectin ligand in secretory endometrium is associated with better endometrial receptivity and facilitates embryo implantation in human being. *Am J Reprod Immunol* 60, 127-134.

Weissman A, Loumaye E, and Shoham Z (1996) Recovery of corpus luteum function after prolonged deprivation from gonadotrophin stimulation. *Hum Reprod* 11, 943-949.

Westergaard LG, Wiberg N, Andersen CY, Laursen SB, Kliem A, Westergaard JG, and Teisner B (1998) Circulating concentrations of placenta protein 14 during the natural menstrual cycle in women significantly reflect endometrial receptivity to implantation and pregnancy during successive assisted reproduction cycles. *Hum Reprod* 13, 2612-2619.

Wilcox AJ, Baird DD, and Weinberg CR (1999) Time of implantation of the conceptus and loss of pregnancy. *N Engl J Med* 340, 1796-1799.

Wolf DP and Mastroianni L, Jr. (1975) Protein composition of human uterine fluid. *Fertil Steril* 26, 240-247.

Yanaihara A, Otsuka Y, Iwasaki S, Koide K, Aida T, and Okai T (2004) Comparison in gene expression of secretory human endometrium using laser microdissection. *Reprod Biol Endocrinol* 2, 66.

Yanaihara A, Otsuka Y, Iwasaki S, Aida T, Tachikawa T, Irie T, and Okai T (2005) Differences in gene expression in the proliferative human endometrium. *Fertil Steril* 83 (Suppl 4), 1206-1215.

Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, Suzuki H, Amano T, Kennedy G, Arai H, Aoki J, and Chun J (2005) LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* 435, 104-108.

Yih MC, Spandorfer SD, and Rosenwaks Z (2005) Egg production predicts a doubling of in vitro fertilization pregnancy rates even within defined age and ovarian reserve categories. *Fertil Steril* 83, 24-29.

Zeleznik AJ (1998) In vivo responses of the primate corpus luteum to luteinizing hormone and chorionic gonadotropin. *Proc Natl Acad Sci USA* 95, 11002-11007.

Zhang B, Schmoyer D, Kirov S, and Snoddy J (2004) GOTree Machine (GOTM): a web-based platform for interpreting sets of interesting genes using Gene Ontology hierarchies. *BMC Bioinformatics* 5, 16.

Zhou XL, Lei Z M, and Rao CV (1999) Treatment of human endometrial gland epithelial cells with chorionic gonadotropin/luteinizing hormone increases the expression of the cyclooxygenase-2 gene. *J Clin Endocrinol Metab* 84, 3364-3377.

Zhu LJ, Cullinan-Bove K, Polihronis M, Bagchi MK, and Bagchi IC (1998) Calcitonin is a progesterone-regulated marker that forecasts the receptive state of endometrium during implantation. *Endocrinology* 139, 3923-3934.

SUMMARY

Chapter 1.

This chapter describes the normal menstrual cycle with the ovarian hormones affecting endometrial structure, and a critical view on the gold standard to study endometrium: endometrial dating according to the Noyes criteria (Chapter **1.1.1**); implantation and the 'implantation window' concept (Chapter **1.1.2**); regulation of endometrial maturation and implantation (Chapter **1.2**) of the necessary markers of receptivity (Chapter **1.3**). Then the problems experienced whilst studying the impact of ovarian stimulation on endometrium are viewed (Chapter **1.4**), with a possible solution by studying endometrial secretion fluid (Chapter **1.5**). A brief review of the composition of the human uterine secretions obtained *in vivo* by flushing or aspiration of the uterine cavity. Finally, the objectives of this study are considered (Chapter **1.6**).

Chapter 2.

The contemporary approach to ovarian stimulation for in vitro fertilization (IVF) treatment results in supraphysiological levels of steroids during the follicular and luteal phase of the menstrual cycle. These sex steroids act directly and indirectly to mature the endometrium, influencing receptivity for implantation. Corpus luteum function is distinctly abnormal in IVF cycles, and therefore luteal support is widely used. Various reasons may underlay the defective luteal phase, including (a) ovarian hyperstimulation per sé, (b) gonadotropin-releasing hormone (GnRH) analogue co-treatment and (c) human chorionic gonadotropin (hCG) to induce final oocyte maturation. The recent introduction of GnRH antagonist co-treatment for the prevention of a premature LH rise during the late follicular phase, will allow for different approaches to ovarian stimulation for IVF. However, a recent meta-analysis showed that implantation rates may be compromised by using GnRH antagonists in currently employed regimens. The development of endometrium, receptive to embryo implantation, is a complex process and may be altered by inappropriate exposure to sex steroids in terms of timing, duration and magnitude. New approaches to the assessment of endometrial receptivity are now required. Novel approaches to ovarian stimulation aimed at adjusted GnRH antagonist regimens and achieving a more physiological luteal phase endocrinology are now appearing in the literature and may represent an important step in the improvement of the overall health economics of IVF.

Chapter 3.

To study if there is an optimal response to ovarian stimulation in terms of number of oocytes for achieving embryo transfer and pregnancy in a first in vitro

fertilization (IVF) cycle, a retrospective nationwide Dutch cohort study was performed.

In 12 IVF clinics 7,422 women undergoing their first IVF cycle, treated in The Netherlands between 1990 and 1995 for subfertility, and between 18 to 44 years of age. Embryo transfer and pregnancy rate in relation to the number of oocytes obtained was analyzed. The relationship was also studied in subgroups according to IVF indication, age and fecundity.

In the entire cohort, the embryo transfer rate, clinical pregnancy rate per transfer and pregnancy rate per started cycle rose to a maximum of 85%, 28% and 24% respectively when 10–14 oocytes were retrieved. In women with ovulatory subfertility an optimum embryo transfer and pregnancy rate was observed when 24 or more oocytes were obtained. An optimal number of oocytes for achieving embryo transfer and pregnancy beyond which outcomes worsen was demonstrated.

