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INFERTILITY AND ITS TREATMENT:

ASSOCIATION WITH OVARIAN GRANULOSA CELL TUMOUR

AND IMPACT ON VASCULAR ENDOTHELIAL GROWTH FACTOR,

LEPTIN AND SELECTED TUMOUR MARKERS IN SERUM

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ACADEMIC DISSERTATION

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" Great successes never come without risks"

Flavius Josephus

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals. In addition, some unpublished material is presented.

I Unkila-Kallio, L., Leminen, A., Tiitinen, A. and Ylikorkala, O. (1998). Nationwide data on falling incidence of ovarian granulosa cell tumours concomitant with increasing use of ovulation inducers. *Hum Reprod*, 13, 2828-2830.

II Unkila-Kallio, L., Tiitinen, A., Wahlstrom, T., Lehtovirta, P. and Leminen, A. (2000). Reproductive features in women developing ovarian granulosa cell tumour at a fertile age. *Hum Reprod*, 15, 589-593.

III Unkila-Kallio, L., Vuorela-Vepsalainen, P., Tiitinen, A., Halmesmaki, E. and Ylikorkala, O. (2000). No cyclicity in serum vascular endothelial growth factor during normal menstrual cycle but significant luteal phase elevation during an in vitro fertilization program. *Am J Reprod Immunol*, 43, 25-30.

IV Unkila-Kallio, L., Andersson, S., Koistinen, H.A., Karonen, S.L., Ylikorkala, O. and Tiitinen, A. (2001). Leptin during assisted reproductive cycles: the effect of ovarian stimulation and of very early pregnancy. *Hum Reprod*, 16, 657-662.

V Unkila-Kallio, L., Tiitinen, A., Alfthan, H., Vuorela, P., Stenman, U. and Ylikorkala, O. (2000). Effect of an in vitro fertilization program on serum CA 125, tumor-associated trypsin inhibitor, free beta-subunit of human chorionic gonadotropin, and common alpha-subunit of glycoprotein hormones. *Fertil Steril*, 74, 1125-1132.

ABBREVIATIONS

ACOG	American College of Obstetrics and Gynaecology
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ART	assisted reproductive technologies
ASRM	American Society of Reproductive Medicine
BMI	body mass index, weight (kg) / height^2 (m^2)
BRCA	breast cancer associated mutation
CA 125	cancer antigen 125
CC	clomiphene citrate
CI	confidence interval
E2	oestradiol
EGF	epidermal growth factor
ESHRE	European Society of Human Reproduction and Embryology
ET	embryo transfer
FET	transfer of a frozen-thawed embryo
FIGO	International Federation of Gynaecology and Obstetrics
FSH	follicle-stimulating hormone
GCT	granulosa cell tumour
GnRH	gonadotrophin-releasing hormone
GPHα	free α-subunit of glycoprotein hormones
hCG	human chorionic gonadotrophin
hCGβ	free β-subunit of hCG
hMG	human menopausal gonadotrophin
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
LH	luteinizing hormone
OC	oral contraceptives
OHSS	ovarian hyperstimulation syndrome
OPU	oocyte pick-up / oocyte retrieval
OR	odds ratio
PCOS	polycystic ovary syndrome
RR	relative risk
SIR	standardized incidence ratio
TATI	tumour-associated trypsin inhibitor
TGFβ	transforming growth factor β
VEGF	vascular endothelial growth factor

I INTRODUCTION

Infertility, defined as failure to conceive within 1 year of normal cohabitation, occurs in approximately 10-20% of couples (Barbieri, 1999a). The main cause of female infertility is anovulation. Thus the introduction of the ovulation inducers clomiphene citrate (CC) and gonadotrophins after the mid-1960ies brought about a revolution in the treatment of infertility. The next breakthroughs in infertility treatment were the development of various assisted reproductive technologies (ART): *in vitro* fertilization (IVF) – embryo transfer (ET) technique (Edwards *et al*., 1969; Steptoe and Edwards, 1978), cryopreservation techniques (Trounson and Mohr, 1983), ovum donation programmes (Lutjen *et al*., 1984) and intracytoplasmic sperm injection (ICSI) (Palermo *et al*., 1992). At present, almost 80% of couples complaining of infertility can be helped to achieve a child.

Initially, the ovulation inducers and high technology infertility treatments were thought to be specific and not to cause any marked untoward side effects. Therefore, reports on a possible association between infertility treatment and ovarian cancer in the early 1990ies caused great concern (Whittemore *et al*., 1992; Willemsen *et al*., 1993; Rossing *et al*., 1994). Theoretically, infertility treatments may trigger the development of ovarian cancer by causing multiple traumata on the ovarian surface (Fathalla, 1971; Casagrande *et al*., 1979) and/or by inducing abnormally high levels of gonadotrophins (Stadel, 1975) and oestrogens (Cramer and Welch, 1983). The data linking infertility treatment to genital cancer were weakened by the knowledge that nulliparity itself is a risk factor for cancer of the ovary, breast and endometrium (Kvale *et al*., 1991), and this aspect had not always been adequately considered in the first epidemiological studies. In the context of using gonadotrophins, the ovarian granulosa cell tumour (GCT) attracts special interest, for in rodents, this tumour develops in the presence of high concentrations of gonadotrophins (Biskind *et al*., 1952; Tennent *et al*., 1990; Risma *et al*., 1995). Hence reports of its occurrence in infertile women (Willemsen *et al*., 1993; Rossing *et al*., 1994; Unkila-Kallio *et al*., 1997) might imply an association with infertility treatments. Thus, the GCT could serve as a model for assessing the risks of ovulation inducers.

This study was conducted to elucidate the impact of infertility and of its treatment on the occurrence of GCT, and the influence of an IVF programme on factors that may be of relevance for the formation and detection of a tumour.

II REVIEW OF THE LITERATURE

1. INFERTILITY

A woman (a couple) is said to be infertile if pregnancy is not achieved within 12 months despite regular intercourse (Barbieri, 1999a). One-third of cases of infertility are related to female factors, one-third to male factors, and one-third to a combination of the two. The most common cause of infertility is anovulation, followed by male factors and tubal occlusion / abnormalities (Table 1). However, in spite of thorough examinations, the cause of infertility still remains unexplained in approximately 10-20% of infertile couples (Collins, 1995).

Eighteen years ago the prevalence of infertility in females between 25 and 45 years of age in Finland was 17% (Rantala and Koskimies, 1986). This is in close accord with the infertility rate of 13% in women between 15 to 44 years of age in the USA (Mosher and Pratt, 1987). Thus, infertility was one of the most common gynaecological disorders, and it now seems to be increasing (Stephen and Chandra, 1998). This increase may be a due to postponement of childbearing beyond the most fertile years and, indeed, in Finland the mean age at the first delivery has increased from 24.6 years in 1965 to 27.6 years in 1995 (personal communication, M Gissler, Finnish Birth Register). The age of the female (and the oocytes) has a great impact on female fertility: in the Hutterites, a Swiss colony in Canada among whom contraception is not used, infertility occurred in 11% of women after the age of 34, in 33% by the age of 40 and in 87% at the age 45, the rate of primary infertility being low (2.4%) (Tietze, 1957). The western lifestyle and standard of living may nowadays impair fertility not only in the female, but also in the male, because, at least in some countries, sperm quality has deteriorated (Andersen *et al*., 2000; Jensen *et al*., 2000). Thus it is likely that in future demands for treatment of infertility will increase.

Table 1. Causes of infertility based on a meta-analysis of 21 publications and 14 141 infertile couples (Collins, 1995).

Cause of infertility	%
Anovulation	27
Tubal factor	22
Endometriosis	5
Uterine / cervical factor	4
Male factor	25
Unexplained	17

2. TREATMENT OF INFERTILITY

The choice of treatment depends on the age of the patient, the cause of infertility and duration of the infertility, the severity of endometriosis, the results of prior treatments and the wishes of the couple (Crosignani and Rubin, 2000). The couple is informed about the timing of intercourse (Wilcox *et al*., 1995) and, as general health measures, the debilitating effects on fertility of both smoking (Rantala and Koskimies, 1987; Zinaman *et al*., 2000) and female overweight (Clark *et al*., 1998; Silva *et al*., 1999). In addition, hyperprolactinaemia and disorders of the thyroid should be treated appropriately (Arojoki *et al*., 2000). As regards the infertility itself, surgery is usually recommended if the cause is endometriosis (Marcoux *et al*., 1997), uterine abnormalities, ovarian tumours, or resistant anovulation in patients with the polycystic ovary syndrome (PCOS) (Tiitinen *et al*., 1993a; Anttila *et al*., 1998; Tulandi and al Took, 1998). For example, in patients with PCOS, the cumulative rate of conception within 12 months after ovarian drilling with diathermy exceeded 50%, and, if the pre-operative luteinizing hormone (LH) level was above 10 IU/L, was even 89% (Li *et al*., 1998).

The most effective treatment for all types of infertility is assisted reproduction in the form of IVF or ICSI (Hull, 1994; Forti and Krausz, 1998). Both treatments require the use of drugs that induce multiple ovulation. In clinical practice, therefore, not only patients with anovulatory infertility but also infertile patients with ovulatory cycles are exposed to ovulation inducers (Donderwinkel *et al*., 2000).

2.1. Induction of ovulation

The majority of cases of female infertility are caused either by anovulation or by various aberrations in ovulation (Crosignani *et al*., 1999; Yen, 1999a; Yen and Laughlin, 1999b) (Tables 1 and 2). The majority of ovulatory disorders are due either to derangement of ovarian function (45%, e.g. PCOS and premature ovarian failure) or to hypothalamic dysfunction (38%, mainly weight loss, exercise, stress); the minority (17%) are caused by pituitary disease (Barbieri, 1999a). Thus, for these patients, the introduction of ovulation inducers in the1960ies was a major breakthrough.

2.1.1. Physiology and principles of induction of ovulation

Ovulation is a complex process, involving a cyclic cascade of neuroendocrine signals, pituitary hormones and ovarian steroid and protein hormones. In addition, to guarantee the success of ovulation, a number of autocrine and paracrine regulators function both in the pituitary and in the ovaries (Figure 1 and Table 3) (Büscher *et al*., 1999; Nash *et al*., 1999; Hull and Harvey, 2000). Follicular growth before the preantral stage is independent of gonadotrophins (Aittomäki *et al*., 1996), but the selection of a cohort of follicles in the early stage of a given menstrual cycle and the final selection of the dominant follicle are highly dependent on follicle-stimulating hormone (FSH) (Figures 1 and 2). The secretion of FSH and LH from pituitary gonadotrophes is caused by the pulsatile secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus and, as a result, the FSH and LH secretions are also pulsatile. FSH enhances oestrogen production from the granulosa cells by stimulating aromatase activity in these cells. It also regulates the development and number of its own receptors on the granulosa cells, and induces the formation of LH receptors on the theca cells. LH, in turn, induces the production of androgens from cholesterol in the theca cells, and these androgens are then converted into oestrogens through aromatization in the granulosa cells (Figure 2). Thus, both FSH and LH action contribute to oestrogenicity during the menstrual cycle. (Yeh and Adashi, 1999)

Oestrogen and inhibin regulate the FSH secretion of the pituitary by negative feedback. Oestrogen also affects its own production by increasing aromatase activity or by inhibiting androgen production. The oestrogen peak in the blood that precedes ovulation is followed by an LH surge, which triggers ovulation approximately 36 hours later. However, the exact mechanism of ovulation is not

well understood (Büscher *et al*., 1999). the follicular fluid surrounding the cumulus oophorus contains leucocytes and a great number of local auto- and paracrine regulators (Table 3), of which at least prostaglandins, angiotensin II, plasmin and interleukins seem to be necessary for ovulation (Anttila *et al*., 1998; Büscher *et al*., 1999). After ovulation, the oestrogen levels fall when the granulosa cells become luteinized and vascularized and start producing progesterone under the control by LH (Yeh and Adashi, 1999; Geva and Jaffe, 2000).

In anovulatory patients ovulation is induced artificially by administration of either CC or gonadotrophins, which increase the levels of FSH, the aim being to induce the growth of one to two follicles (Crosignani *et al.*, 1999). In hypergonadotrophic hypogonadism induction of ovulation does not succeed because, in this condition, endogenous FSH is already high. Treatment of infertile women with regular periods with ovulation inducers (i.e. women with unexplained infertility, minimal endometriosis or sometimes mild male factor infertility) is based on the assumption that ovulation inducers lead to the development of one or several follicles with improvement of ovarian and endometrial endocrine environment and so increase the likelihood that at least one follicle will lead to ovulation, conception and pregnancy. This treatment carries an increased risk of multiple pregnancy if two or more ova become fertilized (Isaksson and Tiitinen, 1997; Forti and Krausz, 1998; Hughes *et al*., 2000b). In assisted reproduction (See 2.2.1) high doses of ovulation inducers are administered after pituitary down-regulation in order to achieve multiple mature oocytes. This ovarian stimulation is called controlled ovarian hyperstimulation, and is used at present for treatment of all types of infertility (Forti and Krausz, 1998).

2.1.2. Clomiphene citrate

The capacity of CC to induce ovulation was demonstrated in 1961 (Greenblatt *et al*., 1961). This perorally administered drug is a non-steroidal oestrogen agonist/antagonist synthesized in 1956 (Table 4). It blocks hypothalamic oestrogen receptors and thus creates a hypo-oestrogenic state in the hypothalamus. This leads to an increase in the pulse frequency of GnRH, which in turn increases FSH and LH secretion, leading finally to the growth of one or several follicles (Barbieri, 1999a).

Table 2. Aetiology of anovulation and its relation to gonadotrophin and oestradiol levels (Yen, 1999a; Yen, 1999b).

Cause of anovulation	FSH	LH	E2
Hypothalamic			
GnRH deficiency	↓	↓	↓
Idiopathic			
Kallmann´s syndrome			
GnRH receptor gene mutations	N	N	↓
Dysfunction of pulsatile GnRH secretion	N/↓	N/↓	↓
Puberty, lactation			
Weight loss, exercise, stress			
Tumours of the hypothalamic area: craniopharyngeoma, germinoma, histiocytosis, sarcoidosis, lymphoma haemochromatosis, Wegener´s granulomatosis			
Head injury			
Irradiation (head, neck)			
Pituitary			
Defect of the gonadotrophs	↓	↓	↓
Pituitary surgery			
Pituitary necrosis / interruption of vascularity			
Sheehan´s syndrome			
Pituitary apoplexy			
Dysfunction of the gonadotrophes			
Empty sella syndrome	↓	↓	N/↓
Lymphocytic hypophysitis, autoimmune	↓	↓	N/↓
Pituitary adenomas and tumours	↑/N/↓	↑/N/↓	N/↓
Ovarian			
Ovarian failure	↑	↑	↓
Genetic, autoimmune, cytotoxic chemotherapy, irradiation (pelvic)			
Inappropriate ovarian-pituitary feedback			
Use of oral contraceptives	↓	↓	N
Use of constant doses of oestrogen	↓	↓	N
Excess of functional oestrogens and androgens	↓	↓	↑
Androgen or oestrogen-producing tumour	↓	↓	↑
Aromatase excess and deficiency syndromes	↓/↑	↓/↑	↑/↓
Gene mutations of FSH, LH, and oestrogen receptors	↑	↑	N/↓
Inappropriate feedback secondary to combined central-peripheral dysfunctions			
Polycystic ovary syndrome	N	N/↑	N/↑
Cushing´s syndrome	N	N	N
Thyroid hormone disorders	N	N	N
Excess of prolactin or growth hormone	N	N	N/↓

N: normal level; ↑ : elevated level; ↓ : decreased level;
E2: oestradiol; FSH: follicle-stimulating hormone; GnRH: gonadotrophin-releasing hormone;
LH: luteinizing hormone

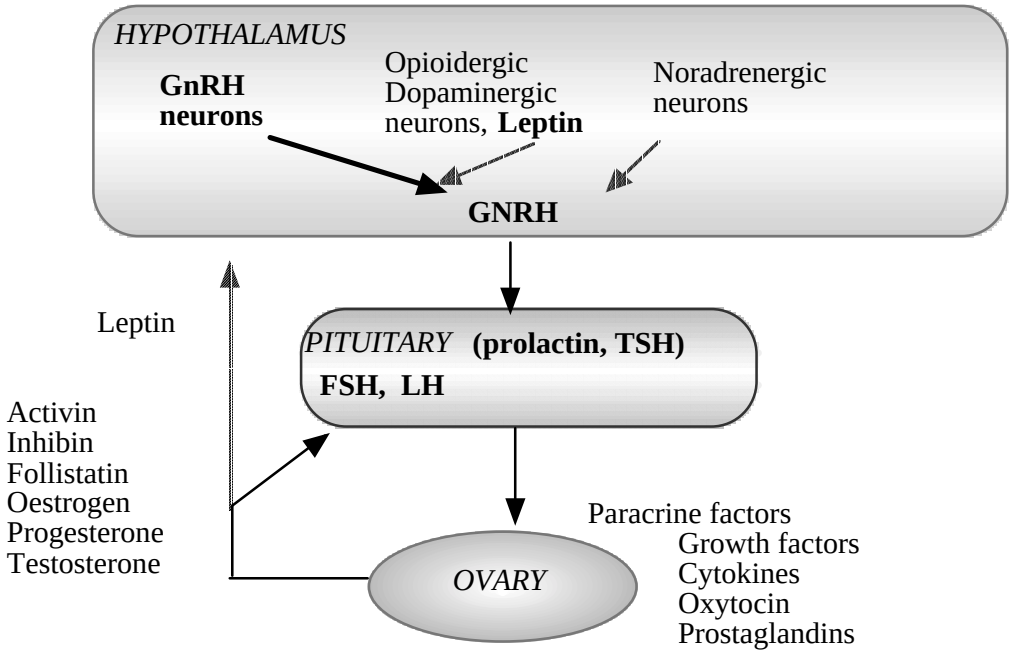


Figure 1. Regulation of ovarian function.

FSH: follicle-stimulating hormone; GnRH: gonadotrophin-releasing hormone; LH: luteinizing hormone; TSH: thyroid-stimulating hormone

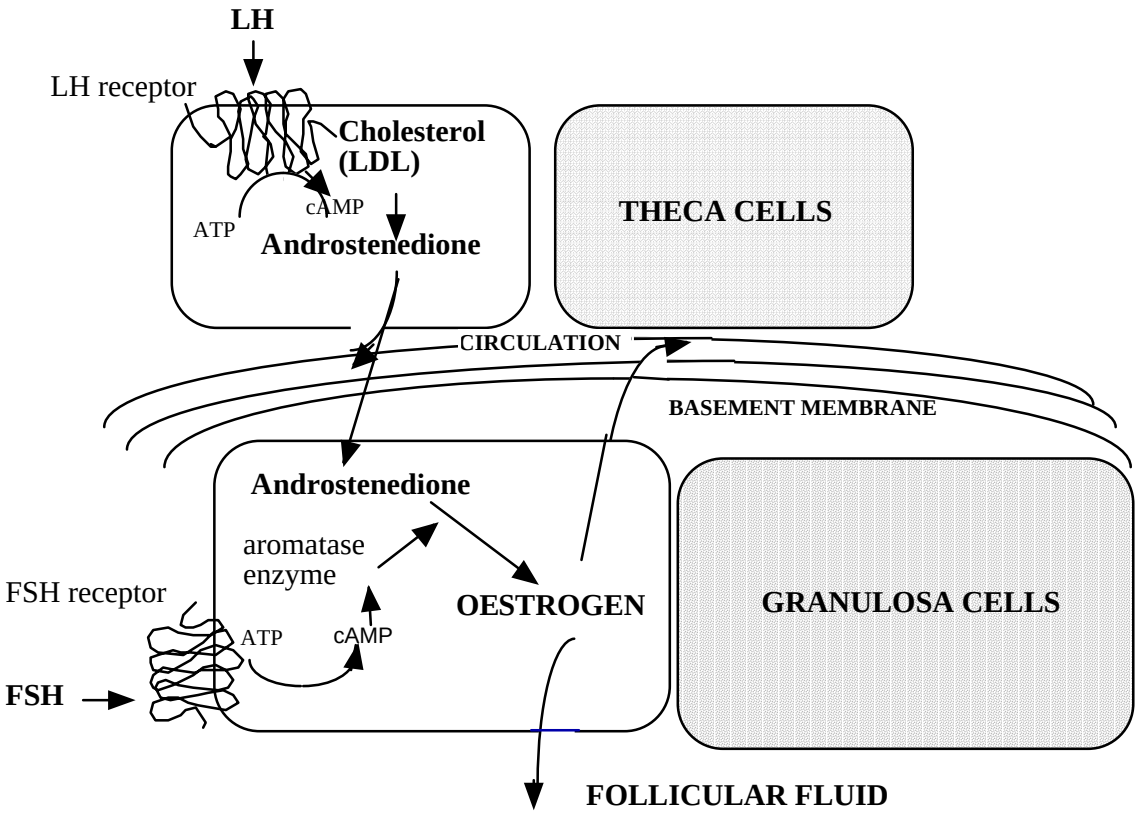


Figure 2. The two-cell / two-gonadotrophin hypothesis of follicular oestrogen production.

ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; LDL: low density lipoprotein; LH: luteinizing hormone; FSH: follicle stimulating hormone

Table 3. Some endocrine and paracrine ovarian regulators and their effects in the ovary and on ovarian tumour growth *in vitro* (Büscher *et al.*, 1999, Nash *et al.*, 1999; Yeh and Adashi, 1999; Doraiswamy *et al*, 2000).

	Putative effects in the ovary	Putative effect on the growth of ovarian tumours
Steroids		
Oestradiol	proliferation of Gcells, antiatretic	effect modulated by cytokines
Growth hormone	modulates follicular growth	
Progesterone	inhibits Gcell apoptosis	concentration-dependent effect
Testosterone and other androgens	substrates for oestrogen synthesis promote progesterone synthesis, induce atresia (high concentration)	
Peptides		
Basic fibroblast growth factor	AGF, mitogenic, prevents atresia, progesterone production	AGF, increased serum levels
Epidermal growth factor	inhibits differentiation of Gcells	stimulates
Transforming growth factor α	AGF, inhibits Gcell proliferation	
IGF1	AGF, prevents atresia	
IGF 2	regulation of corpus luteum	
Leptin	AGF, regulates IGF-mediated effects	?
TGF β	AGF, modulates the effect of inhibins and activins, post-ovulatory healing, prevents atresia	AGF, inhibitis epithelial / stimulates mesenchymal cell growth, immunoinhibition, TGFβ2 conditions precancerous epithelial cells?
Activin	stimulates FSH secretion	
Follistatin	binds to activin	
Inhibin	suppresses FSH secretion	
Vascular endothelial growth factor	AGF, vascular permeability	AGF
Cytokines of leucocyte origin		
IL-1	follicle growth, suppresses premature luteinization, inhibits progesterone secretion	stimulates, induces IL-6 secretion
IL-2	facilitatates cell mediated immunity	?
IL-6	reduces FSH binding capacity, postovulatory healing	stimulates, cell attachment and migration inhibits , apoptosis, increased serum levels
IL-8	targets leucocytes, upregulated by TGF-β?	AGF, stimulates, migration of lymphocytes
Tumour necrosis factor α	AGF, inhibits progesterone secretion regression of CL?,stimulated by FSH induces atresia	stimulates, induces IL-6 secretion
Granulocyte-macrophage colony-stimulating factor	AGF	stimulates, overcomes some effects of TGFβ increased serum levels
Other Substances		
Corticotrophin-releasing factor	regulates thecal-stromal cells	
Plasminogen activator	ovulation ?	
Prostaglandins	ovulation ?	
Renin / Angiotensin II	ovulation?	

