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# ENDOMETRIOSIS AND OVARIAN RESERVE – INFLAMMATION AND PROGNOSTIC MARKERS

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# ABSTRACT

**Introduction:** Endometriosis is a common, benign gynecological disease, associated with pelvic pain and infertility. It is generally thought to be caused by retrograde shedding of menstrual tissue with subsequent attachment to peritoneal surfaces. Endometriosis involves an altered inflammatory response and TNF seems to be an important pro-inflammatory mediator.

Current medical treatment implies hormonal suppression associated with several side-effects. During treatment, pregnancy is either impossible or contra-indicated. Infertile women with endometriosis may conceive after assisted reproduction. However, during IVF, women with endometriosis often show a poor response to ovarian hyperstimulation and have lower pregnancy rate. This suggests that women with endometriosis have reduced ovarian reserve. Identifying diminished ovarian reserve is important during infertility treatment. Several factors have been proposed as markers of ovarian reserve.

**Aims :** To study the effects of a TNF-inhibitor on induced endometriosis and pregnancy outcome in an experimental animal model; to evaluate the significance of two markers for ovarian reserve, FSH-receptor polymorphisms and AMH, in relation to infertility, endometriosis and inflammation.

**Material and methods:** Endometriosis was induced in 18 female baboons and the extent of disease was measured during laparoscopy. The animals were randomized to either TNF-inhibitor (c5N, n=11) or placebo (n=7) for 25 days. The effects were evaluated by laparoscopy. 16 of the baboons received an additional 3 infusions of c5N (n=9) or placebo (n=7). Subsequently, timed mating was commenced. Pregnancy outcome was evaluated after 9 cycles. Single nucleotide polymorphisms (SNPs) at pos 680 (exon 10) in the FSH-receptor was analysed in 68 infertile women using PCR and DNA sequencing. 15 of these women had FSH-levels >10 IU/ml on cycle day 3 or after clomiphene challenge test (CCCT). AMH was measured in serum and follicular fluid from 72 women with endometriosis (n=34) and tubal factor infertility (n=38). In addition, several cytokines and growth factors were analysed in follicular fluid during IVF.

**Results:** Total surface area and volume of endometriotic lesions was significantly reduced in animals treated with c5N compared to placebo. The strongest effect was recorded for red lesions. No adverse effects were observed on the menstrual cycle in either group. Pregnancy rates and cycle fecundity rate (CFR) were comparable in both groups after timed mating. Women with FSH-receptor variant Serine/Serine at pos 680 had significantly higher FSH after CCCT but no differences in receptor distribution were observed. Women with endometriosis had lower AMH in serum and higher amounts of TNF in follicular fluid than women with tubal factor infertility. Women with endometriosis produced fewer small follicles and had a lower fertilization rate after IVF.

**Conclusion:** The results support a central role for TNF in endometriosis. Women with endometriosis seemingly have a diminished ovarian reserve, related to increased inflammatory activity. Inhibition of TNF could represent a novel principle for the treatment of this common disease. Serum levels of AMH may aid the clinician to identify poor responders among women with endometriosis prior to IVF treatment. Also, certain FSH-receptor variants may be a characteristic for a subset of infertile women.

**Key words:** *Endometriosis, infertility, baboon, animal model, FSH, AMH, ovarian reserve*



In memory of Gabriel Fried

*Ingenting är omöjligt  
Gunde Svan*



# LIST OF PUBLICATIONS

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This thesis is based on the following papers which are referred to in the text by their Roman numerals:

- I. Falconer H, Andersson E, Aanesen A, Fried G. Follicle-stimulating hormone receptor polymorphisms in a population of infertile women. *Acta Obstet Gynecol Scand.* 2005;84:806-11.
- II. Falconer H, Mwenda JM, Chai DC, Wagner C, Song XY, Mihalyi A, Sims P, Kyama C, Cornillie FJ, Bergqvist A, Fried G, D'Hooghe TM. Treatment with anti-TNF monoclonal antibody (c5N) reduces the extent of induced endometriosis in the baboon. *Hum Reprod.* 2006;21:1856-62.
- III. Falconer H, Mwenda JM, Chai DC, Song XY, Cornillie FJ, Bergqvist A, Fried G, D'Hooghe TM. Effects of anti-TNF-mAb treatment on pregnancy in baboons with induced endometriosis. *Fertil Steril.* 2008;89 (E-pub ahead of print 2007; Aug 30)
- IV. Falconer H, Sundqvist J, Gemzell-Danielsson K, von Schoultz B, D'Hooghe TM, Fried G. AMH, FSH and TNF in follicular fluid and serum from infertile women with endometriosis. Submitted 2008



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# LIST OF ABBREVIATIONS

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AMH	Anti-müllerian hormone
ASRM	American society for reproductive medicine
CCCT	Clomiphene citrate challenge test
CFR	Cycle fecundity rate
COH	Controlled ovarian hyperstimulation
CPP	Chronic pelvic pain
EC	Endometrial cell
ET	Embryo transfer
FF	Follicular fluid
FSH	Follicle stimulating hormone
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GnRH	Gonadotropin releasing hormone
IFN- $\gamma$	Interferon gamma
IL	Interleukin
IVF	In vitro fertilization
mAb	Monoclonal anti-body
MMP	Matrix metalloproteinase
OPU	Ovum pick up
ORT	Ovarian reserve test
PDGF	Platelet derived growth factor
PF	Peritoneal fluid
POF	Premature ovarian failure
rAFS	Revised American Fertility Society classification of endometriosis
RANTES	Regulated upon activation, normal T-cell expressed and secreted
SNP	Single nucleotide polymorphism
TGF- $\beta$ 1	Serum transforming factor $\beta$ 1
TIMP	Tissue inhibitor of MMP
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor



# INTRODUCTION

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## ENDOMETRIOSIS – A GYNECOLOGICAL ENIGMA

Endometriosis is defined as the presence of endometrial-like tissue outside the uterus, which induces a chronic, inflammatory reaction. The condition is found in women of reproductive age, from all ethnic and social groups. The associated symptoms may severely impair general physical, mental and social well-being (*Kennedy et al., 2005*). Endometriosis is associated with dysmenorrhoea, deep dyspareunia, chronic pelvic pain, cyclical or perimenstrual symptoms with or without abnormal bleeding, infertility and chronic fatigue. However, some affected women remain asymptomatic.

The prevalence of endometriosis is estimated to be around 10%–15% of all women of reproductive age (*Zhao et al., 1998; Nothnick and D'Hooghe 2003; Hemmings et al., 2004*), although recent data suggest that the prevalence may be lower (*Pugsley and Ballard 2007*). Endometriosis is found in 20%–50% of all women with infertility (*Zhao et al., 1998; Winkel 2000; Kyama et al., 2004*), and in 25%–70% of women and adolescents with chronic pelvic pain or pelvic pain and dysmenorrhoea (*Propst and Laufer 1999; Gambone et al., 2002*).

The diagnosis of endometriosis may be suspected from the occurrence of symptoms alone. Frequently these symptoms are similar or identical to those of other gynaecological or gastrointestinal disorders. As yet, imaging techniques such as ultrasound and magnetic resonance imaging (MRI) do not have satisfactory diagnostic accuracy (*Kennedy et al., 2005*). Several serum markers, including CA-125 and combined cytokines, have been proposed as diagnostic blood tests (*Kruitwagen et al., 1991; Bedaiwy et al., 2002; Falconer et al., 2005*). Still, the only way to conclusively diagnose endometriosis is laparoscopic inspection of the pelvis with histological confirmation in uncertain cases. Disease severity, based on the observations from laparoscopy, is usually classified into four stages (I–IV or minimal to severe) (*ASRM, American Society for Reproductive Medicine 1997*). There is no strict correlation between the classification grade and the type or severity of pain symptoms, but a negative correlation has been reported between the grade of endometriosis and pregnancy rate following surgery (*D'Hooghe et al., 2003*).

The diagnosis of endometriosis in women has traditionally been made by the presence of typically puckered black or blue lesions found at laparoscopy. However, endometriosis has been diagnosed in up to 90% of cases with more subtle lesions (red or white), and red lesions are considered to be the most active type (*Nisolle and Donnez 1997*), with the highest likelihood of histological confirmation. Neoangiogenesis is believed to be one of the most important pathogenetic factors for endometriosis. Studies of the stromal vascularization have shown that red lesions have a significantly higher capillary mean surface area when compared to typical black/blue or white lesions (*Nisolle et al., 1993*). Red lesions may later turn in to black lesions, which are considered characteristic for advanced endometriosis (*Donnez 1993; Nisolle et al., 1993; Donnez et al., 1998*). White lesions are assumed to be healed or latent lesions but may also remodel into more active lesions (*D'Hooghe et al., 1992*).



**Fig 1.** Laparoscopic pictures of different endometriotic lesions (Left: typical blue-black lesion, Middle: white lesion, Right: red lesion).

## PATHOGENETIC ASPECTS OF ENDOMETRIOSIS

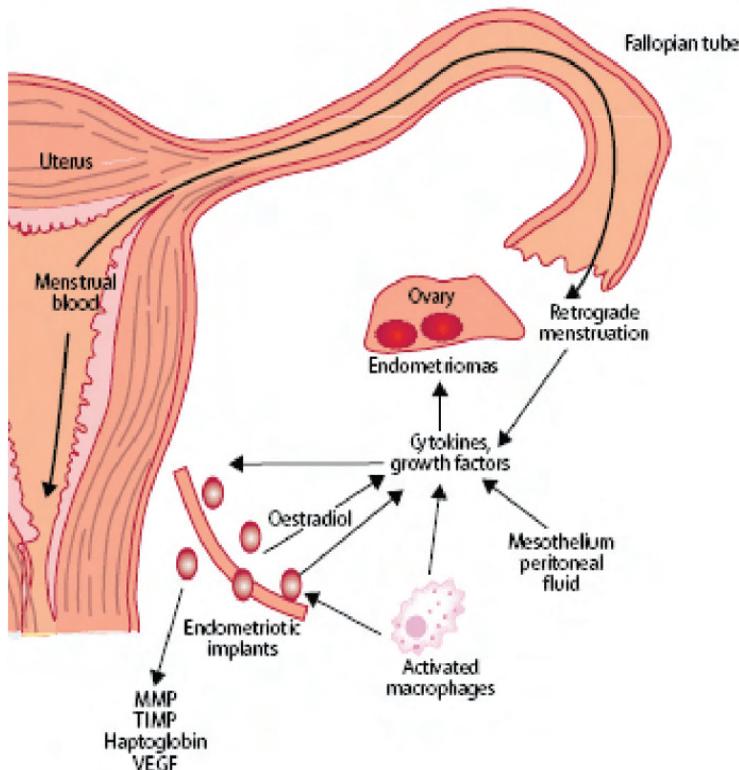
### Retrograde menstruation

Endometriosis was initially described as adenomyotic lesions in the rectovaginal septum and round ligament, occurring along the Müllerian tract (*Cullen 1896; Cullen 1920*). Since the Müllerian ducts arise from coelomic epithelium, the metaplasia hypothesis was proposed. According to this hypothesis, ectopic endometrium and peritoneal endometriosis results from *in situ* metaplasia of totipotential mesothelial serosal cells (*Meyer 1919; Gruenwald 1942*). Hence, the metaplasia hypothesis could explain the occurrence of endometriosis in the absence of menstruation. However, it is weakened by the fact that endometriosis almost entirely occurs in the presence of endometrium and that disease in men is limited to case reports.