Obtaining more than 14 oocytes was associated with declining pregnancy rates per embryo transfer. The optimal range of retrieved oocytes for IVF outcomes was higher in women with ovulatory subfertility.

Chapter 4.

In order to determine the effects of ovarian stimulation with recombinant FSH and co-treatment with GnRH antagonist on endometrial gene expression during the putative 'window of implantation', endometrial biopsies were obtained for microarray analysis in the natural and stimulated cycle of the same subjects.

Four oocyte donors less than 40 years of age, with regular menstrual cycles of 25**–**35 days duration, no anatomical uterine abnormalities, and proven previous fertility participated. Endometrial tissue sampling was performed 5 days after the spontaneous ovulation in the natural cycle, and the ovum pick up in the hyperstimulated cycle (recFSH 150 IU sc, GnRH antagonist 0.25 mg daily sc; 10000 IU sc hCG; no P supplementation was provided after ovum pick-up). First and second-strand cDNAs were prepared according to the Affymetrix microarray preparation protocol. Samples were hybridized to Affymetrix HGU133 Plus 2 arrays and scanned using the HR3000 scanner. Data were analyzed using GeneSpring version 7.2 as well as the GOTM software. Default data normalization was performed using GeneSpring normalization algorithms, and data were then subject to ANOVA statistical evaluation as well as fold change filtration. Select genes were further analyzed by real-time PCR using the SYBR green method. Samples were run in triplicate and fold change was calculated. Statistical significance was determined by a log-transformed paired *t* test of the data.

142 genes were significantly upregulated according to the applied criteria, and 98 significantly downregulated. The principal observations were the marked upregulation of SCYB 13, DKK1, insulin-like growth factor binding proteins 4 and 5, and Homeobox C6. Gene ontogeny (GO) categories of upregulated genes included those involved in cell adhesion, T-cell receptor signaling, regulation of signal transduction, cell growth, proliferation and programmed cell death. GO

catagories of down regulated genes indicated reduced transmembrane receptor protein kinase activity following ovarian stimulation.

When normal fertile women are exposed to ovarian stimulation using recombinant FSH in combination with GnRH antagonist, extensive changes occur in expression of genes governing a wide variety of processes important to endometrial maturation and implantation. Supraphysiological levels of P and E present in the early luteal phase of the non-P supplemented stimulated cycle may be responsible for additional expression of progesterone modulated genes.

Chapter 5.

Studies of endometrial receptivity during the implantation window of IVF treatment cycles are difficult, because endometrial biopsy performed prior to embryo transfer disrupts embryo implantation. The feasibility of assessing endometrial quality from protein patterns in endometrial fluid has been established in previous studies, in both non-conception and conception cycles. Here we report a prospective matched control study designed to test the safety of the procedure of collecting endometrial secretory material during IVF treatment cycles.

Endometrial secretion was obtained transcervically by aspiration just prior to embryo transfer in 66 women undergoing IVF treatment (study group). Pregnancy rates following this procedure were compared with 66 control patients matched for age, ovarian stimulation protocol, number of collected oocytes and number of high quality embryos available for transfer.

Respective biochemical and ongoing pregnancy rate per embryo transfer were 36% and 33% in patients who underwent endometrial secretion aspiration, compared to 33% and 30% respectively the control group (P = 0.84 and P = 0.85). The technique employed provided sufficient endometrial fluid for protein pattern analysis.

Uterine fluid aspiration prior to IVF embryo transfer is a safe method for obtaining sufficient material for uterine secretion electrophoresis, thus allowing analysis of protein patterns serving as receptivity markers during treatment cycles. This technique may offer a novel tool for assessing endometrial receptivity *in vivo* without affecting implantation rates.

Chapter 6.

Endometrial secretion fluid and endometrial tissue were sampled five days after spontaneous ovulation in 31 normo-ovulatory women. Progesterone receptor (PR), Ki-67 expression and the Noyes criteria were used to date endometrial biopsies. The endometrial samples were analysed for protein content, GdA, LIF and Progesterone (P) levels. In addition, 1D gel electophoresis protein patterns were determined. All data were correlated to E and P serum concentrations.

Endometrial maturation assessed by the combination of histological Noyes criteria and immunohistochemical PR and Ki-67 staining patterns correlated

significantly with GdA levels (R = 0.376, P = 0.048) in endometrial fluid samples and serum E (R = 0.568, P = 0.006) and P (R = 0.408, P = 0.023) levels. Endometrial secretion and serum P levels were significantly correlated. However, endometrial secretion protein content and LIF levels were not significantly correlated with endometrial tissue dating parameters or serum E and P levels.

GdA levels in the endometrial fluid correlated significantly with the degree of endometrial maturation assessed by the combination of histological and immunohistochemical criteria. The measurement of GdA levels in endometrial secretion samples may provide a method for assessing endometrial maturation in potential conception cycles without disrupting implantation.

Chapter 7.

To assess ovarian stimulation effects on endometrial tissue in the same patient, 10 healthy, proven fertile, normal and regular cycling (25–35 days) oocyte donors for IVF were monitored by transvaginal ultrasound to detect spontaneous ovulation in the natural cycle, and to time the oocyte pick-up in the stimulated IVF cycle. Endometrial secretion fluid was aspirated and endometrial tissue biopsied 5 days after ovulation and oocyte pick-up. Half of subjects received exogenous progesterone luteal support. Assessment of endometrial maturation in the luteal phase of a spontaneous natural cycle and stimulated IVF cycle, based on endometrial tissue histology dating, cellular immunohistochemistry (Ki-67, estrogen (ER) and progesterone receptor (PR) expression), and endometrial secretion markers (LIF, GdA, P, and USE-PAGE protein expression profile).