AGF: angiogenetic factor; CL: corpus luteum; FSH: follicle-stimulating hormone; G cell: granulosa cell; IGF: insulin-like growth factor; IL: interleucin; TGFβ: transforming growth factor β

In the presence of normal or moderately elevated gonadotrophin levels, as seen, for example, in women with PCOS, the drug of choice for treatment of anovulation is CC (Hughes *et al*., 2000a). In the presence of hyper- or hypogonadotrophic hypo-oestrogenism, CC fails to cause ovulation because of lack of oestrogen feedback from the hypothalamus and hypophysis. The dose of CC can be adjusted to the ovarian response, and ovulation occurs approximately 5 to 12 days after the last dose of CC. The starting dose is 50 to 100 mg/day for 5 days, beginning on the third, fourth or fifth cycle day, but the dose can be increased to 200 mg/day depending on the ovarian response. The half-life of CC is approximately 5 days. (Barbieri, 1999a)

With a daily dose of 50 mg of CC, approximately 50% of anovulatory women will ovulate. An additional 25% of patients can be induced to ovulate by increasing the dose of CC to 100 mg daily. If ovulation occurs after CC intake, the conception rate may be as high as 25% per cycle in the first few cycles, but this tends to decline after the first 3 to 6 months of treatment (Barbieri, 1999a). Thus CC treatment is recommended to be given during four to six consecutive cycles (Tiitinen et.al, 1993b; Isaksson and Tiitinen, 1997). It is known that, apart from the ovulation following CC intake, the cervical mucus may not be optimal and the endometrium may remain thinner than 7 mm, factors that are both associated with a poor implantation rate. In addition, following CC intake, the histology of the endometrium in the luteal phase shows a reduction in glandular density (Sereepapong *et al*., 2000), vasomotor flushes are frequent and the risk of multiple pregnancy is increased (Table 6).

Another antioestrogen, tamoxifen, can also be used for induction of ovulation. It is given at 20 to 40 mg/day for 5 days, beginning on day 3 of the cycle (Borenstein *et al*., 1989). In some countries, tamoxifen is used if CC fails to induce ovulation, but in Finland the next step after CC failure is usually the use of gonadotrophins. Continuous use of tamoxifen is known to associate with increased risk of endometrial cancer and, although the annual risk is low, 2/1000 tamoxifen treated women, tamoxifen is classified as an endometrial carcinogen (Cardosi and Fiorica, 2000; Marttunen *et al*., 2001). There have also been some other antioestrogens on the market, e.g. cyclofenil, but their efficacy for inducing ovulation has not been established (Hughes *et al*., 2000a).

2.1.3. Gonadotrophins

Commercially available gonadotrophin preparations contain injectable FSH and human chorionic gonadotrophin (hCG); the latter is used as an LH substitute. These glycoprotein hormones contain a common α-subunit and a hormone-specific β-unit that dictates their biological specificity (Halvorson and Chin, 1999) (Figure 3).

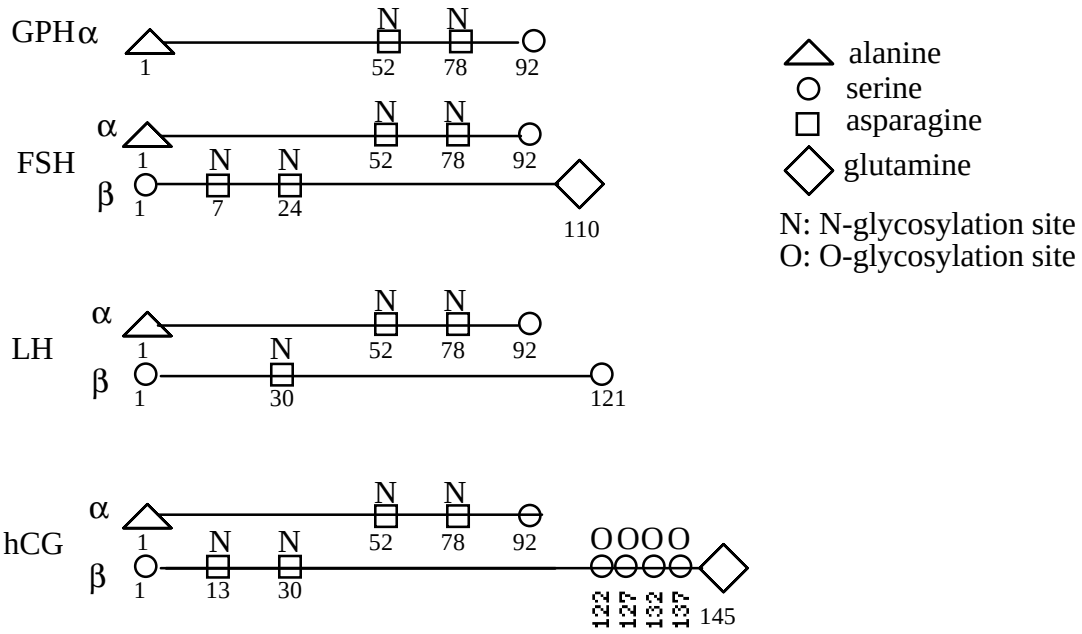


Figure 3. Structures of the common α-glycoprotein hormone (GPHα), the follicle-stimulating hormone (FSH), luteinizing hormone (LH), and human chorionic gonadotrophin (hCG).

Table 4. Drugs used for induction of ovulation and in assisted reproduction in Finland.

Generic name, Route of administration	Introduction to Finland	Usual dosage Ovulation induction Dose/ day (range)	Superovulation for ART Dose/ day (range)
Anti-oestrogens			
clomiphene citrate, p.o.	1966	100 (50-250) mg	150mg (100-250mg)
Gonadotrophins			
hCG	1958		
urinary hCG i.m./ s.c.	1966	5000 IU	5000 IU or 10 000 IU
FSH			
human menopausal urinary, i.m.	1966	75-225 IU	150 (75-450) IU
highly purified, s.c.	1994	75-225 IU	150 (75-450) IU
recombinant, s.c.	1997	37.5-100 IU	150 (50-450) IU
GnRH agonists	late 1980s		
buserelin, nasal spray			1.2 (0.6-1.2) mg
nafarelin, nasal spray			0.8 (0.4-0.8) mg
goserelin, s.c. implant			3.6 mg / 4 weeks
leuprorelin, s.c.			3.75 mg / 4 weeks
triptorelin, i.m.			3.75 mg / 4 weeks
GnRH antagonists	1999		
cetrorelix, s.c.			0.25 mg
ganirelix, s.c.		0.25 m	g

ART: assisted reproductive technologies; FSH: follicle-stimulating hormone; GnRH: gonadotrophin-releasing hormone; hCG: human chorionic gonadotrophin; i.m.: intra-muscular; OI: ovulation induction; p.o.: per os; s.c.: subcutaneous

Gonadotrophins were first used for induction of ovulation in 1958, with FSH isolated from pituitary extract (Gemzell *et al*., 1958) (Table 4). Then, in the early 1960ies, FSH isolated from the urine of postmenopausal women proved to cause ovulation (Lunenfeld *et al*., 1962). In addition to FSH and LH these early preparations also contained large amounts of urinary proteins, cytokines, growth factors, transferrins and other factors that could modulate ovarian function (Gast, 1995). In the 1990ies, highly purified urine preparations containing significantly more FSH than LH became available. However, even such purified preparations contained enough urinary proteins to cause allergic reactions in some patients (Odink *et al*., 1995; Albano *et al*., 1996). These proteins were then eliminated by purifying the urine with the help of FSH monoclonal antibodies and the subsequent preparations contained approximately 95% of FSH. The latest progress in this field was the development of recombinant FSH in the late 1990ies, when it was found possible to produce pure FSH containing no LH or other proteins. This preparation is suitable for subcutaneous administration, making self-administration possible (Shoham and Insler, 1996; Tulppala *et al*., 1999) (Table 4). Gonadotrophins are used for induction of ovulation in the following groups of patients:

a) with hypogonadotrophic hypo-oestrogenic anovulation
(treatment with a preparation containing both FSH and LH)
b) with polycystic ovaries if CC has failed (treatment with a pure FSH preparation)
c) with unexplained infertility, mild endometriosis or mild male factor infertility (treatment with either preparation)
d) undergoing controlled ovarian hyperstimulation for IVF or other ART. (The preparation is chosen according to the cause of infertility and the prior response to ovarian stimulation.)

Induction of ovulation with gonadotrophins should be carefully monitored by assessments of the serum oestradiol (E2) level and of the growth of both follicles and endometrium. At present, the programme most commonly used is the one with low-dose FSH or FSH/LH. In this programme, FSH is initiated with a daily dose of 50-150 IU, and the dosage is adjusted after 6 or 7 days, according to the ovarian and endometrial response. When the serum E2 is between 0.6 – 0.9 nmol/L / follicle and the diameter of the largest follicle is in the range of 16 to 18 mm, 5000 IU of hCG is administered to mimic the LH surge and cause rupture of the follicle, with ovulation approximately 36 hours later (Barbieri, 1999a). Another possibility is the step-down programme, which starts with a high dose of FSH (150-300 IU) for 3 to 4 days for the selection of follicles, after which the dose is reduced to maintain follicular development (Fauser *et al*., 1993). Either of these regimens can be used for induction of ovulation in patients with PCOS or for lean young women who are prone to develop the ovarian hyperstimulation syndrome (OHSS, See 3.1). The most common adverse effects of the use of gonadotrophins are this syndrome and multiple pregnancy (Table 6).

2.1.4. Gonadotrophin-releasing hormone

Native GnRH is essential for normal ovulation. Synthetic native-like GnRH is available and can be given intravenously in a pulsatile fashion (14 µg per pulse every 90 min) to induce the growth of one follicle (Filicori *et al*., 1994; Bayram *et al*., 2000). The half-life of this GnRH is short, from 2 to 4 minutes, and thus its administration is demanding and very expensive. Therefore it is not used in clinics, at least in Finland.

By replacing one amino acid (glycine) of the native GnRH with the unnatural D-amino acid and ethylamide groups, GnRH agonists have been produced which have 2.5 times longer half-lives and are up to 200 times more effective in releasing gonadotrophins from the pituitary than native GnRH. Administration of GnRH agonists causes an initial increase in LH and FSH secretion ("agonistic, flare-up phase") but, if this is continued at the same dosage for several days, desensitization of the pituitary GnRH receptors ensues, and results in suppression of FSH and LH secretion ("antagonistic phase") and in hypo-oestrogenism. These GnRH agonists have gained wide clinical use (Table 4) not only in reproductive medicine but also in the treatment of endometriosis, fibroids and hormone-dependent tumours.

GnRH agonists can be used in combination with induction of ovulation to avoid premature luteinization of the developing follicles (Homburg *et al*., 1993). With the aid of GnRH agonists, it is possible to time ovarian stimulation and ovulation or oocyte retrieval better than is the case with the natural LH surge. It has been shown that the use of the GnRH agonist in IVF / ICSI cycles improves the outcome of treatment (Hughes *et al*., 1992). With the use of the GnRH agonist, side-effects are infrequent and mild (Tapanainen *et al*., 1993) though OHSS may be a more frequent phenomenon than on stimulation without the GnRH agonist (Table 6).

A further modification of the GnRH molecule has resulted in the development of GnRH antagonists that totally abolish the effect of GnRH. These GnRH antagonists block pituitary function rapidly without any flare-up phenomenon and may therefore be used in IVF programmes to prevent the LH surge (Bouchard and Fauser, 2000; Devroey, 2000).

2.2. Assisted reproduction technologies

In ART (Table 5), the oocytes and/or sperms are handled outside the body and then transferred into the female body. To guarantee the availability of an embryo for transfer, an attempt is made to cause several oocytes to mature with the help of ovulation inducers. The most conspicuous progress in ART has been made by the use of the GnRH agonist, transvaginal ultrasound (Wikland *et al*., 1989), cryopreservation techniques (Trounson and Mohr, 1983; Tiitinen *et al*., 1995), and ICSI (Palermo *et al*., 1992). The major factor predicting the outcome of ART is advancing age of the female: in women over 38 years old, ART results in deliveries in 11%, while in younger women the rate is 33% (Van Voorhis and Syrop, 2000). In Finland, the total number of treatments initiated increases by approximately one thousand per year. In 1997, 19 Finnish centres provided 7336 IVF/ICSI and frozen embryo transfer (FET) treatment cycles, so that 2.4% of all newborns were the results of ART (Gissler

and Tiitinen, 1999). Nowadays summary data on clinical outcomes after ART are collected nationally, internationally from Europe (European, 2001) and from the USA (ASRM, 2000) and also from the World (Tarlatzis and Bili, 2000). The pregnancy and delivery rates can be given per initiated cycle, but usually the results per oocyte retrieval /OPU or per ET are favoured.

Table 5. Assisted reproduction techniques (ART) with delivery rates / embryo transfer (ET) in 1997 in the USA, in Europe and in Finland.

ART method	Delivery rate / ET[1] % USA[2]	Europe[3]	Finland[4]
IUI *Intrauterine insemination*	5-15		
Transfer of prepared (washed) spermatozoa into uterine cavity			
IVF-ET *In vitro fertilization and embryo transfer*	30	21	21
Aspiration of oocytes, fertilization and follow-up of cleavage in culture, transcervical transfer of embryos into the uterine cavity at the 2- to 8-cell stage or at the blastocyst stage			
ICSI *Intracytoplasmic sperm injection*	29	22	22
Fertilization of oocytes by injecting one sperm into the nucleus of an oocyte and transcervical transfer of the embryo			
Special techniques for obtaining sperm:			
TESE Testicular sperm extraction			
MESA Microsurgical epididymal sperm aspiration			
GIFT *Gamete intrafallopian tube transfer*	30	-	-
Sperms and oocytes are inserted into the fallopian tube under laparoscopy			
ZIFT *Zygote intrafallopian transfer*	30	-	-
Fertilized oocytes are inserted into the fallopian tubes under laparoscopy			
FET Transcervical transfer of frozen-and-thawed embryos	19	12	13
Other techniques			
Cryopreservation			
Freezing of sperm, oocytes or embryos for further use			
Assisted hatching			
Drilling of zona pellucida to improve embryonic development			
Prolonged culture of embryos			
Follow-up of embryos to the 8-cell or the blastocyst stage before ET			
Blastomere biopsy			
From a 6- or 8-cell embryo, one or two blastomeres are taken for prenatal diagnosis of a hereditary disease			

[1] mean number of embryos replaced (% multiple births): the USA 4 (39%); Europe 53% • 3 (29%); Finland 1.7 (22%)
[2] Data of 73 069 cycles, Society for Assisted Reproductive Technology (SART) Clinical Outcome Reporting System, reported by SART and American Society for Reproductive Medicine (ASRM, 2000)
[3] Data of 203 893 cycles, European IVF Monitoring Programme, reported by European Society for Human Reproduction and Embryology (European, 2001)
[4] Data of 7336 cycles, Finnish IVF Registry, reported by National Research and Development Center for Welfare and Health (Gissler and Tiitinen, 1999)

2.2.1. Controlled ovarian hyperstimulation

Because the pregnancy rate obtained with IVF in normal cycles is low (12%), a part of ART is hyperstimulation of the ovaries with gonadotrophins (Daya *et al*., 1995). The controlled hyperstimulation programme usually includes pituitary down-regulation with a GnRH agonist and administration of gonadotrophins (Figure 4). The ovarian response is monitored by serial measurements of serum E2 and by measurements of the count and size of follicles with the aid of transvaginal ultrasound examination and the dosage of gonadotrophins is adjusted accordingly. Injection of gonadotrophins for 8 to 10 days usually results in the development of 3 to 15 follicles in both ovaries. When at least three follicles, each exceeding 16 mm in diameter, have developed, hCG (5 000-10 000 IU) is injected subcutaneously, and the oocyte pick-up (OPU) is performed transvaginally 34-36 hours later. To ensure endometrial receptivity, progesterone is administered continuously after ET.

Ovarian hyperstimulation causes a 4-fold increase in serum FSH, a 20-fold increase in E2, a 1.4-fold increase in androstenedione and a 2.6-fold increase in testosterone concentrations from suppressed levels (Bützow *et al*., 1999; Fanchin *et al*., 2000). After ovarian hyperstimulation, the level of E2 in the blood is 4- to 10-fold higher (Fishel and Jackson, 1989) and that of progesterone as much as 10-fold higher than during a normal menstrual cycle (Fanchin*et al*., 2000).

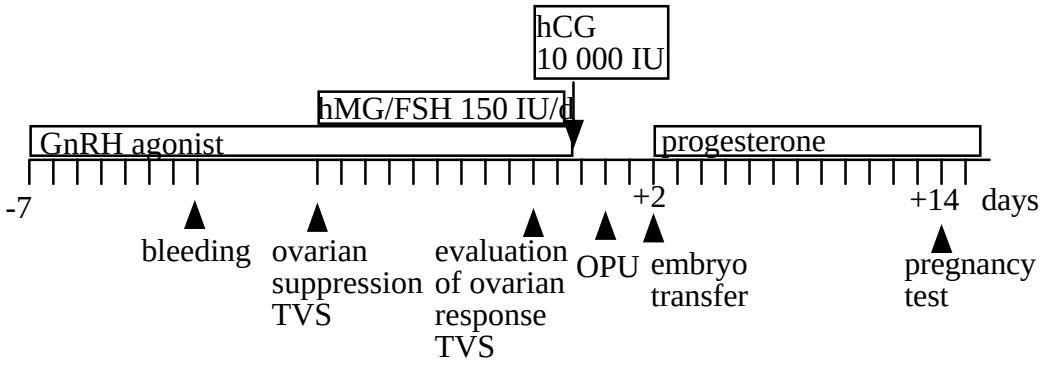


Figure 4. A model for ovarian hyperstimulation with long pituitary downregulation with the GnRH agonist.

FSH: follicle-stimulating hormone; GnRH: gonadotrophin-releasing hormone;
hCG: human chorionic gonadotrophin; hMG: human menopausal gonadotrophin;
OPU: ovum pick-up / ovum retrieval; TVS: transvaginal ultrasound

2.2.2 *In vitro* fertilization

In vitro fertilization (IVF) was originally developed for treatment of infertility that was caused by tubal occlusion (Edwards *et al*., 1969). The first IVF baby in the world was born in 1976 in England (Steptoe and Edwards, 1978) and the first one in Finland in 1984 (Koskimies *et al*., 1984). Since then, this technique has spread to treatment of almost all types of infertility. In 1992-97, for example, 28% of Finnish couples treated with IVF had tubal infertility, 19% some other female infertility, 15% male infertility, 14% combined causes, and 18% unexplained infertility (Gissler and Tiitinen, 1999). IVF is most successful if several oocytes are retrieved with the aid of controlled ovarian stimulation. The retrieved oocytes are then fertilized with specifically prepared semen and the 4- to 8-cell-stage embryo(s) are transferred transcervically into the uterus on the 2nd or 3rd day after OPU (Steptoe and Edwards, 1978). For luteal support, natural progesterone is administered daily either intravaginally or intramuscularly after ET. Before the introduction of natural progesterone for clinical use, luteal support was achieved by giving hCG injections (at repeated dosages of 1500-10 000 IU), which stimulated the production of endogenous progesterone (Barbieri, 1999a).

The pregnancy rate with IVF varies from clinic to clinic, and is dependent on the experience of the IVF clinic, on the age of the female, and on the number and quality of embryos replaced. In 1997 the mean pregnancy rate per ET was 37% in the USA (ASRM, 2000), ranged from 18 to 45% in Europe and was 27% in Finland (European, 2001) (Table 5) and is known to be the same for at least the first three attempts (Croucher *et al*., 1998). The pregnancy rates are comparable to normal fecundability (25-30%) in one cycle. However, early pregnancy failure with ART is common (20%), and some of the pregnancies are ectopic (2-4%), so the live birth rate varies from 20 to 30% (Gissler and Tiitinen, 1999; ASRM, 2000) (Table 5). If there are surplus embryos, they are cryopreserved and can be thawed in future natural cycles or in cycles induced with oestrogen and progesterone with or without pituitary suppression with a GnRH analogue. The pregnancy rates with FET are somewhat lower than with transfer of fresh embryos, and the multiple pregnancy rate is also lower (Table 5). Thus, one IVF treatment may allow several attempts to induce pregnancy.

2.2.3. Intracytoplasmic sperm injection

The technique of injecting a single sperm under the microscope directly into the cytoplasm of the oocyte is called ICSI (Palermo *et al*., 1992). This method has meant a revolution in the treatment of male infertility, because, almost always, a single sperm can be retrieved, if not from ejaculated semen, then from the testis or epididymis. In addition, ICSI is indicated for couples with fertilization problems in IVF treatment. Fertilization and pregnancy rates are comparable with those in standard IVF procedures (Tarlatzis and Bili, 2000) (Table 5). The ovarian hyperstimulation programme for ICSI is similar to that of conventional IVF. However, some concern has arisen over ICSI, because severe male infertility may be caused by chromosomal aberrations (2% of cases of male infertility) and, if ICSI is used, these aberrations may be carried to male offspring. This may be the only specific risk of ICSI, because in all other respects infants born after ICSI are comparable with other children (Tarlatzis and Bili, 2000).

2.2.4. Donated gametes, donated embryos and surrogacy

Nowadays sperm, oocytes and embryos can all be donated. Donations can be made anonymously, but donors must be healthy and free of any sexually transmitted disease such as human immune deficiency virus, or hepatitis B or C. Because of reduced oocyte quality and an increased risk of aneuploidy with advancing maternal age, the age of female donors is usually restricted to 35 years. Donation of sperm has a long history, because sperm can easily be obtained by masturbation and the properly prepared sperm can be frozen for later use in intrauterine inseminations or in IVF/ICSI programmes (Lashen *et al*., 1999). Cryopreservation of the oocytes still has problems and is very rarely used (Kazem *et al*., 1995; Goud *et al*., 2000). Thus, donation of oocytes requires the use of IVF/ICSI programmes: the volunteer undergoes an ovarian hyperstimulation programme and OPU, the oocytes are inseminated with the sperm of the infertile male, and the recipient infertile female receives the embryo(s) in a cycle primed to the volunteer donor's cycle (Söderström-Anttila and Hovatta, 1995). Donated embryos are usually obtained from couples who have undergone successful IVF or ICSI programmes and do not have any need for their frozen embryos (Lindheim and Sauer, 1999; Van Voorhis *et al*., 1999; Kingsberg *et al*., 2000). In surrogacy, the embryo(s) of the infertile couple derived by IVF/ICSI are transferred into the uterus of a volunteer female (Goldfarb *et al*., 2000), and, after delivery, the new-born is adopted by the infertile couple.