The most widely accepted pathogenetic factor of endometriosis is retrograde menstruation (*Sampson 1927*). According to the Sampson hypothesis, endometriosis is caused by retrograde menstruation with intraperitoneal spilling of endometrial cells and subsequent adhesion to and implantation on the peritoneal surface. Retrograde menstruation is known only to occur in women and in non-human primates and in a few exceptional cases, such as the elephant shrew and the bat (*D'Hooghe and Debrock 2002*). Several observations support the Sampson hypothesis of retrograde menstruation. Firstly, epidemiological studies have shown that women are more prone to develop endometriosis if the cycle is short or the menstrual flow is long (*Cramer et al., 1986; Arumugam and Lim 1997; Vercellini et al., 1997*). Secondly, endometriosis is more common among women with obstructed menstrual outflow (*Pinsonneault and Goldstein 1985; Olive and Henderson 1987*). Thirdly, non-human primates with experimental obstruction of the menstrual outflow (e.g. cervical ligation), develop endometriosis (*Te Linde and Scott 1950; D'Hooghe et al., 1994*). These observations strongly suggest that retrograde menstruation is of utmost importance in the pathogenesis of endometriosis.

The main critic against the Sampson hypothesis is that retrograde menstruation occurs in most women. Retrograde menstruation has been reported in as many as 76% (*Liu and Hitchcock 1986*), 82% (*Blumenkrantz et al., 1981*) and 90% (*Halme et al., 1984*) of investigated women. A correlation between the extent of endometriosis and the amount of endometrium used for induction, has been observed during experimental studies in the baboon (*D'Hooghe et al., 1995*). This finding implies that the amount of endometrium may be a crucial pathogenetic factor in the early onset of the disease. However, the development of endometriosis involves not only reflux of endometrium but also adhesion to the peritoneum, proliferation and angiogenesis. It is clear that many other factors apart from retrograde menstruation are necessary in the

formation of endometriotic lesions in the pelvis. Prior to adhesion and implantation, refluxed endometrial cells in the pelvis need to escape normal apoptosis and the cleansing capability of the peritoneal environment. Endometrium of women with endometriosis is believed to be abnormal, predisposed to successful establishment of ectopic disease. (*Giudice and Kao 2004*). Apoptosis of the endometrium seems to be impaired in women with endometriosis and genomic alterations have been detected in eutopic endometrium (*Gebel et al., 1998; Wang and Guo 2004*). Furthermore, a lack of adequate immune surveillance in the peritoneum is thought to be a cause of the disorder.



**Fig 2.** Schematic view of events occurring during retrograde menstruation in the pelvis (adapted from *Giudice 2004*).

### Immunology and inflammation

The concept of endometriosis as a mechanical disease (retrograde menstruation) has been modified over the past 15 years by new insight gained from studies demonstrating an increased local and systemic inflammatory activity in women with endometriosis (*Akoum et al., 1996; Pizzo et al., 2002; Wieser et al., 2002*). Several cytokines and chemokines, such as interleukin (IL)-1, IL-6, IL-8, IL-15, RANTES (regulated on activation, normal T expressed and secreted), TGF- $\beta$ 1 and tumor necrosis factor (TNF), are abundantly expressed in the peritoneal fluid (PF). High serum concentrations of IgG, IgA, and IgM autoantibodies and antibodies to endometrium have been reported (*Wild and Shivers 1985; Nothnick 2001*). There is also evidence of compromised natural-killer-cell (NK-cell) activity in peritoneal fluid in women with endometriosis, which can lead to decreased surveillance of ectopic tissue (*Oosterlynck et al., 1991*). Furthermore, factors involved in the interaction between refluxed endometrial cells and the immune system, such as sICAM-1 (soluble intercellular adhesion molecule), are increased in peritoneal fluid (*Maeda et al., 2002*).

Cytokines are low molecular weight proteins or glycoproteins typically synthesized by peritoneal macrophages, monocytes, ectopic endometrial implants or mesothelial cells of the peritoneum. The activity of macrophages and monocytes in PF seems to be increased in women with endometriosis (Zeller *et al.*, 1987). Apart from cytokines, increased amounts of numerous growth factors (GM-CSF, VEGF, PDGF) have been detected in serum and PF. It has been shown that the level of certain cytokines and chemokines (IL-6, IL-8, RANTES, TNF) in the PF correlate with the severity of endometriosis (Harada *et al.*, 2001; Pizzo *et al.*, 2002). There is strong evidence that cytokines are involved in many of the early steps in endometriosis development and also during progression of the disease. RANTES is a potent chemoattractant for monocytes and macrophages, which is considered important in the early inflammatory reactions in the pelvis (Hornung *et al.*, 1997). IL-1, TNF and IFN- $\gamma$  are capable of upregulating the expression of ICAM-1, which may facilitate adhesion of endometrial cells to the peritoneum (Rothlein *et al.*, 1988). IL-8 and TGF- $\beta$ 1 have been shown to exert growth-promoting effects on endometrial and endometriotic cells (Iwabe *et al.*, 2000; Loverro *et al.*, 2001). IL-15, a novel cytokine with immunoregulatory and angiogenetic properties, is important in stromal decidualization and stimulates uterine NK-cell proliferation. Aberrant expression of IL-15 may contribute to implantation failure associated with endometriosis (Kao *et al.*, 2003). Angiogenesis is thought to be an important pathogenetic factor and several angiogenic factors, such as IL-1, IL-6, IL-8 and VEGF, have been identified in women with endometriosis (Taylor *et al.*, 2002).

#### *TNF*

TNF (tumor necrosis factor, also known as tumor necrosis factor alpha) is a 26kDa cytokine with pleiotrophic, pro-inflammatory effects. It belongs to the TNF superfamily (TNFSF) which also includes lymphotoxin (LT), Fas ligand (FasL) and other related proteins. The main functions of the TNFSF are regulation of proliferation and apoptosis. TNF acts on two different receptors, TNFRI (p55/CD120a) and TNFRII (p75/CD120b). The receptors are present on all cell types apart from erythrocytes and both receptors also exist in soluble forms. Actions mediated through TNFRI are related to apoptotic processes while proliferative effects are mediated through TNFRII. As secreted from endometriotic lesions, activated T-cells and mesothelial cells, TNF stimulates production of IL-6, IL-8 and RANTES, which in turn promote neoangiogenesis and recruitment of macrophages, T-cells and eosinophils. TNF is thought to be a primary local signal which initiates and modulates apoptosis during menstruation (Tabibzadeh 1996).

TNF is elevated in both PF and serum in women with endometriosis (Eisermann *et al.*, 1988; Bedaiwy *et al.*, 2002). Little is known about the activity of TNF in the ovaries, although a few reports suggest that TNF is elevated in follicular fluid (Carlberg *et al.*, 2000; Wunder *et al.*, 2006). TNF has been shown to increase the adherence of cultured stromal cells to mesothelial cells (Zhang *et al.*, 1993). Iwabe et al. demonstrated that TNF stimulates proliferation of endometriotic stromal cells through induction of IL-8 gene and protein expression. It was concluded, that TNF may be one of the essential factors in the pathogenesis of endometriosis (Iwabe *et al.*, 2000). The significance of TNF in the pathogenesis of endometriosis is furthermore supported by the proliferative effect of TNF observed on endometrial cells from women with endometriosis but not on cells from healthy controls (Braun *et al.*, 2002). Also, TNF in high concentrations seems to affect sperm motility in vitro and may have embryotoxic effects (Hill *et al.*, 1987a; Hill *et al.*, 1987b). These findings indicate that TNF may have additional roles in the development of endometriosis-associated infertility.

## **Establishment of ectopic endometrium**

Upon escaping normal breakdown and immunosurveillance, refluxed endometrium needs to attach to the peritoneum in order to form ectopic endometrial implants. It has been speculated that eutopic endometrium from women with endometriosis can attach to and invade peritoneal surfaces. Matrix metalloproteinases (MMP) degrade extracellular matrix components and MMP 7 and 11 are normally expressed in the endometrium during menstrual breakdown (*Osteen et al., 1999*). MMPs are suppressed by progesterone during the secretory phase and are upregulated by TNF and IL-1 (*Sillem et al., 2001*). In women with endometriosis, a relative progesterone resistance has been observed in both eutopic and ectopic endometrium (*Attia et al., 2000*). This together with an overexpression of TNF and IL-1 leads to a persistent expression of MMPs which may enable retrograde endometrial tissue to invade peritoneal surfaces. TNF may also contribute to a decreased expression of tissue inhibitors of MMPs (TIMPS) which could further enhance this process.

As described earlier, progression of endometriosis is promoted by several cytokines and growth factors. Also, the endocrine sensitivity of eutopic endometrium has been well known for more than 30 years. The estrogen-dependency of the disease is also the basis of current medical treatment. It has become evident that the local production of estrogens is an important element in the pathogenesis of endometriosis. Aromatase is the key enzyme in the biosynthesis of estradiol, catalysing conversion of androstenedione and testosterone, derived from ovarian and adrenal sources, to estrone and estradiol, respectively. Aromatase is expressed in ovarian granulosa cells, placental syncytiotrophoblasts, adipose tissue, skin fibroblasts, and brain, but is normally absent from endometrium. However, substantial expression of aromatase has been shown in endometriotic lesions and to less extent in eutopic endometrium of women with endometriosis (*Zeitoun and Bulun 1999*). This local expression of aromatase and subsequent production of estradiol sustains endometriotic lesions and stimulates progression of the disease. Once endometrial tissue attaches and invades peritoneal surfaces, neoangiogenesis is essential to promote growth of endometriotic implants. During laparoscopy, active endometriotic lesions are easily recognized by their rich vasculature. Vascular endothelial growth factor (VEGF) and other angiogenetic factors, such as IL-1, IL-6, IL-8 and platelet-derived growth factor (PDGF), are abundant in ectopic endometrium (*Taylor et al., 2001*). Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor and it has been demonstrated that VEGF is estrogen- and progestin-responsive (*Shifren et al., 1996*).

## **One disease – three entities?**

During laparoscopy, different forms of endometriosis can be observed in the pelvis. Peritoneal endometriosis appears as superficial implants and is characterized by typical blue-black, red or white lesions. Ovarian disease is easily recognized as uni- or bilateral endometriomas. Deep infiltrating disease is commonly found in the pouch of Douglas and in the rectovaginal septum, with or without involvement of the urinary bladder and bowel. All forms may be present in the same woman. Whether peritoneal endometriosis represents an early form of the disease, which may later progress into ovarian and deep infiltrating disease, is unknown. It has been speculated that peritoneal disease in many cases rather reflects a physiological phenomenon caused by retrograde menstruation (*Koninckx 1994*). However, the severity of disease corresponds poorly to symptoms like pelvic pain. Also, fertility may be decreased in women with only limited peritoneal endometriosis. Studies of the gene expression in ectopic and eutopic endometrium

have revealed differences between the various forms of endometriosis (*Matsuzaki et al., 2006*). The clinical presentation of endometriosis together with evidence of biochemical disparity could suggest that the disease constitutes three different entities. This may be relevant in future etiological research and may aid the clinician to individualize treatment.

## TREATMENT OF ENDOMETRIOSIS

Endometriosis-associated pain can be treated both surgically and medically. Surgical ablation of endometriotic lesions should be performed during diagnostic laparoscopy as this reduces pain in minimal to moderate disease (*Jacobson et al., 2001*). Postoperatively, hormonal treatment should be considered (*Parazzini et al., 1994; Vercellini et al., 1999*). The role of surgery in advanced disease is debated, but it is generally believed that endometriosis-associated pain can be reduced by removing the entire lesions in severe and deeply infiltrating disease (*Kennedy et al., 2005*). Medical treatment is based on hormonal suppression, and progestins, danazol, gestrinone or GnRH-agonists appears to be effective in treating pain associated with endometriosis, though their side effects and cost profiles differ (*Prentice et al., 2000a; Prentice et al., 2000b; Davis et al., 2007*). In uncomplicated cases, medical treatment can be offered without definitive, surgical diagnosis. However, conception is not possible during medical treatment and symptoms are likely to recur following treatment cessation. The increasing understanding of the pathogenesis of endometriosis has led researchers to focus on novel molecular targets for the development of future treatment options. Clinical trials with aromatase inhibitors, anti-progesterone e.g. mifepristone and selective progesterone receptor antagonists (SPRMs) have shown positive effects on pelvic pain associated with endometriosis (*Kettel et al., 1998; Soysal et al., 2004; Chwalisz et al., 2005*). Figure 3 illustrates some characteristics of current medical treatment for endometriosis as compared to the vision of an “ideal treatment” for the future.

