

Effects of the levonorgestrel-releasing intrauterine system and a gonadotrophin releasing hormone agonist in the symptomatic treatment of minimal to moderate endometriosis.

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Abstract

Endometriosis is a chronic inflammatory disease that responds to steroidal manipulation. The gonadotrophin-releasing hormone (GnRH) agonist is the gold standard for the treatment of endometriosis. The levonorgestrel-releasing intrauterine system (LNG-IUS) has been shown in small pilot studies to be an acceptable and effective symptomatic treatment option.

The studies in this thesis were in two parts. The first was investigating the mechanisms by which the LNG-IUS was effective in the symptomatic treatment of endometriosis. The symptoms were improved by significant down regulation of estrogen (ER) and progesterone (PR) receptors as well as the reduction in mast cells number present in the ectopic endometrium ($P<0.05$).

The second was a comparative randomized study comparing the efficacy of the LNG-IUS and the GnRH agonist. This was via the ER and PR and the clinical outcome of the patients. The ER and PR were down-regulated by the GnRH agonist ($P<0.05$) in the ectopic endometrium while the ER, PR-A and the stromal compartment of PR-B were down-regulated ($P<0.05$) by the LNG-IUS. There were also improvements in the clinical symptoms as seen in the visual analogue scale (VAS) score in both treatment groups ($P<0.05$). When the concentration levels of Interleukin-6 (IL-6) and Soluble Intracellular Adhesion molecule-1 (sICAM-1) in the peritoneal fluid (PF) were compared before and after treatment with the LNG-IUS and the GnRH agonist there was a significant difference in IL-6 levels ($P<0.05$) with the LNG-IUS but not with the GnRH agonist and no differences in the sICAM-1 levels with the use of either medication.

In conclusion the LNG-IUS improves symptoms of endometriosis via the down regulation of ER, PR and reduction in mast cells number. The efficacy of the LNG-IUS was comparable to that of the GnRH agonist as evidence by the patients' clinical outcome and their effects on ER and PR.

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Abbreviations

AFS	American Fertility Society
Ag	antigen
ASRM	American Society for Reproductive Medicine
BMD	Bone mineral density
BMI	body mass index
BSA	bovine serum albumin
BUS	B-upstream segment
CA-125	Cancer antigen 125
cAMP	cyclic adenosine monophosphate
CCR1	Cognate chemokine receptor 1
CD54	Cluster of Differentiation 54
COCs	Combined oral contraceptives
COX-2	cyclo-oxygenase-2
CPP	chronic pelvic pain
Cyr61	Cysteine-rich Cytochrome 61
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DES	diethylstilbestrol
DEXA	Dual Energy X-ray Absorptiometry
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
Ec	Ectopic Endometrium
ECM	extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERE	estrogen response elements
ER- β	Estrogen Receptor Beta

ER- α ,	Estrogen Receptor Alpha
Eu	Eutopic Endometrium
FSH	Follicle Stimulating Hormone
FFPE	Formalin fixed paraffin embedded
GnRH	Gonadotrophin-releasing hormone
GnRHa	gonadotropin-releasing hormone analogue
HCL	hydrochloric acid
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPO	hypothalamo-pituitary ovarian
hsCRP	high sensitivity C-reactive protein (hsCRP)
ICAM-1/CD54	intercellular adhesion molecule 1/cluster of differentiation 54
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
IVP	Intravenous pyelogram
KIR	Killer cells inhibitor receptor
LFA-1	Lymphocyte function-associated antigen- 1
LH	Luteinizing Hormone
LHRH agonist	luteinizing hormone releasing hormone agonist
LNG	Levonorgestrel
LNG-IUS	Levonorgestrel-releasing intrauterine system
LNR	Leicestershire, Northamptonshire and Rutland
LSSVM	Least squares support vector machines
mAb	monoclonal antibody
MC	Mast cells
MCP-1	Monocyte chemotactic protein-1
MMP	Matrix metalloproteinase

MMPs	matrix metalloproteinases
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NaCl	sodium chloride
NK	natural killers
NRS	normal rabbit serum
NS	Not statistically significance
PBAC	Pictorial blood assessment chart
PBMC	peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	PBS containing 0.1% Triton
PCBs	polychlorinated biphenyl
PCR	polymerase chain reaction
PF	peritoneal fluid
PID	pelvic inflammatory disease
PKA	protein kinase A
PMs	Peritoneal macrophages
POD	Pouch of Douglas
PR	Progesterone Receptor
PR-B	Progesterone receptor-B
PRE	progesterone response elements
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RCOG	Royal College of Obstetricians and Gynaecologists
sICAM	soluble intracellular adhesion molecule
SEM	Standard error of the mean
SERMs	Selective estrogen-receptor modulators

SF-1	steroidogenic factor-1
SPRMs	Selective progesterone-receptor modulators
SR	Slow Release
TAF	transcription activation function
TBP-1	TNF-binding protein-1
TBS	Tris-buffered saline
TGF- β	Transforming growth factor- β
Th1	T helper cell type 1
TIMPs	Tissue inhibitors of metalloproteinases
TNF	Tumour necrosis factor
TVS	Transvaginal ultrasound
USP	United States Pharmacopeia
VAS	visual analogue scale
VEGF	vascular endothelial growth factor
VRS	verbal rating scale

Publications arising from the thesis

Papers

1). Engemise S, Willets JM, Emembolu JO, Konje JC (2011). The effect of the levonorgestrel-releasing intrauterine system, Mirena[®] on mast cells (MCs) numbers in women with endometriosis undergoing symptomatic treatment. *Euro J Obstet Gynecol Reprod Biol*.

2). Engemise S, Willets JM, Taylor AH, Emembolu JO, Konje JC (2011). Changes in glandular and stromal estrogen and progesterone receptor isoform expression in eutopic and ectopic endometrium following treatment with the levonorgestrel-releasing intrauterine system (Mirena[®]). *Euro J Obstet Gynecol Reprod Biol*.

3). Engemise S, Gordon C, Konje JC. Endometriosis, easily missed. (Jun 2010). *BMJ*

Abstract arising from the thesis

1). Effect of Levonorgestrel-releasing intrauterine system (LNG-IUS[®]) on the glandular and stromal estrogen and progesterone receptors in eutopic endometrium (EUE), ectopic endometrium (ECE) and normal peritoneum (NP) in endometriotic patients. *At the 56th Annual Meeting of the Society-for-Gynecologic-Investigation, Date: MAR 17-21, 2009 Glasgow, Scotland*

Chapter I: Introduction

1.1 Introduction

The human endometrium is a highly dynamic tissue which undergoes cycles of growth, differentiation, shedding and regeneration throughout the reproductive life of women (Garry et al, 2009). Endometriosis is a common, benign, estrogen-dependent, chronic gynaecological disorder mostly associated with pelvic pain and infertility. The cells in ectopic glands and stromal behave in the same way as eutopic endometrium, so every month they grow during the menstrual cycle and then shed blood. Normally before a period, the endometrium thickens in order to receive the blastocyst. When pregnancy does not happen, this breaks down and bleeds, leaving the body as menstrual blood. Endometrial tissue outside the uterus goes through the same process of proliferation, thickening and bleeding, but this remains contained setting the stage for inflammation followed by pain, swelling, bleeding, scar and adhesion formation (Pan et al, 2008).

There is no consensus on the histological origin of endometriosis. This disease was first described by Daniel Shroen a German Physician in 1690 in a book entitled *Dusputatio Inaguralis Medica de Ulceribus Ulceri*, in which he depicted sores throughout the stomach, bladder, intestines and broad ligament which had the tendency to form adhesions that linked visceral areas together. Later, in 1860 Carl von Rokitansky a fellow German wrote a detailed description of what we now term endometriosis (Knapp, 1999). This condition appears to be a gynaecological manifestation of a syndrome that may be the tip of a much larger invisible iceberg-one that represents a whole range of health problems that have underlying hormonal and/or immune dysregulation. Women with endometriosis

present with a range of symptoms in addition to those traditionally associated with the condition. However, the most common symptoms include: painful periods, pelvic pain, pain during sexual intercourse, subfertility or infertility, low backache, cyclical haematuria, cyclical dyschezia and rarely haemoptysis and obstruction to bowel or the renal tract. The associated symptoms of chronic pelvic pain (CPP), dysmenorrhoea, dyspareunia and infertility can impact on the physical, mental and social wellbeing of the patient.