These treatments with donated gametes, embryos or surrogacy are options for infertile couples who are unable to produce gametes of their own, who have severe genetic aberrations, or who have such severe anatomical abnormalities that they cannot have children of their own by other treatments. Thus, the most common indication for donated sperm is azoospermia; for ovum donation, ovarian insufficiency (Lutjen *et al*., 1984); and for surrogacy, absence or abnormality of the uterus (Raziel *et al*., 2000). With donated sperm, the rates of pregnancy and pregnancy outcomes are good (Lashen *et al*., 1999). With ovum donation programme, the pregnancy rate per ET is high (46%), but miscarriages are frequent (Foudila *et al*., 1999). The ongoing pregnancies are associated with an increased risk of first trimester bleeding (53%), pregnancy-induced hypertension (31%), and a high rate of caesarean delivery (57%) (Söderström-Anttila *et al*., 1998; Foudila *et al*., 1999). Live birth rates with surrogacy are similar to conventional IVF (Goldfarb *et al*., 2000; Serafini, 2001).

The use of donated gametes, embryos and surrogacy has aroused much public concern, and many ethical experts regard them as inappropriate. Thus, in many countries these policies are forbidden by law (Fasouliotis and Schenker, 2000). In Finland, no legislation exists so far and both surrogacy and oocyte donation programmes have been practised (Söderström-Anttila and Hovatta, 1995; Hovatta, 2000).

3. ADVERSE EFFECTS OF INFERTILITY TREATMENT

Several adverse effects due to the use of ovulation inducers are to be expected and are caused by either hypo- or hyperoestrogenism, which are essential parts of the different treatment regimens (Derman and Adashi, 1994; Schenker and Ezra, 1994) (Table 6). The most severe complication of induction of ovulation is OHSS, which develops in 1-10% of patients, the severe forms of which may be associated with thromboembolism (Whelan and Vlahos, 2000). Adverse effects resulting from OPU or ET are rare (< 1 %) (Schenker and Ezra, 1994). However, superovulation and transfer of two or more embryos in IVF/ICSI cycles carries the risk of a multiple pregnancy and its risks (see below) (Schenker and Ezra, 1994; Jacobs and Agrawal, 1998). In singleton pregnancies after IVF, the rate of babies small for gestational age is doubled compared to babies born after normal conceptions (Koudstaal *et al*., 2000). Children born after IVF/ICSI have not been shown to have increased risk of malformations (Tarlatzis and Grimbizis, 1999; Tarlatzis and Bili, 2000) or cancer (Lerner-Geva *et al*., 2000).

3.1. Ovarian hyperstimulation syndrome

Both the use of gonadotrophins and / or CC can lead to OHSS (Golan *et al*., 1989). The major abnormality in OHSS is increased capillary permeability, resulting in serious fluid-balance aberrations. The ovarian renin-angiotensin system, the vascular endothelial growth factor (VEGF), histamine, prostaglandins and various cytokines have been suggested to be responsible for OHSS (Whelan and Vlahos, 2000). Of these factors, VEGF, which is present in human granulosa cells and follicular fluid, stimulates angiogenesis, and increases vascular permeability, appears to be one of the most likely agents in the pathophysiology of OHSS (Levin *et al*., 1998; Whelan and Vlahos, 2000).

The risk of OHSS is highest in young (age < 25 years) and lean (BMI <20 kg/m^2) patients with history of allergy, and in patients with PCOS or previous OHSS. Treatment with a GnRH agonist with gonadotrophins, with high levels of serum oestradiol (• 7 nmol/L) and with a large number of follicles (> 15 follicles), and luteal support with hCG leading to pregnancy are also risk factors for OHSS. The symptoms occur with increasing serum levels of hCG and thus usually regress within the first week after hCG injection but are aggravated again if pregnancy ensues. The clinical findings in OHSS include cystically enlarged ovaries, which cause abdominal discomfort and pain. The capillaries in the ovaries and also those in other parts of the body leak protein-rich fluid, causing ascites, pleural effusion and hypovolaemia. The severity of OHSS is classified according to these clinical signs (Table 7). Hypovolaemia can be so grave that it leads to oliguria, electrolyte imbalance, respiratory failure, disseminated coagulopathy and even to death (Whelan and Vlahos, 2000). The risk of thromboembolism in OHSS is caused by hypercoagulative changes, but this risk is further increased by pressure of the enlarged ovaries against the large pelvic veins (Kaaja *et al*., 1989; Jacobs and Agrawal, 1998). It is curious that thromboses can develop in both the veins (Loret de Mola *et al*., 2000) and the arteries (El Sadek *et al*., 1998; Ludwig *et al*., 1999b; Yoshii *et al*., 1999).

Table 6. Adverse effects of ovarian stimulation and *in vitro* fertilization
(Derman and Adashi, 1994; Jacobs and Agrawal, 1998; Barbieri, 1999a; Barbieri, 1999b; Hughes et al., 2000b)

Factor and adverse effect	Frequency (%)
1. Ovulation inducers	
Clomiphene citrate	
Vasomotor flushes	10-20
Visual disturbances	1-3
Headache	1
Adnexal tenderness	5
Cyst formation	< 5
Cervical mucus abnormality and luteal phase defect	59
Ovarian hyperstimulation syndrome	NA
Multiple pregnancy, twins	<10
• triplets	<1
Gonadotrophins:	
Allergic reactions	NA
Ovarian hyperstimulation syndrome	1-10
Severe form	<1-2
Hypercoagulable state, thromboembolism	
Multiple pregnancy	15
Gonadotrophin-releasing agonists:	NA
Hypo-oestrogenism	
Sweating, flushes, Sore vagina	
Nasal congestion	
2. Oocyte pick-up	
Vaginal or intra-abdominal bleeding	<1
Infection	<1
Immunological reactions (ovarian antibodies)	NA
3. Transfer of • 2 embryos	
Multiple pregnancy:	25-50
Preterm delivery *vs* singleton	2- 3 fold
Pre-eclampsia *vs* singleton	5-10 fold

NA: percentage not availablle, vs: versus

Prevention of OHSS is based on the identification of high-risk patients and individualizing their treatment regimen. There is some evidence that a high serum level of VEGF may predict the risk of OHSS (Geva and Jaffe, 2000; Chen *et al.*, 2000). Moreover, interleukins 6 and 8 (Chen *et al*., 2000), inhibin A and B (Enskog *et al*., 2000) or CA 125 (Scarpellini and Scarpellini, 1992; Ozaksit *et al*., 1993) can also be helpful in assessing the risk of OHSS. In patients judged to be at increased risk of OHSS, the dosage of ovulation inducers has to be kept at the lowest possible and the ovarian response has to be monitored even more carefully than usual. In addition, if signs of OHSS are present, it is wise to forego ET and instead to cryopreserve embryos for further use (Tiitinen *et al*., 1995). It is possible that in the future the risk of OHSS can be further reduced by using GnRH antagonists instead of GnRH agonists, or totally abolished by the use of *in vitro* maturation of oocytes (Suikkari *et al*.,

2000). If, however, severe OHSS ensues, anti-angiogenetic agents, such as VEGF antagonists or VEGF synthesis inhibitors, may provide effective treatments in future (Whelan and Vlahos, 2000). Regardless of the modalities for prevention and treatment of OHSS, the recovery of the ovaries after severe OHSS has to be followed carefully, because OHSS may indicate an increased risk of ovarian malignancy (Atlas and Menczer, 1982).

3. 2. Multiple pregnancy

The rate of spontaneous twins is about 1% of all pregnancies and that of triplets 0.01-0.02%. With the use of CC, the overall rate of twinning increases to 6-10% and with the use of gonadotrophins for induction of ovulation it rises to 10-40%, depending on the quality of monitoring the treatment. Of interest is, that with the use of ovulation inducers monozygotic twinning seems to be increased 3-fold. With ART in Europe, the overall rate of multiple births is still high (Table 5; twins 26%, triplets 4%, quadruplets 0.2%) (European, 2001) and in Finland it is 27% for IVF, 23% for ICSI, and 17% for FET (Gissler and Tiitinen, 1999).

Multiple pregnancy is associated with medical, financial, psychological and social risks (Elster, 2000), especially with an increased risk of maternal complications and fetal loss caused by prematurity. Low birthweights (<2500g) are common (24%) among children born after IVF or ICSI treatment; most of them are being due to multiple pregnancies. With triplets or higher-order multiples, multifetal pregnancy reduction (induced abortion) has been performed to improve the outcome for mother and children (Elster, 2000). This procedure, which raises ethical concerns and carries an increased risk of losing the pregnancy, is more common in countries where transfer of multiple embryos is common. In Scandinavia, a general consensus exists that the best way to reduce multiple pregnancies with ART is single embryo transfer, which can give clinically comparable results (pregnancy rate / ET from 20 to 39% (Vilska *et al*., 1999; Strandell *et al*., 2000).

Table 7. Classification of ovarian hyperstimulation syndrome (OHSS) and need for hospitalization (Golan *et al.*, 1989; Whelan and Vlahos, 2000)

Symptom	Classification of OHSS		
or finding	Mild / Grade 1-2	Moderate / Grade 3	Severe / Grade 4-5
Abdominal	Distension/discomfort, Nausea, vomiting, and/or diarrhoea	Grade 1-2 +Ascites by ultrasound	Grade 3 +Clinical evidence of ascites
Ovarian	Size 5-12 cm		
Respiratory	-		Hydrothorax, tachypnea
Haematology	-	Mild haemoconcentration	Severe haemoconcentration Coagulation abnormalities
Renal			Diminished renal perfusion
Hospitalizatio*n*	Not required	Individual judgement	Necessary

4. INFERTILITY, INFERTILITY TREATMENTS, AND RISK OF GYNAECOLOGICAL MALIGNANCIES

The most common cancer in women is breast cancer, accounting for approximately 30% of all cancers, whereas genital cancers account for 15%. Accordingly, in 1997 3324 women in Finland were diagnosed as having breast cancer, 663 women had endometrial cancer, 620 women had ovarian or fallopian tube cancer or borderline tumours, 160 women had cancer of the uterine cervix, and 106 women had cancers of other genital origin (Finnish Cancer Registry, 1997). Malignancies originating from the breast, the endometrium or the ovary may, to some extent, have endocrine aetiology, oestrogen appearing to have the most conspicuous impact (Henderson *et al*., 1993) (Table 8). Furthermore, nulliparity and advanced age at the first delivery are common risk factors for these three cancers (Kvale *et al*., 1991). Therefore it is no wonder that infertility itself is a risk factor for these cancers, especially in view of the fact that infertility is often linked with elevated levels of oestrogens and/or gonadotrophins (Brinton *et al*., 1989; Anttila *et al*., 1994). This brings us to ovulation inducers, which can induce multiple follicles and stimulate release of gonadotrophins and/or oestrogen (Balen, 1995; Meirow and Schenker, 1996; Rodriguez *et al*., 1998a). Gonadotrophin receptors are expressed on all the cells of ovarian inclusion cysts but in only 50-60% of cells of ovarian cancers; this may suggest a role for gonadotrophin in ovarian cancer biology (Ala-Fossi *et al*., 1999; Konishi *et al*., 1999). The roles of FSH and LH may be different in this respect, because LH has been shown to block the cell-proliferating effect of FSH on ovarian cancer cell lines (Zheng *et al*., 2000).

The report of a 27-fold risk of ovarian cancer after infertility treatment (Whittemore *et al*., 1992) together with numerous case reports of infertile patients with gynaecological cancers (Bandera *et al*., 1995; Tarlatzis *et al*., 1995; Unkila-Kallio *et al*., 1997) have demonstrated a need for further studies on the risk of genital malignancies in infertile patients, and such data are now available from Australia, Denmark, Israel, Italy and the USA, (Rossing *et al*., 1994; Venn *et al*., 1995; Bristow and Karlan, 1996; Rossing *et al*., 1996a; Parazzini *et al*., 1997; Garland *et al*., 1998; Modan *et al*., 1998; Rodriguez *et al*., 1998a; Weiss *et al*., 1998; Venn *et al*., 1999) (Tables 10a and 10b). Radiotherapy treatment for infertility has not been associated with increased risk of gynaecologic malignancies (Ron *et al*., 1999).

4.1. Ovarian malignancies

The incidences of ovarian malignancies in the USA and Northern Europe are the highest in the world, being 10.9/100 000 in Finland or 14.7/100 000 when borderline tumours and tubal cancers are also included (Finnish Cancer Registry, 1997). The risk of developing ovarian cancer before the age of 65 is 0.8%. This risk is reduced to 0.3% if the woman is multiparous or if she has used oral contraceptives (OC) for at least 4 years, but increased to 1.6% if she is nulliparous and has not used OC. Belonging to a family with hereditary cancer syndrome increases the life-time risk to 4.4%.(Hartge *et al*., 1994).

Table 8. Theories of the causes of ovarian cancer (+ = supports, ? = questions the theory)

Theory

Incessant ovulation:

Extravagant, purposeless ovulations cause ovarian trauma and the repair of the trauma induce genetic alterations within the surface epithelium (Fathalla, 1971) or formation of epithelial inclusion cysts and development of neoplastic cells (Resta 1993; Tressera *et al*., 1998; Ness and Cottreau, 1999).

Inflammation:

Cell damage (ovulation) induces secretion of potentially mutagenic cytokines and prostaglandins that may participate in malignant transformation (Ness and Cottreau, 1999; Doraiswamy *et al*., 2000)

+ Longer ovulatory age is associated with ovarian cancer (Casagrande *et al.*, 1979; Schildkraut *et al.*, 1997; Webb *et al.*, 1998)

+ Pregnancies and oral contraceptives decrease the risk of ovarian cancer (Hankinson *et al*., 1992; Adami *et al*., 1994; Hankinson *et al*., 1995)

+ Endometriosis is associated with ovarian cancer (Brinton *et al.*, 1997)

? Anovulatory infertile patients run an increased risk of ovarian cancer (Schildkraut *et al.*, 1996)

? Oral contraceptives or parity do not reduce the risk of mucinous ovarian cancers (Risch *et al*., 1996)

Gonadotrophins:

Gonadotrophins may have direct carcinogenic activity (Cramer and Welch, 1983): gonadotrophins stimulate cell growth and inhibit apoptosis in ovarian epithelial cells and ovarian cancer cells (Konishi *et al*., 1999) and promote the growth of ovarian cancer by inducing angiogenesis (Schiffenbauer *et al.*, 1997).

Oestrogens:

Oestrogens increase granulosa cell proliferation and hence the frequency of mitotic activity, which may lead to malignant transformation (Cramer and Welch, 1983)

+ Gonadotrophin and oestrogen receptors are expressed in ovarian tumours (Konishi *et al*., 1999)

+ The incidences of gynaecological malignancies increases after the menopause (Stadel, 1975)

+ In rodents, high levels of gonadotrophins induce GCT (Biskind *et al*., 1952; Tennent *et al*., 1990)

+ Excess of oestrogens and androgens may increase the risk of ovarian cancer, whereas progesterones may be protective (Risch, 1998, Rodriguez *et al*., 1998b)

+ The use of non-contraceptive oestrogens / hyperoestrogenism increase the risk of ovarian endometrioid cancer (Risch *et al.*, 1996; Zanetta *et al.*, 2000)

+ Prolonged use (• 10 years) of oestrogen replacement therapy associates with increased risk of ovarian cancer mortality (Rodriguez *et al*., 2001)

+ GnRh analogues show promise for therapy of ovarian cancers (Schally 1999b)

? Oestrogen replacement therapy was not associated with ovarian cancer in a meta-analysis (Coughlin *et al.*, 2000)

Infection:

Pelvic inflammatory disease may induce secretion of potentially mutagenic cytokines and prostaglandins that may induce malignant transformation (Ness and Cottreau, 1999)

+ Tubal ligation and hysterectomy reduce the risk of ovarian cancer (Hankinson *et.al.*, 1993)

+/? The association of pelvic inflammatory disease with ovarian cancer is inconsistent (Risch and Howe, 1995; Parazzini *et al*., 1996)

Genetic inheritance:

Approximately 3-13% of ovarian cancers are familial, either site-specific ovarian cancers, hereditary breast ovarian cancers, or hereditary nonpolyposis colon cancers / ovarian cancer syndrome / Lynch syndrome II (DePasquale *et al*., 1998) .

GCT: granulosa cell tumours

Ovarian malignancies in Danish women between 18 and 59 years of age originated from germ cells (3-4%), sex-cord stromal cells (3-4%) or ovarian epithelial cells (80-90%) or were unclassifiable or metastases (6%) (Mosgaard *et al*., 1997b). Of these malignancies, those of stromal origin, especially GCTs, and epithelial cancers are thought probably to be associated with infertility treatments (Tarlatzis *et al*., 1995).

4.1.1. Granulosa cell tumour

Granulosa cell tumour (GCT, Figure 5) is the most common of the sex-cord stromal tumours of the ovary and it is often capable of producing steroids, especially oestrogen (Table 9) (Serov *et al*., 1973; Young and Scully, 1994). Approximately 2-3% of all ovarian tumours are GCTs (Young and Scully, 1994; Platz and Benda, 1995). Their incidence ranges from 0.58 / 100,000 in Israel to 1.6 / 100,000 in Sweden (Stenwig *et al*., 1979; Björkholm and Silfversward, 1980; Ohel *et al*., 1983).

Granulosa cell tumours can be divided into juvenile and adult types, which are both typically of low malignant potential, but have a tendency to recur even 30 years after primary treatment (Hines *et al*., 1996). All GCTs should therefore be classified and followed up like ovarian malignancies (Fox *et al*., 1975). Juvenile GCTs account for 5% of all GCTs and are usually associated with precocious puberty (Cronje *et al*., 1998). The more common adult type of GCT is typically diagnosed in postmenopausal women complaining of bleeding, an abdominal mass or haemoperitoneum. The symptoms of GCT are mostly related to hyperoestrogenaemia (pubertas praecox, menorrhagia, or postmenopausal bleeding with endometrial hyperplasia or cancer) (Ohel *et al*., 1983) or to high levels of inhibin (secondary amenorrhea) (Lappohn *et al*., 1992). The histology of a GCT is complex, and often specific stainings are needed for a final diagnosis (Gitsch *et al*., 1991; Choi *et al*., 2000). Assessment of inhibin, (Kauppila *et al*., 1992; Burger *et al*., 2000; Ala-Fossi *et al*., 2000) and/or Müllerian inhibiting substance (Lane *et al*., 1999) in the serum can be of help in the follow-up of patients with GCT.

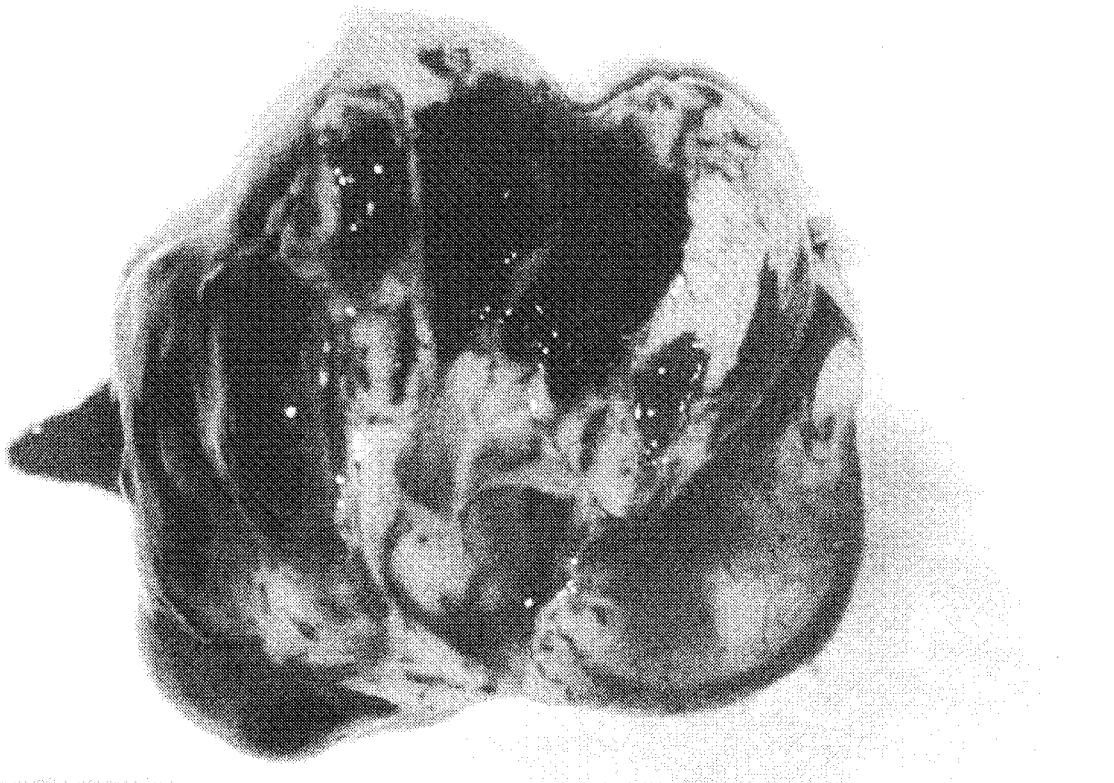
In rodents, GCT development can be induced by high levels of gonadotrophins (Biskind *et al*., 1952), especially if inhibin production is prevented (Matzuk *et al*., 1996; Beamer *et al*., 1998; Rilianawati *et al*., 1999). The association of high gonadotrophins and GCT has led to a search for activating mutations of the FSH receptor in these tumours, but so far none has been found (Fuller *et al*., 1998). Recently, oestrogen β-receptor was detected in GCT, implying that oestrogen may have a role in the pathogenesis of GCT (Chu *et al*., 2000). In addition, chromosomal aberrations (trisomy 12, genomic imprinting) may be causative factors, at least in juvenile GCT (Fletcher *et al*., 1991; Beamer *et al*., 1998). Because ovulation inducers increase gonadotrophin levels, it has been suggested, on the bases of case reports in infertile women, that the use of ovulation inducers may cause an increase in GCTs in infertile women (Willemsen *et al*., 1993). Of the other sex-cord stromal tumours, Sertoli-Leydig cell tumour in an infertile patient has been reported (Chou *et al*., 1997). On the other hand, it has been shown that each pregnancy reduces the risk of GCT by 16% (Adami *et al*., 1994). Unfortunately, most of the epidemiological studies on ovarian malignancies lack specific data on GCTs and studies on GCT do not include the data on infertility treatments.

Table 9. Some charachteristics of sex-cord stromal tumours and other steroid-secreting ovarian tumours (Serov, 1973; Young and Scully, 1994).