<p><b>Current medical therapy</b></p> <ul style="list-style-type: none"> <li>• Suppression of estradiol relieves pain</li> <li>• Not curative</li> <li>• Moderate-severe side-effects</li> <li>• Does not allow pregnancy</li> <li>• Does not improve fertility</li> </ul>	<p><b>The ideal treatment</b></p> <ul style="list-style-type: none"> <li>• High efficacy on endometriosis-associated symptoms</li> <li>• No adverse effects on bone</li> <li>• High tolerability</li> <li>• Allows pregnancy</li> <li>• Improves fertility</li> </ul>
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**Fig 3.** Characteristics of current medical treatment and a future “ideal treatment”.

### *TNF-antagonists*

TNF is implicated in the pathogenesis of many chronic inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis and Crohn’s disease. The introduction of TNF antagonists represents a significant advance in the therapy of these conditions (*Valesini et al., 2007*). The effects of TNF can be neutralised either by monoclonal antibodies (infliximab, adalimumab) or by blocking soluble TNF receptors (etanercept). Recent studies suggest that apoptosis of monocytes and lymphocytes could be the mechanistic basis of TNF inhibitors (*Di Sabatino et al., 2004; Shen et al., 2005*). Furthermore, studies indicate that anti-TNF reduces pathological neovascularization (*Gardiner et al 2005*) and inhibits the production of GM-CSF (granulocyte–macrophage colony-stimulating factor) by mucosal T cells (*Agnholt et al 2004*). Thus, anti-TNF may act on several possible pathophysiological mechanisms in endometriosis.

The role of TNF antagonists in endometriosis have been investigated in a small number of preclinical and experimental studies. The proliferative effects of TNF on endometrial cells was blocked by the anti-TNF agent etanercept (*Braun et al., 2002*). In both rats and baboons, TNF-antagonists have been demonstrated to prevent the onset of induced endometriosis (*D'Antonio et al 2000, D'Hooghe et al 2005*).

## **INFERTILITY**

Infertility is a common condition and approximately 10% of couples have difficulty conceiving a child. In young, healthy couples, the probability of conception in one reproductive cycle is typically 20 to 25%, (cycle fecundity rate, CFR) and in 1 year it is approximately 90% (*Gnoth et al., 2003*). In clinical practice, an evaluation is commonly recommended after 1 year of unprotected intercourse without conception, the standard clinical definition of infertility. After the introduction of in vitro fertilization (IVF) in 1978, there has been a dramatic improvement in the management of female infertility. The most common causes of infertility are shown in Table 1.

Male	20-25 %
Tubal	25-30 %
Anovulation	20 %
Endometriosis	5-10 %
Unexplained	25-30 %

**Table 1.** Distribution of causes of infertility.

## **Endometriosis and infertility**

Endometriosis is associated with a lower pregnancy rate, not only from natural ovulatory cycles, but also after intrauterine insemination or after in vitro fertilization and embryo transfer (*Barnhart et al., 2002; D'Hooghe et al., 2003*). CFR in women with endometriosis is 2-10% (*Hughes et al., 1993*) and there is a higher prevalence of endometriosis in infertile women compared with fertile women (*Strathy et al., 1982*). Other reports have confirmed that infertile women are 6 to 8 times more likely to have endometriosis than fertile women (*Verkauf 1987*). Several mechanisms have been proposed to explain the association between endometriosis and infertility. These include severe adhesions in the pelvis, increased inflammatory activity in peritoneal fluid and impaired implantation (*Schenken et al., 1984; Lessey et al., 1994; Lebovic et al., 2001*).

As judged from randomized trials, surgical ablation of endometriotic lesions plus adhesiolysis may improve fertility in patients with minimal-mild endometriosis (*Jacobson et al., 2002*). Also, in the absence of randomized trials, there is consensus that surgery is probably effective to improve fertility in women with moderate to severe endometriosis (*Kennedy et al., 2005*). Merely ovarian suppression is not effective and should not be offered as treatment for the indication of endometriosis-associated subfertility; conception is either impossible or contraindicated during medical treatment of endometriosis (*D'Hooghe et al., 2003*). Intrauterine insemination could be considered in minimal-mild disease (*Tummon et al., 1997*) although IVF is more appropriate in advanced cases or after other treatments have failed. As yet, it remains controversial whether surgery should be performed prior to IVF in infertile women with endometriosis.

## Ovarian reserve

Ovarian reserve is a term used to describe the functional potential of the ovary and reflects the number and quality of available oocytes. The ovarian reserve, constituted by the size of the ovarian follicle pool and the quality of the oocytes therein, declines with increasing age, resulting in the decrease of a woman's reproductive capacity (*te Velde et al., 1998*). Ovarian reserve can be considered normal in conditions where stimulation with the use of exogenous gonadotropins will result in the development of at least 8–10 follicles and the retrieval of a corresponding number of healthy oocytes at follicle puncture (*Fasouliotis et al., 2000*). Women with a poor response, so-called 'low responders' (*Ferraretti et al., 2000*), are said to have a diminished ovarian reserve (*Scott and Hofmann 1995; Sharara et al., 1998*). Several factors are believed to be involved in a low response, including increasing age, pelvic adhesions, ovarian disease and immunological factors. Basal FSH, FSH-receptor polymorphisms and AMH, anti-mullerian hormone (also known as Mullerian inhibiting substance, MIS), have been proposed as markers of ovarian reserve (*Tanbo et al., 1992; Gromoll et al., 1996; Visser et al., 2006*). The purpose of ovarian reserve tests (ORTs) is to identify women with diminished ovarian reserve for their age.

AMH is produced by preantral and small antral follicles and is a member of the TGF $\beta$ -family (transforming growth-factor beta). AMH is thought to play a role in the initial recruitment and in the selection of the dominant follicle (*Visser et al., 2006*). The expression of AMH is regulated independently by gonadotropins and androgens and it was demonstrated that TNF exerts an inhibitory effect on AMH-expression in mice testes (*Hong et al., 2003*). A poor response in IVF is associated with low levels of S-AMH and data suggest that AMH is superior to FSH as an indicator of ovarian reserve (*van Rooij et al., 2005; Al-Qahtani and Groome 2006*). The accuracy of predicting pregnancy is in general limited for all available ORTs (*Broekmans et al., 2006*). However, a recent study suggests that FSH receptor genotype is associated with pregnancy outcome after IVF (*Klinkert et al., 2006*). Several authors have previously reported that polymorphisms in the FSH-receptor may be of clinical relevance during infertility investigation (*Gromoll et al., 1996; Sudo et al., 2002; Laven et al., 2003*). Whether these genetic alterations contribute to severely diminished ovarian reserve, or premature ovarian failure (POF), is unknown.

## ANIMAL MODELS IN ENDOMETRIOSIS RESEARCH

For apparent reasons, studies to determine the onset, ethiology and progression of endometriosis are difficult to perform in women. Although endometriosis can be diagnosed through laparoscopy, at the time of diagnosis, the duration of disease is unknown. In a multifactorial disease like endometriosis, adequate control groups are difficult to define. Finally, for ethical reasons, it is difficult to carry out invasive studies in humans. For these reasons, relevant alternative models are needed for endometriosis research (*Falconer et al., 2005*).

Both rodents and non-human primates have extensively been used in endometriosis research. It is obvious that a close phylogenetic relationship between animal and human is preferred. Even if it is possible to study certain aspects of a disease in rodents, it is not clear whether these findings can be transferred to humans. The use of primates has made it possible to induce endometriosis, with lesions similar to those seen in humans. This is important for research purposes since spontaneous endometriosis in primates most commonly is minimal to mild. The ability to induce moderate to severe disease is crucial for the study of pathogenesis, endometriosis-associated

subfertility and drug effects. Endometriosis research in animal models can address most aspects of the disease, including pathogenesis, infertility and effects of new drugs.

### **Small animals**

Rats, rabbits and hamsters have been used as models for induction of endometriosis. The disease has been induced using both auto- and xenotransplants. Autologous transplants have been performed in rabbits, rats and hamsters, using uterine tissue removed at mini laparotomy (*Schenken and Asch 1980; Vernon and Wilson 1985; Steinleitner et al., 1991*). Syngenic mice have been used in the same fashion (*Somigliana et al., 1999*). This non-physiological method of induction has caused several problems such as adhesions interfering with fertility. Development of nude mice and SCID (severe combined immuno deficiency) has made it possible to successfully implant human endometrium, thereby making it a more attractive model for the study of endometriosis.

The advantages of using rodents compared to primates are low cost and ease of maintenance. However, these benefits probably do not make up for the disadvantages. The most important drawbacks are that rodents lack a menstrual cycle and do not have spontaneous endometriosis. The rat has spontaneous ovulation but its luteal phase is shorter than in humans, while the rabbit lacks a luteal phase. To study endometriosis in rodents, the disease must invariably be induced. Induced endometriosis in rodents differs significantly from the disease in humans, with rodents developing more cystic lesions that do not show the rich variations of lesions that can be seen in primates. It has also been questioned whether the peritoneal environment of rodents contains the paracrine stimuli necessary for neoangiogenesis.

### **Non-human primates**

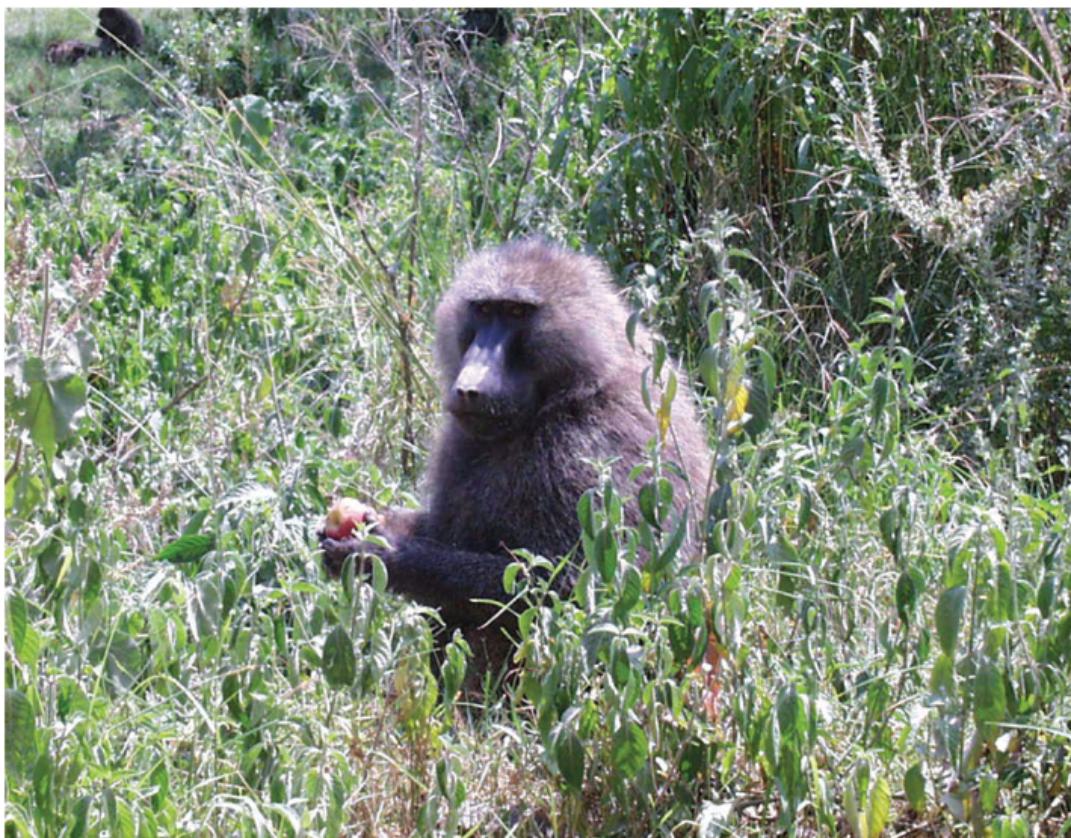
Non-human primate studies have been carried out using several species including rhesus macaques, cynomolgus monkeys and baboons. The great apes (chimpanzee, gorilla, urangutang) are closest to humans in many anatomical and physiological aspects of reproduction. However, since all of them are protected, endangered species in the wild, they cannot be used for research.