No significant alterations in endometrial Noyes histology dating criteria, expression of cellular Ki-67, PR and ER, and differences in the LIF, GdA, P levels or protein profile expression in secretion fluid were shown in the natural and stimulation cycles. Comparing stimulation cycles with and without luteal progesterone supplementation, showed significant lower Ki-67 (P = 0.03) and ER (P = 0.04) but unchanged PR expression. In the stimulated cycles in which luteal support was provided no difference was observed in secretory expression of LIF, GdA, or P compared to those without supplementation. Differences in concentrations between stimulated cycle and natural cycle in each patients showed significant positive correlations between P changes in secretions with P and E levels changes in serum (R_P = 0.71, P = 0.04; R_E = 0.74, P = 0.04), GdA changes in secretions (R_{GdA} = 0.81, P = 0.01), but not LIF changes in secretions (R_{LIF} = 0.69, P = 0.06), and negative correlations with cellular ER expression changes (R_{ER} = -0.74, P = 0.04). Ovarian stimulation resulted in a higher incidence of suboptimal profile expression (3 out of 8 patients) compared to expressions in natural cycles (1 out of 8 patients).

Ovarian stimulation has little impact on tissue derived markers of endometrial maturation, and per sé on LIF, GdA, and P concentrations in the uterine cavity during the window of implantation. However, luteal support, only provided in the early secretory phase for a short time, resulted in significant differences in cellular maturation markers. Changes in serum E and P changes the

intracavitary P concentrations, which are associated with GdA levels and cellular ER expression.

Chapter 8.

In this chapter is an overview and discussion of the results and conclusions provided by the strudies, followed by directions for future research and analysis of endometrial maturation and receptivity.

SAMENVATTING

Hoofdstuk 1.

Dit hoofdstuk beschrijft de normale menstruele cyclus en de ovariële hormonen die de structuur van het endometrium beïnvloeden. Verder zal de gouden standaard om het endometrium te bestuderen kritisch beschouwd worden: endometrium datering met behulp van de 'Noyes criteria' (hoofdstuk **1.1.1**), de implantatie en het 'implantation window' concept (hoofdstuk **1.1.2**); regulatie van endometriummaturatie en implantatie (hoofdstuk **1.2**) en de receptiviteitmarkers (hoofdstuk **1.3**). Daarna zullen problemen bekeken endometriumweefselafname tijdens de IVF-behandelingscyclus beschouwd worden (hoofdstuk **1.4**), waarna een mogelijke oplossing volgt door het bestuderen van endometriumsecreet (hoofdstuk **1.5**). Een kort overzicht van de samenstelling van het menselijk endometriumsecreet verkregen door middel van spoelen of aspiratie. Ten slotte zijn de doelstellingen van dit proefschrift worden uiteengezet (hoofdstuk **1.6**).

Hoofdstuk 2.

Dit hoofdstuk behandelt de moderne aanpak van de ovariële stimulatie tijdens de IVF behandeling resulterend in suprafysiologische serumconcentraties van geslachtshormonen tijdens de folliculaire en luteale fase van de menstruele cyclus. Deze geslachtshormonen zorgen direct en indirect voor het uitrijpen van het endometrium, waardoor ze het implantatieproces beïnvloeden. Het corpus luteum functioneert duidelijk abnormaal in IVF-behandelcycli, en dus wordt luteale ondersteuning middels progesteron- of choriongonadotrofine (hCG) suppletie op grote schaal gebruikt. Verschillende redenen kunnen ten grondslag liggen aan een dysfunctionerende luteale fase: (a) het ovariële hyperstimulatiesyndroom, (b) gelijktijdige behandeling met het gonadotropin-releasing hormoon (GnRH) analoog, en (c) het hCG voor de definitieve eicelrijping. Door recente invoering van GnRH antagonisten in de IVF-behandeling, ter preventie van een premature LH stijging tijdens de late folliculaire fase, zijn verschillende ovariële stimulatieregimes voor de IVF bedacht. Echter, een recente meta-analyse liet zien dat de implantatiekansen mogelijk worden beïnvloed door het gebruik van GnRH-antagonisten. Implantatie van het embryo in het endometrium is een complex proces dat kan veranderen door geslachtshormoonblootstelling wat betreft tijd, duur en bloed/weefselconcentratie. Beoordeling van endometriumreceptiviteit is nodig om nieuwe behandelregimes te beoordelen. Deze nieuwe regimes zijn gericht op het bereiken van meer fysiologische hormoonspiegels gedurende de luteale fase van de IVF-behandelingscyclus en kan een belangrijke stap zijn in verbetering van de zwangerschapskansen. Hierdoor zijn minder behandelingen per vrouw nodig en dit betekent een verbetering van de algehele gezondheidstoestand en een kostenbesparing gezien het aantal verminderde IVF-behandelingen.

Hoofdstuk 3.

Om na te gaan wat de optimale respons is bij ovariële stimulatie met geslachtshormonen, voor wat betreft het aantal te verkrijgen eicellen, voor het tot stand brengen van embryo's en het aantal tot stand gebrachte zwangerschappen in een eerste IVF cyclus, is er gebruik gemaakt van een groot Nederlands cohort vrouwen. Het gaat hier om 7422 vrouwen tussen 18 tot 44 jaar die tussen 1990 en 1995 in één van de 12 Nederlandse IVF-klinieken voor vruchtbaarheidsproblemen werden behandeld. In dit cohort werd de embryoterugplaatsing en zwangerschap in relatie met het aantal verkregen oöcyten geanalyseerd tijdens hun eerste IVF-behandelingscyclus. De relatie werd ook onderzocht in subgroepen met verschillende IVF-indicaties, leeftijdsgroepen en typen vruchtbaarheidsstoornis.