As a condition it is both puzzling and frustrating; puzzling in that little is known about its true prevalence and predisposing factors and frustrating for women because there is no definitive cure and considerable confusion exists regarding optimal methods of therapies; and for physicians because the associated symptoms of pain and altered fertility do not necessarily correlate with the extent of the condition.

Various medical options are available for symptom control in women with endometriosis. These include progestogens (oral and depot), the combine oral contraceptive pill (COCP) and the gonadotropin releasing hormone analogue [GnRH agonist (commonly referred to as the gold standard treatment) (Crosignani et al, 2006)]. Recently Levonorgestrel-releasing intrauterine system (LNG-IUS) has been shown in small studies to have an efficacy similar to that of the GnRH agonist (Petta et al, 2005)

Precisely how the LNG-IUS improves endometriosis-related pain remains unanswered (Lockhat et al, 2004). It is known that systemic and local progestogens reduce the expression of estrogen and progesterone receptors within the endometrium (Critchley et al,

1998; Engemise et al, 2011a). This action may alter the production of local tissue factors, block endometrial DNA synthesis and mitotic activity, and lead to endometrial atrophy in eutopic endometrium, but whether this effect is same in ectopic endometrium (endometriosis) is not known. For this to occur, however the circulating and/or local levels of the progestogen and levonorgestrel must be enough to suppress ovulation. A small study showed that women who reported an improvement in endometriosis-related symptoms 6 months after treatment with the LNG-IUS had peritoneal fluid levels of levonorgestrel approximately two-thirds those of serum (Lockhat et al, 2005a). Whether these high levels persist beyond 6 months is unknown as there are no available studies. However, the effectiveness of the LNG-IUS in these patients has been demonstrated for up to 3 years (Lockhat et al, 2005b) suggesting that levonorgestrel in the system must continue to exert or maintain its effect on ectopic endometrium.

The aims of this study were therefore to firstly to investigate the mechanisms of action of the LNG-IUS on minimal to moderate endometriosis and secondly to compare the efficacy of the levonorgestrel-releasing intrauterine system [Mirena[®] (LNG-IUS)] with that of a gonadotrophin-releasing hormone (GnRH) agonist the gold standard, in the medical management of endometriosis.

It was hypothesized that the efficacy of the LNG-IUS is achieved via actions on estrogen (ER) and progesterone (PR) receptors possibly by decreasing the expression of glandular and stromal ER- α , ER- β and PR in the ectopic endometrium.

A second hypothesis was that mast cells are down regulated after the use of the LNG-IUS in the symptomatic treatment of women with minimal to moderate endometriosis possibly through ER and PR found within the mast cells. Mast cells numbers are reduced following the use of LNG-IUS for the symptomatic treatment of minimal to moderate endometriosis (Engemise et al, 2011b).

1.2. Epidemiology of endometriosis

Few well-conducted studies have reported on the prevalence of endometriosis; infact the true prevalence in women without a previous diagnosis is unknown. This is partly because many women with endometriosis are asymptomatic or have symptoms which may be atypical. The diagnosis is made confidently at laparoscopy and confirmed by histology (Cheong et al, 2006). However a negative histology does not exclude the diagnosis (Chatman & Zbella, 1987).

It is estimated that 5-10% of women of child bearing age suffer from endometriosis (Missmer et al, 2003). The prevalence of the condition is found to be as high as 62% in infertile women, 50% in teenagers with intractable dysmenorrhea, and 4% in asymptomatic women undergoing laparoscopy for tubal ligation (Lessey, 2000; Crosignani et al, 2006). The incidence appears to increase with delayed childbearing, increased menstrual episodes, shorter menstrual cycle length, a family history of the disease, declining use of oral contraception and exposure to certain toxins, such as dioxin (Cramer et al, 1986; Koninckx et al, 1991). An increased risk of endometriosis has also been reported with low parity, low

body mass index (BMI) and in taller women (Koninckx et al, 1991; Sangihaghpeykar et al, 1995). It is reported to be less common in Black Africans and more common in East Asians compared with Caucasians, but it is found in all ethnic groups (Hasson, 1976; Eskenazi et al, 1997).

1.3. Aetiopathogenesis of endometriosis

The aetiology and pathophysiology of endometriosis are not well understood. Classically, three main theories exist to explain the aetiology of endometriosis. The implantation theory of Sampson argues that endometrial cells in retrograde menstruation implant and grow in the peritoneal cavity, consequently forming endometriotic lesions (Sampson, 1927). The coelomic metaplasia theory of Meyer suggests that metaplasia of the coelomic epithelium explains the origin of some types of endometriosis (Meyer, 1919). The metastatic theory, which logically explains the rare endometriotic lesions distant from the uterus, suggests that distant lesions are established by the haematogenous or lymphogenous spread of viable endometrial cells (Halban, 1925).

Other theories such as the induction theory, an extension of the coelomic metaplasia theory proposes that endogenous biochemical or immunological factors induce undifferentiated cells to differentiate into endometrial tissue. Initial evidence in support of this theory came from Levander and Normann who implanted sections of the uterine wall obtained from pregnant rabbits into the subcutaneous tissue of 2-month-old female rabbits stimulated with gonadotrophins immediately before transfer (Levander et al, 1955). They observed cells

characteristic of the endometrium, as well as the formation of cysts in the surrounding tissue (Levander et al, 1955). The exact promoting factors involved in this process were uncertain but estrogens was thought to be essential and recently much attention and interest has focused on the microenvironment of the peritoneal cavity i.e the peritoneal fluid and its constituents.

Although the implantation theory is the most commonly accepted, there are problems with it in that while retrograde menstruation has been demonstrated in 75-90% of menstruating women, only 5-10% of them develop endometriosis (Halme et al, 1984; Liu et al, 1986; Guidice et al, 2004). This would suggest that additional factors beyond the presence of ectopic tissue are involved in the establishment of the condition.

Altered immune function has been proposed as a possible explanation of the discrepancy between the frequency of retrograde menstruation and that of endometriosis. This theory, first postulated by Gleicher et al (1987), states that alteration in the immune system results in a failure to “mop up” ectopic endometrial cells, allowing them to infiltrate the site of the disease (Gleicher et al, 1987; Matalliotakis et al, 1997; Umesaki et al, 1999; Lebovic et al, 2001). Such alterations in endometriosis sufferers have been shown in studies with natural killer (NK) and cytotoxic T cells (Mathur et al, 1982). Aberrations have also been found in immune mediators such as tumour necrosis factor- α , interferon- γ and polyclonal B-cell autoantibodies (Mathur et al, 1982). Some of these autoantibodies are organ-specific such as antiendometrial and antiovarian antibodies (Gleicher et al, 1989).

It has also been suggested that in ectopic sites endometrial cells express antigenic properties that cause some autoantibodies to form and bind to the endometrial tissue and thus lead to a suboptimal immune response for clearing ectopic tissue from the peritoneal cavity. This association between autoantibody abnormalities and endometriosis could explain endometriosis-related infertility. An increased risk of pregnancy loss has also been clearly associated with the presence of abnormal non-organ specific (Glinioer et al, 1991) as well as organ-specific autoantibodies (Hang et al, 1983). However the presence of these antibodies has also been demonstrated in other pelvic pathologies (pelvic inflammatory disease, adhesions) and therefore their significance in endometriosis, has not yet been ascertained.