Primates develop spontaneous disease and endometriosis has been reported in over 10 different primate species. Not all of them are suitable as animal models for endometriosis research, and due to the specific demands, the baboon has been developed at the Institute of Primate Research, Nairobi, Kenya as a model for the study of endometriosis. Most of the endometriosis research in non-baboon species has been conducted in primates with induced endometriosis. The first attempts to induce endometriosis in rhesus monkeys were carried out by Jacobson in 1926 (*Jacobson 1926*). In this study, minced endometrial tissue was obtained after hysterotomy of the anterior uterine wall and was sown in the abdomen, or placed beneath the peritoneum in a few specific locations. This method resulted in implantation of endometrium on the surface of the uterus. A new method for the induction of endometriosis involved repositioning of the cervix into the abdominal cavity, thereby allowing intra-abdominal menstruation (*Te Linde and Scott 1950*). Endometriosis has also been successfully induced in the cynomolgus monkey (*Schenken et al., 1987*).

#### *The baboon model*

The baboon has become the mostly used non-human primate model in the last 10-15 years. The suitability of the baboon as a model in endometriosis research is due to a number of factors.

Firstly, baboons adapt well in captivity with maintained menstrual cycling and fertility. Secondly, the menstrual cycle of the baboon is very similar to humans, generally given to be 31 to 34 days, with a range of between 20 and 50 days (*Bambra 1993*). Thirdly, the baboon shows a cyclic inflation and deflation of the perineal skin, enabling non-invasive follow-up of menstruation, follicular and luteal phases. Fourthly, adult female baboons weigh about 10-20 kg, a size allowing repeated blood sampling and performance of complicated surgical procedures. Fifthly, transcervical curettage in the baboon differs not significantly from the procedure in humans. The cervical channel of the baboon mimics the human, except for size. The rhesus macaque on the other hand, has a spiral shaped channel, which makes it difficult to perform transcervical procedures. The endometriotic lesions in the baboon, both spontaneous and induced, are similar to lesions observed in humans, both in terms of shape and form but also in location. This, together with advantages shared with other non-human primates (genetics, spontaneous disease), makes the baboon the current gold standard for endometriosis research.



# AIMS

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The general aim of this clinical and experimental study was to reveal important aspects on the pathophysiology of endometriosis and infertility. In addition to explore the feasibility of a novel treatment modality against endometriosis.

The specific aims of this thesis were:

- To explore whether inhibition of TNF has a therapeutic potential against endometriosis in a baboon model.
- To evaluate effects from TNF inhibition on endometriosis-associated infertility
- To elucidate the possible association between polymorphisms in the FSH-receptor and reduced ovarian reserve
- To study markers for ovarian reserve and inflammation in women with endometriosis

# MATERIAL AND METHODS

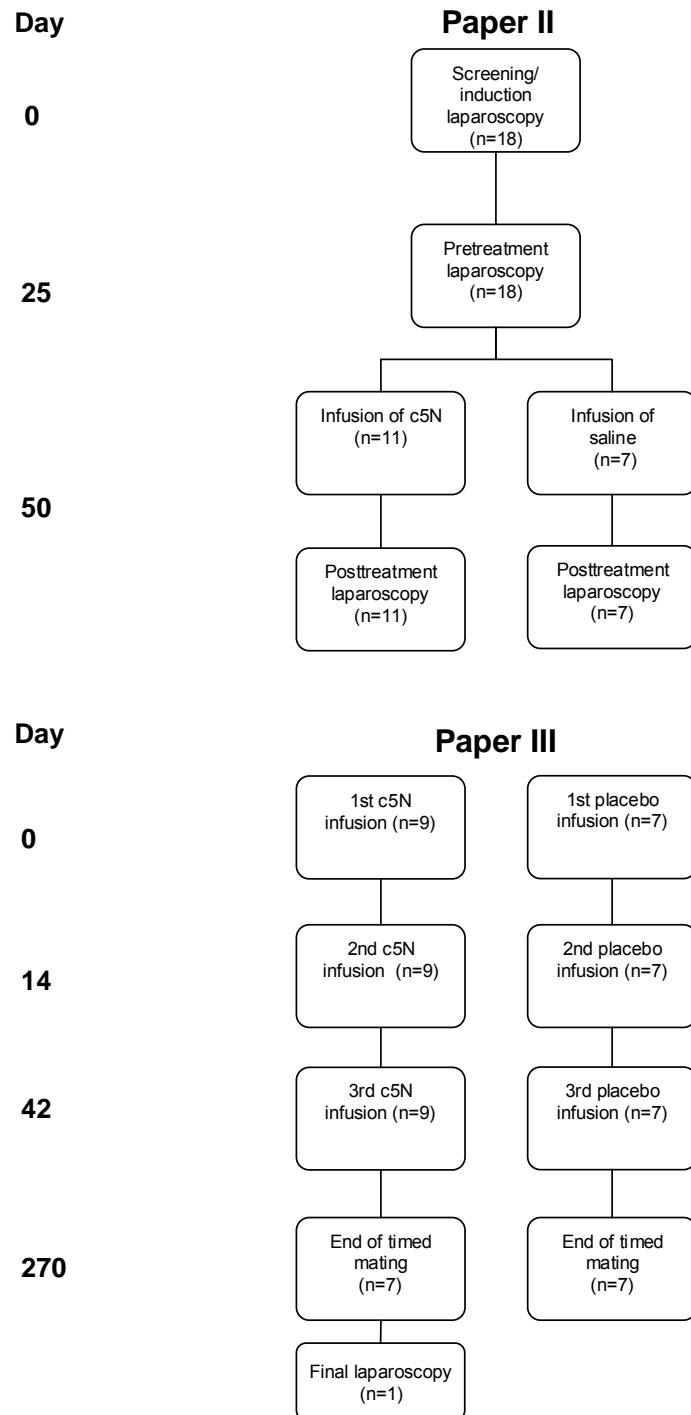
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## **ANIMALS (II, III)**

A total of 18 adult female baboons (*Papio anubis*) with proven fertility in the wild were selected to participate in the experimental studies. All 18 animals were used in the first study (II) and 16 of these in the second (III). A flow chart for these experiments is shown in Figure 4. Their exact age was not known, but the animals had been in captivity at the Institute of Primate Research (IPR, Kenya) between 10 and 42 months (median 10 months). The animals were all trapped in the wild and kept in quarantine for 3 months. During quarantine, the animals were screened for Tuberculosis (TBC), Simian T-Lymphotropic Virus-1 (STLV-1) and Simian Immunodeficiency Virus (SIV). All animals had a normal menstrual cycle (mean  $\pm$  SD of  $39 \pm 7$  days) prior to the study. The stage of the menstrual cycle was monitored daily by animal technicians through visual observation of inflation/deflation of the perineal skin. No hormonal assays were performed during this study. 9 male baboons of proven fertility with a normal sperm analysis were used for the second experimental study (paper III). The Institutional Scientific and Ethical Committee (ISERC, Nairobi, Kenya) of IPR reviewed and approved the study protocol. In study III, the power calculation was based on a previous study showing that the cycle pregnancy rate was significantly lower in baboons with stage III-IV endometriosis and in animals with stage II disease than in those with stage I endometriosis or with a normal pelvis (*D'Hooghe et al., 1996*). It was hypothesized that following induction all baboons would develop stage III to IV endometriosis, since previous inductions with a standardized weight of endometrium resulted in endometriosis stage III to IV endometriosis in 50% of baboons (*D'Hooghe et al., 2006*). In this context the expected CFR was estimated to be 5%, assuming that all baboons would develop moderate or severe endometriosis. The power analysis was then performed with the assumption that c5N treatment would restore the CFR in baboons with induced endometriosis from 5 % to 20 %. To test this hypothesis, it was calculated that 59 cycles had to be studied in each group to obtain statistical significance at a level of  $p < 0.05$ . For practical reasons, all animals were exposed to 9 mating cycles, or until the occurrence of pregnancy.

## **MONITORING OF THE MENSTRUAL CYCLE (II, III)**

The menstrual cycle was monitored through daily observations of the perineal skin (sex skin) (*D'Hooghe et al., 1991; Bambra 1993*). The menstrual cycle in the baboon is divided into 8 different stages, with stage 7 being the menstrual phase, stage 1-5 the follicular phase and 6, 0 the luteal phase (*Hendrickx 1971*). The progressive inflation of the perineum throughout the cycle correlates with the follicular growth and the subsequent deflation (stage 6 and 0) to the luteal phase. Ovulation normally occurs 3 days prior to perineal deflation with a margin of error of 2 days (*Hendrickx 1971*). An additional stage, stage 8, is similar to stage 0 (however more reddish) and becomes apparent after approximately 40 days of gestation (*Altmann 1970*). No hormonal assays were performed during the experimental studies.



**Fig 4.** Flow chart illustrating the design of the experimental studies.

## **STUDY AGENT (II, III)**

As infliximab does not bind to monkey TNF, a mouse-human version of a murine anti-TNF mAb (c5N, IgG2a subtype), was employed in the studies. c5N is chimerized in order to reduce immunogenicity and the antibody binds and neutralizes both monkey and human TNF. c5N was produced and purified by Centocor (Malvern, PA, USA). Drug pharmacokinetics was not evaluated during these trials. The dosage used in the studies was equivalent to the dose of infliximab (Centocor, Malvern, PA, USA) administrated to humans for Crohn's disease (5 mg/kg). Both c5N and placebo were administrated as an intravenous infusion over 2 hours. During infusion of test agent, a veterinarian documented respiration frequency, pulse and temperature every 15 minutes.

## **SURGICAL PROCEDURES (II, III)**

Anaesthesia and laparoscopies were performed as described previously (*D'Hooghe et al., 1991*). Peritoneal fluid (PF) was aspirated before the baboon was placed in the Trendelenburg position and stored for future analysis. A single investigator (HF) performed all laparoscopies.

The induction laparoscopy was performed on the first or second day of menstruation. During this laparoscopy, and prior to the induction process, all animals were screened for the presence of pelvic abnormalities. Transcervical curettage with a Novak curette was performed as previously described (*D'Hooghe et al., 1995*). The endometrial tissue was weighed and minced through an 18-gauge needle. The average weight of the endometrial tissue was  $1,65 \pm 0,70$ g ( $1,80 \pm 0,55$  and  $1,55 \pm 0,79$ g for the placebo and c5N group respectively). The tissue was then seeded under laparoscopic vision into the pelvic area. Induction sites were standardized to the uterovesical fold, urinary bladder, pelvic walls, anterior side of broad ligament, uterus and the pouch of Douglas. The ovaries were excluded due to the risk of extensive formation of tubal/ovarian adhesions, which could impair the inspection at follow-up laparoscopies.