In het gehele cohort steeg het aantal embryoterugplaatsingen, klinische zwangerschappen per terugplaatsing en het aantal zwangerschappen per cyclus tot een maximum van respectievelijk 85%, 28% en 24% indien 10 tot 14 eicellen werden verkregen bij de punctie. Bij vrouwen met ovulatoire vruchtbaarheidsproblematiek werd een optimaal percentage embryoterugplaatsingen en zwangerschappen waargenomen bij 24 of meer verkregen eicellen. Bij meer dan die 24 eicellen werd een lager aantal embryoterugplaatsingen en zwangerschappen waargenomen.

Het verkrijgen van meer dan 14 oöcyten werd geassocieerd met een stabiele of een dalende kans op zwangerschap per embryoterugplaatsing. Het optimale aantal te verkrijgen eicellen, om zo optimale IVF-resultaten te verkrijgen, was hoger bij vrouwen met een ovulatoir vruchtbaarheidsprobleem.

Hoofdstuk 4.

Om het het effect te bepalen van ovariële stimulatie met recombinant FSH samen met GnRH antagonist op genexpressie in endometrium tijdens de 'implantation window', werd endometrium biopsie verricht in een natuurlijke cyclus en in een gestimuleerde IVF-cyclus van diezelfde vrouwen.

Vier eiceldonoren jonger dan 40 jaar, met een regelmatige menstruele cyclus van 25 - 35 dagen, zonder anatomische afwijkingen van de uterus en bewezen vruchtbaarheid werden gevraagd deel te nemen aan dit onderzoek. Endometriumweefsel werd verkregen 5 dagen na de spontane ovulatie in de natuurlijke cyclus, en 5 dagen na de eicelpunctie in de gestimuleerde IVF-behandelingscyclus (recFSH 150 IE per dag sc, GnRH antagonist 0,25 mg per dag sc; 10.000 IE sc hCG, geen P suppletie werd verstrekt na de eicelpunctie). DNA werd voorbereid volgens het Affymetrix microarray protocol. Weefselmonsters werden gehybridiseerd met 'Affymetrix HGU133 Plus 2' arrays en gescand met behulp van de HR3000 scanner. Data werden met behulp van 'GeneSpring 7.2', alsmede de GOTM software geanalyseerd. Standaardgegevens normaal gloeien werden uitgevoerd met behulp van GeneSpring normaal algoritmen en deze gegevens werden vervolgens onderworpen aan ANOVA statistische evaluatie alsook mate van verandering. De geselecteerde genen

werden verder geanalyseerd door middel van real-time PCR met behulp van de groene SYBR methode. De monsters werden in drievoud geanalyseerd en de mate van verandering werd berekend. Statistische significantie werd bepaald door een log-getransformeerd gepaarde *t*-test van de gegevens.

Van alle onderzochte genen kwamen 142 genen significant versterkt en 98 significant verminderd tot expressie volgens de gebruikte criteria. De belangrijkste observaties waren de significant versterkte expressies van SCYB 13, DKK1, 'insulin-like growthfactor binding proteins' 4 en 5, en Homeobox C6. Gen ontogenie (GO) categorieën van de genen die versterkt tot expressie komen, waren die die betrokken zijn bij celadhaesie, T-cel-receptor signaaltransductie, regulering van de signaaltransductie, celgroei, proliferatie en geprogrammeerde celdood. GO categorieën van de genen met verminderde expressie gaven een verlaagde 'transmembrane receptor proteïne kinase' activiteit na ovariële stimulatie.

Wanneer vruchtbare vrouwen worden blootgesteld aan ovariële stimulatie met recombinant FSH in combinatie met een GnRH-antagonist, worden veranderingen waargenomen in expressie van genen die een breed scala van processen aansturen belangrijk voor endometriummaturatie en implantatie. Suprafysiologische serumconcentraties van P en E in de vroeg luteale fase van de niet P gesupplementeerde stimulatie cyclus kunnen verantwoordelijk worden gehouden voor de extra uitdrukking van P gemoduleerde genen.

Hoofdstuk 5.

Endometriumafname uitgevoerd voorafgaand aan de embryoterugplaatsing tijdens/net voor de 'implantation window' in een IVF-behandelingscyclus, verstoort het implantatieproces van het embryo in het endometrium. Een prospectieve 'matched control' studie werd verricht om het effect van endometriumsecreetaspiratie tijdens een IVF-behandelingscyclus op de zwangerschapskans te testen.

Endometriumsecreetaspiratie werd verricht net voor de embryoterugplaatsing bij 66 vrouwen die een IVF-behandeling kregen (studie groep). Zwangerschapskans in deze groep werd vergeleken met 66 controle-patiënten, 'gematched' op leeftijd, ovariëel stimulatie protocol, aantal verzamelde oöcyten en het aantal hoge kwaliteit embryo's beschikbaar voor terugplaatsing.

De biochemische en klinische zwangerschappen per embryotransplantatie waren respectievelijk 36% en 33% bij patiënten die een endometriumsecreetaspiratie ondergingen, vergelijkbaar met respectievelijk 33% en 30% in de controle groep (P = 0,84 en P = 0,85). Daarnaast werd voldoende endometriumsecreet opgenomen voor analyse.

Endometriumsecreetaspiratie voorafgaand aan een embryoterugplaatsing tijdens een IVF-behandelingscyclus is een veilige methode voor het verkrijgen van voldoende materiaal voor endometriumsecreetanalyse om zo eiwit-patronen te vinden die als receptiviteitsmarkers tijdens IVF-behandelingscycli kunnen dienen. Deze techniek kan een nieuw instrument zijn voor de beoordeling van

endometriumreceptiviteit *in vivo* zonder effect op implantatie en zwangerschapskansen.

Hoofdstuk 6.