Spuijbroek *et al* (1992) postulated that the initial pathogenetic phase of endometriosis formation is an invasive process requiring extracellular matrix (ECM) breakdown. This matrix undergoes periodic remodelling in the normal endometrial tissue during the menstrual cycle. The remodelling process which is modulated by mammalian matrix metalloproteinases (MMPs) is achieved by the degradation of the structural components of ECM (Page-McCaw et al, 2007). Precise regulation of MMPs is essential in orchestrating the physiological functioning of the endometrium. Derangement of MMP regulation is considered to be a critical factor in the development of pathological conditions such as endometriosis (Hulboy et al, 1997; Sillem et al, 1998). Inhibition of ectopic endometrial lesion formation was noticed following suppression of the activity of metalloproteinases in nude mice, leading to the suggestion that MMP may be of importance in the pathogenesis of endometriosis (Bruner et al, 1997). Collette et al. (2006) reported an increased activity of MMP-9 in the endometrium of women with endometriosis and concluded that

misexpression of matrix degrading enzymes might enable retrograde endometrial tissue to invade the peritoneal surface.

Some researchers consider endometriosis to be a local pelvic inflammatory process, with an altered function of peritoneal immune-related cells which are unable to eliminate the ectopic tissue (Wilson et al, 1994). Women with this condition have been shown to have impaired activation of peritoneal T cells predominantly Th1 (T helper cell type 1) inflammatory cells and decreased natural killer (NK) cell activity in the peritoneal fluid (PF) (Antsiferova et al, 2005).

The successful implantation and growth of endometriotic cells is widely believed to be modulated by the proangiogenic factor vascular endothelial growth factor (VEGF), the main stimulus for angiogenesis and increased vessel permeability in endometriosis (Taylor et al, 2002). It is known that VEGF is strongly expressed by endometriotic lesions, activated macrophages and neutrophils (McLaren et al, 1996; Shifren et al, 1996; Mueller et al, 2000).

Molecular anomalies such as the aberrant expression of cytochrome P-450 aromatase have been described in eutopic and ectopic endometrium of some women with endometriosis (Noble et al, 1996). The cytochrome P-450 aromatase enzyme is responsible for catalysing the conversion of androstenedione into estrone, so its expression in endometriotic tissue could contribute to the development of endometriosis. Aromatase expression level is highest in ovarian granulosa cells in premenopausal women, while after menopause adipose

tissue is the major site for expression (Grodin et al, 1973; Bulun et al, 1994a). Since endometriosis is hormone dependent, the presence of aromatase in extra-ovarian sites such as the ectopic lesions where it catalyzes the conversion of circulating androstenedione to estrone and subsequently converted to estradiol will result in the promotion of the growth of the lesions (**Fig.1.I**). However, Velasco *et al.* (2006) found that aromatase expression was preferentially detected in the secretory phase of the menstrual cycle and does not seem to be related to the degree or severity of endometriosis.

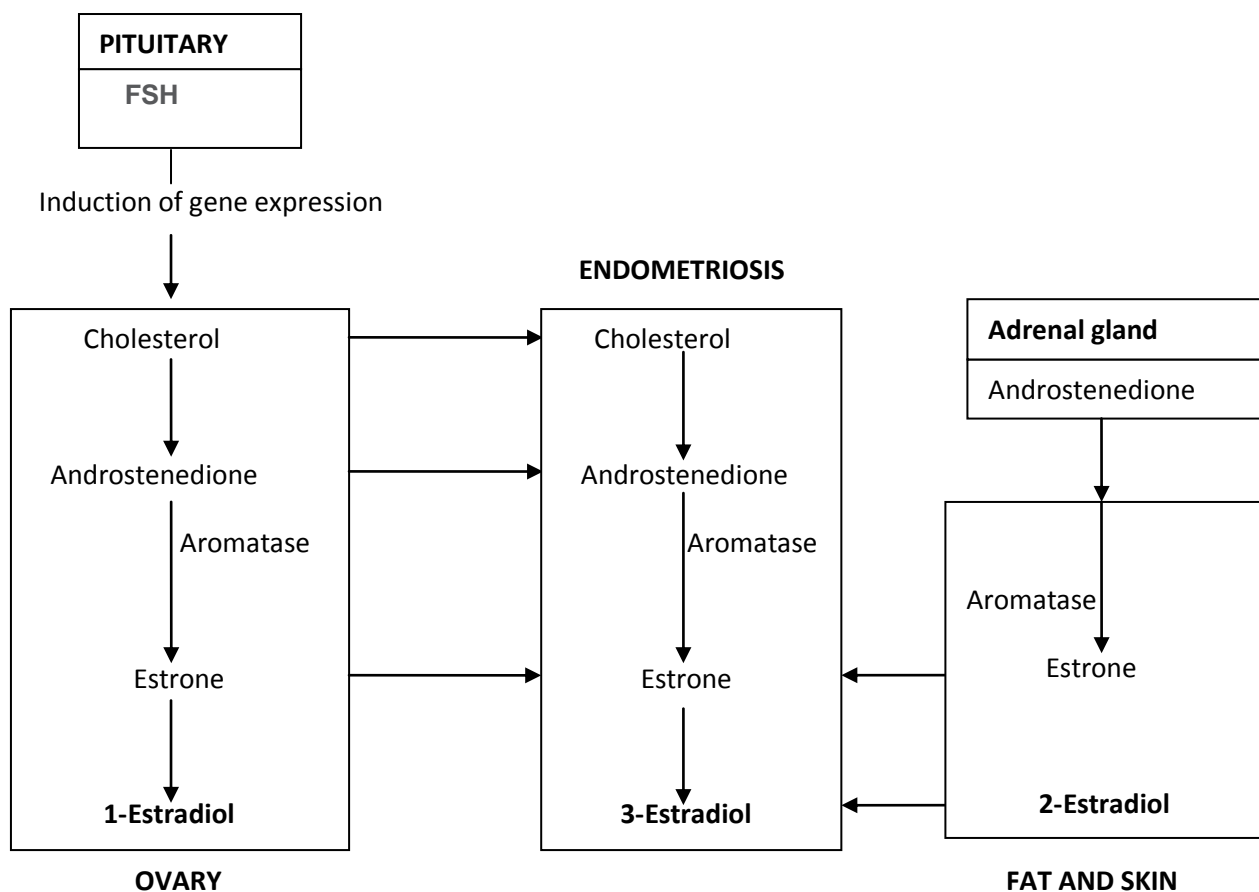


Fig.1.1. The Role of Aromatase enzyme in three sources of estradiol, biologically active estrogen, in endometriotic tissue.

Studies of siblings and first degree relatives, estimate that the risk of having endometriosis is 7% for an affected first degree relative suggesting a complex and polygenic nature of inheritance (Simpson et al, 1980). Linkage studies have also shown a susceptibility locus on Chromosome 10q26 (Treloar et al, 2005). Women with endometriosis have altered endometrial gene expression patterns compared to women without (Giudice, 2003; Kao et al, 2003). Cysteine-rich Cytochrome 61 (Cyr61) stimulates adhesion and angiogenesis and its expression is altered in women with endometriosis. In normal endometrium, Cyr61 is expressed in the proliferative phase but disappears by the mid-secretory phase. In women with endometriosis, however, Cyr61 expression persists throughout the cycle potentially contributing to endometriotic lesions (Absenger et al, 2004). Anti-estrogens appear to inhibit Cyr61 expression, thus, the regulation of Cyr61 could potentially play a therapeutic role in the treatment of endometriosis (Sampath et al, 2002; Absenger et al, 2004).

Recent aetiopathological theories of endometriosis relate to progesterone resistance and environmental agents such as polychlorinated biphenyls (PCBs) and dioxins (Osteen et al., 2005). Nutritionally, wheat has also been implicated as a potential exacerbator of endometriosis but this remains to be proven (Shepperson, 2004).

The myriad of presentations, appearances, both micro and macroscopic, variable distribution and nature of endometriotic lesions imply that no single theory can explain the pathogenesis of this complex condition. It was precisely for this reason that the composite theory (**Fig. 1.2.**) proposed by Nisolle and Donnez (1997) recognized that a combination of risk factors and mechanisms was involved in the formation/propagation of endometriosis. The predominant mechanisms involved in the pathogenesis of endometriosis are

implantation, direct invasion and coelomic metaplasia for peritoneal lesions, rectovaginal nodules and endometriomas respectively. Superimposed on these are various factors including immunological predisposition, genetic/familial, hormonal and environmental factors.