The first video-laparoscopy was performed to stage the extent of induced endometriosis on day 25 after induction and was labelled the "pre-treatment laparoscopy". A detailed pelvic map was constructed, with systematic photographic and video documentation of the pelvis during each laparoscopy. Adhesions involving ovary, Fallopian tube and cul-de-sac (ASRM-adhesions) were graded according to the revised classification system of the American Society for Reproductive Medicine. Other adhesions that were not related to the ovary, Fallopian tube and cul-de-sac and that were observed between individual peritoneal endometriotic lesions and pelvic organs were recorded separately. The surface area of an endometriotic lesion (and an endometriotic lesion-related adhesion) was determined by multiplying length (mm) x width (mm) or, in cases of a circular lesion, by using the formula  $\pi \times r^2$ . The volume of a lesion was estimated by multiplying surface area ( $mm^2$ ) x depth (mm). Lesion area and volume were calculated from measurements made with a lateral trocar with a 1 mm hole at the tip. Only a diagnostic laparoscopy was performed; no endometriosis biopsies were taken.

The second video-laparoscopy was performed on day 25 after c5N/placebo administration and was defined as the posttreatment laparoscopy. Lesions were documented according to size, type and localization on video, photo prints and individual pelvic maps. At least one and at most two, representative biopsies of an endometriotic lesion (preferentially red or blue-black) were taken by scissors and electrocoagulation from each baboon for pathologic confirmation of the disease. Only easily accessible lesions were excised, frozen in liquid nitrogen and stored at -80° C.

A final laparoscopy was performed in study III in all non-pregnant animals after 9 timed mating cycles. During this procedure, endometriotic lesions were documented according to size, type and localization.

### **TIMED MATING (II, III)**

The timed mating process was carried out as previously described (*D'Hooghe et al., 1996; Stevens 1997*). Male baboons (1 male/female) were introduced in the female cages during the preovulatory phase and remained in the cage until the perineal skin of the female baboons showed signs of deflation (luteal phase, stage 6). Male baboons were selected based on availability and on their ability to establish pregnancy. Baboons were observed for pregnancies based on amenorrhea and a deep pink colour of the perineal skin (stage 8). Suspected pregnancies were all confirmed with abdominal ultrasound. All pregnancies during the study were terminated preterm by hysterotomy after the ultrasound confirmation of pregnancy, and the baboons were subsequently euthanized as per the IPR standard Operating Procedure.

All timed mating cycles were evaluated after the termination of the study. Due to unpredicted circumstances, such as temporary limited availability of male baboons and irregular menstrual cycles, mating cycles were excluded from statistical analysis if there was a reasonable possibility that timed mating did not occur in optimal conditions. These suboptimal conditions were defined as: a duration of timed mating shorter than 4 days, cancellation of timed mating more than 2 days prior to the initiation of luteal phase (expected ovulation), or the presence of an abnormal cycle, defined as a non-menstrual cycle with the duration of either follicular or luteal phase deviating more than 1 standard deviation (SD) from the average length for that baboon.

### **CLINICAL MATERIAL (I, IV)**

The clinical material in study I comprised a total of 68 women who underwent investigation for infertility at the infertility unit (RMC) at the Karolinska University Hospital, Sweden. The causes of infertility included male factor, endometriosis, tubal factor, anovulation and unknown infertility. They were divided into two groups based on their FSH-levels. The cut-off for the normal group was set at  $\text{FSH} \leq 10 \text{ IU/L}$ . The pathological group, defined as a  $\text{FSH} > 10 \text{ IU/L}$  on cycle day 3 or a pathological clomiphene citrate challenge test (CCCT), comprised 16 women. The normal group with  $\text{FSH} \leq 10 \text{ IU/L}$ , consisted of 52 women. A CCCT was performed in patients with age  $\geq 35$  years, unknown cause of infertility or poor response on previous FSH-stimulation. A total of 38 women underwent CCCT (55.9 %); 29 in the normal population (55.8 %) and 9 in the pathological (56.3 %). Venous blood samples were collected for hormone analysis and analysis of the FSH-receptor. Each patient received a full explanation of the purpose of the study and gave written informed consent. The study was approved by the local ethics committee.

In study IV, consecutive follicular and serum samples were collected from women attending the infertility unit (RMC) at the Karolinska University Hospital, Sweden. From this prospective cohort, a total of 34 women with laparoscopically verified endometriosis were identified. A second group of 38 women with tubal factor infertility and with no signs of endometriosis upon laparoscopy were used for comparison. The groups were similar with respect to age, BMI, smoking and duration of infertility. All women underwent IVF and embryo transfer as per standardised protocol. The study protocol was reviewed and granted by the regional ethics committee.

## OVARIAN STIMULATION PROTOCOL (IV)

All patients followed a standard long GnRHa-FSH protocol for stimulation as described earlier (*Fried et al., 1996*). Gonadotropin releasing hormone agonist (GnRHa) (Suprefact®, Hoechst AB, Stockholm, Sweden) was administered as a nasal spray  $6 \times 200 \mu\text{g}/\text{day}$  starting on day 21 of the menstrual cycle and was reduced to half the dose when FSH injections were commenced. Downregulation was verified by vaginal ultrasound scanning, followed by administration of 75–300 IU/day of recombinant FSH (Gonal-F®, Serono Nordic AB, Sollentuna, Sweden) subcutaneously (s.c.). Starting dose was adjusted according to the same rules for all patients. The initial dose of FSH was generally 150 IU up to age 35, and 225 IU above this age, unless response to previous FSH stimulation at IVF indicated otherwise. Follicular development and endometrial growth were monitored by vaginal ultrasonography using a Siemens Sonoline SI/200 in combination with blood samples for serum estradiol assays. When an adequate stimulation was achieved, i.e. a controlled rise of serum estradiol and a leading follicle diameter of at least 17 mm, 10,000 IU hCG (Profasi®, Serono Nordic AB, Sollentuna, Sweden) was given s.c. Approximately 35 h later OPU was performed by transvaginal ultrasound-guided follicle aspiration. IVF, ET, and pregnancy follow-up were performed as described elsewhere (*Fried et al., 1996*). Luteal phase support was given using 400 mg micronized progesterone (Progesteron MIC APL, Sweden) as vaginal suppositories 3 times daily until a pregnancy test was performed and if found positive, continued for 8 weeks after embryo transfer. Pregnancy was defined as a serum hCG level  $> 10 \text{ IU/L}$  at 2 weeks after ET and subsequently rising. Clinical pregnancy was defined as presence of at least one intrauterine fetus with regular heart beats.

## HORMONE ASSAYS (I, IV)

Serum and follicular fluid concentrations of AMH were determined by enzyme immunoassay (EIA) (A16507, Immunotech, Marseille, France). Detection limits and intra- and interassay coefficients of variation were 150 pM, 12.3 % and 14.2 %. The plate was read at 450 nm. Serum concentrations of FSH were assayed by the Central Laboratory for Clinical Chemistry, Karolinska Hospital. The assay is standardized by both the World Health Organization's Second International Reference preparation of FSH for Bioassay, number 78/549, and the World Health Organization's Second International Reference Preparation of human menopausal gonadotropin. Estradiol and FSH in follicular fluid were determined by chemiluminescent enzyme immunometric assay (LKE21 resp. LKFS1), run on IMMULITE® automatic analyser (Diagnostic Products Corporation, California, USA). Samples for analysis of estradiol were diluted 1:1000 in E2 sample diluent (LE2Z, Diagnostic Products Corporation, California, USA). Detection limits and intra- and interassay coefficients of variation were 55 pmol/L, 15 % and 16 % for estradiol; and 0.1 IU/L, 3.7 % and 6.7 % for FSH. All hormone assays were done according to manufacturer's protocol.

## CYTOKINE ASSAYS (IV)

TNF levels in follicular fluid were determined by IRMA, immunoradiometric assay, (KIC1751, Biosource, California, USA) and the assays were performed according to manufacturer's protocol. Detection limits and intra- and interassay coefficients of variation were 5 000 pg/ml, 6 % and 7 %.

Levels of IFN- $\gamma$  (interferon gamma), VEGF (vascular endothelial growth factor), GM-CSF (granulocyte macrophage colony stimulating factor), IL-6, IL-8, IL-10 and IL-15 were determined with a Bio-Plex™ human cytokine panel (Bio-Rad Laboratories Inc., California, USA). The method combines the principle of a sandwich immunoassay with fluorescent bead-based technology, allowing individual and multiple analyses of up to 100 different analytes in a single microtiter well. The assay was done in 96-well microplate format according to manufacturer's protocol. The plate was read with Bio-Plex® suspension array system (Bio-Rad Laboratories). Analysis of experimental data was done using five-parametric curve fitting using Bio-Plex Manager version 4.1.1 from BioRad Laboratories.

### **ANALYSIS OF FSH-RECEPTOR POLYMORPHISMS (I)**

Genomic DNA was prepared from peripheral blood by rapid purification procedure as follows: 50  $\mu$ L frozen blood was thawed, mixed with 400  $\mu$ L freshly prepared 0.17M  $\text{NH}_4\text{Cl}$ , inverted and left in room temperature for 20 min. The samples were spun for 30 sec, and the pellet washed 3 times in cold 0.9% NaCl. Finally, the pellet was resuspended in 200  $\mu$ L 0.05 M NaOH, boiled for 10 min and neutralized with 25  $\mu$ L 1 M Tris-HCl, pH 8.0. (*Lundberg Giwercman et al., 1998*). PCR primers for the FSH receptor were designed according to routine methods. The primer pair used was: GCAAGTGTGGCTGCTATGAA (5'-3') and GTGACATACCCTCAAAGGC (5'-3', complementary), corresponding to nucleotides 1991-2011 and 2220-2240 of the human FSH receptor open reading frame.

PCR was performed on the resulting genomic DNA (1 $\mu$ L) using 0.3  $\mu$ L of Dynazyme DNA polymerase (In Vitro AB, Solna, Sweden) in a 25  $\mu$ L reaction mixture containing 2.5 $\mu$ L dNTP, 1.5  $\mu$ L each of specific primers, 2.5  $\mu$ L Dynazyme 10x Buffert and 15.7  $\mu$ L H<sub>2</sub>O. PCR conditions were one cycle of 96°C for 3 minutes, one cycle of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minutes (35 cycles each). The PCR was performed on a Perkin Elmer DNA Thermal Cycler (Perkin Elmer, Waltham, USA). PCR products (6  $\mu$ L mixed with loading buffer dye) were separated on a 1.5% agarose gel (Agarose NA, Pharmacia) at 140 V and visualized by ethidium bromide staining. The identities of the PCR products were verified by sequencing. The PCR products were gel purified using the ExoSAP-IT (USB Corp, USA) and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Corp.) at the automated sequencing facility at Molecular Genetics, Department of Cell and Molecular Biology at Karolinska Institutet. Using BLAST search ([www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/)) the FSH receptor primer products were found to be identical (99%) to XM 002212.3 H. Sapiens follicle stimulating hormone receptor (FSHR).

**STATISTICS**

All statistical analyses were performed with the statistical package Graphpad Prism and Statmate (Graphpad Software Inc, San Diego, CA). A p-value < 0.05 was considered significant.

	Student's T-test	ANOVA	Wilcoxon signed rank test	Mann-Whitney U test	Fisher's exact test	Spearman	Kaplan-Meier
Paper I	X	X					
Paper II	X		X	X			
Paper III	X			X	X		X
Paper IV				X	X	X	

Table 2. Overview of the different statistical methods used for each study.