Sex-cord stromal tumours	Cell origin	Steroid secretion E	P	A	I	Mean age, years	Percentage of OT	Malignancy
Androblastoma (arrhenoblastoma or Sertoli-Leydig cell tumour)	Sertoli, Leydig fibroblasts	(+)	(+)	+		25	<0.5	20%
Fibroma	fibroblasts	-	-	-	-	48	4	very rare, fibrosarcoma
Granulosa cell tumour	granulosa theca	+	(+)	(+)	+	50-55	2	all, low grade
Gynandroblastoma	ovarian and testicular						very rare	
Lipoid cell tumour	Leydig, hilus	-	-	+		>50	<0.5	no
	adrenal		+	+			<0.5	20-25
Theca cell tumour, thecoma	theca	+		(+)		>50		almost never
Other ovarian tumours with occasional steroid secretion								
Mucinous cystadenocarcinoma	Epithelial	+	+	+				
Dysgerminoma	Germ		+	+				
Teratoma	Germ	+	+					

A: androgen; E: oestrogen; I: inhibin; OT: ovarian tumours; P: progesterone

Figure 5. Typical granulosa cell tumour with solid and cystic structure, cysts filled with clotted blood. Courtesy of Dr. Anssi Tenhunen



4.1.2. Epithelial cancers

More than 90% of ovarian cancers arise from a single epithelial cell (Bast *et al*., 1995), preferably in ovarian inclusion cysts (Tresserra *et al*., 1998). It is commonly believed that the onset of cancer of this type is preceded by premalignant stages, such as an epithelial tumour of low malignant potential, a borderline ovarian tumour (Scully, 2000), or an endometriotic cyst (Vercellini *et al*., 2000), and further, because morphological precursors of ovarian epithelial cancer are evident (Resta *et al*., 1993) and the risk factors for these tumours are similar to those for invasive ovarian cancer (Harlow *et al*., 1988; Eltabbakh *et al*., 1999). Interestingly, the borderline tumours seem more frequently to be associated with infertility (Mosgaard *et al*., 1998; Duska *et al*., 1999), or its treatments (Harris *et al*., 1992; Parazzini *et al*., 1998) than the invasive cancers (Tables 10a and 10b). The epithelial cancers are classified as serous type (80-90%), mucinous, endometrioid, clear cell, and Brenner tumours. Cigarette smoking, the classical risk factor for many cancers, is a risk factor only for mucinous epithelial ovarian cancer (odds ratio, OR 2.9, 95% confidence interval, $CI_{95\%}$ 1.7,4.9) (Marchbanks *et al*., 2000).

From epidemiological studies on ovarian cancer it is known that the risk of ovarian cancer decreases with long-term use of combined types of OC or GnRH agonists (Henderson *et al*., 1993), whereas low parity, infertility (Rodriguez *et al*., 1998a) and infertility treatments probably increase the risk (Whittemore *et al*., 1992). This study by Whittemore had used combined data from small-scale USA case-control studies and its finding of a 27-fold risk in treated nulliparous infertile women received much criticism, as the study lacked data on the causes of infertility, or the treatment received and suffered from the small number of cases. In subsequent epidemiological study on infertile patients, the use of CC for 12 months or more was associated with an increased risk of ovarian cancer in both anovulatory and ovulatory infertility (relative risk [RR] 11.1, $CI_{95\%}$ 1.5-82.3) (Rossing *et al*., 1994), but studies thereafter have not supported this relationship (Mosgaard *et al*., 1997b) (Tables 10a and 10b). In a Danish study of 684 women with ovarian cancer (all types), only 4.1% had undergone hormonal ovarian stimulation (Mosgaard *et al*., 1997b), i.e., not more frequently than the control subjects. The Danish authors, like the others, concluded that the risk factors for ovarian cancer were infertility and nulliparity, not the treatment of infertility (Mosgaard *et al*., 1997b). The largest study so far has included 29,700 women referred for IVF treatment in Australia (Venn *et al*., 1999). This study showed an increased risk of ovarian cancer in patients with unexplained infertility (Table 10b). In addition to unexplained infertility, anovulatory infertility has also been related to an increased risk of ovarian cancer (OR 2.5, $CI_{95\%}$ 1.1-5.9) (Rossing *et al*., 1994), especially if body mass index (BMI) was low in patients with PCOS (Schildkraut *et al*., 1996). Patients with endometriosis have been found to have an increased risk of ovarian cancer (standardized incidence ratio [SIR] 1.9, $CI_{95\%}$ 1.3-2.8) regardless of fertility, the risk has been highest with ovarian endometriosis (SIR 4.2, $CI_{95\%}$ 2.0-7.79) (Brinton *et al*., 1997) and the increased risk has also been associated with the use of non-contraceptive oestrogens (Risch *et al*., 1996). Further, patients who carry a hereditary or sporadic mutation linked to ovarian cancer may run a higher risk of ovarian cancer when they undergo ovarian hyperstimulation (Nieto *et al*., 1999).

Table 10a. Epidemiological studies on borderline ovarian tumours, infertility , infertility treatments and parity. Risk ratios are given as odds ratios (OR) or standardized incidence ratios (SIR) with 95% confidence limits in parentheses. Significant findings are bolded.

Case-control	Diagnosis of tumour		Cases /controls			OR for		
1st author, year	Year, Country	Age in years	No. / No.	Infertile %	Treated %	Infertility	Ovulation inducers	Nulliparity
Harris 1992	1976-86 USA, 5 studies		223 / 3149	19 /12	5/1a	**1.9 (1.3-2.7)**	**4.0 (1.1-13.9)a**	
Shushan 1996	1990-93 Israel	36-64	36 / 408	10/29	28/7		**3.5 (1.2-10.1)b all OI**	
							9.4 (1.7-52.1)c hMG	
Parazzini 1998	1986-91 Italy	23-64	93 / 273	3/0	4/0	-	**27.5 (1.98 - ≈)**	
Mosgaard 1998	1989-94 Denmark	<60	231 / 1721	23 / 14	7 / 3	**1.7 (1.2-2.4)**	**2.2 (1.2-3.9)**	**1.6 (1.1-2.4)**
	nulliparous		37 / 111				3.0 (0.7-12.3) CC / hCG	
Case series:								
Gotlieb 1998	25 years Israel	12-78	82 / -	13/-	10/-	no causative link		

Cohort	Infertility	Follow-	Patients	No. of tumours	SIR (95% CI)	Effect on risk
1st author, year		up year	No.	detected / expected		
Rossing 1994	1974-85 USA	1991	3837	5 / 2	**3.3 (1.1-7.8)**	
	exposed to OI		135			0.9 (0.1-6.1) for hMG/hCG

CC: clomiphene citrate; hCG: human chorionic gonadotrophin; hMG: human menopausal gonadotrophin; No.: number; OI ovulation inducers;
abased on three studies: 19 out of 88 cases infertile and 95 out of 752 infertile controls; b adjusted for age, parity, body mass index, region of birth, education, family history, interviewer; chMG alone

Table 10b. Epidemiological studies on ovarian cancer and infertility, infertility treatments and parity. Risk ratios are given as odds ratios (OR) or standardized incidence ratios (SIR) (95% confidence interval), significant findings are bolded.

Study	Diagnosis of cancer	Cases /Controls			OR for		
1st author, year	Year, country, age (years)	No. / No.	Infertile(%),	Treated (%)	Infertility	Ovulation inducers	Nulliparity
Joly 1974	1957-65 USA	399 / 1153	22/16		**1.5**	-	**1.3**
Booth 1989	1978-83 UK	235 / 451	14 / 8		**1.9**	-	1.7
Whittemore 1992	1956-86 USA, (3 studies)	622 / 1101	15 /12	3 / 1	1.0 (0.8-1.4)	**2.8 (1.3-6.1)**	
	in nulligravida	88 / 101a	39 / 23	14 / 1	2.1 (1.0-4.2)	**27.0 (2.3-315.6)**	
Risch 1994	1989-92 Canada	450 / 564	17 / 11		0.7		**2.6**
Franceschi 1994	1991-93 Italy, 18-75	195 / 1339	2 / 3	1 / 1	0.8	0.7 (0.2-3.3)	1.5
Shushan 1996	1990-93 Israel, 36-64	200b / 408	17 / 11	12 / 7	1.6	1.3 (0.6-2.7)	**3.8**
						3.2 (0.9 -11.8) for hMG	
Mosgaard 1997	1989-94 Denmark, 18-59	684c/1721	22 / 16	4 / 3	**1.5 (1.2-2.0)**	0.8 (0.4-1.3)d	**1.5-2.0**
						0.6 (0.2-1.3)e	**2.7 (1.3-5.5)** *vs* all
Parazzini 1997	1983-91 Italy, 22-74	971 / 2758	1 / 2	0.5 / 0.4	**2.6 (1.5-4.6)**	1.1 (0.4-3.3)g *vs* all	ns
						0.5 (0.1-3.6) *vs* infertile	

Study	Infertility Cohort / End of follow-up			No. of cancers	SIR (95% CI)	Effect on risk	No. of cancers
1st author, year	Time, Country	/ year, mean age	No.	detected / expected			detected / expected
Ron 1987	1964-74 Israel	81	2575	4 / 2.4	2.1 ns	Unclassified infertility 6.1 (1.0-20.0)	2 / 0.3
Brinton 1989	1935-64 USA		2335	11 / 8.6	1.3 ns	Progesterone deficiency associated with an increased risk	
Rossing 1994	1974-85 USA	91 42	3837	11h / 4.4		**2.5 (1.3-4.5) CC-use SIR 3.1 (1.4-5.9)**	11.9 / 2.9
	subcohort					CC use≥ **12 mo RR 11.1 (1.5-82.3)**h 2	
Venn 1995	1978-92, Australia	93	10358	6 / 3.6	1.7 (0.8-3.7)	**Unexplained infertility SIR 7.0 (2.9-16.8)**	5 / 0.7
	exposed to OI		*5564*	*3 / 1.8*	*1.7 (0.6-5.3)*		
Modan 1998	1964-74 Israel	91	2496	12 / 7.2	1.6 (0.8-2.9)	CC use SIR 2.7 (1.0-5.8)	6 / 2.3
	exposed to OI		*1309*	*6 / 3.5*	*1.7 (0.6-3.8)*		
Venn 1999	1978-93 Australia	93	29700	13 / 13.1	1.0 (0.6-1.7)	**Unexplained infertility SIR 2.6 (1.1-6.4)**	5 / 1.9
	exposed to OI		*20583*	*7 / 8.0*	*0.9 (0.4-1.8)*	Duration of exposure, no effect	
Potashnik 1999	1960-84 Israel	94 44	1197	2 / 2.2	0.9 (0.1-3.3)		
	exposed to OI		*780*	*1 / 1.5*	*0.7 (0 -3.8)*		

No.: number; mo: months; OI: ovulation inducers; *vs*: versus, a: 11 of the cases and 6 of the controls had used hMG alone, OR 3.2 (0.9-11.8); b: 164 invasive and 36 borderline epithelial ovarian cancers; b2: relative risk; c: 595 epithelial, 24 stromal, 23 germ cell and 5 unspecified ovarian tumours, OR adjusted for age, residence, use of oral contraceptives and intrauterine device, menopausal status, previous cancer, familial cancer, hormone replacement therapy, and body mass index; d: treated nulliparous; e: treated parous women; f: nulliparous infertile women without treatment; g: adjusted for age, education, parity, oral contraceptives, infertility, use of fertility drugs h: including 4 invasive epithelial cancers, 5 borderline tumours, 2 granulosa cell tumours; adjusted for parity, use of OC, age at 1st birth, body weight, age at menarche, type of infertility.

4.2 Endometrial cancer

In Finland in 1997, the incidence of endometrial cancer was 14.3 / 100,000 (Finnish Cancer Registry, 1997). Long-term exposure to oestrogen action alone increases the risk of endometrial cancer (Henderson *et al*., 1993; Beral *et al*., 1999) as does also tamoxifen (Cardosi and Fiorici, 2000). In addition, patients with a hereditary tendency to nonpolypotic colorectal cancer or endometrial cancer are at increased risk of developing endometrial cancer (Hakala *et al*., 1991; Lynch *et al*., 1998). Tubal sterilization has no effect on this risk (Lacey *et al*., 2000). Endometrial cancer at age • 45 year of age has been related to obesity (• 90 kg, OR 5.5, $CI_{95\%}$ 2.9-10.6), infertility (OR 3.6, $CI_{95\%}$ 1.3-9.9), family history of colonic carcinoma (OR 5.0, $CI_{95\%}$ 1.3-19.1) and nulliparity (OR 2.8, $CI_{95\%}$ 1.1-7.2) (Farquhar *et al*., 1999). Epidemiological studies in infertile patients raise especially those with progesterone deficiency (SIR 9.4, $CI_{95\%}$ 5.0-16.0) and patients with PCOS or obesity (Modan *et al*., 1998; Klip *et al*., 2000) with increased risk which is independent of infertility treatment (Modan *et al*., 1998). In addition, unexplained infertility appears to be a risk factor, although its impact is not so strong (SIR 4.6 ,$CI_{95\%}$ 1.9-11.0) and was reduced in IVF-treated patients (Venn *et al*., 1999). However, within 12 months after IVF the risk of endometrial cancer after IVF may be increased (Venn *et al*., 1999).

4. 3. Cervical cancer

In Finland, the incidence of cervical cancer, which was 3.9 / 100,000 in 1997 has been decreasing until the last few years, when it has started to increase again. Among women with a history of infertility, the risk of cervical cancer is low (SIR 0.5, $CI_{95\%}$ 0.4-0.7), although tubal occlusion may predispose to this risk (Rossing *et al*., 1996a). This finding is supported by a recent association between *Chlamydia trachomatis* infections and cervical cancer (Koskela *et al*., 2000). As regards infertility treatments, it is known that patients treated with CC have a reduced risk of cervical cancer (RR 0.4, 95% $CI_{95\%}$ 0.2-0.8) (Rossing *et al*., 1996a).

4.4. Breast cancer

The incidence of breast cancer in Finnish females was 78.6 /100,000 in 1997 (Finnish Cancer Registry, 1997). Advanced age at the first pregnancy (Apter, 1996), nulliparity, infertility (Weiss *et al*., 1998), and obesity are well-established risk factors for breast cancer. In addition, endometriosis, regardless of infertility, appears to be an independent risk factor for breast cancer (SIR 1.3, $CI_{95\%}$ 1.1-1.4) (Brinton *et al*., 1997). In contrast, young age at the first full-term pregnancy, multiparity, leanness and physical activity appear to protect against breast cancer (Hoffman-Goetz *et al*., 1998).

The stimulatory effect of E2 on neoplastic mammary cells *in vitro* is well established (Fuqua *et al*., 2000), but the roles of gonadotrophins and progesterone are less clear (Bernstein *et al*., 1995; Thompson and Ambrosone, 2000). Earlier studies on infertile women have suggested an increased risk of breast cancer in patients with progesterone deficiency, but later studies did not confirm this association. Epidemiological data have so far failed to show any association between infertility

treatments and breast cancer (Rossing *et al*., 1996b; Westhoff, 1996; Ricci *et al*., 1999). Furthermore, the prospective Nurses´ Health Study II on 116,678 women between 25 and 42 years of age showed a reduced risk of breast cancer in women with ovulatory infertility (RR 0.41, $CI_{95\%}$ 0.2-0.9) (Garland *et al*., 1998). According to one study, however, it may be that within 12 months after IVF the risk of breast cancer is slightly increased (SIR 2.0, $CI_{95\%}$ 1.2-3.2) (Venn et al., 1999). Infertile patients carrying breast cancer gene 1 or 2 mutations (BRCA1, BRCA2) may form a special subgroup, because pregnancy in these patients increases, rather than decreases, the risk of breast cancer (Jernstrom *et al*., 1999). Thus these patients need counselling and perhaps a more conservative approach towards infertility treatments (Armstrong *et al*., 2000).

5. BIOREGULATORS SECRETED BY GRANULOSA CELLS

Granulosa cells surrounding the developing oocyte are separated from the stroma by a basement membrane (Figure 2). Because the granulosa cell layer is avascular, the regulation of granulosa cell function occurs mainly through paracrine or autocrine mechanisms (Geva and Jaffe, 2000). During follicular development, the number of granulosa cells increases 160-fold, there being approximately 47 million such cells in a mature follicle (Gougeon, 1986). In addition to E2, granulosa cells secrete several bioregulators which operate locally in a paracrine and autocrine fashion (Table 3). Yet it must be acknowledged that, although these bioregulators function *in vitro*, we have not enough evidence to be sure that they function *in vivo* (Yeh and Adashi, 1999). During luteinization, granulosa cells undergo rapid neovascularization to form the corpus luteum (Geva and Jaffe, 2000). The corpus luteum begins to secrete progesterone and other substances that are essential for endometrial maturation, implantation and the maintenance of early pregnancy. If pregnancy does not occur, no hCG is available and the corpus luteum becomes avascular and non-functional within 14 days. The regulation and function of the human corpus luteum is still not fully understood, but paracrine and autocrine bioregulators may be significant (Reynolds and Redmer, 1999).

5.1. Inhibins and activins

Inhibins consist of an α-subunit linked to either a β_A-subunit to form inhibin A ($\alpha\beta_A$) or to a β_B-subunit to form inhibin B ($\alpha\beta_B$) (McLachlan *et al*., 1987). Inhibins selectively suppress the synthesis and secretion of FSH from the pituitary, whereas activins, which are homodimers of inhibin β-subunits ($\beta_A\beta_A$, or $\beta_B\beta_B$), stimulate FSH secretion. During a normal menstrual cycle (Ala-Fossi *et al*., 1998) and during ovarian stimulation the level of inhibin B in the serum increases in relation to the E2 level and follicular development whereas the level of inhibin A is elevated during the luteal phase (Hall and Welt, 2000). In patients with GCT and in patients with mucinous ovarian cystadenocarcinomas, the levels of inhibins are elevated and thus the level of inhibin can serve as a tumour marker in the diagnosis and follow-up of these patients (Burger *et al*., 2000).

5.2. Vascular endothelial growth factor

Vascular endothelial growth factor stimulates angiogenesis and enhances vascular permeability (Ferrara *et al*., 1992). It exists in several isoforms; the most abundant form present in most tissues is $VEGF_{165}$ (Geva and Jaffe, 2000). From animal studies we know, that VEGF is essential for angiogenesis of the corpus luteum and further, for progesterone production and endometrial maturation (Ferrara *et al*., 1998; Fraser *et al*., 2000). Of the female reproductive organs, VEGF has been detected in follicular fluid (Lee *et al*., 1997), granulosa cells (Yan *et al*., 1993; Laitinen *et al*., 1997), theca cells (Kamat *et al*., 1995; Suzuki *et al*., 1998), endometrial cells (Charnock-Jones *et al*., 1993; Shifren *et al*., 1996), and placental cells (Sharkey *et al*., 1993; Vuorela *et al*., 1997). The synthesis of VEGF is stimulated, at least *in vitro*, by gonadotrophins and oestrogen (Shifren *et al*., 1996; Laitinen *et al*., 1997; Huang *et al*., 1998; Neulen *et al*., 1998), and by hypoxia (Geva and Jaffe, 2000). These phenomena may also occur *in vivo*, because patients with PCOS (Agrawal *et al*., 1998) and patients with OHSS (Rizk *et al*., 1997) exhibit high serum levels of VEGF. It has been suggested that high VEGF concentrations in the serum on the day of hCG administration (Geva *et al*., 1999) predict the risk of OHSS (Artini *et al*., 1998; Ludwig *et al*., 1999a) but the data are not consistent (Chen *et al*., 2000). On the other hand, it is known that the levels of VEGF in the serum correlate to those measured from follicular fluid (Lee *et al*., 1997) and in follicular fluid, elevated levels of VEGF at time of oocyte retrieval have associated with reduced fertility and suboptimal embryo development (Friedman *et al*., 1998; Barroso *et al*., 1999). Studies on VEGF in patients with endometriosis suggest a role for VEGF as an aetiologic factor in endometriosis (Pellicer *et al*., 2000). Thus, the role of VEGF in ART awaits further confirmation

The data on the circulating levels of VEGF during a normal menstrual cycle are not uniform. Some authors have reported fairly stable levels (Lee *et al*., 1997; Yamamoto *et al*., 1997b; Chung *et al*., 1998) whereas others have found a decrease during the luteal phase of the menstrual cycle (Heer *et al*., 1998). In normal cycles a close dynamic relationship exists between serum progesterone and VEGF concentrations in the follicular fluid (Anasti *et al*., 1998). When interpreting these serum data, it has to be acknowledged that VEGF is also synthesized by platelets (Banks *et al*., 1998; Wartiovaara *et al*., 1998), vascular smooth muscle cells (Tischer *et al*., 1991) and macrophages (Berse *et al*., 1992). These sources are affected by the collection of blood samples and therefore they may well contribute to the VEGF level in the serum (Banks *et al*., 1998) and cause erroneous readings in the assay if the blood sample has not been collected gently.

A role for VEGF as a tumour marker/prognostic factor in breast and ovarian cancers has been proposed (Yamamoto *et al*., 1997a; Tempfer *et al*., 1998; Gasparini, 2000; Shen *et al*., 2000). The preoperative serum VEGF concentration has been an independent prognostic factor for ovarian malignancy when stage, histological grade and lymph node involvement were included in the analysis (Tempfer *et al*., 1998), but not if CA 125 was added to multivariate analysis (Obermair *et al*., 1998). On the other hand, patients with malignant (especially serous) ovarian cystic tumours had significantly

higher levels of VEGF both in the follicular fluid and in the serum than patients with benign ovarian tumours (Boss *et al*., 2001). Taken together, the present data suggest that VEGF is an important factor in physiological and unphysiological angiogenesis in the genital organs (Geva and Jaffe, 2000) and it is possible that VEGF has potential to become a tumour marker for ovarian tumours.

5.3. Leptin

Leptin acts as a signal between the hypothalamus and the adipose tissue, which is the main energy reserve (Zhang *et al*., 1994; Montague *et al*., 1997; Himms-Hagen, 1999). In addition, it may have a permissive role in female puberty (Apter, 1997), because its levels increase at puberty and are two to three times higher in women than in men (Koistinen *et al*., 1998). Administration of leptin to leptin-deficient, infertile obese mice not only reduced their weight but restored their fertility (Barash *et al*., 1996). In a leptin-deficient girl, administration of leptin induced detectable LH pulses, which are essential for the initiation of menstrual cycles (Farooqi *et al*., 1999). Thus, leptin seems to be a regulator in human reproduction. In addition, it is an angiogenetic factor (Bouloumie *et al*., 1998) and may have a role in haemopoiesis and immunity (Gonzalez *et al*., 2000).

Most of the leptin present in human blood is secreted from white fat cells, but in women of fertile age a minor part may also come from the granulosa cells (Antczak *et al*., 1997) and during pregnancy also from the placental cells (Masuzaki *et al*., 1997). The secretion of leptin is pulsatile and seems to be synchronized with the LH pulses (Licinio *et al*., 1998). Leptin receptors are expressed in the anterior pituitary, in some other parts of the brain, in the ovarian granulosa and theca cells, and in the secretory endometrial cells (Gonzalez *et al*., 2000). During a normal menstrual cycle, the leptin levels in the serum rise during the follicular phase and reach their peak during the luteal phase (Hardie *et al*., 1997; Shimizu *et al*., 1997; Mannucci *et al*., 1998; Messinis *et al*., 1998; Riad-Gabriel *et al*., 1998; Cella *et al*., 2000). However, not all data are in line with this conclusion, since stable levels of leptin have been reported during the menstrual cycle (Teirmaa *et al*., 1998; Cella *et al*., 2000). The regulation of leptin secretion in humans is complex (Figure 1), but E2 is thought to have a stimulatory role (Mannucci *et al*., 1998; Messinis *et al*., 1998; Strowitzki *et al*., 1998; Bützow *et al*., 1999; Stock *et al*., 1999; Lindheim *et al*., 2000; Zhao *et al*., 2000). The production of leptin from trophoblasts (Masuzaki *et al*., 1997) contributes to the raised serum levels of leptin during pregnancy (Laivuori *et al*., 2000).