Endometriumsecreet en weefsel werden afgenomen 5 dagen na een spontane ovulatie bij 31 normo-ovulatoire vrouwen. De combinatie van het progesteron receptor (PR), de Ki-67 expressie en de histologische 'Noyes criteria' werd gebruikt voor de datering van het endometriumweefsel. In het endometriumsecreet werden de concentraties bepaald van het eiwit, GdA, LIF en P. Daarnaast werd het eiwitpatroon bepaald mbv. 1D gelelectroforese. Alle gegevens werden gecorreleerd met E en P concentraties in het serum.

Endometriummaturatie, beoordeeld door de combinatie van de histologische 'Noyes criteria' en de immunohistochemische kleuringen PR en Ki-67 was sterk gecorreleerd met GdA in endometriumsecreet (R = 0,376 P = 0,048) en serum E (R = 0,568 P = 0,006) en P (R = 0,408 P = 0,023) concentraties. De concentraties van P in het endometriumsecreet en P in het serum waren significant gecorreleerd. Echter, het eiwitgehalte en de LIF concentratie in endometriumsecreet waren niet significant gecorreleerd met de endometriumweefselmaturatie, en de serumconcentraties van E en P.

GdA concentratie in het endometriumsecreet blijkt sterk gecorreleerd met het niveau van maturatie van het endometrium, beoordeeld door de combinatie van histologische en immunohistochemische criteria. Bepaling van GdA in het endometriumsecreet kan een methode zijn om endometriummaturatie te bepalen zonder dat het effect heeft op de zwangerschapskans in cycli waarin de mogelijkheid bestaat dat een embryo implanteert in het endometrium.

Hoofdstuk 7.

Om het effect van ovariele stimulatie te bepalen bij dezelfde onderzoekspatient, werden 10 gezonde eiceldonoren met een regelmatige menstruele cyclus van 25 - 35 dagen, een normale uterus en bewezen vruchtbaarheid gevraagd deel te nemen aan dit onderzoek. Endometriumsecreet werd geaspireerd en endometriumweefsel gebiopteerd 5 dagen na de eisprong en de eicelpunctie in respectievelijk de natuurlijke cyclus en IVF-behandelingscyclus. De helft van de proefpersonen kreeg exogene P suppletie. Beoordeling van endometriummaturatie in de luteale fase van een natuurlijke cyclus en een IVF-behandelingscyclus werd uitgevoerd met behulp van een combinatie van histologische 'Noyes criteria', cellulaire immunohistochemie (Ki-67, ER en PR expressie), en endometrium secretiemarkers (LIF, GdA, P en eiwitexpressiepartroon).

Er werd geen verandering van endometriummaturatie, cellulaire expressie van Ki-67, PR en ER, concentraties van LIF, GdA, en P, of eiwitpatroonverandering in endometriumsecreet aangetoond wanneer de natuurlijke cyclus en IVF-behandelingscyclus werden vergeleken. In IVF-

behandelingscycli met en zonder luteale P suppletie bleek aanzienlijk lagere expressie van Ki-67 (P = 0,03) en ER (P = 0,04) op te treden. In de IVF-behandelingscycli werd geen verschil waargenomen in celullaire PR expressie en endometriumsecreetconcentraties van LIF, GdA, en P bij de patienten met of zonder P suppletie.

Concentratieverschillen tussen de IVF-behandelingscyclus en de natuurlijke cyclus bij elke patiënt, vertoonden significante positieve correlaties tussen P concentratieveranderingen in secreet met serum P en E concentratie-veranderingen (R_P = 0,71 P = 0,04; R_E = 0,74 P = 0,04), en GDA veranderingen in secreet (R_{GdA} = 0,81 P = 0,01). Daarentegen tussen P serumconcentratieveranderingen en LIF concentratieveranderingen in secreet geen relatie waargenomen (R_{LIF} = 0,69 P = 0,06), en met cellulaire ER expressieveranderingen een negatieve correlatie (R_{ER} = -0,74 P = 0,04). Ovariële stimulatie resulteerde in een hoger aantal suboptimale eiwitexpressiepatronen (3 van de 8 patiënten) in vergelijking met de eiwitexpressiepatronen in de natuurlijke cycli (1 van de 8 patiënten).

Ovariële stimulatie heeft weinig effect op endometriummaturatie, op LIF, GdA, en P concentraties in de baarmoederholte gedurende de 'implantation window'. Echter, kortdurende luteale ondersteuning in de vorm van exogeen P suppletie, leidt tot aanzienlijke verschillen in cellulaire rijping markers. Veranderingen in serumconcentraties E en P, veranderen de intracavitaire P concentraties, die op hun beurt weer het GdA niveau en de ER expressie beïnvloeden.

Hoofdstuk 8.

Dit hoofdstuk is een overzicht en beschouwing van de resultaten en conclusies van de eerder beschreven studies, gevolgd door een advies voor toekomstig onderzoek en analyse van endometriummaturatie en receptiviteit in het kader van vruchtbaarheidsproblematiek.

PUBLICATIONS,ABSTRACT& PRESENTATIONS

PUBLICATIONS

Van der Gaast M, Macklon N. Endometrial receptivity from a clinical perspective.
TFO **2001**;15:26-35.

Van der Gaast MH, Beckers NG, Beier-Hellwig K, Beier HM, Macklon NS, Fauser BC. Ovarian stimulation for IVF and endometrial receptivity - the missing link.
Reprod BioMed Online **2002**;5:36-43.

Van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM, Macklon NS. Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates.
Reprod BioMed Online **2003**;7:105-109.

Van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM, Macklon NS. Endometrial secretion for receptivity research.
TFO **2003**;15:24-25.

Van der Gaast MH, Eijkemans MJC, Van der Net JB, Burger CW, Van Leeuwen FE, Fauser BC, Macklon NS. Optimum number oocytes for a successful first IVF treatment cycle.
Reprod BioMed Online **2006**;13:476-480.