# RESULTS AND DISCUSSION

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## EFFECTS OF ANTI-TNF-MAB ON INDUCED ENDOMETRIOSIS (II)

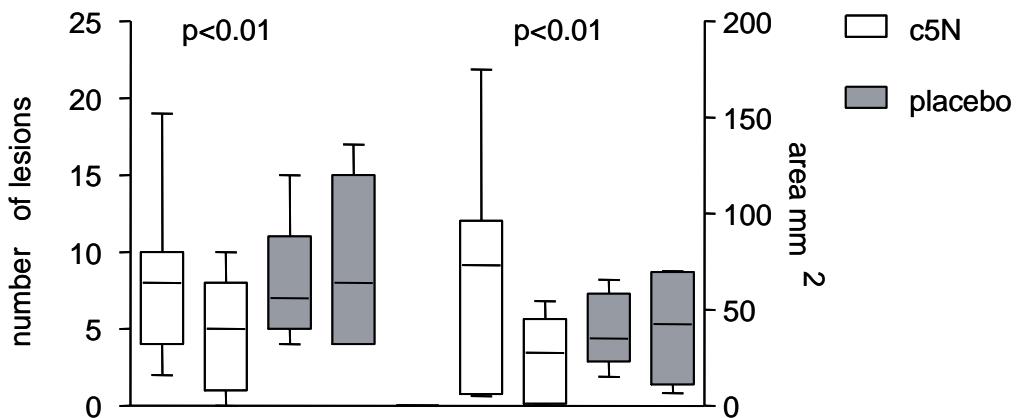
Increasing evidence suggest that TNF exercise a pivotal role in the early onset and progression of endometriosis (*Bullimore 2003*). Promising results have been reported on the use of various TNF-inhibitors in endometriosis models (*D'Antonio et al., 2000; Barrier et al., 2004; D'Hooghe et al., 2006*). In this study, we tested the hypothesis that a TNF monoclonal antibody would reduce the extent of induced endometriosis in the baboon.

In the baboons treated with anti-TNF-mAb, a significant reduction of both surface area and volume of endometriotic lesions was observed when compared to the pretreatment laparoscopy. In contrast, no significant changes were observed in the placebo group (Table 3). Furthermore, in the c5N group, the number and surface area of red lesions was significantly decreased when compared to the pretreatment laparoscopy (Fig 5).

	c5N (n=11)		Placebo (n=7)	
	Pre	Post	Pre	Post
Total surface area (mm <sup>2</sup> )	105.0 ± 18.9	78.6 ± 15.6*	71.3 ± 8.6	75.2 ± 10.6
Total volume (mm <sup>3</sup> )	111.5 ± 19.6	86.3 ± 20.5*	62.9 ± 10.0	70.0 ± 14.1
No. total lesions	16.6 ± 1.4	18.6 ± 1.8*	16.7 ± 1.5	18.7 ± 1.6*
rAFS score	11.6 ± 1.4	9.6 ± 1.2	9.1 ± 1.4	10.3 ± 1.3
No. Adhesions	2.2 ± 0.7	2.5 ± 0.7	2.4 ± 1.2	3.3 ± 1.4

**Table 3.** Mean values (± SD) for total surface area, volume and number of endometriotic lesions at pre- and posttreatment laparoscopies in baboons treated with either c5N or placebo. Also, rAFS score and number of adhesions are given. \*p<0.05

Remodelling is an important concept in endometriosis. This term describes the change in phenotype between endometriotic lesions during the progression of disease (*D'Hooghe et al., 1992*). It has been shown that red lesions are more active than white or the typical blue-black lesions, with a higher surface area of capillary vessels (*Nisolle et al., 1993*). White lesions on the other hand are considered healed or latent while typical blue-black lesions represent more advanced disease. The remodelling from one type of lesion into another was clearly seen in this study. In support of the favourable effect of c5N treatment, the remodelling from white lesions to red lesions or typical blue-black lesions was less pronounced in the c5N group than in the placebo group. These results suggest that c5N is capable of reducing induced peritoneal endometriosis in the baboon model. The effect was most clearly seen on the number and surface area of red lesions. Also, the apparent disruption of the natural progression of endometriosis could support the anti-proliferative effects of TNF inhibitors observed in recent in vitro experiments (*Braun et al., 2002*).



**Fig 5.** Change in number (left) and surface area (right) of red lesions between pre- and post-treatment laparoscopies in baboons treated with either c5N or placebo. Box and whisker plots representing median values with fifty percent falling within the box. The whiskers represent range.

A major drawback with current treatment of endometriosis is the inability of treated women to conceive. All treatment modalities affect the menstrual cycle and pregnancy is either impossible or contra-indicated during medication. A secondary endpoint in study II was the effects on the menstrual cycle. The menstrual cycles of the baboons were carefully monitored 6 months before, during and 2 months after the study. No significant alterations in cycle length, when compared to the average pre-study cycles, were observed in either treatment group (Table 4). This suggests that the reproductive potential (i.e., ovulation) is preserved during treatment with c5N.

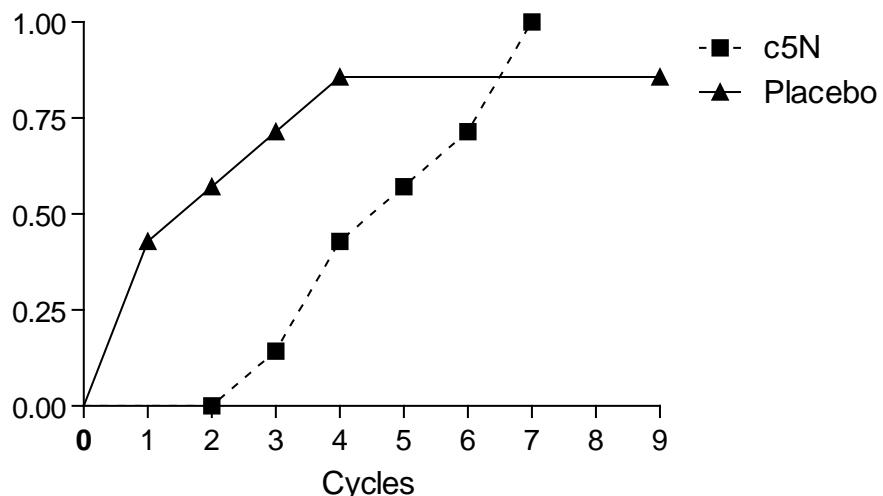
	Cycle length at baseline	Cycle length after induction	
Placebo	39.0±5.4	39.8±8.7	ns
c5N	35.4±5.3	35.8±6.6	ns

**Table 4.** Menstrual cycle length (days, means ± SD) before the onset of study II compared to cycle length after the induction of endometriosis (mean from 3 cycles).

The finding of systemic and local inflammation in women with endometriosis (Agic *et al.*, 2006) suggests similarities with other chronic, inflammatory disease such as rheumatoid arthritis and ankylosing spondylitis. However, current medical therapy is focused on hormonal suppression with moderate to severe side-effects as a result. Novel therapy in preclinical and clinical studies is mostly directed towards the refinement of hormonal therapy (aromatase inhibitors, mifepristone, SPRMs) or unspecific pathogenetic targets (angiogenesis inhibitors). The introduction of compounds with specific anti-inflammatory properties, e.g. TNF-inhibitors, may challenge current treatment paradigms of endometriosis. TNF-inhibitors have significantly improved quality of life in patients with rheumatic disease (Olsen and Stein 2004) and it is reasonable to believe that this effect could apply also to women with endometriosis. To date, no clinical studies on the use of TNF-inhibitors in endometriosis are available.

### EFFECTS OF ANTI-TNF-MAB ON PREGNANCY (III)

The purpose of this experimental study (III) was to test the hypothesis that anti-TNF-mAb improves fertility in non-human primates with endometriosis. 16 female baboons received a total of four intravenous infusion of either c5N (anti-TNF-mAb, n=9) or placebo (saline, n=7). After 9 months of timed mating, pregnancy outcome was analysed and compared between the groups. The overall mating ratio was 85.4 % and was comparable between the c5N (84.8 %) and placebo (86.3 %) groups. Two animals in the c5N-group died during the study and were excluded from statistical analyses. The total pregnancy rate was 100% (7/7) in the c5N group and 86% (6/7) in the placebo group. The overall cycle fecundity rate (CFR) was 22.4 % with no significant difference between the c5N (18.4 %) and placebo (30.0 %) groups. Similarly, the mean time to pregnancy was comparable in the c5N ( $4.2 \pm 2.3$  cycles) and placebo ( $2.9 \pm 2.5$  cycles) groups. Also, the cumulative pregnancy rate within 9 cycles was comparable in the c5N group (100%) and the placebo group (86%; Fig 6).



**Fig 6.** Cumulative pregnancy rate within 9 cycles in baboons with induced mild endometriosis treated with c5N (n=7) or with placebo (n=7).

We were thus unable to confirm our hypothesis since pregnancy outcome was similar in both groups. There are a few methodological aspects which may contribute to this result. The most apparent is the small sample size. The power calculation was performed under the assumption that the induction of endometriosis would cause a more severe degree of disease in the animals. The average adapted ASRM score was 10 for both groups which corresponds to ASRM group II. Although previous data suggest that stage II disease impairs fertility in baboons to some extent (*D'Hooghe et al., 1996*), current results indicate that fertility was normal in the studied animals. It is also possible that the short duration of disease in these animals was insufficient to severely affect fertility. However, all animals in the c5N eventually became pregnant which supports the hypothesis that TNF-inhibitors preserve reproductive potential during treatment of endometriosis. This is further supported by a retrospective study of women with Crohn's disease treated with TNF-inhibitors, where similar pregnancy outcome was observed compared to non-treated women (*Katz et al., 2004*).

There are a limited number of randomized, prospective drug trials in endometriosis-associated infertility treatment. The vast majority of fertility improving studies has focused on the refinement of IVF protocols. Of the existing RCTs, none have demonstrated an improvement of pregnancy outcome after medical treatment (*Hughes et al., 2007*). An interesting study was published by Balasch et al, where the potential fertility enhancing effects of the immunomodulating drug penoxifylline was studied (*Balasch et al., 1997*). Although unable to demonstrate any significant improvement, this study represents an attempt to explore the potential of anti-inflammatory compounds in endometriosis-associated infertility. It was concluded from this and other similar studies that the number of patients were insufficient to reach significant power. Also, several of these studies have included women with multiple causes of infertility which makes it difficult to isolate the effect of endometriosis as a cause of infertility. In summary, no beneficial effects of c5N were observed on fertility in baboons with induced endometriosis. However, all animals eventually became pregnant during treatment. This suggests that the reproductive potential is preserved during treatment of endometriosis with a TNF-inhibitor.

### **UNEXPECTED EVENTS DURING EXPERIMENTAL STUDIES (II, III)**

Four of the 11 baboons treated with c5N during the experimental studies died, whereas no health problems were observed in the placebo group. It is reasonable to speculate whether c5N contributed to the deaths of these animals. Treatment with TNF-inhibitors can be associated with some severe adverse events, including activation of latent infections. Malignancies (including lymphoma) and deaths have been reported in patients treated with TNF blocking therapies. Today, there is no final evidence that these adverse events are increased in anti-TNF treated patients when compared to the baseline risk of the treated population (*Colombel et al., 2004*). Thus, rheumatoid arthritis patients do have an increased risk of lymphoma and death, regardless their treatment modality. Similarly, the baseline risk of infection is increased in most patients treated with TNF blockade due to the severity and duration of inflammation and due to associated co-morbidities. (*Colombel et al., 2004*). The histopathological analyses of tissue biopsies from the deceased animals in these studies were inconclusive regarding the definite cause of death and toxicity studies are required to clarify the potential contribution of c5N in the deaths of the animals. However, commercially available TNF-inhibitors for treatment of Crohn's disease and ulcerative colitis (infliximab), rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis (infliximab, adalimumab, and etanercept), and psoriasis (infliximab and etanercept) are generally well tolerated and severe adverse events are rare in humans (*Blonski and Lichtenstein 2006*) and in previous studies in baboons (*Barrier et al., 2004; D'Hooghe et al., 2006*). Nonetheless, all patients need to be carefully screened for infection, including tuberculosis, before starting anti-TNF therapy.