It is not known whether leptin has a role in human infertility. The data on leptin levels in women with PCOS, a disease often associated with obesity and anovulatory infertility, are inconsistent (Brzechffa *et al*., 1996; Caro, 1997; Rouru *et al*., 1997; El Orabi *et al*., 1999; Jacobs and Conway, 1999; Sir-Petermann *et al*., 1999). In patients with endometriosis, the levels of leptin in the serum and peritoneal fluid have been found to be higher than (Matarese *et al*., 2000) or similar to (Matalliotakis *et al*., 2000) those in matched control subjects. Leptin levels were lower in advanced-stage endometriosis than in early stage endometriosis (Matarese *et al*., 2000) and treatment with GnRH agonist (leuprolide) and danazol increased the leptin levels (Matalliotakis *et al*., 2000). Leptin receptors are, indeed, present in endometrial cells (Kitawaki *et al*., 2000) making it seem probable that leptin has a role, not only in pathophysiology of the endometriosis, but also in the infertility caused by endometriosis, probably in unexplained infertility, and also in implantation and thus in predicting the outcome of ART (Mantzoros *et al*., 2000). Recently, subfertile ovulatory patients with the endometrial maturation defect were shown to be deficient in functioning leptin receptors, in spite of adequate concentrations of serum hormones and normal steroid hormone receptors (Alfer *et al*., 2000). However, these data are sparse and at present no consensus exists with regard to the impact of leptin in human infertility. In addition,

the ability of leptin to induce angiogenesis (Bouloumie *et al*., 1998) and the stimulatory effect of hypoxia on its secretion (Barroso *et al*., 1999) may link leptin with tumours, probably at least with breast cancer (Tessitore *et al*., 2000).

6. TUMOUR MARKERS ASSOCIATED WITH GENITAL CANCERS

Tumour markers are compounds (usually proteins) produced by malignant cells which appear in serum in such large quantities that their assessment is possible. Thus, measurement of these markers may help the diagnosis and follow-up of these patients (Lindblom and Liljegren, 2000).

6.1. Cancer antigen 125

Cancer antigen 125 (CA 125) is a high-molecular-weight glycoprotein (Bast *et al.*, 1981), the release of which is linked to the epidermal growth factor (EGF) and cell surface proteases (O'Brien *et al*., 1998). The main source of pathological CA 125 production is malignant ovarian cells. Ca 125 is also expressed in the coelomic epithelium (the mesothelial cells, endometrium, fallopian tubes, endocervix and amniotic cells) and in the epithelial cells of ovarian cysts and their papillary projections (Zeimet *et al*., 1998). Thus elevated levels of CA 125 (• 35 IU/L) can be seen in both benign and malignant diseases (Table 11) (Halila *et al*., 1986; Lehtovirta *et al*., 1990; Bast *et al*., 1998; Abrao *et al*., 1999). However, the levels are much more higher in cancer, especially in the serous type of ovarian cancer, cancer of the Fallopian tubes and endometrial adenocarcinomas (Bast *et al*., 1998).

There is no consensus as regards the role of CA 125 in ovarian function. Hyperoestrogenism may either increase (Lanzone *et al*., 1990; Zweers *et al*., 1990; Ozaksit *et al*., 1993) or cause no change in the level of CA 125 (Phocas *et al*., 1994). Yet patients with OHSS express high levels of serum CA 125 (Jager *et al*., 1987; Scarpellini and Scarpellini, 1992; Ozaksit *et al*., 1993). In addition, follicle fluid is rich in CA 125 (Mordel *et al*., 1992), but ovarian volume is not a determinant of CA 125 in serum (Granberg *et al*., 1990). However, the levels of gonadotrophins or gonadal steroids are not consistently correlated with serum CA 125 (Lehtovirta *et al*., 1990; Jimena *et al*., 1993; Ozaksit *et al*., 1993; Bon *et al*., 1999). The role of CA 125 in predicting the likelihood of pregnancy after IVF-ET is also disputable (Miller *et al*., 1996; Brandenberger *et al*., 1998; Noci *et al*., 1999; Baalbergen *et al*., 2000). As is evident from the above, healthy reproductive organs produce CA 125 but, in malignancy, this production is enhanced. Moreover, the impact of ovarian hyperstimulation and/or of IVF on CA 125 levels has not been established so far.

6.2. Tumour-associated trypsin inhibitor

Tumour-associated trypsin inhibitor (TATI) is a 6 kD peptide isolated from the urine of a patient with ovarian cancer (Stenman *et al*., 1982). Later, TATI was found to be identical to the pancreatic secretory trypsin inhibitor (Bartelt *et al*., 1977). One of TATI´s effects is to inhibit the proteolytic activity of trypsin and other proteases and so prevent the tissue damage that is caused by trypsin release (Stenman *et al*., 1991; Marchbank *et al*., 1998). In addition, TATI stimulates the growth of human endothelial cells and stimulates mucosal repair at sites of injury (Marchbank *et al*., 1998).

In healthy subjects, TATI is present in low concentrations, and removal of the pancreas does not bring this level to zero (Halila *et al*., 1985). Mucus-producing cells in the gastro-intestinal tract, lungs, liver, kidney, ovary and breast also produce TATI (Marchbank *et al*., 1998). In patients with mucinous ovarian tumours and also in those with some other malignancies serum levels of TATI are raised (Table 11) (Stenman *et al*., 1991; Pectasides *et al*., 1996; Venesmaa *et al*., 1998). Moreover, it is known that endometriosis (Medl *et al*., 1997) and renal failure (Tramonti *et al*., 1998) increase the levels of TATI in serum. However, TATI levels have not been studied in serum during normal menstrual cycles nor has its response to ovarian stimulation.

6.3. Free β-subunit of human chorionic gonadotrophin

Human chorionic gonadotrophin is composed of α- and β-subunits. The β-subunit of hCG (hCGβ) has 84% homology with the LHβ-subunit (Figure 3) and thus both subunits can bind to a common gonadal receptor (Schally *et al*., 1999a). Intact hCG stimulates progesterone production in the corpus luteum during the first trimester of pregnancy, but the free α- and β-subunits lack gonadotrophic activity (Albanese *et al*., 1996). These subunits are secreted by the pituitary and the placenta, and their carbohydrate structure depends on their source (Blithe and Iles, 1995). Intact hCG and the β-subunit are excellent markers for trophoblastic diseases (Alfthan and Stenman, 1996; Vartiainen *et al*., 1998). In addition, both subunits are also secreted in a number of non-trophoblastic malignancies (Table 11) (Braunstein *et al*., 1978; Iles *et al*., 1990; Alfthan *et al*., 1992; Webb *et al*., 1996).

In non-pregnant women, hCG is secreted in a pulsatile fashion, similarly to LH (Odell and Griffin, 1987). In IVF cycles resulting in pregnancy, the level of intact hCG begins to rise 7 to 8 days after ET and doubles in 1.9 days and peaks 6 to 10 weeks later. The secretion of hCGβ in relation to total hCG is highest during the first 2 weeks of pregnancy, then gradually decreasing from the 4th to the 12th week after the last menstrual period (Alfthan *et al*., 1988). The fact that the levels of free hCGβ may be high in IVF pregnancies may cause false-positive alarms on serum screenings for Down's syndrome (Wald *et al*., 1999). However, the impact of IVF on the two hCG subunits has not yet been established.

6.4. Common free α-subunit of glycoprotein hormones

The common free α-subunit of glycoprotein hormones (GPHα) is encoded by a single gene, while β-subunits of the different glycoproteins are encoded by several different genes (Albanese *et al*., 1996). GPHα is present in serum of both pregnant and non-pregnant women. In non-pregnant women, GPHα is derived from the pituitary gonadotrophes or thyrotrophes and its secretion is regulated by GnRH and probably also by oestrogen (Albanese *et al*., 1996). The biological function of GPHα is not fully known. It is postulated that it might play a role as a paracrine regulator of prolactin secretion (Blithe and Iles, 1995) or human endometrial cell differentiation (Nemansky *et al*., 1998).

During the menopause the levels of GPHα rise 10-fold, as also during the use of GnRH agonists, while the use of a GnRH antagonist totally blocks GPHα secretion (Fluker *et al*., 1994). During pregnancy, GPHα is derived from the placental syncytiotrophoblasts and the levels increase (Korhonen *et al*., 1997). In addition, in patients with malignancies the level of GPHα may be elevated (Table 11) (Honegger *et al*., 1995). However, the excretion of GPHα during IVF programmes or in different types of infertility has not been studied.

Table 11. Some tumour markers used for the diagnosis and follow-up of gynaecological malignancies.

Tumour marker	Upper normal limit	Half-life	Causes of elevations in serum (references in text)
Cancer antigen 125	35 IU/mL	5-10 days[1]	Menstruation, I trimester pregnancy, endometriosis, fibroids, Adenomyosis, pelvic inflammatory disease, surgery, Peritonitis, pleuritis, hepatitis, mesotheliomas, immature teratomas Cancer of the ovary, fallopian tubes, endometrium, breast, colon, lung, pancreas, and lymphomas.
α-subunit of GPH	< 31 pmol/l[2]	< 6 hours[3]	Menopause, GnRH analogues, bronchogenic tumours Pituitary tumours, carcinoids, insulinomas, cancer of the cervix
β-subunit of hCG	< 1 pmol/L	23 hours[3]	Pregnancy, trophoblastic diseases, Cancer of the ovary, cervix, pancreas, biliary duct, bladder
TATI	< 2 nmol/L	6-8 min[4]	Smoking, endometriosis, renal failure, mucinous ovarian tumours Cancer of the ovary, pancreas, lungs, bladder

GnRH: gonadotrophin-releasing hormone; GPH: glycoprotein hormone; hCG: human chorionic gonadotrophin; TATI: tumour-associated trypsin inhibitor. [1]Bidart *et al*., 1999; [2] age < 45 years for age; •45 years upper normal limit < 57.9 pmol/L (Alfthan H, unpublished data); [3]Korhonen *et al*., 1997; [4]Marchbank *et al*., 1998

III AIMS OF THE STUDY

The present study was undertaken to investigate the impact of the use of ovulation inducers on the occurrence of GCTs in Finland, and the impact of an IVF programme on agents which are either derived from ovaries or otherwise associated with gynaecological tumours.

The specific aims were:

1. to study the incidence of GCT in relation to the use of ovulation inducers in Finland in 1965 - 1994,

2. to elucidate the reproductive features of patients with GCT at fertile age,

3. to study the effect of an IVF programme on serum levels of VEGF and leptin,

4. to study the effect of an IVF programme on serum levels of the following tumour markers: CA 125, TATI, hCGβ and GPHα,

5. and to study the influence of very early IVF-pregnancy on the serum levels of VEGF, leptin, CA 125 and TATI.

IV SUBJECTS AND METHODS

These studies were undertaken during 1995-1999 with the approval of the Ethics Committee of the Department of Obstetrics and Gynaecology, University Central Hospital, Helsinki. The patients in studies III-V signed their consent after being informed of the purpose, nature and possible risks of the study.

1. SUBJECTS

1. 1. Study I

The study on the incidence of GCT in Finland included all the patients who were diagnosed as having had GCT and were reported to the Finnish Cancer Registry in 1965-1994 (Table 12). This register covers almost 100% of the cancer patients in this country (Teppo *et al*., 1994). For comparison, all the patients with ovarian malignancies during the same time period were also studied. Sales statistics of ovulation inducers (CC, hMG, FSH) and OCs were collected from the National Agency for Medicines and from the Institute for Medical Statistics and the units (packages) sold were calculated to indicate the number of courses as follows: one course of CC means 100 mg of CC daily for 5 days, that of hMG/FSH 150 IU of FSH daily for 10 days, and one cycle of OC the use of OC for 28 days (Table 12).

1.2. Study II

The reproductive histories of patients with GCT were analysed among a group of 146 patients with GCT diagnosed at Helsinki University Central Hospital during the 40-year period 1956 - 1996 (Table 12). The data included 14 patients with mixed granulosa-theca cell tumours with a clear granulosa cell component. The staging of the tumours was standardized according to FIGO criteria (ACOG, 1992). Menstrual history, occurrence of infertility, use of any hormones and clinical symptoms were recorded from the patients´ charts. The reproductive characteristics of patients with GCT at a fertile age (from 16 to 45 years, n=50) were compared with those who had GCT later in life (from 46 to 75 years, n=96). In addition, the subgroup of patients who had been at a fertile age since 1966 and could have been users of ovulation inducers (n=41) was analyzed separately. The results concerning fertility were compared with the data of the two population–based databanks (Table 12) on fertility features in Finland in general (Nikander, 1992) or among women participating in the Pap screening programme in Helsinki (Rantala and Koskimies, 1986). The age of menarche and body mass index were compared to national averages (Widholm and Kantero, 1971; Pietinen *et al*., 1996).

1.3. Studies III-V

The entire study population consisted of 71 consecutive patients, who were infertile because of either tubal occlusion (n=31), endometriosis (n=10) or some unknown cause(s) (n=30) and who took part in their first IVF treatment at our clinic (Study V). Subgroups of 37 (Study III), and of 69 (Study IV) of the original 71 infertile patients were investigated for VEGF and leptin, respectively (Table 12). For the VEGF analysis (Study III), patients with term pregnancies and patients for whom 4-6 serum samples were available so that at least one sample was taken after OPU, were included randomly. The subgroup for leptin analysis included all patients with OPU who had 4 (n=2), 5 (n=8) or 6 (n=51) serum samples for analysis (Study IV).

The relevant clinical characteristics, such as weight, height, parity, medications, smoking and family history of cancer were recorded. In study IV the patients with a BMI of less than 19.4 kg/m^2 were classified as "underweight", those with a BMI between 19.5 kg/m^2 to 26.5 kg/m^2 as "normal weight", and those with a BMI of 26.5 kg/m^2 or more as "overweight"(Bianco *et al*., 1998).

The control population consisted of 10 healthy women (Study III), who did not use any hormonal contraceptives, intrauterine device or medication. In study V, one of the original 10 control women was excluded because of her apparent infertility (Table 12). The distribution of patients and controls among the various study groups is described in detail in the original papers.

Table 12. Study subjects (data given as mean ± standard deviation).

Patients, Controls, Materials by Study	Source of Data	No. of Subjects	Time Period	Age, years	Body Mass Index , kg/m^2
I a) Females with GCT	Finnish Cancer Registry	590	1965 - 1994	all ages	NA
b) Females with ovarian cancer	Finnish Cancer Registry	12650	1965 - 1994	all ages	NA
c) Sales statistics of ovulation	National Agency for Medicines	-	1981 - 1994	-	-
inducers and oral contraceptives	Institute for Medical Statistics	-	1965 - 1994	-	-
II a) Patients with GCT	HUCH, Dept. Obst. & Gyn.	146	1956 - 1996	53.0±15.3	25.6±4.9
b) Nikander, 1992	Population study, Finland	5105	1989	22 - 55	NA
c) Rantala & Koskimies, 1986	Pap screening, Helsinki area	4710	1982	30, 35, 40	NA
III a) Infertile women and IVF	HUCH, Dept Obst. & Gyn.	37	1995 - 1996	32.9±4.1	23.4±4.1
b) Healthy controls	volunteers	10	1996	35.3±4.7	21.7±2.7
IV Infertile women and IVF	HUCH, Dept Obst. & Gyn.	69	1995 - 1996	33.2±3.9	22.9±3.6
V a) Infertile women and IVF	HUCH, Dept Obst. & Gyn.	71	1995 - 1996	33.3±3.8	23.0±3.5
b) Healthy controls	volunteers	9	1996	34.8±4.9	21.4±2.8

GCT: granulosa cell tumour; Dept. Obst. & Gyn.; Department of Obstetrics and Gynaecology, HUCH: Helsinki University Central Hospital; IVF: *in vitro* fertilization programme; NA: not available

2. PROTOCOL

2.1. Ovarian stimulation protocol

The IVF programme consisted of long pituitary down-regulation with a GnRH agonist (nafarelin, Synarela®, Syntex, Södertälje, Sweden) which was initiated at a daily dose of 800 µg during the approximated midluteal phase of a preceding spontaneous menstrual cycle. The phase of the cycle was determined by calculating midluteal phase from the known length of previous menstrual periods and the luteal phase was confirmed by a transvaginal ultrasound finding of a secretory endometrium and absence of follicles, or presence of a corpus luteum. In the presence of ovarian suppression (defined as a double-layer of endometrium • 5 mm thick, and serum E2 concentrations of less than 0.1 nmol/L), the dose of the GnRH agonist was halved and ovarian stimulation was started by giving randomly either urinary gonadotrophins (Humegon® n=15, Organon, Oss, the Netherlands, or Pergonal® n=19, Laboratories Serono S.A., Aubonne, Switzerland, containing equal amounts of FSH and LH) or purified urinary FSH (Fertinorm HP® n=16, Laboratories Serono S.A., or Follegon® n=19, Organon) at a daily dose of 150 IU (n=58) or 225 IU (n=11). The dosage was adjusted after the first 5 days according to the ovarian response. Human chorionic gonadotrophin (Pregnyl®, Organon) was injected (10 000 IU) when at least three follicles exceeded 16 mm in diameter. A transvaginal OPU was performed 36 hours later. Ova were fertilized *in vitro*, and a maximum of two embryos were transferred 2 (n=48) or 3 days (n=11) after OPU. In four patients, fertilization failed to produce any embryos , and in an additional four patients, ET was not done because of predictive signs of OHSS (serum E_2 • 15 nmol/L and the presence of more than 20 follicles). After ET, 600 mg of micronized progesterone (Lugesteron®, Leiras, Turku, Finland) was given daily intravaginally for 2 weeks. A consistently rising hCG level was judged to indicate clinical pregnancy, whereas a transient rise in hCG followed by profuse bleeding within 2 weeks was regarded as a subclinical abortion (Table 17). If pregnancy was not achieved, the patient was asked to come for a follow-up visit in the follicular phase (pd 8-10) of her second menstrual cycle after IVF, when her ovaries and uterus were investigated by transvaginal ultrasound (Study V).

2.2. Timing of blood sampling and assays used

Before, during and after ovarian stimulation, frequent blood samples were collected in the morning after an overnight fast, as indicated in Table 13. The blood sample taken prior to IVF treatment (sample I) is called "baseline or basal" in the IVF patients. In addition, eight random patients (four with tubal occlusion and four with unexplained infertility) provided blood samples every second or third day during treatment with a GnRH agonist to permit a study of the effect of ovarian suppression on the levels of pituitary hormones (Study V). Ten healthy control women provided blood samples every second or third day during one menstrual cycle (10–14 samples per subject). The day of ovulation in the menstrual cycle was determined from the LH surge and progesterone rise. The cycle was divided into the menstrual phase (days from –15 to -9, n = 21 samples), the follicular phase (days -8 to -3, n = 20), the periovulatory phase (ovulation ± 2 days, n=17), and the luteal phase (days +3 to +

14, n = 40). The assays used are described in Table 14. The conversion factor for E2 was 1 pmol/L = 3.671 pg/mL, and that for progesterone 1 nmol/L = 3.180 ng/mL.

3. STATISTICAL ANALYSES

Variables that were not normally distributed by the Kolmogorov-Smirnov normality test were logarithmically (log_{10}) transformed for parametric testing. Comparisons between groups were performed with the chi square test (categorical variables), Student's two-tailed unpaired t test (continuous variables, two groups), analysis of variance (ANOVA, continuous variables, several groups) or analysis of covariance (ANCOVA). For within-group analysis, Student's two-tailed paired t test or repeated measures analysis of variance with Fisher's PLDS (Study IV) or with the Bonferroni-Dunn post hoc test were used (Study V). Relations between continuous variables were calculated with linear or multiple regression. A *P* value of < 0.05 was considered significant.

Table 13. Timing of collection of blood samples during the *in vitro* fertilization programme in studies III-V.

Blood sampling Number	Time (mean ± SD)	Study III VEGF	Study IV Leptin	Study V Tumour markers
I	Prior to start of GnRH agonist (cycle day 22.3 ± 0.3)	37	66	71
II	At ovarian suppression	37	66	69
III	After 4 to 5 days of ovarian stimulation			69
IV	3 to 5 days before OPU	21	62	62
V	At OPU	37	66	67
VI	At ET, 2 to 3 days after OPU			66
VII	5 to 6 days after OPU			31
VIII	8 to 9 days after OPU	20	64	63
IX	10 to 12 days after OPU			30
X	14 days after OPU	36	60	60
XI	Second cycle after IVF (cycle day 9.6±1.8)			42

ET: embryo transfer; GnRH: gonadotrophin-releasing hormone; IVF: *in vitro* fertilization programme; OPU: ovum pick-up; SD: standard deviation; VEGF: vascular endothelial growth factor

Table 14. Characteristics of assays used.

Factor	Principle of assay	Source of reagents	Intra- and interassay CV% (in the concentration range)	
CA 125	Immuno1®immunoanalyser OC 125 and M 11 antibodies	Bayer Corp., Tarrytown, NY, USA Centocor, Malvern, PA, USA	< 4	(15-490 kU/L)
FSH	Fluoroimmunoassay, AutoDELFIA™	DELFIA, Wallac, Turku, Finland	< 3	(2.6-44.5 IU/L)
GPHα	Fluoroimmunoassay	in-house method[1]	< 10	(10-1000 pmol/L)
hCGβ	Fluoroimmunoassay	in-house method[2]	< 10	(1.0–5000 pmol/L)
hCG dimer	Fluoroimmunoassay	DELFIA	< 8	(7-940 IU/L)
Leptin	RIA	Linco Research, St.Charles, USA	< 5	(2.8-15.6 µg/L)
LH	Fluoroimmunoassay, AutoDELFIA™, “LHspec”	DELFIA	< 4	(0.3–42.0 IU/L)
Oestradiol	RIA	Orion Diagnostica, Espoo, Finland	< 9	(0.4-9 nmol/L)
Progesterone	RIA	Orion Diagnostica	< 10	(2.1–45 nmol/L)
TATI	RIA	Orion Diagnostica	< 11	(1-60 nmol/L)
TSH	Fluoroimmunoassay, ACS:180 autoanalyser	Ciba Corning, Halstead, Essex, UK	< 7	(0.6-26 mIU/L)
VEGF	EIA, Quantikine™	R&D Systems, Abingdon, UK	< 9	(15-2000 pg/mL)

CV: coefficient of variation; EIA: enzyme immunoassay; FSH: follicle stimulating hormone;

GPHα: common α-subunit of glycoprotein hormones; hCG: human chorionic gonadotrophin;

LH: luteinizing hormone; RIA: radioimmunoassay; TATI: tumour-associated trypsin inhibitor;

TSH: thyroid-stimulating hormone; VEGF: vascular endothelial growth factor;

[1] Alfthan *et al*., 1988; [2] Korhonen *et al*., 1997

V RESULTS

1. INCIDENCE OF GRANULOSA CELL TUMOUR IN FINLAND (Study I)

The incidence of GCTs rose from 0.74 / 100,000 women in 1965-69 to 0.82 / 100,000 in 1970-74, but thereafter the incidence fell by 40% to 0.47 / 100,000 women in 1985-94 (Figure 6). At the same time, the consumption of CC increased 13-fold from 1966 to 1988. Gonadotrophins (hMG, FSH) were used minimally up to the late 80s, after which their use showed a sharp 200-fold rise. At the same time the incidence of all ovarian malignancies, including GCT, rose by 28% from 10.6 / 100,000 to 13.6 / 100,000, although the use of combined OCs steadily increased from the 1980s to the 90s, showing a 5-fold rise since 1966. The decrease in the incidence of GCTs occurred mainly in women aged 15 to 29 and in those aged 45 to 74 years (Table 15).