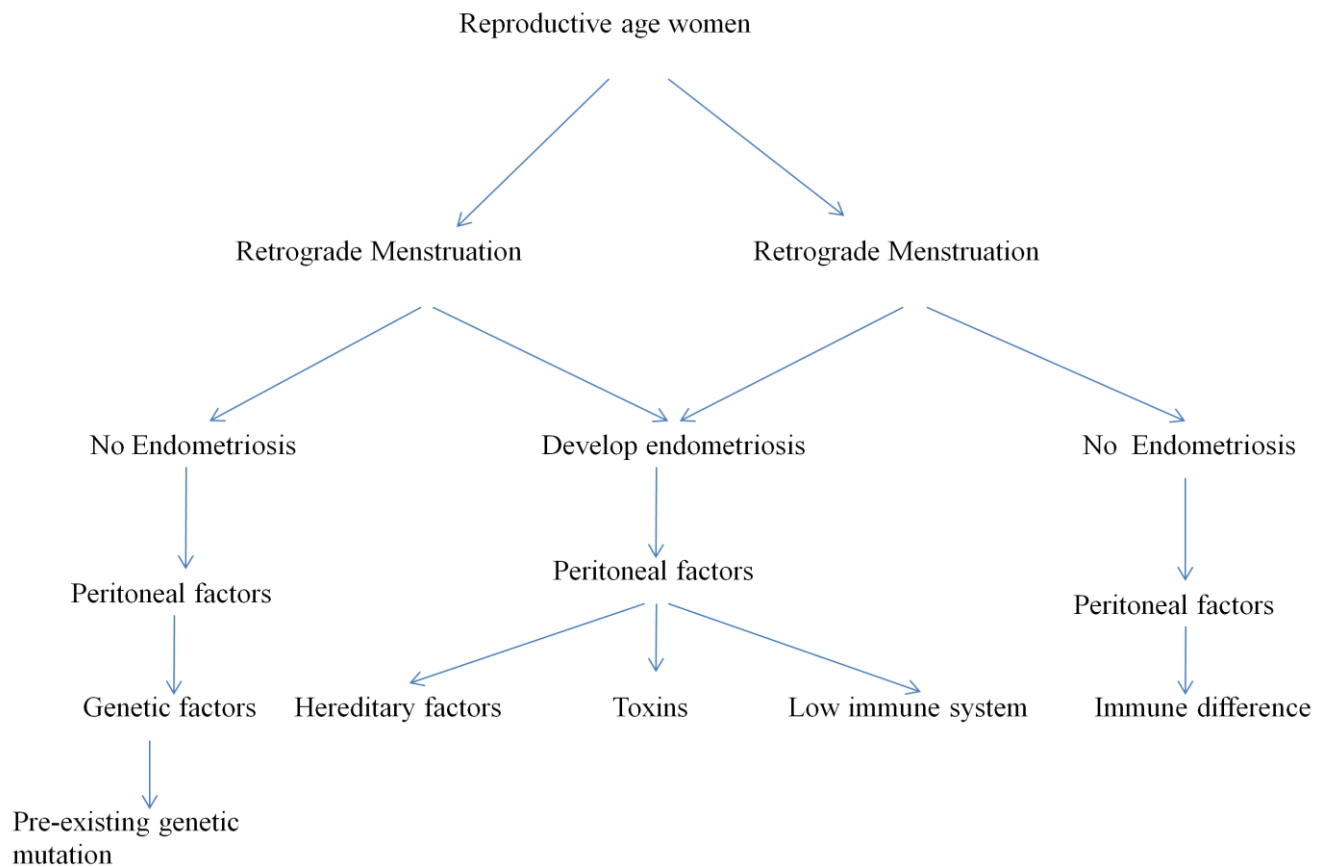


Fig. 1.2. Composite Theory of endometriosis

1.4. Endometriosis as an epigenetic disease.

As a multifactorial disease, endometriosis involves both hormonal and immunological aberrations. Incidentally, epigenetics has now been shown to play roles in both hormonal and immunological aberrations (Rosser et al, 2011). Although the possibility that certain susceptibility genes may be responsible for increased risk of endometriosis in perhaps a small portion of women, means some aspects of endometriosis may be explained from an epigenetics perspective. There is indeed accumulating evidence that various epigenetic aberrations exist in endometriosis (Rosser et al, 2011).

The first piece of evidence came from a study showing that the putative promoter of HOXA10 in the endometrium from women with endometriosis is hypermethylated as compared with that from women without endometriosis (Wu et al, 2005). HOXA10 is a member of a family of homeobox genes that serve as transcription factors during development and has been shown to be important for uterine function. It is expressed in human endometrium, and its expression is dramatically increased during the midsecretory phase of the menstrual cycle, corresponding to the time of implantation and increased circulating progesterone (Troiano and Taylor, 1998). This suggests that HOXA10 may have an important function in regulating endometrial development during the menstrual cycle and in establishing conditions necessary for implantation (Taylor et al, 1998).

In the endometrium of women with endometriosis, however, HOXA10 gene expression is significantly reduced, suggesting that there may well be some defects in uterine receptivity

(Gui et al, 1999; Taylor et al, 1999), which may be responsible for reduced fertility in these women. As promoter, hypermethylation is generally associated with gene silencing; the observed HOXA10 promoter hypermethylation provides a plausible explanation as to why HOXA10 gene expression is reduced in the endometrium of women with endometriosis (Wu et al, 2005). The HOXA10 promoter hypermethylation coinciding with reduced HOXA10 expression has been demonstrated also in induced endometriosis in baboons (Kim et al, 2007), and in mouse (Lee et al, 2009). HOXA10 hypermethylation, accompanied by overexpression of DNA (cytosine-5)-methyltransferase 1 (*DNMT1*) and DNA (cytosine-5)-methyltransferase 3 beta (*DNMT3B*), has been reported recently in mice prenatally exposed to diethylstilbestrol (DES), a known endocrine disruptor (Bromer et al, 2009).

The second piece of evidence came from the study demonstrating that the promoter of progesterone receptor-B (PR-B) is hypermethylated in endometriosis (Wu et al, 2006b). In addition, the PR-B promoter hypermethylation is concomitant with reduced PR-B expression, providing support for the role of epigenetic aberration in PR-B down-regulation. It is well-known that there is a general tendency towards progesterone resistance in endometriosis (Giudice and Kao, 2004). It is also known that PR-B is down-regulated in endometriosis (Attia et al, 2000) and may be responsible for, at least in part, progesterone resistance since progesterone is mediated through its receptors, including PR-B. PR-B promoter hypermethylation thus provides a plausible explanation as to why PR-B is persistently down-regulated in endometriosis.

While the evidence in support of the paradigm outlined above seems compelling, it does not entirely explain the concept of progesterone resistance in the eutopic endometrium in patients with endometriosis for two reasons. First, suppression of endometriotic lesions and associated inflammation, for example upon prolonged treatment with GnRH-analogues or after surgical ablation of the lesions, would suffice to restore normal steroid hormone responses and cure the disease, which is ostensibly not the case. Second, and as mentioned before, there is compelling evidence that endometriosis is associated with perturbed gene expression in purified eutopic endometrial stromal cells that are maintained in culture, irrespectively whether stimulated or not with progesterone or other differentiation cues (Klemmt et al, 2006; Minici et al, 2008; Aghajanova et al, 2010 & 2011).

These observations strongly infer that progesterone resistance is likely to be as much a consequence of changes in the epigenetic chromatin landscape of endometrial cells as the result of intrinsic defects in PR or other signal transduction pathways. Inflammatory signals are established epigenetic modifiers, which raises the possibility that cyclic menstruation is important for ‘programming’ hormonal responses in the uterus, especially prior to pregnancy (Brosens et al, 2009). Moreover, animal experiments demonstrated that induction of pelvic disease has long-lasting consequences for the chromatin landscape of eutopic endometrial cells by altering DNA methylation and histone tail modifications in proximal promoter regions of progesterone-dependent genes (Kim et al, 2007; Guo, 2009; Lee et al, 2009). For example, as demonstrated by Kim and co-workers that induction of endometriosis in the baboon resulted in a gradual decrease in endometrial HOXA10 expression, a homeobox transcription factor involved in endometrial development and differentiation. Importantly, this down-regulation was only significant 6–12 months after

the induction of endometriosis and corresponded to increased methylation of the proximal promoter of HOXA10 (Kim et al, 2007).