### **FSH-RECEPTOR POLYMORPHISMS AND OVARIAN RESERVE (I)**

The influence of age on female fertility is well known. It has previously been shown that natural fertility starts to decline after the age of thirty, with progression to eventual sterility at a mean age of 41 (*Spira 1988; Wood 1989*). A woman's age is also directly related to success in IVF treatment (*Templeton et al., 1996*). Individual response to exogenous FSH is variable. Elevated serum levels of FSH at cycle day 3 or after CCCT is associated with a poor follicular development during COH (*Sharara et al., 1998*). These tests are widely used in clinical practice to identify poor responders. Previous data suggest that ovarian response to exogenous gonadotropins may be related to FSH-receptor genotype (*Perez Mayorga et al., 2000*). Two

common single nucleotide polymorphisms (SNPs) have been identified in the FSH-receptor exon10; threonine or alanine at position 307 and serine or asparagine at position 680. These two positions are in linkage disequilibrium, resulting in three distinct receptor variants (Asn/Asn, Asn/Ser, Ser/Ser at position 680). A relationship between the FSH-receptor variant Ser/Ser680 and diminished ovarian reserve has been proposed by several authors (*Gromoll et al., 1996; Sudo et al., 2002; Laven et al., 2003*). The primary aim of study I was to explore the distribution of FSH-receptor polymorphisms in a population of infertile women. The secondary goal was to compare the distribution of FSH-receptor variants between women with normal and pathological FSH-levels (at cycle day 3 or after CCCT).

The overall distribution of FSH-receptor genotypes demonstrated a predominance of the homozygous variants (Asn/Asn 35%, Asn/Ser 24%, Ser/Ser 41%). The distribution of receptor variants seems to differ between studied populations. Whereas several authors have reported a dominance of the heterozygous variant (*Gromoll et al., 1996; Sudo et al., 2002; Jun et al., 2006*), others have demonstrated results similar to ours (*Laven et al., 2003; Loutradis et al., 2006*). These differences probably reflect the ethnic variations between populations. It is well known that the distribution of gene polymorphisms varies in different ethnical populations and this is one major factor of bias that has given concern regarding SNP data analysis (*Clayton et al., 2005; Weir et al., 2005*). Here, we observe no differences in the distribution of FSH-receptor variants between women with apparent normal ovarian reserve and women with diminished FSH at cycle day 3 or 10. The analysis of receptor variants in relation to normal FSH revealed a significantly higher mean FSH at cycle day 10 in the Ser/Ser-group compared to cycle day 3 and also compared to the other receptor variants at cycle day 10 (Table 5).

<b>Population</b>	<b>Asn/Asn</b>	<b>Asn/Ser</b>	<b>Ser/Ser</b>
<b>Normal FSH cd 3</b>	5.6±1.9	6.7±1.3	5.7±1.7
<b>Normal FSH cd10</b>	6.9±1.9	6.3±1.7	8.3±2.8*

**Table 5.** Mean levels ( $\pm$  SD) for FSH (IU/L) according to FSH receptor polymorphisms in 52 women with apparent normal ovarian reserve for cycle day 3 (cd3) and cycle day 10 (cd10). In the Ser/Ser-group, FSH at cd10 was significantly higher than at cd 3 ( $p<0.05$ ). Also in this group, FSH levels at cd10 were increased compared to the Asn/Asn and the Asn/Ser-groups ( $p<0.01$ ).

IVF outcome was not reported in paper I as the data collection exceeded the time frame of this project. A recent analysis of IVF data showed that 42 of the 68 women went through a total of 85 IVF-cycles (*Falconer and Fried 2007, unpublished*). During controlled ovarian hyperstimulation, a significantly higher dose of exogenous FSH was required in the Ser/Ser-group as compared to the Asn/Asn- and the Asn/Ser-groups (Table 6). No other significant differences were recorded between the groups, although women with FSH-receptor variant Ser/Ser seemingly reached lower estradiol and produced fewer oocytes at OPU.

These results suggest that certain FSH-receptor variants correlate poorly with the subset of infertile women where pathological FSH-levels were identified. The pathology behind these changes is clearly a complex trait with multiple mechanisms. However, women with normal basal FSH had significantly higher FSH levels on cycle day 10 after CCCT in the Ser/Ser-group. Therefore, a reduced ovarian responsiveness may be more common in women with Ser/Ser receptor variant.

This is supported by our recent analysis of IVF data and also by several other studies (*Gromoll et al., 1996; Sudo et al., 2002; Laven et al., 2003*). Recently, Klinkert et al reported FSH receptor genotype to be associated with pregnancy outcome (*Klinkert et al., 2006*).

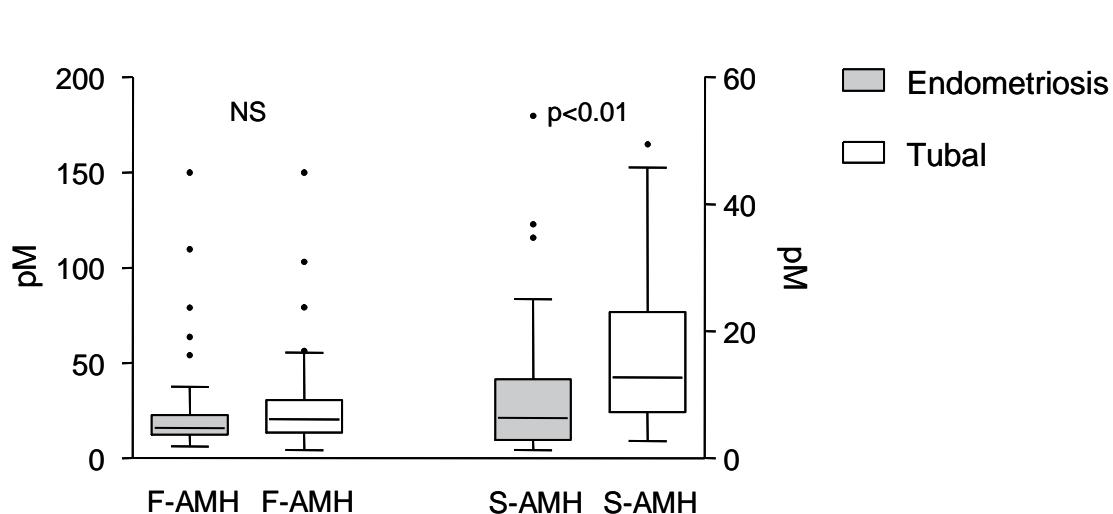
	<b>Asn/Asn (n=10)</b>	<b>Asn/Ser (n=12)</b>	<b>Ser/Ser (n=20)</b>	
<b>Cycles</b>	22	23	40	
<b>FSH total (IU)</b>	2125 (875-3375)	2275 (925-3400)	2775 (1250-5400)	p<0.05
<b>E2 at OPU (pmol/l)</b>	2915 (844-5543)	2720 (1024-8105)	2425 (778-4433)	NS
<b>Oocytes</b>	7 (2-13)	6 (3-12)	4 (1-10)	NS
<b>Pregnancy rate/cycle</b>	24 %	26 %	18 %	NS

**Table 6.** Analysis of IVF data from 85 cycles according to FSH-receptor genotype. Values represent median and range.

It is possible that genotyping of the FSH-receptor may aid the clinician to identify a group of women with a latent or subclinical reduction of ovarian reserve. Also, analysing FSH-receptor polymorphisms may be a helpful tool to determine dose of FSH during controlled ovarian hyperstimulation.

#### AMH, FSH, TNF AND OVARIAN RESERVE (IV)

The significance of serum AMH and basal FSH as markers of ovarian reserve has been evaluated in several studies on infertile women in general (*Scott and Hofmann 1995; Visser et al., 2006*). Women with endometriosis respond less well to controlled ovarian hyperstimulation, but there are few studies about the ovarian reserve in this group. Increasing evidence suggest a link between endometriosis-associated infertility and inflammation (*Halis and Arici 2004*).



**Fig 7.** Levels of AMH in follicular fluid (left) and serum (right) for women with endometriosis (n=34) and women with tubal factor infertility (n=38). Box and whisker plots representing the median values with fifty percent falling within the box. Whiskers represent non-outlier range.

The aim of study IV was to analyze the levels of AMH in relation to IVF outcome and inflammatory activity in the follicles. Analyses of AMH in serum and follicular fluid showed lower amounts of AMH in serum from women with endometriosis whereas no differences were observed in follicular fluid (Fig 7). Also, women with endometriosis produced fewer small follicles (<12 mm) during COH and had a significantly lower fertilization rate. Serum AMH data are consistent with recent findings by Lemos et al, who reported on lower cycle day 3 levels of AMH in women with minimal to mild endometriosis (*Lemos et al., 2007*).

We also found increased amounts of TNF and other important cytokines and growth factors in women with endometriosis (Table 7). There are numerous reports on increased amounts of TNF in the peritoneal fluid (*Bullimore 2003*), but little is known about follicular TNF levels in women with endometriosis. In a recent publication, no differences were detected in follicular fluid from women with endometriosis compared to women with unexplained infertility (*Kilic et al., 2007*). This discrepancy could reflect the heterogeneity of the chosen control groups and it cannot be ruled out that the ovarian environment in unexplained infertility is altered.

	<b>Endometriosis</b>	<b>Tubal factor</b>	
<b>F-TNF (pg/mL)</b>	40.0 (7.71-95.8)	30.8 (10.9-112.8)	p<0.05
<b>F-IL6 (pg/mL)</b>	12.7 (5.6-59.0)	12.1 (4.7-36.9)	NS
<b>F-IL8 (pg/mL)</b>	128 (33.3-431)	125 (70.8-369)	NS
<b>F-IL10 (pg/mL)</b>	1.49 (0.33-3.81)	2.12 (0.61-7.21)	p<0.05
<b>F-IL15 (pg/mL)</b>	22.7 (4.72-49.2)	17.0 (4.16-62.9)	p<0.05
<b>F-VEGF (pg/mL)</b>	102 (357-3930)	871 (119-4591)	NS
<b>F-GM-CSF (pg/mL)</b>	224 (68.1-472)	187 (15.2-337)	p<0.05

**Table 7.** Concentrations of cytokines and growth-factors in infertile women with endometriosis (n=34) and women with tubal factor infertility (n=38). (Values represent median and range; F=follicular fluid).