Macklon NS, **Van der Gaast MH**, Hamilton A, Fauser BC, Giudice LC. The impact of ovarian stimulation with recombinant FSH in combination with GnRH antagonist on endometrial transcriptome in the window of implantation.
Reprod Sci **2008**;15:357-365.

Van der Gaast MH, Classen-Linke I, Beier-Hellwig K, Krusche CA, Fauser BC, Beier HM, Macklon NS. Impact of ovarian stimulation on mid-luteal endometrial tissue and secretion markers of receptivity.
Reprod BioMed Online **2008**;17:553-563.

Van der Gaast MH, Macklon NS, Beier-Hellwig K, Krusche CA, Fauser BC, Beier HM, Classen-Linke I. The feasibility of a less invasive method to assess endometrial maturation - comparison of simultaneously obtained uterine secretion and tissue biopsy.
*BJOG **2009**;116:304-312.*

ABSTRACTS AND PRESENTATIONS RELATED TO THIS THESIS

Van der Gaast MH, Beier-Hellwig K, Fauser BCJM, Beier HM, Macklon NS. Towards non-invasive assessment of Endometrial Receptivity.
European Society for Human Reproduction and Embryology. ESHRE Wintercourse, Amsterdam, December **2001**.

Van der Gaast MH, Macklon NS, Beier-Hellwig K, Fauser BCJM, Beier HM. Aspiration of uterine fluid prior to embryo transfer is a safe method for receptivity analysis by uterine secretion electropheresis.
2nd International Workshop on Human Implantation, Madrid, May **2001**.

Classen-Linke I, **Van der Gaast MH**, Krüsche CA, Beier-Hellwig K, Fauser BCJM, Beier HM, Macklon NS. Transfer cycle biopsies from hyperstimulated cycles correlate with the expected luteal phase stage.
2nd International Workshop on Human Implantation, Madrid, May **2001**.

Van der Gaast MH, Beier-Hellwig K, Fauser BCJM, Beier HM, Macklon NS. Endometrium secreet voor onderzoek van receptiviteit.
VFS (Vereniging voor Fertiliteitsstudies), Nijmegen, November **2002**.

Van der Gaast MH, Beier-Hellwig K, Classen-Linke I, Fauser BCJM, Beier HM, Macklon NS. Correlation between endometrial receptivity as measured by PAGE analysis of protein profiles in endometrial secretions, and analysis of endometrial biopsies in the same subject. A prospective, blinded study.
3rd International Workshop on Human Implantation, Madrid, June **2003.**

Van der Gaast MH, Beier-Hellwig K, Classen-Linke I, Fauser BCJM, Beier HM, Macklon NS. Endometrium - Het stiefkind van de infertiliteit.
Gynaecongres NVOG (Nederlandse Vereniging voor Obstetrie & Gynaeologie), Rotterdam, May **2004**.

Van der Gaast MH, Beier-Hellwig K, Classen-Linke I, Fauser BCJM, Beier HM, Macklon NS. Bepaling van effecten van ovariële hyperstimulatie op het endometrium: een geïntegreerde benadering.
VFS (Vereniging voor Fertiliteitsstudies), Antwerp, April **2005**.

Van der Gaast MH, Beier-Hellwig K, Classen-Linke I, Fauser BCJM, Beier HM, Macklon NS. Assessing the impact of ovarian stimulation on endometrial maturity: an integrated approach.
21st Annual meeting of the European Society for Human Reproduction and Embryology. ESHRE Annual Meeting, Copenhagen, June **2005**.

Macklon NS, **Van der Gaast MH**, Hamilton A, Fauser BCJM, Giudice LC. The impact of ovarian stimulation with recombinant FSH in combination with GnRH antagonist but no luteal support on endometrial gene expression in the window of implantation.
23rd Annual meeting of the European Society for Human Reproduction and Embryology. ESHRE Annual Meeting, Lyon, July **2007**.

DANKWOORD

Het dankwoord wordt het laatst geschreven bij het afsluiten van een promotietraject, maar door menigeen als eerste gelezen..... Velen ben ik dank verschuldigd bij het tot stand brengen van dit proefschrift, maar enkelen meer dan dat en die worden hier genoemd.

Professor NS Macklon, beste *Nick*. Het is ruim 10 jaar geleden dat ik werd aangenomen als fertiliteitsarts waar jij net hoofd van de IVF-afdeling was geworden. Na een jaar hebben we de eerste stappen gezet om embryo-implantatie en endometriumreceptiviteit te onderzoeken. Het was een lange weg met valkuilen, vallen en opstaan, en het proberen van allerlei technieken. Dit onderzoek zou nooit afgerond zijn zonder jouw vasthoudendheid, inspanning, optimisme en relativeringsvermogen. Kortom, jouw hulp is voor mij van grote waarde geweest. Niet alleen dat dit onderzoek afgerond is, maar ook voor datgeen wat ik nu word, een gynaecoloog. Je hebt niet alleen ervoor gezorgd dat ik een opleidingsplaats heb gekregen, maar je hebt ook de helft van mijn kinderen op de wereld gezet. Dank voor dit alles.

Professor BCJM Fauser, beste *Bart*. Altijd op de achtergrond gedurende mijn promotietraject maar wel altijd met goede en pragmatische ideeën en plannen over hoe dit onderzoek aangepakt kon worden. Daarnaast de vrijheid die je mij en Nick gaf om iets nieuws te proberen en dat te onderzoeken heb ik zeer gewaardeerd. Verder vond ik de klinische fertiliteitsbesprekingen inspirerend en de gevleugelde woorden "bij twijfel niet doen" blijven nog dagelijks in mijn achterhoofd spoken. Dank voor deze periode.