Perhaps the most important piece of evidence supporting the proposition that endometriosis is an epigenetic disease comes from a study demonstrating that *DNMT1*, DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*) and *DNMT3B*, the three genes coding for DNMTs that are involved in genomic DNA methylation, are all overexpressed in endometriosis (Wu et al, 2007b). Since these genes are involved in *de novo* as well as maintenance methylation (two general classes of enzymatic activities in the cell that are responsible for DNA methylation), their aberrant expression suggests that aberrant methylation may be widespread in endometriosis. As methylation is closely linked with chromatin remodeling, the aberrant expression of these genes may also signal that there are aberrant epigenetic changes, other than methylation, in endometriosis.

Further evidence comes from studies on the role of the orphan nuclear receptor, steroidogenic factor-1 (SF-1), which plays a key role in the development and function of steroidogenic tissues. This transcriptional factor which is essential for activation of multiple steroidogenic genes for estrogen biosynthesis is usually undetectable in normal endometrial stromal cells but is aberrantly expressed in endometriotic stromal cells. Xue et al. (2007b) showed that SF-1 promoter has increased methylation in endometrial cells yet in endometriotic cells it is hypomethylated. They also found that ER- β promoter is hypomethylated in endometriotic cells, which accounts for its overexpression (Xue et al, 2007a).

1.5. Staging of endometriosis

The management of various disorders, cancer in particular, benefits from the adoption of a staging of the disorder. This allows immediate comprehension of the severity of the condition, helps to guide therapeutic strategies, permits the formulation of a reliable prognosis and is an indispensable scientific tool in clinical trials (Canis et al, 1993). A staging system should be based on the natural history of the disease, its local invasiveness, the presence of metastases and lymphatic involvement in the case of cancer or functional and organic consequences in the case of endocrine, metabolic or non-cancerous conditions. The *sine qua non* in designing a staging system is a proven progression through sequential steps of increasing severity that are causally linked with the outcome of interest (Canis et al, 1995). Whether these criteria are met in the case of endometriosis is unclear. Moreover, the staging has not been demonstrated to be predictive with regards to response to treatment in terms of reproductive results, pain reduction and recurrence rate (Brosens et al, 1993).

A laparoscopy is the gold standard reference for the diagnosis of endometriosis, and staging of the disease is performed using this procedure. The current staging of endometriosis (revised American Fertility Society (rAFS) 1997; American Society for Reproductive Medicine (ASRM), 1997) based on visual findings, is simple and concise for easy implementation in routine practice and is sufficiently analytical and descriptive to allow clear comparison of anatomicopathological modifications with time or following treatment as well as unbiased circulation of clinical and scientific information with limited intra- and inter-observer variability [(Buttram, 1985; Candiani, 1986; Canis et al, 1993 & 1995;

Hornstein et al, 1993; Rock, 1995; Lin et al, 1998) **Appendix 1**]. However, in addition to being an essential tool used to provide an accurate assessment of the pelvic condition, such a scheme provides intrinsic prognostic properties in terms of consistent and predictable reproductive outcomes according to the various stages of the endometriosis and in relation to specific treatments (Rock et al, 1981; Adamson et al, 1982; Guzick et al, 1982; Palmisano et al, 1993; Schenken and Guzick, 1997). Moreover, the likelihood of symptomatic recurrence and disease relapse associated with the available therapeutic options should be reliably estimated (Buttram, 1987; Weitzman and Buttram, 1989; Groff, 1991; Olive, 1992; Damario and Rock, 1997; Hoeger and Guzick, 1997 & 1999; Stovall et al, 1997).

There are several major unsolved problems relating to the design of a useful staging system for endometriosis. It is unclear whether endometriosis is a single disease with multiple pathological manifestations, that is, peritoneal, ovarian and deep (Brosens, 1994; Brosens et al, 1994a & 1994b) or if these lesions are expressions of different aetiologies (Nisolle and Donnez, 1997). All the classifications are based on visual findings, but what is seen is not always active endometriosis (Sturgis and Call, 1954; Brosens et al, 1993b; Dubuisson and Chapron, 1994; Brosens, 1997; Evers et al, 2005; Marchino et al, 2005). Many lesions are consequences of previous implants now healed (Brosens et al, 1985; Brosens, 1993) or, as in the case of ovarian cysts, their diameter may not be proportional to the amount of active endometrium present (Brosens, 1994; Brosens et al, 1994b).

The behaviour of endometriosis is unpredictable, sometimes self-limiting in its spread or even regressing (Brosens et al, 1994a; Bergqvist, 1995; Moen, 1995). Furthermore, it is

very difficult to stage a disorder with reference to more than one outcome, that is, fertility, pain and recurrence. This would require the demonstration that different lesions carry different but consistent prognosis. Such a supposition may be unfounded, because the foci that cause pain may not necessarily be the same that affect fertility and vice versa (AFS, 1993; Rock, 1993; Vercellini, 1997). Finally, if scores are attributed arbitrarily and not empirically derived (Guzick et al, 1982), the concept of a point system itself may be criticized, because the same bias could have a systematic and repeated effect on diagnosis and classification of the disorder.

Concern over the reproducibility of the scoring system is directed at the variability in assessing ovarian endometriosis and cul-de-sac obliteration. To improve accuracy of the scoring system, ovarian endometriotic cyst should be confirmed by the presence of the following features (Vercellini, et al, 1991):

- 1) Cyst diameter <12cm;
- 2) Adhesion to pelvic side wall and/or broad ligament;
- 3) Endometriosis on the surface of ovary; and
- 4) Tarry, thick, chocolate-coloured fluid content.

Cul-de-sac obliteration should be considered partial if endometriosis or adhesions have obliterated part of the cul-de-sac, but some normal peritoneum is visible below the uterosacral ligaments. Complete obliteration of the cul-de-sac exists when no peritoneum is visible below the uterosacral ligaments.

1.6 Investigation and diagnosis of Endometriosis

1.6.1 Endometriotic lesions.

Three common sites of endometriotic lesions have been described: peritoneal, ovarian, and rectovaginal (Donnez et al, 2003). It has been suggested that these are considered separate entities with different pathogenesis. Peritoneal implants are classically described as bluish/black powder-burn lesions, with varying degrees of surrounding pigmentation and fibrosis. The dark colouration is as a result of haemosiderin deposition from entrapped menstrual blood, but it is important to realize that most peritoneal implants are not black and may be red, white, yellow/brown or clear. White, opacified areas and red, flame-like lesions contain endometriosis in approximately 80% of cases, while brown patches have endometriosis in approximately 50% of cases (Wiegerinck et al, 1993). However, white peritoneal implants which mimic scar tissue are associated with pain more than either black or red ones and it is believed that the black/red lesions produce prostaglandin $F_{2\alpha}$, which is the cause of pelvic pain. It has been proposed that endometrial implants undergo a process of natural evolution through a number of phases, with a combination of various types of lesions co-existing in the same patient.

Ovarian endometriomas are smooth, dark brownish cysts, which maybe associated with adhesions that lie on the surface of the ovary. They contain brown, dense, chocolate-like fluid and endometriomas larger than 3cm are often multi-locular. The pathogenesis of ovarian endometriomas is a source of controversy. Although there seems to be a consensus on the invagination theory, there are still conflicting views between the implantation theory

and the metaplastic theory (Nisolle et al, 1997; Ferenczy, 1998). The presence of mesothelial invagination in continuum with endometriotic tissue suggests that metaplastic histogenesis of ovarian endometriotic lesions occurs (Ferenczy, 1998).