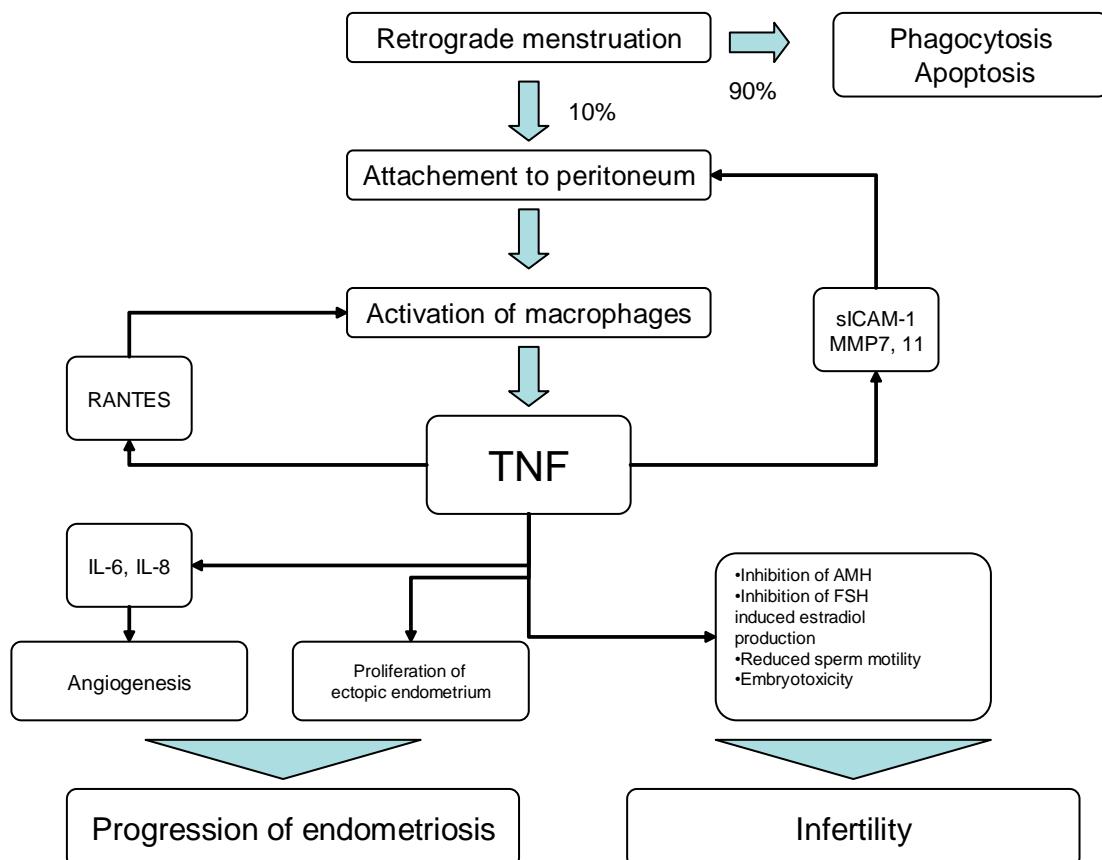
While previous data support a regulatory function of TNF on AMH in testes (*Hong et al., 2003*), we were unable to demonstrate any correlations between these hormones in serum or follicular fluid. It is possible that the regulation of AMH differs between men and women, although the regulatory step SF-1 (steroidogenic factor 1) has been described in both genders (*Shen et al., 1994*). However, negative correlations were observed between AMH in both serum and follicular fluid and FSH in follicular fluid. Thus, high concentrations of FSH in follicular fluid correspond to low levels of AMH in serum and follicular fluid. Since AMH is considered to be regulated independently from gonadotropins, this relationship may merely be a general effect of diminished ovarian reserve.

Taken together, the results suggest that women with endometriosis have a diminished ovarian reserve, presumably on the basis of an increased inflammatory activity in the ovarian follicles. The exact mechanism on how an elevated immune response influences the ovarian reserve is still unknown. However, TNF in follicular fluid correlates with oocyte quality (*Lee et al., 2000*). It is possible that TNF and other pro-inflammatory mediators may have direct effects on oocyte maturation as well as indirect effects on the antral follicle pool.

## TNF – A KEY PLAYER IN ENDOMETRIOSIS PROGRESSION AND INFERTILITY?

The results from this thesis add to current knowledge on the impact of inflammatory response in the early onset and progression of endometriosis. The majority of inflammatory actions seem to pass through the secretion of TNF from activated immune cells and numerous reports confirm the vital role of this potent cytokine (*Bullimore 2003*). Also, central physiological mechanisms in menstruation, follicular development and subsequent ovulation may be regulated by TNF (*Loukides et al., 1990; Tabibzadeh 1996*). In figure 8, an attempt has been made to summarize and integrate the pivotal function of TNF in endometriosis and infertility.

The starting point in this integrated theory is the retrograde reflux of menstrual debris into the pelvic area. In the majority of women, immunosurveillance and normal physiological apoptosis (regulated by TNF) clears the peritoneal cavity from the refluxed endometrium. However, in about 10 % of all women, viable endometrium escapes these protective mechanisms and attach to the peritoneum. This will activate peripheral immune cells (macrophages, monocytes, NK-cells) and initiate the production and secretion of pro-inflammatory cytokines. The release of TNF stimulates the production of RANTES, IL-6 and IL-8. RANTES is a powerful attractant of macrophages and IL-8 is particularly involved in neoangiogenesis; a critical step in the formation of novel blood vessels to support the growing endometriotic implants. TNF has direct effects on the eutopic endometrium and enhances the proliferation of glandular and stromal cells. Also, the production of IFN- $\gamma$ , L-1 and TNF stimulates the production of ICAM-1 which further facilitates the attachment process. This vicious circle is further sustained by dysfunctional NK-cells and deficient cell-mediated immunity.



**Fig 8.** Schematic presentation of the potential role of TNF in endometriosis and related infertility.

Although not so far showed in women, TNF may downregulate the production of AMH from antral follicles. AMH is thought to be involved in the recruitment of antral follicles and in the selection of a dominant follicle. Furthermore, TNF is known to inhibit FSH-induced estradiol production (*Rice et al., 1996*) and may thus interfere with normal steroidogenesis in the ovary. Also, direct effects of TNF on oocyte quality, sperm motility and embryonic development may further contribute to reduced fertility.

Taken together, pathological secretion of cytokines and growth factors seems to initiate a vicious circle of events leading not only to the progression of endometriosis but also to a decrease in reproductive capacity. TNF exerts a crucial role in this chain of negative events.

### **CRITICAL ASSESSMENT AND FUTURE PERSPECTIVES**

The beneficial effects of anti-TNF-mAb on endometriosis observed in the present thesis were limited to peritoneal disease. No animals developed severe disease or endometriomas and thus, the effects of anti-TNF on stage IV and ovarian endometriosis remains unknown. Apart from a post-mortem study on baboons with spontaneous endometriosis, ovarian disease is rarely seen and is difficult to induce (*D'Hooghe 1997; Dick et al., 2003*). However, the striking effects from only short-term treatments on peritoneal endometriosis support the notion of a critical role for TNF in endometriosis. The results from paper II are in keeping with several other experimental studies and justify clinical trials.

The somewhat disappointing results of the fertility study (III) are most likely due to the limited number of animals in combination with the induction of only stage I and II endometriosis. Compared to previous fertility trials at the Institute of Primate Research (*D'Hooghe et al., 1996*), cycle fecundity rate may be considered close to normal in the studied animals. This in turn implies that stage II disease is associated with normal fertility. However, normal fecundity in baboons depends on several circumstances such as breeding conditions, size of colony and time in captivity. Stevens reported pregnancy rates as high as 80% under optimal conditions in US breeding colonies (*Stevens 1997*). This number was based on more than 1400 matings and is probably more accurate than data from IPR. This suggests that the studied animals in paper III had significantly reduced fertility on the basis of endometriosis.

While we were unable to demonstrate positive fertility effects of TNF-inhibitors, no adverse effect was observed on the menstrual cycle during treatment. This is an interesting finding, since current medical treatment is associated with temporary sterility. Endometriosis mostly affects younger women and future medical therapy should aim to preserve and improve reproductive capacity during treatment.

An issue of special concern was the unexpected deaths of 4 baboons during the experimental studies. Although unconfirmed, it cannot be ruled out that the deaths of these animals were related to anti-TNF-mAb treatment. Treatment with TNF-inhibitors is generally considered safe, although severe adverse events, e.g. malignancies and activation of latent infections, have been reported (*Colombel et al., 2004*). However, at present, TNF-inhibitors are widely used in clinical practice against several chronic diseases, such as rheumatoid arthritis and Crohn's disease. It should be recalled that the c5N preparation in the present work, was synthesized specifically for use in the baboon model. Thus, it is possible that the dosage was inappropriate during the experiments and no toxicological studies were performed. These unexpected events further emphasize the need and importance of animal models in the development of novel drug therapy,

especially with powerful compounds such as monoclonal antibodies.

In the light of modern high-throughput SNP technologies, paper I suffers from a limited number of subjects. This is true for a majority of similar association studies published the last 10 years. However, modern multiplex technologies have not been available at a reasonable cost until recently. In the early 2000s, simple PCR technique was still the prevailing analytic method. The difficulties to interpret and extrapolate multiple association findings have been addressed in several reviews. The simplicity to identify a variety of genetic markers through PCR has led to a large number of association studies with sometimes contradictory results. Gambaro et al discussed the risk of premature conclusions in population association studies, since complex diseases often arise from interactions between several genetic and environmental factors (*Gambaro et al., 2000*). The inability to replicate many results clearly illustrates the shortcomings of recent association studies. It was concluded that this may be due to publication bias, failure to attribute results to chance and inadequate sample size (*Colhoun et al., 2003*). The advances in this field the last 2-3 years have further been boosted by the completion of the international HapMap project (*Frazer et al., 2007*). The purpose of this gigantic project was to record all existing SNPs in the genome. The resulting map will help researchers to better plan and execute association studies. In spite of these shortcomings, the results from paper I are in keeping with some larger studies and support the notion that polymorphisms in the FSH receptor may contribute to diminished ovarian reserve.

In paper IV, certain methodological circumstances apply. The follicular fluid was obtained from luteinized follicles during OPU in stimulated cycles. Whether artificial luteinization alters follicular content or not is uncertain but it is unlikely that normal preovulatory conditions are maintained. However, for ethical reasons, it would not be possible to retrieve follicular fluid from normal ovulatory cycles. Most studies on follicular fluid physiology suffer from the same problem. It could also be argued that pooling of the follicular fluid for each patient represent a methodological issue since oocyte maturation differs between individual follicles. This could lead to misinterpretation of AMH assays. Pooling of follicular fluid has been used in a number of studies and current knowledge on AMH physiology is largely based on such results. From a technical point of view, the increased amount of fluid will allow for multiple analyses.

The rapidly evolving data on the central role of immunology and inflammation in the pathogenesis of endometriosis has initiated the development of new interesting therapeutic principles. Tailored therapy against specific pathogenetic targets represents an attractive concept for the future. This strategy should ensure a high success rate and limit unwanted effects. Future studies on endometriosis treatment should be directed towards the refinement of compounds with anti-inflammatory properties. Both experimental and clinical trials are necessary in this development.

In spite of the advances in the study of endometriosis and pathogenetic mechanisms, the vast field of genetics and endometriosis needs further attention. To date, more than 100 genetic association studies have been published with highly contradictory results (*Falconer et al., 2007*). Inadequate sample size and study design are major drawbacks in the majority of these studies. The lessons learned through these studies will provide an excellent basis for future genetic studies in endometriosis. It is clear that future genetic studies need careful planning and collaboration between multiple centres.

# GENERAL CONCLUSIONS

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This thesis adds some new data to further elucidate the pathogenesis of endometriosis and related infertility. The results support a central function of TNF and the potential use of TNF-inhibitors in the treatment of endometriosis. Furthermore, both FSH-receptor polymorphisms and AMH may be useful as markers of ovarian reserve.

In summary:

- Inhibition of TNF reduced the extent of induced peritoneal endometriosis in baboons. The strongest effect was seen on red lesions, which are considered to be the most active manifestations of the disease. During and following treatment, the menstrual cycle was unchanged. This suggests that the reproductive potential is maintained during treatment.
- All animals in the baboon model conceived during ongoing treatment with anti-TNF-mAb. The pregnancy rate did not differ as compared to non-treated animals. However, this finding suggests that inhibition of TNF will not compromise fertility. Whether treatment with anti-TNF-mAb can also improve endometriosis-associated infertility remains unclear.
- Inhibition of TNF may represent a novel principle for the treatment of endometriosis. The potential value and safety of different TNF antibodies and/or receptor blocking agents should be further explored in clinical trials.
- Women with endometriosis seem to have a diminished ovarian reserve, as indicated by low levels of AMH and a less favourable IVF outcome. High levels of TNF and other pro-inflammatory cytokines in follicular fluid, suggest diminished ovarian reserve in women with endometriosis to be related to increased inflammatory activity. Thus, anti-inflammatory drugs may have a therapeutic effect against infertility related to endometriosis.
- Serum levels of AMH could aid the clinician to identify low responders among women with endometriosis prior to IVF treatment. Also, certain FSH-receptor variants may be a characteristic for a subset of infertile women.



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