Table 15. Incidence and number of ovarian granulosa cell tumours in Finland in 1965-94 by 5-year periods and age.

Time	Incidence / 100,000 in age groups, years						
period	<15	15-29	30-44	45-59	60-74	75	No.
1965-69	0.07	0.29	0.54	2.19	2.16	1.60	108
1970-74	0	0.26	0.95	2.06	2.47	0.93	122
1975-79	0.04	0.20	0.93	2.13	1.47	1.50	109
1980-84	0.04	0.10	0.56	1.60	1.44	0.76	81
1985-89	0	0.02	0.56	1.41	1.21	1.84	84
1990-94	0.09	0.08	0.64	0.84	0.84	0.62	86
Total	0.04	0.16	0.67	1.71	1.78	1.21	
No. (%)	6 (1)	30 (5)	103 (17)	216 (37)	186 (32)	49 (8)	590 (100)

No.: number

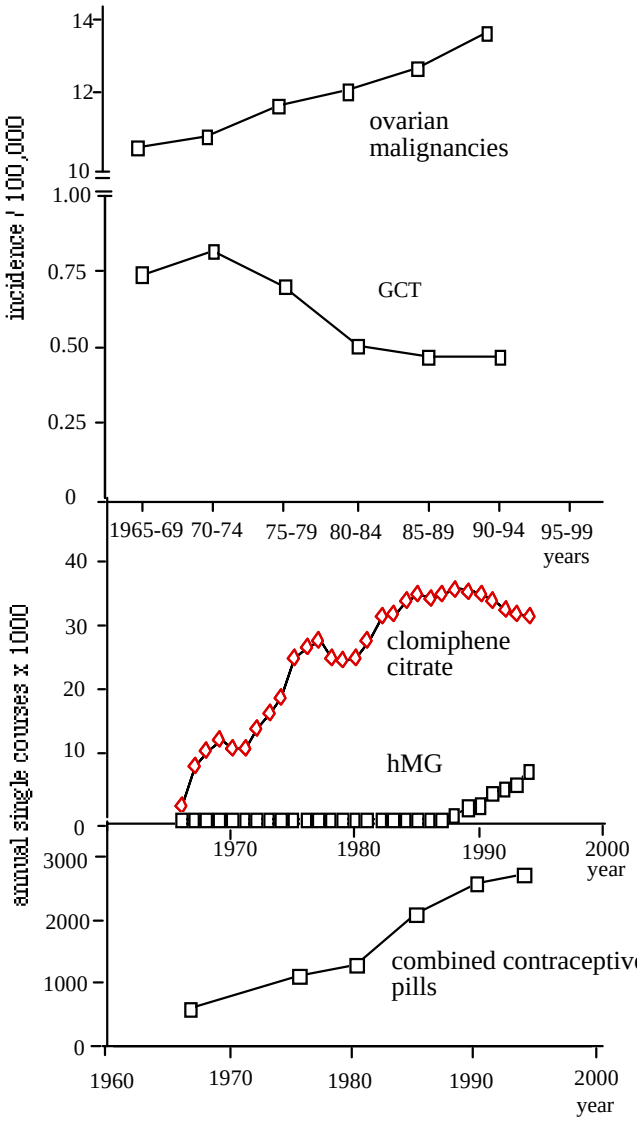


Figure 6. Incidence of ovarian malignancies and granulosa cell tumour (GCT) in 5-year periods as well as the number of annual courses of clomiphene citrate, human menopausal gonadotrophins (hMG) and combination contraceptive pills in Finland in 1965-94.

2. REPRODUCTIVE FEATURES OF PATIENTS WITH GRANULOSA CELL TUMOUR (Study II)

Of the 146 women studied, 50 had GCT diagnosed at a potentially fertile age (Table 16), and 118 after the introduction of ovulation inducers in 1966. The age at diagnosis for all cases ranged from 16 to 87 years, with a mean age of 53.0 years. The majority of GCTs (82%) occurred in women between 35 and 75 years of age and over half of the patients were postmenopausal (56%), the median time from menopause to GCT diagnosis being 13 years (range 0 - 36). Four women (5%) had reached the menopause before the age of 40.

The age at menarche (range 10 - 18 years) was comparable to the national average if GCT occurred at a fertile age, but the frequency of nulliparity was almost twice as high as in Finland generally (Table 16). If GCT was diagnosed after the fertile age, the range of ages at menarche was similar, and over half of the patients were multiparous (53%), corresponding to the national average. In the GCT series as a whole, three women (2%) were pregnant at the time of GCT diagnosis, whereas 12 women (9%) had experienced infertility, mainly anovulatory infertility (58%). The frequency of primary infertility was high in the women with GCT at a fertile age (23%), as the national average was 9%. Of the infertile women, five had used fertility drugs for 1 to 10 months, but none had participated in an IVF programme. Thus the frequency of users of ovulation inducers was 4.2% (5/118). The tumours in women with a history of use of ovulation inducers were detected at stage I and mostly within a year after the first use of an ovulation inducer. After removal of the tumour, all five women became pregnant normally. The number of patients with GCT at a fertile age showed no increase during the four decades studied (24% in 1956-66, 28% in 1967-76, 22% in 1977-86 and 26% in 1987-96).

The most common gynaecological symptom of GCT at a fertile age was oligo-amenorrhea (45%) whereas the most common symptom at an older age was dysfunctional bleeding (71%). Hyperplasia of the endometrium was encountered in all age groups, but was more frequent if GCT was diagnosed after the age of 45 (Table 16). Cancer of the endometrium was detected only in patients with GCT after the fertile age.

Table 16. Reproductive characteristics and endometrial histology of 146 patients with a granulosa cell tumour in relation to fertile age (• 45 years). The population-based data (PBD) are given for reference with corresponding age-adjusted data of the current series.

	•45 years 50 (34) No. (%)	> 45 years 96 (66) No. (%)	Total	Data available No. (%[1])	Subgroup[2] No. (%)	PBD No.(%)
Menarche (mean, years)	13.4	14.4	14.0	114 (78)		13.2[a]
Age at diagnosis (median, years)	37.0	61.0	54.0	146 (100)		
Body mass index (mean, kg/cm2)	24.8	26.0	25.6	142 (97)	25.5,	25.1[b]
BMI •30	6 / 48 (13)	16 / 94 (17)	22 (15)		11 / 86 (13)	14%[b]
Parity, No.	50	92		142 (97)	62	
Mean parity	1.1	2.0	1.7		1.3	1.8[c]
Nulliparous	25 (50)	21 (23)	46 (32)		27 (39)	24%[c]
Primiparous	9 (18)	22 (24)	31 (22)		11 (18)	17%[c]
Multiparous	16 (32)	49 (53)	65 (46)		27 (44)	59%[c]
Infertility	11 / 48 (23)	1 / 88 (1)	12 (9)	135 (93)		-
age <40, primary infertility					7 / 31 (23)	9%[c2]
age 30-40, total infertility					6 / 26 (23)	15%[d]
Histology of the endometrium, No.	41	86	127 (87)			
Endometrial hyperplasia	8 (20)	30 (35)	38 (30)			
Endometrial polyp	3 (7)	11 (13)	14 (11)			
Adenomatous hyperplasia	1 (2)	10 (12)	11 (9)			
Cancer	- (-)	10 (12)	10 (8)			
Pregnancy	3 (7)	-	3 (2)			
Normal	26 (63)	25 (29)	54 (42)			

No.: number; [1] percentages calculated from the data available; [2] analysis of the same age groups as the reference. Subgroups in the GCT series corresponding to the age group of the reference study: Pietinen *et al*., 1996 (n=86; age 30-59 years in our series); Nikander, 1992 (n=62; age 22-51 years in our series); Nikander , 1992 (n=31; age<40 years in our series); Rantala and Koskimies, 1986 (n=26; age 30-40 years in our series)

[a]Widholm and Kantero, 1971 (n=8111 girls, 10-20 years in 1969); [b]Pietinen *et al*, 1996 (n=734, 30-59 years in Helsinki area in 1992); [c]Nikander, 1992 (n=4155, 22-51 years in 1989); [c2]Nikander, 1992 (n=836 nulliparous <40 years); [d]Rantala and Koskimies, 1986 (n=4202, 30, 35 or 40 years in 1982).

3. STUDIES ON *IN VITRO* FERTILIZATION CYCLES AND ON VERY EARLY PREGNANCY

In all, 71 cycles with controlled ovarian hyperstimulation were studied. Of them, four patients failed to become stimulated so 67 patients finally underwent OPU. Embryo transfer was performed in 59 patients, because the programme failed to produce transferable embryos in four women and an additional four patients experienced no ET because of signs of severe OHSS (Table 17). All the patients were treated at the outpatient clinic. Of the 59 patients with ET, 18 (31%) achieved clinical pregnancies, 13 delivered at term (22%), and three of them had twins (23%). Of the 49 patients not achieving pregnancy, 42 (79%) participated in the follow-up study 2 months after IVF treatment. The patients undergoing IVF and normal cycles were comparable in regard to age, BMI, and pre-treatment FSH level (Tables 12 and 17).

Table 17. Clinical characteristics (mean ± SD) of the study populations.

	Total series	Causes of infertility			Controls
		Tubal occlusion	Pelvic endometriosis	Unexplained infertility	
Patients beginning GnRHa	*71*	*31*	*10*	*30*	*9*
Basal FSH (IU/L)[1]	5.9 ± 2.0	6.0 ± 1.7	5.7 ± 1.2	6.0 ± 2.7	5.8 ± 1.8
Ovarian suppression (days)	16.6 ± 4.7	15.8 ± 2.1	16.5 ± 2.9	17.4 ± 6.7	
Patients beginning hMG	*69*	*30*	*9*	*30*	
- duration (days)	10.7 ± 1.4	10.9 ± 1.1	10.2 ± 1.0	10.7 ± 1.7	
- gonadotrophin use (IU)	2002 ± 639	1955 ± 553	1933 ± 613	2070 ± 734	
Maximal oestradiol (pg/mL)	1202 ± 1202	1453 ± 1589	1335 ± 1195	986 ± 594	182 ± 85 *
(nmol/L)	4.5 ± 4.4	5.3 ± 5.8	4.9 ± 4.4	3.6 ± 2.2	0.7 ± 0.3
Patients with OPU	*67*	*29*	*9*	*29*	
Number of follicles	16.1 ± 8.6	16.7 ± 9.7	18.0 ± 8.4	15.0 ± 7.5	
Number of oocytes	11.4 ± 6.3	11.8 ± 7.1	12.9 ± 6.8	10.5 ± 5.4	
Fertilization (%)	53 ± 27	61 ± 26	51 ± 27	44 ± 25	
Patients with ET	*59*	*25*	*7*	*27*	
Progesterone[2] (ng/mL)	21.2 ± 19.3	23.3 ± 25.3	19.4 ± 11.5	22.9 ± 17.0	12.0 ± 4.6
(nmol/L)	69.7 ± 62.7	70.5 ± 76.6	58.7 ± 34.9	72.2 ± 53.1	37.6 ± 15.6
Subclinical abortion	5	1	-	4	
1st trimester miscarriage	5	4	-	1	
Delivery	13	6	2	5	
Patients with follow-up[3]	*42*	*17*	*7*	*18*	

BMI: body mass index; ET: embryo transfer; FSH: follicle-stimulating hormone; GnRHa: gonadotrophin-releasing hormone agonist; hMG: human menopausal gonadotrophin; OPU: ovum pick-up;
[1] early luteal phase of a normal cycle prior to treatment [2] 8 days after OPU / controls: 8 days after ovulation
[3] follow-up visit 2 months after *in vitro* fertilization programme
* $p < 0.05$; no other comparisons between different groups were significant (ANOVA)

3.1. Vascular endothelial growth factor (Study III)

Prior to IVF treatment, the midluteal VEGF levels in serum ranged from 10 pg/mL to 679 pg/mL. No differences were observed in the serum levels between the different infertility groups or between the IVF patients and the control population.

IVF Cycle: Ovarian suppression did not affect VEGF levels in serum. However, preceding and following OPU, the serum VEGF levels rose and were significantly higher at 8-9 days after OPU (range 31-796 pg/mL) as compared with those in the controls or with those during the midluteal phase prior to IVF treatment in the same patients. (Figure 7)

Midluteal pretreatment VEGF was related to the BMI ($r = 0.53$, $P < 0.01$) and to the progesterone ($r = 0.53$, $P < 0.05$), and these two factors explained 40% of the midluteal increase in the VEGF level. When the IVF patients and the controls were pooled, the midluteal VEGF correlated with the peak follicular phase E2 ($r = 0.40$, $P < 0.05$) and with progesterone at the luteal phase ($r = 0.43$, $P < 0.05$).

Very early pregnancy: The IVF-induced rise in the VEGF level was seen both in the women who became pregnant and in those who failed to do so. At 12 days after ET, the mean levels of VEGF seemed to be higher in patients with term pregnancies (range 64 - 823 pg/mL) than in the non-pregnant patients (range 20 -487 pg/mL), but the difference was not significant. (Figure 7, at lower right).

Normal cycle: Concentrations of VEGF ranged from 23 pg/mL to 389 pg/mL. No cyclicity in VEGF could be detected (Figure 7). The levels of VEGF were independent of age, BMI, or the levels of FSH, E2, or progesterone.

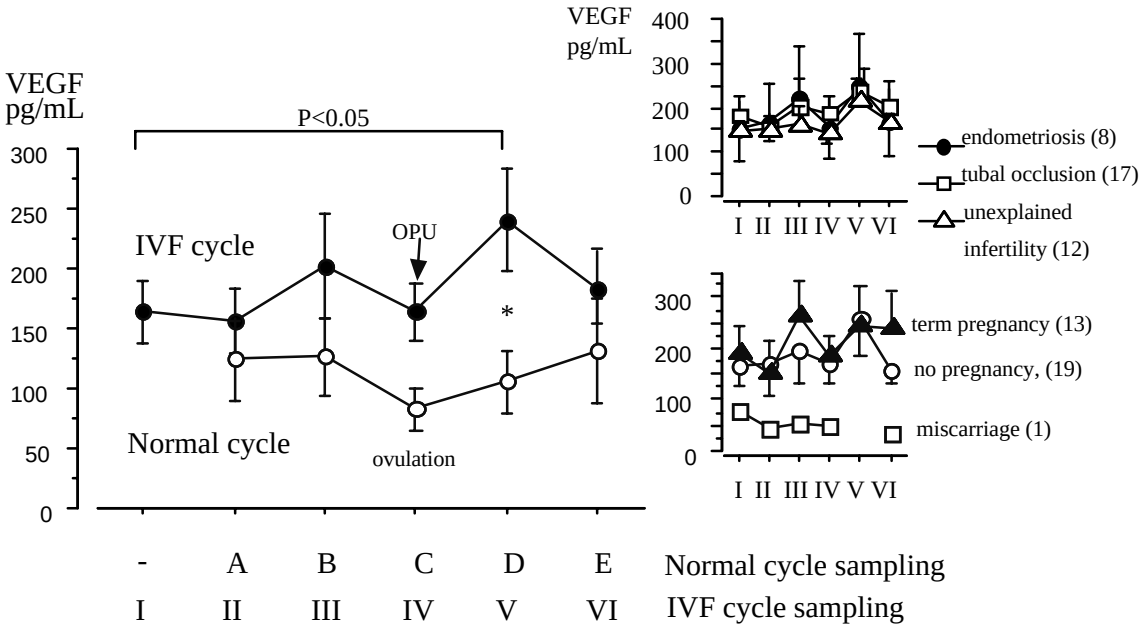


Figure 7. Serum vascular endothelial growth factor (VEGF) levels (mean±SE) in 37 infertile women undergoing an IVF programme and in 10 healthy women with ovulatory cycles. * $P < 0.05$ between cycles, other P values refer to IVF cycles.
At upper right are the levels of VEGF in IVF cycles with respect to cause of infertility and at lower right those with respect of pregnancy result; number of patients in parentheses.

I midluteal phase of the previous cycle
II ovarian suppression
III 3-5 days before ovum pick-up (OPU)
IV day of OPU
V 8-9 days after OPU
VI 12-14 days after OPU

A day 2-4 of menstrual bleeding
B 2-3 days before ovulation
C day of ovulation
D 8-9 days after ovulation
E 11-14 days after ovulation

3. 2. Leptin (Study IV)

Basal leptin levels correlated strongly with BMI (r= 0.85, $P < 0.0001$), Accordingly, the levels were lower in patients who were underweight (6.4 ± 0.8 µg/L, range 3.1-11.9 µg/L) than in those with normal weights (12.0 ± 0.7 µg/L, range 5.0-25.3 µg/L, $P < 0.01$), or in those who were overweight (25.7 ± 2.8 µg/L, range 15.5-39.3 µg/L, $P < 0.0001$). The difference in leptin between the normal and the overweight groups was significant ($P < 0.0001$). Basal leptin levels in unexplained infertility (12.1 ± 1.5 µg/L), in endometriosis (13.1 ± 3.0 µg/L), and in tubal occlusion (14.0±1.3 µg/L) did not differ significantly. Neither age (•35 years / > 35 years), type of infertility (primary / secondary), nor smoking (yes/no) were significant factors for basal leptin levels. When all the patients were considered as a single group, both serum E2 (r = 0.16, $P = 0.01$) and BMI (r = 0.89, $P < 0.001$) were significant factors for the midluteal phase leptin level.

IVF cycle: Ovarian suppression was associated with a fall in leptin of 21 ± 4% ($P < 0.01$) (Figure 8). This did not correlate with falls in LH (46 ± 5%) or E2 (91 ± 1%). Relatively, the fall in leptin seemed higher in the patients with normal weight (21 ± 4%) or overweight (26 ± 7%) than in the underweight patients (12 ± 10%), but the differences between these changes were not significant. When E2 production was suppressed (E2 < 0.1 nmol/L), E2 correlated positively with leptin (r = 0.20, $P = 0.01$).

Ovarian stimulation was followed by significant increases in leptin concentrations (Figure 8). After 4 to 5 days of ovarian stimulation, this rise was on average 45 ± 6% and reached a maximum of 76 ± 8% at the time of OPU. A rise of 20% or more in leptin occurred during stimulation in 56% of the underweight patients, in 83% of those with normal weight and in every overweight patient ($P < 0.05$). No linear relation existed between daily serum leptin and E2 or progesterone during ovarian stimulation, or between leptin and the number of ovarian follicles. The rise in leptin during stimulation with human menopausal gonadotrophin (containing LH) (90 ± 11%) did not differ significantly from that induced with purified FSH (62 ± 10%) ($P = 0.07$). The increases in leptin during ovarian stimulation were similar in all types of infertility (Figure 8).

Luteal phase: In the presence of high progesterone (69.7 ± 7.8 nmol/L) 8 days after OPU, the leptin levels were similar to those seen at OPU (Figure 8). The level of leptin did not correlate with that of progesterone but correlated positively with E2 (r=0.17, $P < 0.05$). The midluteal level of leptin after ovarian stimulation (16.5 ± 1.5 µg/L) was 28 ± 7% higher than at the same phase of a normal cycle prior to IVF treatment (13.1 ± 0.9 µg/L, $P < 0.001$).

Very early pregnancy: Twelve days after ET, when endogenous hCG becomes detectable if pregnancy occurs, the level of leptin fell ($P < 0.01$; Figure 8). The relative decrease in leptin levels from OPU to 14 days after OPU (i.e. 12 days after ET) tended ($P = 0.06$) to be larger in women failing to become pregnant (25 ± 4%) and in those with miscarriages (27 ± 7%) than in women whose pregnancies were successful (8 ± 6%; Figure 8). A successful outcome of pregnancy was associated with a higher level of leptin in the first days of gestation (18.7 ± 4.8 µg/L; Figure 8) than in those experiencing a first trimester miscarriage (10.0 ± 1.9 µg/L, $P < 0.001$), a subclinical abortion (11.5 ± 3.4 µg/L, $P < 0.05$), or no implantation (11.6 ± 1.2 µg/L, $P < 0.0001$). In the three women with twin pregnancies, the leptin levels (20.4 ± 1.9 µg/L) were comparable with those seen in single pregnancies (18.2 ± 6.3 µg/L). hCG was not an independent factor for leptin (r = 0.12, $P = 0.08$).