Professor HM Beier, beste *Henning*. Jouw visie en "drive", om Rotterdam kundig en enthousiast te maken voor het endometriumsecreet werkte aanstekelijk! De onderzoeksgroep uit Aken, onder andere bestaande uit *Karin, Irmgard en Claudia*, met de discussies bij jullie in Aken en bij ons in Rotterdam zijn een inspiratie geweest om endometriumonderzoek in Rotterdam en later in Utrecht gestalte te geven. Mijn Duits is er geen haar beter van geworden door de discussies in het Engels, maar .... "Vielen dank!"

Professor CW Burger, beste *Curt*. In 1992 kwamen elkaar voor het eerst tegen in mijn doctoraalfase met een onderzoeksproject "Fotodynamisch therapie bij Gynaecologische tumoren" in het toenmalige AZVU. Een project met combinatie van oncologie en gynaecologie waarbij ik toentertijd dacht om oncoloog te worden. Alsof het lot zo beschikt zijn we elkaar weer tegengekomen in Rotterdam, inmiddels m'n opleider en nu ook lid van mijn promotiecommissie. Dank voor het vertrouwen en de adviezen in m'n eerste opleidingsjaar, een jaar waarin de opleiding en het onderzoek tegenstrijdige belangen leken te zijn. Ik ben blij dat je zitting neemt in de promotiecommissie.

Professor J Lindemans en *Professor JA Land* wil ik bedanken voor het beoordelen van het manuscripten en dat u beiden zitting neemt in de commissie.

Doctor JSE Laven, beste *Joop*. De afgelopen tijd was voor mij niet even gemakkelijk door jouw confronterende kritieken ("Be humble!"). We hebben nu een (inmiddels non-smoking) omgangsmodus gevonden. Dank voor de levenslessen hoe het wel (en misschien soms ook niet) moet.

Doctor Eijkemans, beste *Rene*. We hebben wat geworsteld met die OMEGA database. Jouw SPSS kundigheid, onverzettelijkheid, relativeringsvermogen en de wil om anderen statistisch enigzins bij te spijkeren zijn een voorbeeld voor velen!

Als ik aan mijn onderzoeksperiode denk, denk ik aan de periode op de kopkamer op 5 Noord. Lol, lachen, lief en leed zijn de kernwoorden die centraal staan voor die periode. Als enige overgebleven man tussen alle onderzoekende dames heb mij nooit als een vreemde eend in de bijt gevoeld maar als een vis in het water. Intussen is het al weer een hele poos geleden, en iedereen is bezig met z'n opleiding (*Annemarie* en *Christien*), onderzoeksproject (*Anne*, *(B)Esther* en *(H)Esther*), of huisartspraktijk en kinderen krijgen/verzorgen (*Femke*; inmiddels heb je mij ingehaald!), maar dank voor de fijne tijd!

De Follikelboy club: *Arne, Babek, Bernd, Diederick, Evert, Jits*, *Thierry* en later aangevuld door de follikelmeiden ook *Annemarie, Femke en Nicole*. Heel aparte club, bezig met follikels enzo..... maar het endometrium wil ook wat en is volgens mij niet enkel een volgzaam eindorgaan van ovariële hormonen! De vraag is of deze club, nadat het eerst enkel uit mannen heeft bestaan, vrouwen hebben toegelaten, er plek is voor iemand met interesse voor het endometrium!
Diederick, rookmaatje van het eerste uur. Nu in Canada maar nog steeds transatlantisch Skypend contact over de laatste nieuwtjes, feitjes, strategische en tactische keuzen binnen de gynaecologie. Hopelijk kom je binnenkort weer naar Nederland samen met je meiden!
Nicole, jij was de eerste die mij begeleidde in de "wereld van de voortplantingsgeneeskunde" en het maken van follikelecho's. De aanstekelijke drang om onderzoek te doen en dat direct te gebruiken in de dagelijkse fertiliteitpraktijk, hebben mij toentertijd gesterkt om onderzoek te doen op het gebied van embryo-implantatie in de voortplantingsgeneeskunde.
Olivier, jij bent nu de enig overgebleven man van de onderzoeksgroep! Straks m'n (part-time) collega in het Albert Schweitzer Ziekenhuis, en hopelijk vinden we wat tijd om weer meer bij te praten. Succes met je laatste loodjes op het Erasmus MC en aanstaande vaderschap!

Ook dank ik de gynaecologen van Reinier de Graaf Gasthuis voor de geboden mogelijkheden om dit proefschrift af te maken en hun gestelde vertrouwen tijdens de opleiding. Het waren 3 heerlijke jaren! Met name *Kees Jansen* wil ik bedanken voor de getoonde interesse en enthousiasme voor dit onderzoek en de discussies over de fertiliteitpraktijk van alle dag. Verder wil ik het hele

fertiliteitsteam nog bedanken voor de goede en leuke 5 maanden in Voorburg! "Jullie zijn de beste".

In de laatste fase van mijn promotietraject, wil ik ook *Anneke Steensma* niet vergeten te bedanken voor de steun, inzichtelijke gesprekken en de mogelijkheid om nog tijd te steken in de laatste loodjes van dit boekje. Anneke de komende tijd hou ik jou uit de wind!

Elaine Joziasse-Fitzpatrick, dank voor het het kritisch doornemen van het manuscript.

Verder wil ik m'n vrienden *Wieger*, *Janneke*, *Pat*, *Colinda*, *Brigitte*, *Suzanne*, *Ton*, *Frank*, *Marco*, *Boris* en *Patricia* bedanken voor de steun, interesse en vriendschap van de afgelopen tijd. E-i-n-d-e-l-i-j-k i-s h-e-t k-l-a-a-r!! Met name wil *Peter* en *Wim*, studievrienden van het eerste uur, inmidels al weer 22 jaar(!), bedanken dat jullie mijn paranimf willen zijn.

Ton, *Annie* en *Astrid* bedank ik voor hun onvoorwaardelijke steun in de afgelopen jaren op de Zestienhovensekade!