A deep infiltrating endometriosis or rectovaginal endometriotic nodule is localized in the rectovaginal septum and is probably a distinct entity, as opposed to peritoneal and ovarian endometriosis (Nisolle et al, 1997). It has been suggested that it originates from the Müllerian rest cells, which have remained in the rectovaginal septum (Chapron et al, 2006; Signorile et al, 2009). This must be considered as adenomyomas, as they have been shown to consist of smooth muscle with active glandular epithelium and scanty stromal (Ferenczy, 1998). Immunohistochemistry results show poor differentiation and hormonal independence of these lesions and indicate a close relation with their mesodermal Mullerian origin (Ferenczy, 1998).

1.6.2 Methods of diagnosing endometriosis

The symptoms of endometriosis vary widely and tend to be non-specific. Lack of a non-invasive diagnostic test contributes to the long delay between the onset of symptoms and diagnosis. However, with a careful and detailed history, those that are specific to endometriosis can be identified. Careful attention to risk factors especially menstrual and family history, the course of the symptoms, fertility and contraceptive history are useful in increasing the index of suspicion.

Establishing the diagnosis on the basis of these symptoms alone can be difficult because the presentation of endometriosis is so variable and there is considerable overlap with other conditions such as irritable bowel syndrome and chronic pelvic inflammatory disease (PID), conditions whose findings on physical examination maybe as diverse as those of endometriosis. A normal physical examination, however, does not rule out the diagnosis of endometriosis. There is therefore often a delay of several years between the onset of symptoms and a definitive diagnosis (Hadfield et al, 1996; Arruda et al, 2003; Husby et al, 2003). At presentation, the misdiagnosis of PID is commonly made even in the absence of the microbes associated with PID hence it may take between 8-12 years to make a diagnosis of endometriosis (Hadfield et al, 1996).

There is currently no single reliable test to diagnose endometriosis, however; a combination of biochemical markers and clinical assessment is likely to increase the accuracy of diagnosis. Although many attempts have been made to identify serum markers as a way of screening for endometriosis, there is yet to be an adequately sensitive and specific test for this purpose although various biochemical tests are currently being investigated to aid diagnosis. Cancer antigen (CA)-125 has been evaluated in a number of studies and has a sensitivity of 13-60%, and specificity of 93-100% (Chen et al, 1998). The poor sensitivity of CA-125 in the diagnosis of endometriosis means that it is not valuable as a screening test. In severe disease, CA-125 levels may be used to predict the response to medical or surgical treatment in some women. When CA-125 was combined with other plasma biomarkers such as CA-19-9, interleukin (IL)-6, IL-8, tumour necrosis factor-alpha, high sensitivity C-reactive protein (hsCRP), cognate chemokine receptor 1 messenger ribonucleic acid (CCR1 mRNA) and monocyte chemotactic protein-1(MCP-1) obtained

during the secretory phase or during menstruation both minimal-mild and moderate-severe endometriosis were diagnosed with a high degree of sensitivity and specificity (Somigliana et al, 2004; Agic et al, 2008; Mihalyi et al, 2010). Using stepwise logistic regression, Mihalyi et al in (2010), used these biomarkers to diagnose moderate to severe endometriosis with a sensitivity of 100% (specificity 84%) and minimal–mild endometriosis with a sensitivity of 87% (specificity 71%) during the secretory phase of the menstrual cycle. Using least squares support vector machines (LSSVM) analysis, minimal to mild endometriosis was diagnosed with a sensitivity of 94% (specificity 61%) during the secretory phase and with a sensitivity of 92% (specificity 63%) during the menstrual phase of the menstrual cycle.

Anti-endometrial and anti-carbonic anhydrase antibodies have also been investigated for their diagnostic potential in these women, but these are technically difficult and complex to quantify and are also expensive for general application (Brosens et al, 2003).

The gold standard for the diagnosis of endometriosis is laparoscopic inspection, ideally with histological confirmation (Kennedy et al, 2005), although its recognition varies with the experience of the surgeon, especially for subtle, bowel, bladder, ureteral and diaphragmatic lesions (Brosens et al, 2003). There is insufficient evidence to justify timing the laparoscopy at a specific time in the menstrual cycle, but it should not be performed during or within 3 months of hormonal treatment so as to avoid under-diagnosis (Kennedy et al, 2005). Diagnostic laparoscopy is associated with an approximately 30/1000 risk of minor (e.g. nausea, shoulder tip pain) and 0.6-1.8/1000 risk of major complications (bowel injury, bladder) (HarkkiSiren et al, 1997; Chapron et al, 1998).

Imaging technique such as transvaginal ultrasound (TVS) and magnetic resonance imaging (MRI) on the other hand are useful only for the diagnosis of endometriomas and definition of the degree of recto-vaginal infiltration but have no real value in diagnosing peritoneal endometriosis (Moore et al, 2002). TVS may have a role in the diagnosis of disease involving the bladder or rectum (Kennedy et al, 2005). If there is clinical evidence of deeply infiltrating endometriosis, ureteral, bladder and bowel involvement should be assessed. Consideration should be given to performing an MRI or ultrasound scan (transrectal and/or transvaginal and/or renal), with or without intravenous urography (IVU) and barium enema studies depending upon the individual circumstances, to map the extent of the disease which may be multi-focal (Kennedy et al, 2005).

1.7. Treatment Modalities

The treatment of endometriosis has three aims depending on the symptoms and extent of the condition: These include

- a). pain relief,
- b). destruction/remove the lesions and
- c) restoration of anatomy.

An ideal treatment should be one that is least invasive, least expensive, most cost effective and with the least side effects. The treatment of endometriosis is further compounded by the

fact that the overall severity of the condition does not correlate with the frequency and/or severity of symptoms.

Unfortunately, endometriosis is a chronic condition with surgical or medical treatment often providing only temporary relief as recurrence rates of symptoms and indeed lesions tends to be high (Vercellini et al, 2006). Although hysterectomy and bilateral salpingo-oophorectomy had been considered in the past as “curative” the rates of recurrence after such “curative” surgery have been reported to be between 5-10% (Winkel et al, 1999), casting doubts over it being curative in all cases.

It is therefore not surprising in the light of all these factors that there is no single therapeutic option that is most effective and suitable for every woman with endometriosis. Instead there are a variety of approaches to dealing with the symptoms of this condition and for most women, a combination of these approaches is often necessary to alleviate symptoms. These options include

- a) expectant management
- b) medical management or
- c) surgery

1.7.1 Expectant management

This is an option when the diagnosis is incidental and the woman is asymptomatic. Some women with minimal to moderate symptoms may also opt for this approach following a confirmatory diagnostic laparoscopy. It is most effective in women in the perimenopausal period as the symptoms tend to improve with the onset of menopause (Schenken and Malinak, 1982).

1.7.2 Medical management

Most medical treatments options for endometriosis are based on the premise that it is a hormonally (estrogen) responsive disease (Prentice, 2001). As knowledge of the pathophysiology of endometriosis improves, so also is a better understanding of medical and surgical options. An ideal drug for the medical management of endometriosis should remove existing pathology and related adhesions without interfering with the menstrual cycle, be safe in pregnancy, and have few or no side effects. Such a drug does not exist today, and various combinations are commonly offered to individual patients.

The dependence of endometriotic implants on estrogen has led to numerous attempts to ‘hormonally’ simulate pseudomenopause or pseudopregnancy, the two physiological states associated with atrophy of the implants through interruption or suppression of cyclic ovarian hormone production.