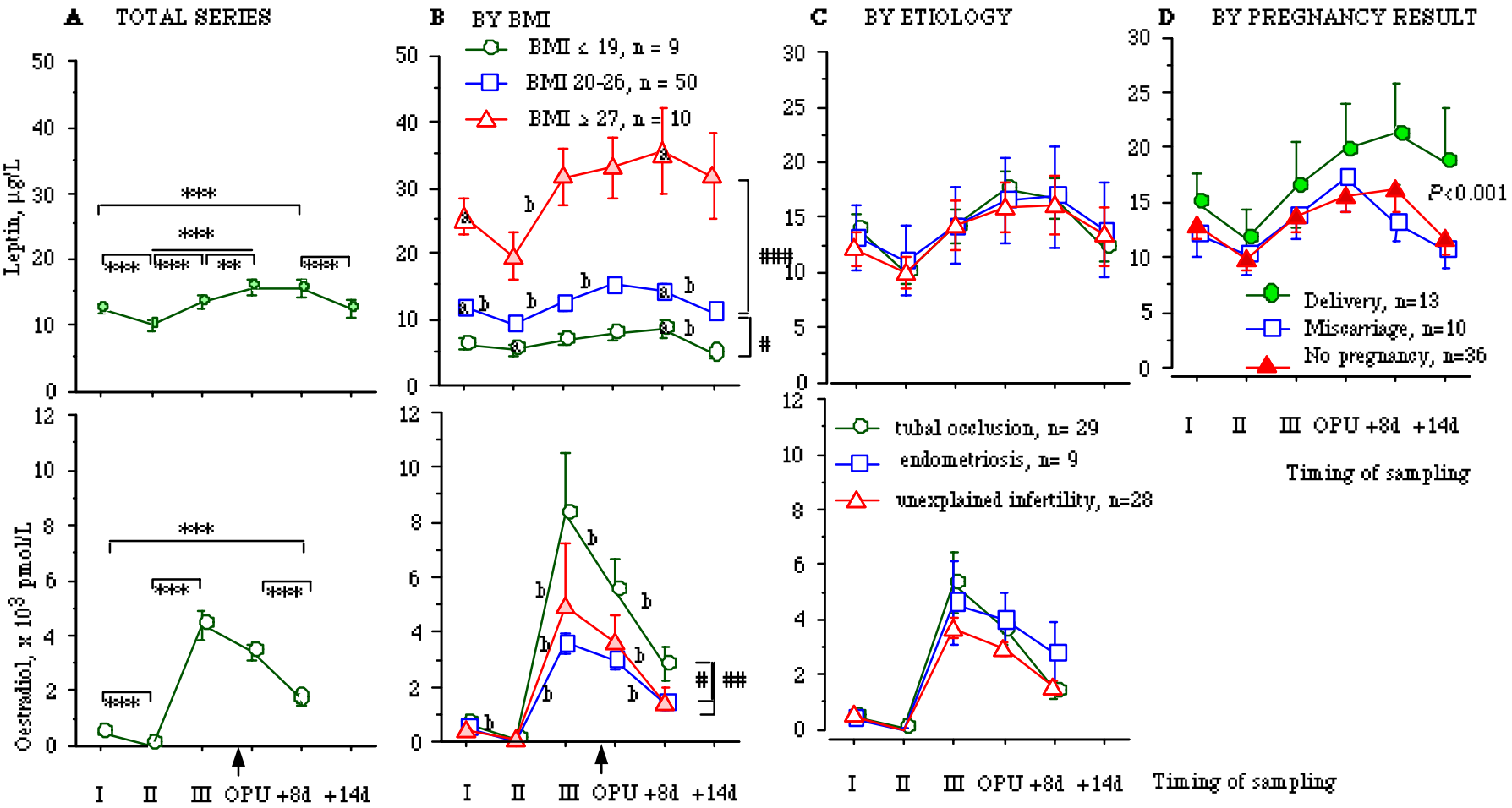


Figure 8. Mean (± SE) serum concentrations of leptin and oestradiol (A), with respect to BMI (B), with respect to cause of infertility (C) and with respect of pregnancy result (D) in 66 women undergoing an IVF programme resulting in embryo transfer for 59 women.
I = midluteal phase, before the start of GnRH agonist, II = ovarian suppression, III = ovarian stimulation, 3-5 days before ovum pick-up (OPU), d = days after OPU. The arrow indicates the time of hCG injection. ANOVA for repeated measurements (BMI grouping as covariate), Fisher's PLSD $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$, the effect of BMI grouping: # $P < 0.05$, ## $P < 0.001$, ### $P < 0.0001$. Panel B: Fisher's PLSD significant between marked points (a), between consecutive points (b). Panel C no difference between groups. Panel D: $P = 0.08$ between groups, 14 days after OPU, ANCOVA (BMI as a covariate) $P < 0.001$, Fisher's PLSD $P < 0.01$ between delivery and miscarriage, $P < 0.001$ between delivery and no pregnancy.

Correlations: Patients who became pregnant as a result of the treatment showed a significant correlation between the ovarian stimulation-induced rise in leptin and the maximal E2 value (Figure 9). Similarly, patients with pregnancies showed positive correlations between the rise in leptin and the number of follicles (r=0.62,*P* = 0.006), and the oocyte count (r = 0.74, *P* < 0.001), and a marginal correlation with progesterone at OPU (r = 0.46, *P* = 0.05).

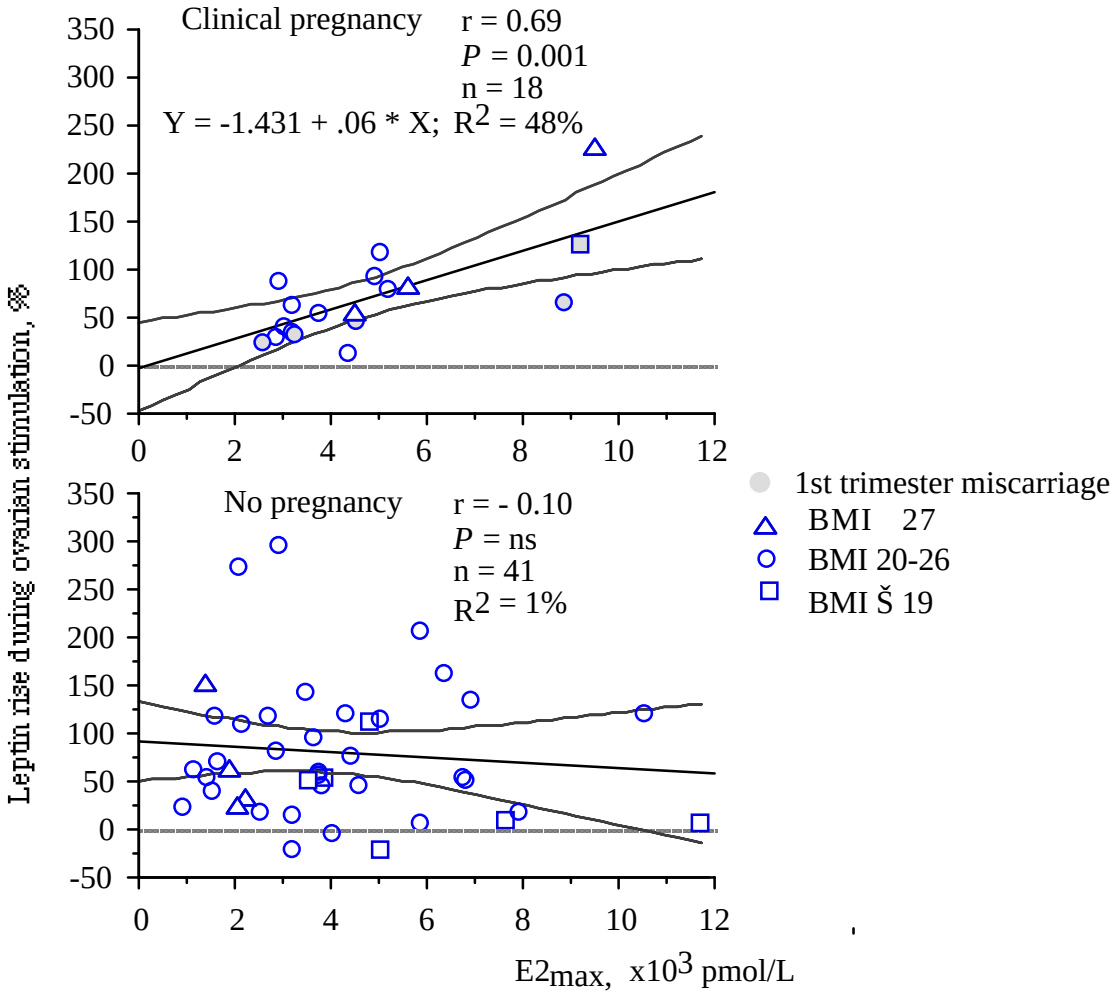


Figure 9. Relations between rises in leptin and peak oestradiols ($E2_{max}$) during ovarian stimulation in women who became clinically pregnant as a result of IVF treatment and those failing to do so.

3.3. Tumour markers (Study V)

3.3.1. Cancer antigen 125

Prior to IVF treatment, the midluteal level of CA 125 was significantly higher in patients with endometriosis than in the other groups (Figure 10). Four of the patients with endometriosis had levels exceeding the upper normal limit of 35 kU/ml in clinical practice (range 61-109 kU/L) (Bidart *et al*., 1999).
IVF cycle: Ovarian suppression caused a 36% ($CI_{95\%}$ 19, 54) increase in CA 125 levels (Figures 10 and 11). This rise was higher (mean 98%, $CI_{95\%}$ 8, 189) in the 11 patients with bleeding at verified suppression than in those without bleeding (24%, $CI_{95\%}$ 11, 38, $P < 0.01$).
Ovarian stimulation was followed by a decrease in CA 125 to the basal level (Figure 10) and this was unaffected by discontinuation of the GnRH agonist or by injection of hCG.
During the luteal phase, CA 125 levels increased significantly above the starting values (mean rise 114%, $CI_{95\%}$ 57, 171). The relative increase in CA 125 appeared greatest in women with tubal occlusion (Figure 11).
Very early pregnancy: Fourteen days after OPU, the CA 125 levels in 18 pregnant women (median 36 kU/L, $CI_{95\%}$ 27, 70) did not differ significantly from those in non-pregnant women (24 kU/L, $CI_{95\%}$ 19, 36; Figure 13).
Two months after IVF: The CA 125 levels were significantly higher (17 kU/L, $CI_{95\%}$ 14, 25) than the pre-treatment levels (15 kU/L, $CI_{95\%}$ 11, 21; $P < 0.01$; Figure 11). The median difference between the levels was 12%, ranging from a decrease of 50% to an increase of 234%. The Ca 125 levels exceeded the 35 kU/L (range 54-203 ku/L) in seven (17%) patients of whom five were infertile on account of endometriosis but two because of unexplained causes. Of these seven patients, three were shown to have ovarian endometriomas and one a dermoid cyst, as evidenced by pelvic ultrasound examination and later by surgery.
Correlations: The basal CA 125 level correlated inversely with BMI ($r = - 0.32$, $P < 0.01$). At OPU, CA 125 correlated with E2 ($r = 0.28$, $P < 0.05$) and at the time of ET both with E2 ($r = 0.40$, $P < 0.01$) and progesterone ($r=0.47$, $P < 0.0001$). At 8 days after OPU, CA 125 correlated with E2 ($r = 0.44$, $P < 0.01$), with the number of follicles ($r = 0.56$, $P < 0.0001$) and with progesterone ($r = 0.26$, $P < 0.05$).

Normal cycle: The Ca 125 level was higher during the menstrual phase. The mean relative increase from the midluteal phase level to the peak level was 34% ($CI_{95\%}$ –3%, 72%).

3.3.2. Tumour-associated trypsin inhibitor

No significant cyclicity in the serum levels of TATI occurred during either the IVF cycles or the normal cycles; neither infertility, pregnancy nor IVF treatment affected the levels of TATI in serum. (Figures 10, 11 and 13).

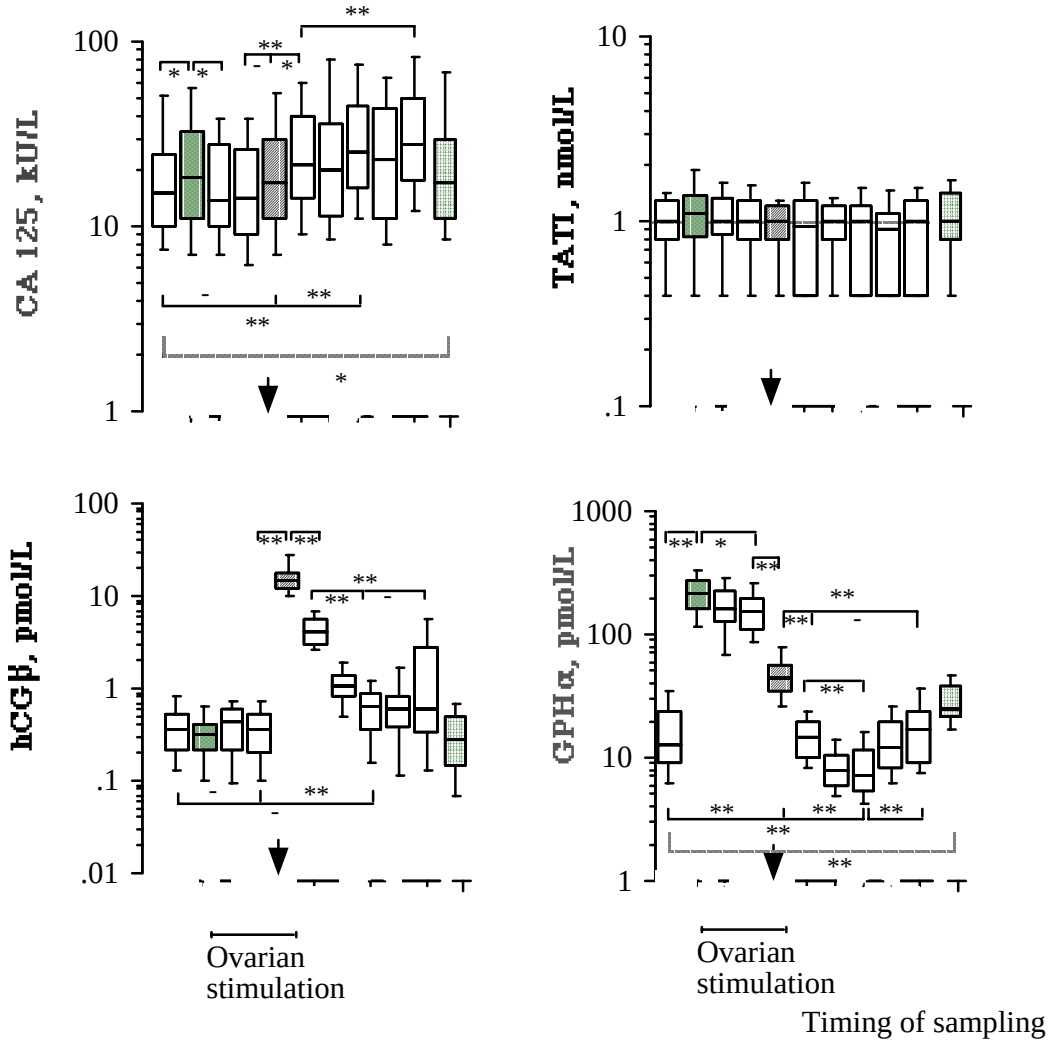


Figure 10. Serum concentrations of CA 125, tumour-associated trypsin inhibitor (TATI), free β-subunit of hCG (hCGβ), and free glycoprotein hormone α-subunit (GPHα) before, during, and 2 months after IVF treatment with long downregulation followed by ovarian stimulation with urine-derived FSH. The arrow indicates injection of hCG (10 000 IU) 36 hours prior to ovum pick-up (OPU).The boxes show the quartiles (25th and 75th percentiles) and the median. Whiskers extend to the 10th and 90th percentiles. Outliers not shown.

ANOVA for repeated measurements with the Bonferroni-Dunn post hoc test (log10-data) *P Š 0.001, ** $P <$ 0.0001, - not significant (solid line: I, II, III, IV,V,VI,VIII, X included), (---: I, II, III, IV, V,VI,VIII, X, XI included for comparison between I and XI).

Timing of sampling:

I: Midluteal phase of prior spontaneous menstrual cycle, (n=71)

II: Ovarian suppression (n=69)

III: Gonadotrophin used for 3-5 days, (n=69)

IV: 3-5 days prior to OPU (n=68)

V: OPU (n=67)

VI: Embryo transfer (n=66)

VII: 5-6 days after OPU (n=32)

VIII: 8 days after OPU (n=65)

IX: 10-11 days after OPU (n=30)

X: 14 days after OPU (n=61)

R: Midfollicular phase 2 months after IVF (n=42)

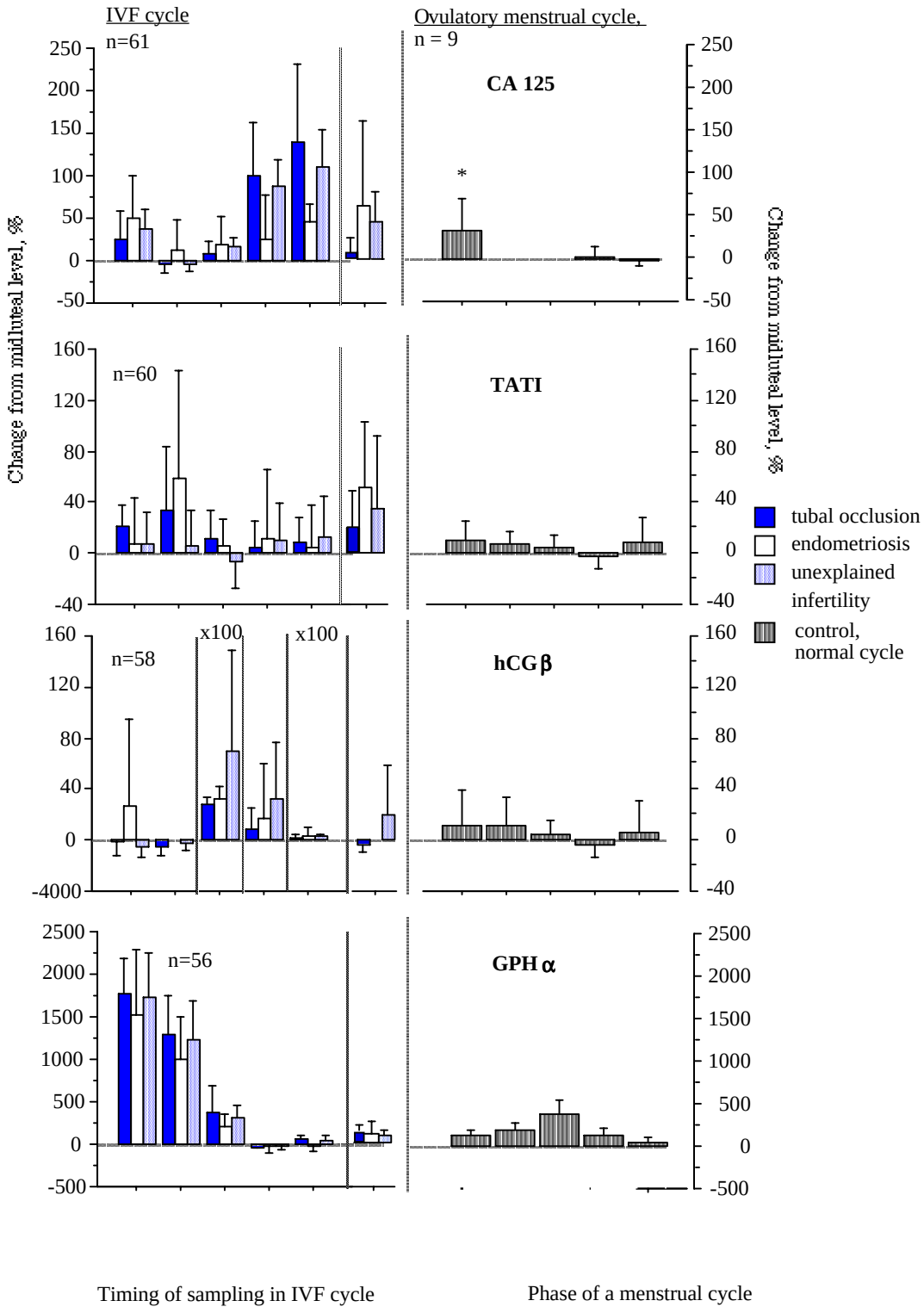


Figure 11. Mean percentage changes (± 95 % confidence limits) in serum CA 125, tumour-associated trypsin inhibitor (TATI), β-subunit of human chorionic gonadotrophin (hCGβ) and free glycoprotein hormone α-subunit (GPHα), from the midluteal phase level of the preceding cycle during an IVF programme and 2 months after IVF, and during normal menstrual cycle in controls. Roman numerals indicate the timing of blood samples, see Figure 10.

3.3.3. β-subunit of human chorionic gonadotrophin

Basal hCGβ levels ranged from undetectable to 2.7 pmol/L and the levels were unaffected by infertility. *IVF Cycle: Ovarian suppression / stimulation* had no effect on hCGβ levels until injection of hCG, which caused a 40% rise in hCGβ . The hCGβ then fell to a normal level within a week after injection. (Figures 10 and 11)
Very early pregnancy: In patients becoming pregnant, the levels began to rise already at 10 to 12 days after OPU (sample IX, Table 13). The median level was 0.8 pmol/L ($CI_{95\%}$ 0.5, 6.2) in patients with clinical pregnancies and 0.4 pmol/L ($CI_{95\%}$ 0.4, 1.31) in non-pregnant patients. At 14 days after OPU the levels of hCGβ were significantly higher in all pregnant patients than in non-pregnant patients, however, successful pregnancies could not be differed from miscarriages (Figure 13).
Two months after IVF: The levels of hCGβ were similar to the pre-treatment levels.

Normal cycle: No significant variation occurred in hCGβ.

3.3.4. Common free α-subunit of glycoprotein hormones

The basal level of GPHα (Figure 3) were unaffected by infertility, but correlated with LH (r = 0.94, $P < 0.0001$).
IVF cycle: The start of *ovarian suppression* with the GnRH agonist induced a rapid rise in GPHα levels, which remained elevated during the entire suppression phase. This was specific for GPHα, because hCGβ and TSH were unaffected and LH and FSH declined after an initial rapid flare-up (Figure 12). The relative increase in GPHα during the suppression phase was 1680%, ($CI_{95\%}$ 1396, 1966) and this rise was greater ($P < 0.01$) in the patients without bleeding (1800%, $CI_{95\%}$ 1497, 2102) than in those with bleeding (747%, $CI_{95\%}$ 282, 1211).
Ovarian stimulation did not affect the levels of GPHα, whereas discontinuation of the GnRH agonist and simultaneous injection of hCG caused a rapid decrease in GPHα (Figures 10 and 11).
Very early pregnancy: At 14 days after OPU, the GPHα levels were significantly ($P < 0.01$) lower (median 9 pmol/L, $CI_{95\%}$ 8,12) than those in the non-pregnant patients (median 20 pmol/L, $CI_{95\%}$ 17, 24). (Figure 13)
Two months after IVF: In all the infertile patients the post-treatment follicular phase GPHα was significantly higher (median level 25 pmol/L, $CI_{95\%}$ 23, 30) than the pre-treatment luteal phase level of GPHα (17 pmol/L, $CI_{95\%}$ 10, 24, $P < 0.0001$) but comparable with the follicular phase level of normal menstrual cycles of the controls (Figures 10 and 11).

Normal cycle: GPHα peaked prior to the LH surge (Figure 11). The mean relative increase from the midluteal phase level to the peak level was 373% ($CI_{95\%}$ 181, 566; Figure 11).

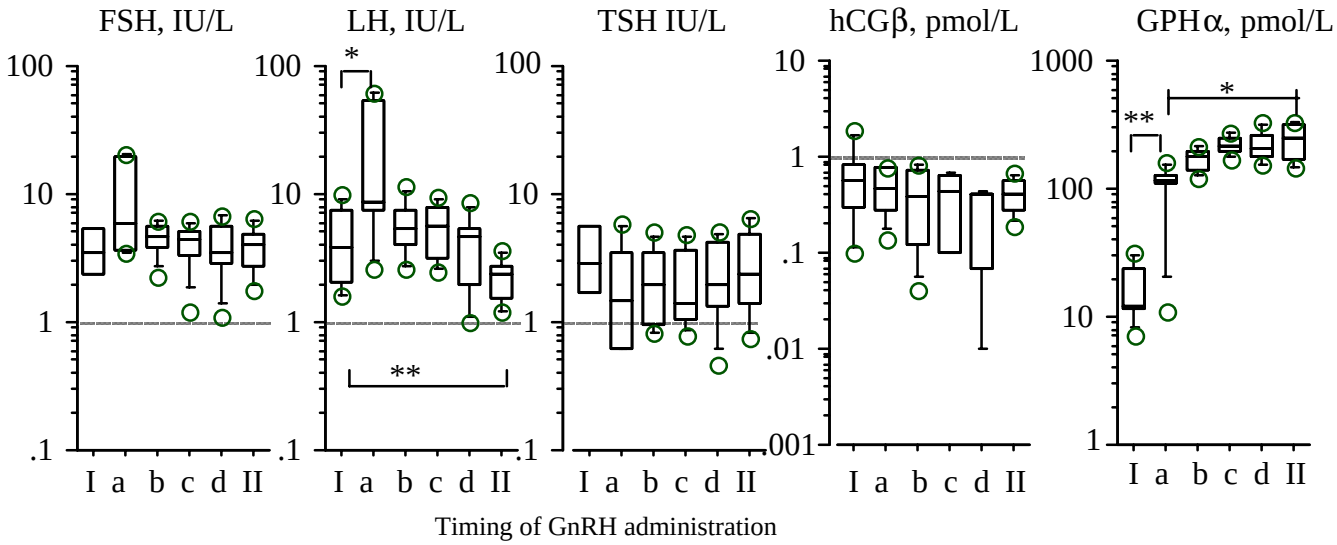


Figure 12. The effect of ovarian suppression with a GnRH agonist (nafarelin) on the serum levels of FSH, LH, TSH, hCG β and GPHα in 8 infertile women.