Guus, broeder (tegen wil en dank?), bedankt dat je de plaatjes heb willen maken. Soms was het moeilijk uit te leggen wat ik precies wilde, maar ik vind dat het fantastisch gelukt is! Dank!

Janny en *Sjerry*, mijn lieve ouders. De afgelopen tijd was niet altijd even gemakkelijk. De tijd dat ik bij jullie was, was vaak ook om weer bij te komen en bij te tanken van allerlei stress en beslommeringen van de werkvloer en onderzoek, in een periode dat onze familie zienderogen groter werd en het alleen maar drukker werd. Ik ben ontzettend trots dat jullie mij altijd onvoorwaardelijk gesteund hebben.

Lieve *Max*, *Janneke*, *Luuk* en *Klara*. Jullie zijn me een stel! Ik kon niet altijd aan jullie verwachtingen voldoen, maar dat wordt anders want de komende tijd is voor jullie! "Papa is back".

"Last but not least"
Lieve, lieve, lieve, lieve, lieve *Chantal*. Jij ben mijn reddingsboei geweest. Hoe jij alles, mij en ons gezin, drijvend heb kunnen houden vind ik bewonderingswaardig! Niet genoemd in een artikel, maar desondanks ben je van onschatbare waarde geweest bij het tot stand brengen van dit boekje. Ik hou van je!

CURRICULUM VITAE AUCTORIS

Mark Hinko van der Gaast was born 15th of March 1968 in Wolvega. In 1987 he graduated from secondary school Rijksscholen-gemeenschap (VWO) in Den Burg on Texel. From 1987 to 1996 he attended Medical School at the Free University (VU, now VUmc) in Amsterdam. During this period he was also a student trainee in the Clinical Research Unit of Internal Medicine and Oncology (Head: Prof.dr. H.M. Pinedo) to study multidrug resistance (MDR) in lung cancer cell lines. After graduation in 1996 he worked as a locum House Officer in Surgery in London and Carmarthen, in the United Kingdom. In 1997 from January until December he worked as resident in Surgery, and Obstetrics and Gynaecology in the Medisch Spectrum Twente hospital in Oldenzaal. Here he met his wife Marie-Chantalle Bus. From January to April 1998 he worked as resident Obstetrics and Gynaecology in Hospital De Heel (now Zaans Medical Centre) in Zaandam. In this same year he started at the Division of Reproductive Medicine (Head: Prof.dr. B.C.J.M. Fauser), Department of Obstetrics and Gynaecology of the University Hospital of the Erasmus MC in Rotterdam. From 2000 and onwards he worked on the studies which are described in this thesis. Also in this period his two first children, Max (2000) and Janneke (2002), were born. In September 2003 he started is residency training in Obstetrics and Gynaecology in the Erasmus MC (Prof.dr. Th.J.M. Helmerhorst and Prof.dr. C.W. Burger). Shortly after the start of his training his two youngest children, the twin Luuk and Klara (2004), were born. His residency training continued in Reinier de Graaf Gasthuis in Delft (Dr. W.A. ter Harmsel and Dr. H.A. Bremer) from September 2005 until September 2008. Now he continued his urogynecology training in the Erasmus MC in Rotterdam (Prof.dr. C.W. Burger and Prof.dr. E.A.P. Steegers) and Albert Schweitzer Hospital in Dordrecht (Dr. G.S. Kooi and Dr. C.J.A. Hogewoning). His residency training will finish in 2010.

LIST OF ABBREVIATIONS

a-FGF	= acidic fibroblast growth factor
b-FGF	= basic fibroblast growth factor
CAM	= cellular adhesion molecules (ICAM, VCAM and NCAM)
CCL / R	= CC chemokine ligand / receptor
CSF-1	= colony stimulating factor 1
COX-2	= cyclooxygenase 2
CXCL / L	= CXC chemokine ligand / receptor
CX3CL / R	= CX3C chemokine ligand / receptor
(c)d	= (cycle) day
DAF	= decay accelerating factor for complement (CD55)
E	= estradiol
ECM	= extracellular matrix
EGF(R)	= epidermal growth factor (receptor)
ER	= estrogen receptor
ET	= embryo transfer (embryo transplantation)
FSH	= follicular stimulating hormone
GADD45	= growth arrest and DNA damage-inducible protein
GdA	= glycodelin A
GnRH	= gonadotropin releasing hormone
gp130	= glycoprotein-130, signal transducing chain of LIFR, IL6R & IL-11R
HB-EGF	= heparin binding epidermal growth factor
hCG	= human chorionic gonadotropin
HGF	= hepatocyte growth factor
hMG	= human menopausal gonadotropin
IFN	= interferon
IGF(BP)	= insulin growth factor (binding protein)
IL	= interleukin
IUD	= intra uterine device
LH	= luteinizing hormone
LIF(R)	= leukaemia inhibitory factor (receptor)
MAG	= mouse ascites Golgi
MAO-A	= monoamine oxidase A
MAP3K5	= mitogen-activated protein kinase kinase kinase 5
MMP	= matrix metalloproteinase
MUC-1	= mucine 1
NO	= nitric oxide
OPN	= osteopontin
OPU	= ovum pick up (egg retrieval)
P	= progesterone
P	= statistic P value
PCOS	= polycystic ovary syndrome
PDGF	= platelet derived growth factor
PlGF	= placental growth factor
PR	= progesterone receptor *or* pregnancy rate
sc	= subcutaneous(ly)
SDS-PAGE	= sodium dodecyl sulfate - polyacrylamide gel electrophoresis
TIMP	= tissue inhibitor of metalloproteinase
TGFβ	= transforming growth factor β
TNFα	= tumor necrosis factor α
TSP	= thrombospondin
TV(U)S	= transvaginal ultrasound
VEGF(R)	= vascular endothelial growth factor (receptor)