1.7.2.1. Pseudo-pregnancy options

1.7.2.1.1 Progestogens

Progestogens given orally or intramuscularly have been shown to be effective in treating the symptoms of endometriosis. Administration of progestogen can induce pseudopregnancy accompanied by resolutions of signs and symptoms of endometriosis in some patients. Therefore, before choosing therapy, health care providers should consider an individual woman's family planning needs and fertility status (Child & Tan 2001). Progestogens have been used as therapy for endometriosis worldwide for more than 40 years (Schweppe, 2001). The rationale for their use includes:

- (a). A suppression of the hypothalamo-pituitary ovarian (HPO) axis (**Fig.1.3**), a process that induces anovulation and thus lowers serum estrogen levels (Luciano et al, 1988).
- (b). exertion of an antiproliferative effect on the endometriosis by causing initial decidualisation of endometrial tissue followed by atrophy (Hague et al, 2002).
- (c). inhibition of angiogenesis (Blei et al, 1993), required for maintenance of endometriotic implants
- (d). decreasing markers involved in the pathogenesis of endometriosis such as peritoneal fluid leukocytes (Haney & Weinberg, 1988),

(e). reducing menstrual flow and sometimes induction of amenorrhoea. (Vercellini et al, 2003a) and

(f). antagonising the effect of estrogens at the receptor levels. (Bukulmez et al, 2008)

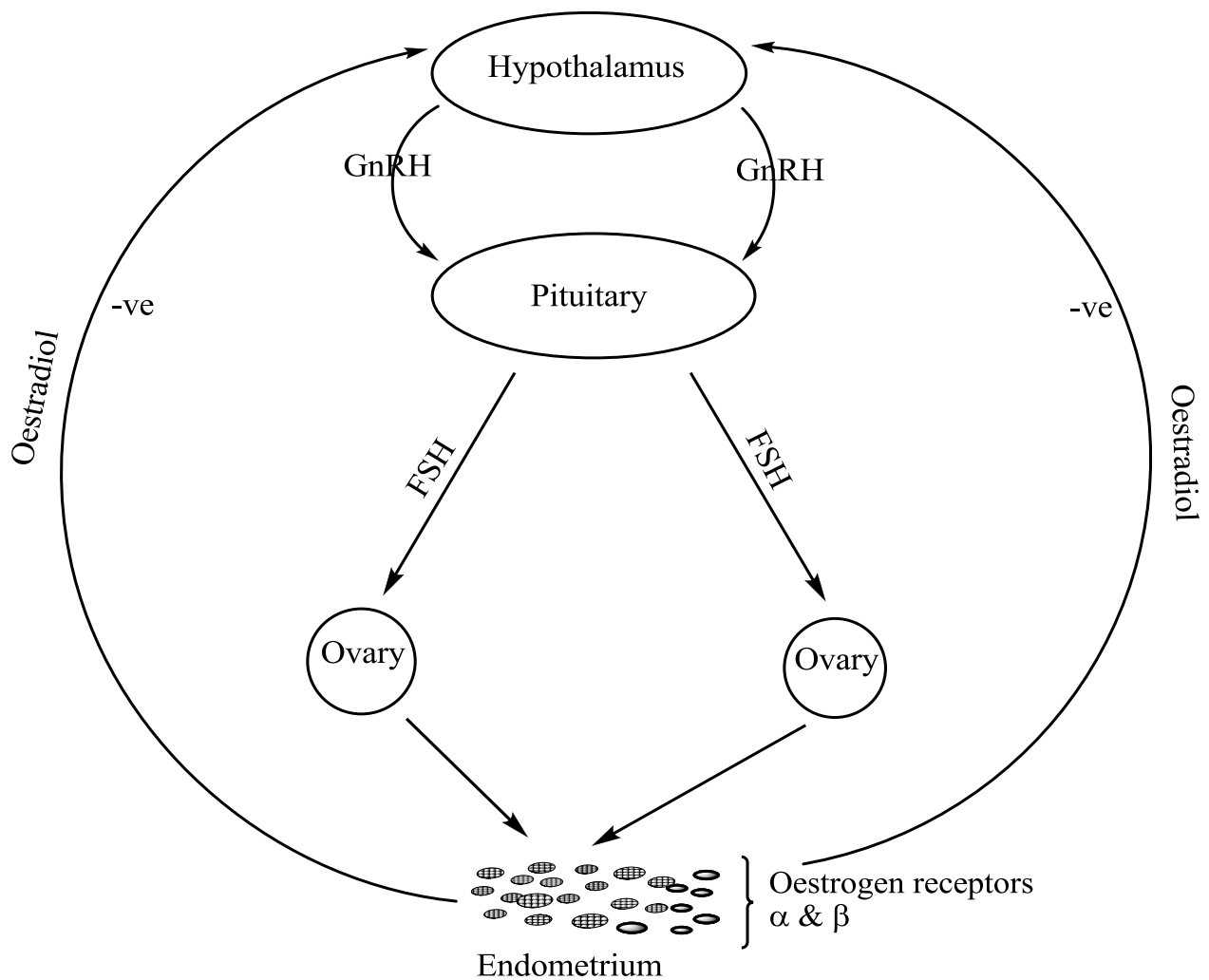


Fig.1.3. A simple diagrammatic representation of the origins, target organs and feedback mechanisms of the principal hormones involved in the hypothalamic-pituitary-ovarian axis.

Progestogens are often considered the first choice for the treatment of endometriosis because they are effective in reducing the American Fertility Society (AFS) scores and pain with efficacy approaching that of either danazol or GnRH agonist in some cases. They are cheap and are associated with fewer side effects than either danazol or GnRH agonist (Vercellini et al, 1997). Although there are various progestogens and different routes of administrations a review of the use of progestogens in the treatment of endometriosis in 1997 concluded that there is no evidence that any single progestogen or any particular dose is preferable to another. In most studies, the effect of treatment has been evaluated after 3 to 6 months of therapy (Moore et al, 1999). Recently dienogest has been shown in small randomised trials to have an efficacy as high as that of GnRH agonist with fewer side effects (Strowitzki et al, 2010).

1.7.2.1.2 Combined oral contraceptives (COCs)

COCs are used widely as therapy for women with chronic cyclical pelvic pain whose cause is suspected or has been confirmed to be secondary to endometriosis. These agents are generally well tolerated with fewer metabolic consequences compared to danazol and GnRH agonists (Rice, 2002; Vercellini et al, 2003a). The use of COCs results in ovulation suppression, decreased gonadotrophin levels, reduced menstrual flow and decidualization of endometriotic implants (Rice, 2002). COCs have also been shown to down-regulate cell proliferation and increase apoptosis in the eutopic endometrium of women with endometriosis (Meresman et al, 2002).

Although COCs have been used extensively in clinical practice for many years for treating endometriosis associated pain, evidence for their efficacy has been largely observational (Rice, 2002; Vercellini et al, 2003a). One small, open-label randomized clinical study comparing goserelin with a cyclic low-dose COC containing ethinyl estradiol and desogestrel found a greater reduction in dyspareunia in the GnRH agonist group and similar relief of non-menstrual pain in both groups (Vercellini et al, 1993). Estrogens in oral contraceptives may potentially stimulate the proliferation of endometriosis. However, any combined oral contraceptive pill containing 30-35 mg of ethinyl estradiol used continuously to achieve amenorrhea or hypomenorrhoea that often occurs in women taking oral contraceptives may be beneficial to women with prolonged, frequent menstrual bleeding, which is a known risk factor for endometriosis (Cramer et al, 1986).

1.7.2.2 Pseudo-menopause options

1.7.2. 2.1 Gonadotropin-releasing hormone agonists (GnRHa)

GnRH agonists are the gold standard medical treatment option for endometriosis (Valle et al, 2003). They induce medical menopause by down-regulating hypothalamic-pituitary GnRH receptors, thus causing decreased gonadotrophin secretion, suppression of ovulation and reduced serum estrogen levels (**Fig.1.3**) (Child et al, 2001; Valle et al, 2003). They can be administered intramuscularly, subcutaneously, or intranasally.

As expected, given their mechanism of action, GnRH agonists are associated with significant hypo-estrogenic side effects. Short-term side effects include menopausal symptoms such as hot flushes, vaginal dryness, loss of libido, and emotional lability. Long-term side effects include a substantial reduction in bone mineral density (BMD) (3.2% reduction in lumbar spine BMD after 6 months and 6.3% after 12 months of continuous treatment), which restricts therapy in most women with these agents alone (without add-back therapy) to a maximum of 6 months' (Child et al, 2001; Valle et al, 2003). Reversibility of this bone loss is equivocal and therefore of concern (Riis et al, 1990; Barbieri, 1992), especially when the treatment is longer than 6 months. The use of "add-back" therapy has been shown to reduce loss of BMD (Child et al, 2001; Valle et al, 2003) and vasomotor symptoms thereby allowing its administration for longer than six months.