Anova for repeated measurements *$P < 0.01$, ** $P < 0.0001$ Bonferroni Dunn post hoc test
Timing of sampling: I midluteal phase, cycle day 21.4 ± 2.2 (mean ± SD) start of the GnRH agonist, a = 2±1 days, b = 4 ± 1 days, c = 7 ± 0.4 days, d = 12 ± 1 days, II = ovarian suppression, 16 ± 3 days use of GnRH agonist.

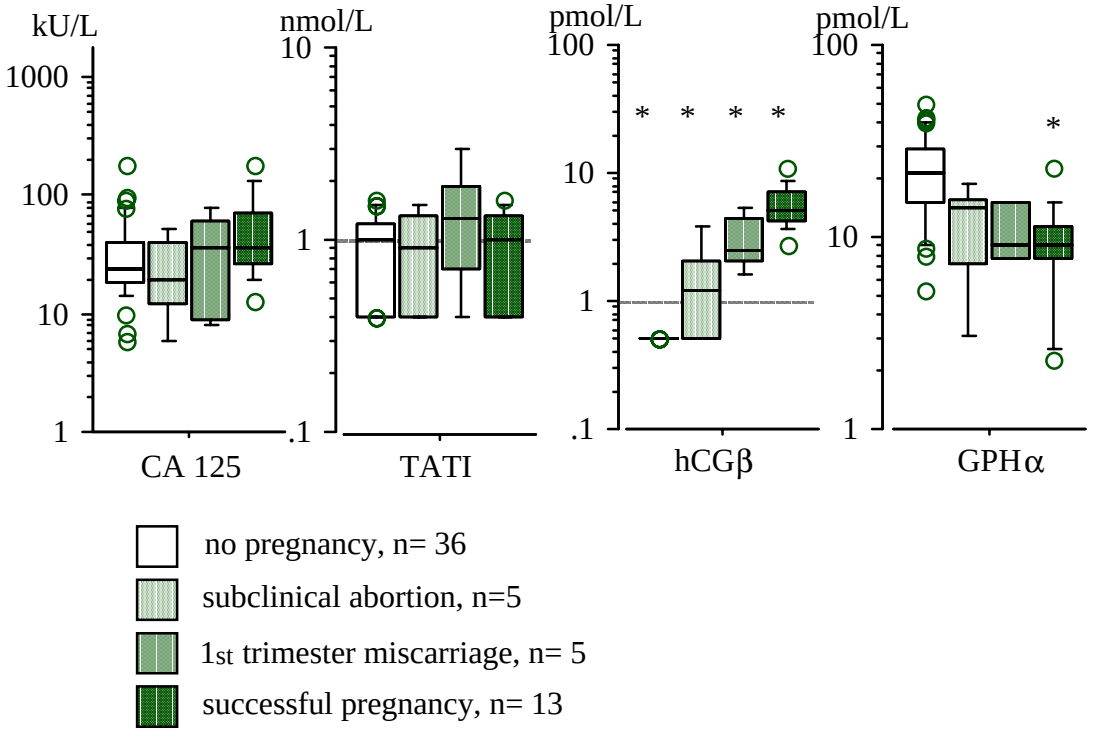


Figure 13. Distribution of CA 125, tumour associated trypsin inhibitor (TATI) , free β-subunit of human chorionic gonadotrophin (hCGβ) and free glycoprotein hormone α-subunit (GPHα) in 59 women 12 days after embryo transfer with reference to pregnancy outcome.

* $p < 0.0001$ ANOVA, all comparisons for hCG β, for GPHα between successful pregnancy and no pregnancy

VI DISCUSSION

Infertility in western populations is on the rise, and this indicates an enhanced need and resources for infertility treatments in future (Mosgaard *et al*., 1995; Chandra and Stephen, 1998; Raitio *et al*., 1998). The willingness of a given infertile couple to use modern infertility treatments depends not only on the efficacy of the treatments but also on the costs, convenience and possible risks of the treatment (Rosen *et al*., 1997). The risks of infertility treatments can be classified as immediate adverse events, which vanish quite soon after the treatment, and long-term consequences, such as the possibility of a tumour. The aim of these studies on ovarian GCT and IVF cycles was to gain new insight into the possible risks of infertility treatments and the physiological changes induced by IVF treatment as practised in Finland for some 20 years and at present.

1. Infertility, infertility treatments and granulosa cell tumour

In Finland the incidence of all ovarian cancers, including borderline tumours and tubal cancers, was rising from 1965 to 1994 (Study I). Therefore the hypothesis was to see a rise in the incidence of GCT during the study period also, especially as CC and gonadotrophins had been introduced concomitantly. In fact, there was a 13-fold rise in the use of CC and a 200-fold rise in the use of FSH / hMG during the study period, these rises being similar to those reported from the USA and Denmark (Wysowski, 1993; Mosgaard *et al*., 1995). Nevertheless, during the same period the incidence of GCT decreased by 40%. This fall occurred predominantly in women between 45 to 74 years of age, and in young women (age 15 -29 years). These young women may account for the rise in the consumption of OCs and whether the use of OCs reduces both GCTs and epithelial ovarian cancers is debatable. Interestingly, no change in the incidence was observed in women in the common childbearing age range, 30-40 years. However, it has to be acknowledged that GCT is a rare tumour and its incidence may fluctuate over quite a wide range without traceable cause-and-effect links. However, our study did not lend support to the claim that ovulation inducers trigger the onset of GCT (Willemsen *et al*., 1993).

Among patients with a GCT, the overall infertility rate was 9% (study II), which is fairly normal for the Finnish population. The frequency of nulliparity was similar to that seen in other large series on GCT (Fox *et al*., 1975; Pankratz *et al*., 1978; Stenwig *et al*., 1979; Björkholm and Pettersson, 1980; Ohel *et al*., 1983). However, in patients with a GCT diagnosed at a fertile age, the rates of infertility and nulliparity were significantly higher than those generally found in the Finnish population. Further, the frequency of anovulatory infertility was double that for the unselected Finnish population (Rantala, 1988) and further, after surgery, spontaneous pregnancies were frequent. These figures imply that ovulation inducers are unlikely to cause GCT, but that the cause of anovulatory infertility may be a pre-existing GCT. However, a stimulatory effect of ovulation inducers on the growth of a pre-existing GCT is possible. Furthermore, the present study found no increase in the frequency of GCT in females of fertile age from 1956-66 to 1987-96, even though, during that time, the use of ARTs has increased markedly. These findings can be taken as further evidence that infertility treatments do not induce the development of GCT.

2. The effect of infertility, an *in vitro* fertilization programme and very early pregnancy

A number of biologically potential mediators take part in the regulation of ovarian physiology and thus, also, in the physiology of the endometrium. The physiology of the genital organs may be altered during aggressive ovarian suppression/stimulation, both of which are essential parts of modern ART. In the present series several of these possible regulators were studied before, during and after the IVF regimen. The recruitment of a rather large number of ovulatory patients with different types of infertility allowed also evaluation of the impact of the type of infertility on the midluteal levels of these bioregulators during natural cycles and whether they responded to the IVF programme. In interpreting the results, it has to be taken into account that not only the ovaries, but also other organs producing these compounds, became exposed to the wide fluctuations in gonadotrophins and sex steroids during the IVF cycles.

2.1 ... on the angiogenetic factors, vascular endothelial growth factor and leptin in the serum

The pretreatment midluteal levels of VEGF were not affected by infertility. During the normal menstrual cycle the levels of VEGF remained unchanged, and this finding is in line with previous data (Lee *et al*., 1997; Yamamoto *et al*., 1997b; Chung *et al*., 1998). In accordance with a previous report (Lee *et al*., 1997) our IVF programme led to significant elevations in serum VEGF during the luteal phase. The elevated level of VEGF may derive from the granulosa cells, corpora lutei, endometrial cells, or other sites capable of producing VEGF (Antczak *et al*., 1997; Geva and Jaffe, 2000). As shown by *in vitro* and *in vivo* studies, it is possible that VEGF production can be stimulated by an LH surge and may reflect the quality of the corpus luteum function (Geva and Jaffe, 2000; Reynolds *et al*., 2000). In a former study (Lee *et al*., 1997) serum VEGF levels were significantly elevated in pregnant patients 11-14 days after ET. However, in our 13 pregnant patients with term deliveries the VEGF levels in serum 12 days after ET revealed only a visual impression of higher levels than in non-pregnant patients. This association gains further support from recent data showing significantly higher levels of VEGF in the serum of patients on becoming pregnant (Jinno *et al*., 2000). A larger IVF series with more pregnant women is needed to confirm that VEGF in serum at time of a very early pregnancy has significance for IVF outcome.

The facts that granulosa cells are able to accumulate and secrete in a special way VEGF, leptin and transforming growth factor-β2 (TGFβ) (Antczak *et al*., 1997) which all are angiogenetic factors (Table 3) and further, VEGF and TGFβ have been associated with gynecological malignancies (Nakanishi *et al*., 1997; Donovan *et al.*, 1997) introduced us to study the role of leptin in infertility and its levels in serum during an IVF programme in relation to E2 levels. However, in the different types of infertility the pretreatment luteal levels of leptin and leptin responses to IVF were similar. Thus, circulating leptin does not appear to be involved in unexplained infertility. The pretreatment luteal-phase level of leptin was highly dependent on BMI, whereas the leptin responses to IVF were independent of BMI. During ovarian suppression, the leptin levels decreased, a phenomenon which may be secondary to hypo-oestrogenism, as is supported by the correlations between leptin and E2

levels prior to the treatment and at suppression. A link between leptin and E2 also gains support from the fall in leptin following ovariectomy (Messinis *et al*., 1999). In addition, ovarian stimulation was accompanied by elevation of leptin, which was probably the consequence of an oestrogen surge. However, during ovarian hyperstimulation, no linear relationship could be demonstrated between same-day E2 and leptin, either in our series or in other studies (Mannucci *et al*., 1998; Riad-Gabriel *et al*., 1998; Teirmaa *et al*., 1998; Bützow *et al*., 1999). The levels of leptin were highest at the time of OPU, when the patients had been exposed to the effect of hMG / FSH and hCG. This may be due, in part, to hCG, because hCG stimulates the release of leptin by the adipocytes, at least *in vitro* (Sivan *et al*., 1998) and can perhaps cause the granulosa cells to release leptin (Antczak *et al*., 1997; Cioffi *et al*., 1997; Karlsson *et al*., 1997). Arguments against this speculation are the fall in leptin levels and the lack of correlation between the levels of leptin and hCG at OPU and on the first days of pregnancy.

In view of the trophoblastic production of leptin (Masuzaki *et al*., 1997; Senaris *et al*., 1997) and of the significant rises in leptin during pregnancy (Hardie *et al*., 1997; Sivan *et al*., 1998; Laivuori *et al*., 2000), it was expected to see rising levels of leptin in women very early in pregnancy. It was therefore surprising that the levels of leptin decreased during the first few days of gestation. The higher levels of leptin in pregnant than in non-pregnant women may be a reflection of stronger oestrogenic stimulation (probably mediated via hCG) on leptin production from the adipocytes or of leptin production from the granulosa cells (Kitawaki *et al*., 1999), or trophoblast cells (Masuzaki *et al*., 1997; Senaris *et al*., 1997).

Interestingly, the increases in leptin and E2 during ovarian stimulation were positively related to successful outcome of the treatment (Figure 11). This is a novel finding and suggests that leptin may have a role in reproduction. The leptin responses to ovarian stimulation in patients who did not become pregnant are in accord with the finding that an increase in leptin may also be associated with a reduced ovarian response (Bützow *et al*., 1999). Leptin has been detected in early human oocytes and thus may also influence the early development of the embryo (Antczak and Van Blerkom, 1997). It remains to be seen, whether the leptin response to ovarian stimulation is a factor in determining the quality of the oocyte or in angiogenesis, hence affecting endometrial receptivity or the function of the corpus luteum. It has been shown that the endometrium of subfertile women lacks leptin receptors, in the presence of normal concentrations of circulating hormones (Alfer *et al*., 2000). It is further known that leptin can stimulate matrix metalloproteinases, which are capable of digesting the extracellular matrices of the host tissue and hence, the increased leptin levels in the serum may reflect regulation of trophoblast invasion in early pregnancy (Bischof *et al*., 2000).

2.2. ... on tumour markers in the serum

In normal cycles, the tumour marker CA 125 was significantly elevated during menstruation and in patients with endometriosis, and these findings are in harmony with previous data (Lehtovirta *et al*., 1990; Mol *et al*., 1998; Bon *et al*., 1999). Interestingly, infertility itself appeared to have no effect on CA 125. It is probable that the increase in serum CA 125 in endometriosis is derived from endometriotic tissue capable of producing CA 125 (Fedele *et al*., 1988). Ovarian suppression stimulated the release of CA 125, whereas ovarian stimulation led to the return of CA 125 to baseline level. However, ET causes a rise in CA 125, probably from the endometrium (Weintraub *et al*., 1990;

Zeimet *et al*., 1993). The positive correlations of CA 125 with sex steroids and with the number of ovarian follicles may imply that the corpora lutei also contribute to the rise in CA 125 in the luteal phase. Some *in vitro* (Zeimet *et al*., 1993; Paoletti *et al*., 1995) and *in vivo* (Gurgan *et al*., 1993; Ozaksit *et al*., 1993) data support this view. It is also possible that the OPU-associated trauma to the peritoneum or to the ovarian surface may have contributed to the luteal rise in CA 125. Regardless of the cause of the rise in CA 125, it was interesting to note that 2 months´ wash-out time was insufficient to normalize serum CA 125 levels. This may imply a prolonged effect of the IVF programme on the synthesis and release of CA 125. The sources of this synthesis cannot be determined from serum measurements, although the explanatory factors may be the activation or growth of endometriotic tissue or the healing process of multiple corpora lutei. Clearly, longer follow-up studies are needed to determine whether repeat IVF programmes cause cumulative increases in serum CA 125 and to reveal the impact of a rise in CA 125 on the risk of future malignancy.

The high levels of GPHα during ovarian suppression with nafarelin are likely to result in release of GPHα from the pituitary (Clayton, 1993). Thus the pituitary cells maintained their capacity to produce GPHα, although the release of LH was blocked by the GnRH agonist. A similar rise of GPHα in the response to the GnRH agonist treatment has been reported in girls with precocious puberty (Lahlou *et al*., 1987) and in patients with fibroids (Bischof *et al*., 1992). The falling levels of GPHα following cessation of the GnRH agonist and injection of hCG support the stimulatory role of the GnRH agonist on GPHα and imply that our hCG preparation contained no free GPHα. During a normal cycle, GPHα secretion peaked prior to LH and thus it appears probable that GPHα plays a role in the initiation of the LH surge. In addition, a role has been proposed for GPHα in the initiation of decidualization of the endometrium (Nemansky *et al*., 1998).

2.3. ... on the risk of gynaecological malignancies

From *in vitro* and animal studies, it is evident that gonadotrophins and perhaps also oestrogens are involved in the development of a number of genital cancers, especially that of breast and endometrial cancer (Cramer and Welch, 1983; Schiffenbauer *et al*., 1997; Konishi *et al*., 1999). The epidemiological data on human females also show that multiple ovulations are associated with ovarian cancer (Webb *et al*., 1998), and infertility with borderline ovarian tumours that may lead to invasive cancer (Scully, 2000). It should, however, be noted that OPU as a part of IVF does not actually cause multiple trauma on the ovarian surface, because the collection needle generally perforates the ovarian surface once, and many ova can then be collected from inside the ovary. In addition, by OPU the follicular fluid, which is rich in growth factors and oestrogens, is thoroughly aspirated, so that the peritoneal cavity and its organs are not exposed to it in large amounts. However, theoretically the formation of multiple corpora lutei and their regression may lead to an increased number of inclusion cysts and to malignant transformation.

On the other hand, the serum concentrations of gonadotrophins are much lower (less than 20 IU/L), during an IVF treatment than after the menopause, when the levels of FSH and LH may reach levels exceeding 100 IU/L. Further, during the menopause women become exposed to these high levels of gonadotrophins for up to 30 years, although these levels tend to decline in old age. Furthermore, during a long-protocol IVF treatment, the only gonadotrophin that is elevated is FSH, as the LH level is depressed by the GnRH agonist, and recombinant gonadotrophins no longer contain LH. In animal studies, especially LH has been linked with tumour growth, owing to animals with a special genetic predisposition (Keri *et al*., 2000). Furthermore, during IVF treatment hormonal balance is optimized to improve endometrial receptivity and thus, patients with anovulatory infertility probably favor the maximized luteal support in terms of decreased risk of endometrial cancer (Mosgaard *et al*., 1997a, Venn *et al*., 1999).

Since Whittemore´s study (Whittemore *et al*., 1992), only the study of Rossing (Rossing *et al*., 1994) has been able to link ovulation inducers (long-term use of CC) with borderline and ovarian cancer, whereas later studies point out that it is infertility, rather than the use of ovulation inducers, that is related to ovarian cancer (Mosgaard *et al*., 1997b; Modan *et al*., 1998; Venn *et al*., 1999). Interestingly the common oestrogen-associated malignancy, breast cancer, has not been associated with infertility treatments, and the role of postmenopausal oestrogen replacement therapy in ovarian cancer is also controversial (Coughlin *et al*., 2000; Rodriguez *et al*., 2001). On the other hand, IVF treatment may, however, transiently increase the risk of uterine and breast cancer (Venn *et al*., 1999). Our finding of persistent elevation of CA 125 at 2 month's after the IVF programme can be seen as lending support for this hypothesis, although the source of the increased secretion of CA 125 is unknown. This may also be supportive to the association of borderline ovarian tumours with infertility and infertility treatments (Table 10a).

In all, it seems likely that, among infertile women, some groups (or individuals) among women with anovulatory infertility (Modan *et al*., 1998) or with unexplained infertility (Venn *et al*., 1999) carry an increased risk of genital cancer. These women may have a pre-existing malignancy in the ovaries or the endometrium that escapes detection until ovarian stimulation with gonadotrophins and oestrogen rise induces its growth to a detectable level. This growth may take advantage from the IVF-induced increase in the circulating levels of special growth factors, like the angiogenetic factors VEGF or leptin. In addition, obese women with or without ovarian endometriosis (Brinton *et al*., 1997; Zanetta *et al*., 2000), lean PCOS patients (Schildkraut *et al*., 1996) or carriers of gene mutations that predispose to genital cancer (Nieto *et al*., 1999) may run an increased risk of genital malignancy, regardless of fertility. In cases with suspicion of a tumour during an IVF programme, it has to be acknowledged that CA 125 and GPHα, although not TATI or hCGβ, are increased during different phases of ovarian hyperstimulation.

VII SUMMARY AND CONCLUSIONS

1. Between 1965-1970 and 1991-1994 the incidence of GCT in Finland fell from 0.74 / 100,000 to 0.47 / 100,000 although, at the same time, the use of CC increased 13-fold and that of gonadotrophins 200-fold. This argues against the suggestion that ovulation inducers play an active role in the aetiology of GCT at least in Finland.

2. Oligomenorrhea, nulliparity and anovulatory infertility were all characteristic of patients with GCT at fertile age in 1956-1996. GCT after the fertile age was characterized by normal parity and by postmenopausal bleeding that frequently associated with endometrial hyperplasia or cancer. Ovulation inducers had been used by 5 among 41 of the patients who had been of fertile age after the introduction of ovulation inducers. The percentage of patients with GCT at fertile age per decade stayed constant during the four decades. These data suggest that GCT at fertile age may be associated with infertility, but a causal link between the use of ovulation inducers and GCT is unlikely.

3. In the IVF group of 37 infertile women, the pretreatment midluteal serum levels of VEGF were similar to those of the controls. During the IVF programme the synthesis of VEGF increased at the luteal phase, whereas during normal cycles, no cyclicity in VEGF in the serum was observed.

The serum leptin levels prior to the IVF programme, and the responses of leptin to the treatment, were similar in 61 patients whose infertility were due to tubal occlusion, endometriosis and unexplained infertility. Ovarian suppression led to a fall in leptin of 21±4%, whereas the level increased by 76±8% during ovarian stimulation paralleling the changes in serum E2 and remained elevated in the midluteal phase of the cycle being 28±7% higher than the pretreatment concentration. The responses of leptin and E2 during stimulation were correlated, but only in patients achieving a clinical pregnancy. This may imply that leptin has a role in regulating reproduction.

The elevated levels of VEGF and leptin in the serum during the luteal phase of an IVF cycle probably reflect the increased need for angiogenesis for the function of the corpus luteum, for endometrial maturation and for trophoblast invasion.

4. Of the tumour markers studied, prior to treatment only the serum levels of CA 125 were higher in patients with endometriosis than in patients with tubal occlusion, unexplained infertility or no infertility. After the start of the GnRH analogue, the release of GPHα increased markedly and remained high until OPU. Ovarian suppression was characterized by a peak in CA 125, ranging from 24 to 98% from the pretreatment level followed by a fall during ovarian stimulation. After OPU CA 125 concentrations rose significantly and remained elevated during the luteal phase (114% from pretreatment level), regardless of the type of infertility. This luteal phase elevation in CA 125 may reflect simply the ovarian trauma and peritoneal irritation caused by OPU or endometrial maturation. The serum levels of TATI or hCGβ were not affected by an IVF programme.

By 2 months after IVF, GPHα had normalized but, in non-pregnant patients CA 125 were 12 % higher than the pretreatment levels. This may imply a more prolonged effect of an IVF treatment on the secretion of CA 125 probably from physiological or endometriotic sources. Longer follow-up studies on IVF patients are needed to see when CA 125 normalizes after an IVF programme.

5. Very early pregnancies, detected at 12 days after ET, did not induce marked changes in the levels of VEGF, CA 125 or TATI in the serum when compared to non-pregnant patients. However, the leptin levels in sera were higher in patients with successful pregnancies than in those experiencing miscarriages or in non-pregnant patients. The increased synthesis of leptin in very early pregnancy may originate from trophoblastic sources.

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