Add-back can be achieved with progestogens such as norethisterone 1.2 mg (Riis et al, 1990), norethindrone acetate 5 mg (Hornstein et al, 1998) and medrogestone 10 mg/day (Sillem et al, 1999), tibolone 2.5 mg/day (Lindsay et al, 1996; Taskin et al, 1997), an estrogen/progestogen combination, e.g. conjugated estrogens 0.625 mg combined with medroxyprogesterone acetate 2.5 mg (Friedman et al, 1993) or norethindrone acetate 5 mg (Hornstein et al, 1998) combined with estradiol 2 mg and norethisterone acetate 1 mg (Franke et al, 2000). Several studies have shown that using hormonal add-back does not lessen the efficacy of the GnRH agonists (Lindsay et al, 1996; Taskin et al, 1997; Sillem et al, 1999; Hornstein et al, 1998).

1.7.2.2.2 Danazol

Danazol is an oral androgenic agent that induces amenorrhoea through suppression of the hypothalamic-pituitary-ovarian (HPO) axis. It directly inhibits steroidogenesis, increases metabolic clearance of estradiol and progesterone, and interacts with endometrial androgen and progesterone receptors. In addition it causes immunologic attenuation of potentially adverse reproductive effects (Barbieri & Ryan, 1981; Hill et al, 1987).

These multiple effects of danazol produce a high-androgen, low-estrogen environment that does not support the growth of endometriosis, and the ensuing amenorrhea prevents new seeding of implants from the uterus into the peritoneal cavity. Doses of 800 mg/day are frequently used in North America, whereas 600 mg/day are commonly prescribed in Europe and Australia. It appears that the absence of menstruation is a better indicator of response than drug dose. A practical strategy for the use of danazol is to start treatment with 400 mg daily (200 mg twice a day) and increase the dose, if necessary, to achieve amenorrhea and relieve symptoms (Wingfield, 1996).

In fact, poor tolerability represents the major drawback of danazol as a treatment for endometriosis. This agent has androgenic, anabolic and hypo-estrogenic properties. The most common side effects include weight gain, fluid retention, acne, oily skin, hirsutism, hot flushes, atrophic vaginitis, breast atrophy, reduced libido, fatigue, nausea, muscle cramps, and emotional instability (Trabant et al, 1990). Deepening of the voice is another potential side effect that is non reversible. The fact that some of the side effects can be

permanent (virilisation, deepening of the voice and clitoral enlargement) led the Royal College of Obstetricians and Gynaecologists (RCOG) to recommend avoiding its use as a treatment option in the United Kingdom (Selak et al, 2001).

1.7.2.2.3. Gestrinone

Gestrinone is a 19-nortestosterone derivative with androgenic, anti-progestogenic, and anti-gonadotropic properties. Although it causes signs and symptoms that mimic the pseudomenopausal state it is commonly classified as an androgen. It creates a hormonal environment that results in the cellular inactivation and degeneration of endometriotic implants but not their disappearance (Brosens et al, 1987). It induces amenorrhea in 50-100% of women which is dose-dependent. The standard dose that has been used is 2.5 mg twice a week, although it has been reported that 1.25 mg twice weekly is equally effective (Hornstein et al, 1990). The side effects are dose-dependent and similar but less severe than those of danazol (Fedele et al, 1989). These include nausea, muscle cramps, and androgenic effects such as weight gain, acne, seborrhoea, oily hair/skin, and irreversible voice changes. Gestrinone is as effective as GnRH agonist for the treatment of pelvic pain associated with endometriosis (Gestrinone Italian Study Group, 1996).

1.7.2.3. Aromatase inhibitors

Estradiol (E₂) is the essential sex steroid responsible for the establishment and growth of endometriosis. The conversion of androstenedione and testosterone to estrone and estradiol

is catalysed by aromatase, which is expressed in a number of human tissues. In the reproductive age, the ovary is the most important site of estradiol biosynthesis, and this takes place in a cyclic fashion. Aromatase (a product of the CYP19 gene) is a cytochrome P-450 haemoprotein-containing enzyme complex that catalyzes the rate-limiting step in estrogen biosynthesis. Aromatase catalyzes the conversion of the C₁₉ steroids (androstenedione and testosterone) to estrogens (estrone and estradiol) (Simpson et al, 1994). This enzyme is expressed in many human cells including ovarian granulosa cells, placental syncytiotrophoblasts, adipose cells, skin fibroblasts, the brain and endometriotic cells (Simpson et al, 1994).

A gene in chromosome 15q21.2 encodes aromatase. During the reproductive years, aromatase activity is located mainly in the ovary and is not found in normal endometrium and myometrium (Bulun et al, 1994b; Bulun et al, 1994c). Eutopic and ectopic endometrium of women with endometriosis however contain aromatase P-450 and thus produce estrogen (Noble et al, 1996; Bulun et al, 2000). The presence of aromatase and the consequent local estrogen production may promote the growth of implanted lesions. In such endometrium, 17 β -hydroxy steroid dehydrogenase type 2 enzyme, which catalysis the conversion of estradiol to estrone, is also deficient (Zeitoun et al, 1998). Prostaglandin E₂ (PGE₂) has been identified as the most potent inducer of aromatase activity in endometriotic cells, and estrogen upregulates PGE₂ formation by stimulating cyclo-oxygenase-2. Thus, a positive feedback loop for continuing local estrogens and PGE₂ production is established in the pathological tissue, possibly leading to the proliferative and inflammatory characteristics of endometriosis. These findings suggest that aberrant expression of aromatase in endometriotic tissue may be involved in the pathogenesis of this

disease, promoting survival and growth of the lesions (Montagna et al, 2008). Furthermore, cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- α) are identified as important factors in the regulation of aromatase activity in fibroblasts derived from ectopic endometrial tissue (Macdiarmid et al, 1994; Purohit et al, 1995). Increases in the peritoneal fluid concentration of IL-6 and TNF- α occur in women with endometriosis, suggesting that they could be involved in the regulation of aromatase expression (Iwabe et al, 2002).

Aromatase inhibitors suppress estrogen production in peripheral tissues such as fat and decrease circulating estrogen levels considerably (Oxholm et al, 2007). Blocking aromatase activity in these extraovarian sites with specific inhibitors may therefore represent a new generation of drug options for the treatment of endometriosis. Some selective third-generation non-steroidal aromatase inhibitors which have been used successfully to treat endometriosis in both postmenopausal and premenopausal women include anastrozole and letrozole (Takayama et al, 1998; Ailawadi et al, 2004; Razzi et al, 2004; Shippen et al, 2004; Soysal et al, 2004; Amsterdam et al, 2005, Verma and Konje, 2009).

Takayama et al. (1998) were the first to report on the treatment of endometriosis using an aromatase inhibitor in a postmenopausal woman. The patient has had a total abdominal hysterectomy and bilateral salpingo-oophorectomy but continued to have a large persistent pelvic mass and severe pelvic pain. The mass and the pain resolved following the use of the aromatase inhibitor anastrozole 1mg daily for 9 month. Since then, several other cases have been reported, the most recent by Mousa et al. (2007).

The doses of aromatase inhibitors used do not suppress ovarian activity in ovulating women (Verma and Konje, 2009). In some studies, anastrozole was administered together with either a GnRH agonist, a progestogen, progesterone or the combined oral contraceptive pill in four phase-II trials (Takayama et al, 1998; Ailawadi et al, 2004; Razzi et al, 2004; Shippen et al, 2004; Soysal et al, 2004; Amsterdam et al, 2005). All four studies showed significant benefits with anastrozole in reducing pelvic pain. In one randomized study of anastrozole with goserelin versus goserelin alone, 54.7% of the patients were symptom free in the anastrozole and goserelin group compared to only 10% of those treated with goserelin alone within 24 months of completing the treatment (Soysal et al, 2004). The combination of letrozole with an oral progestogen or an oral contraceptive produced similar results (Ailawadi et al, 2004; Shippen et al, 2004; Amsterdam et al, 2005).

Oral administration of anastrozole in combination with a progestogen, the cyclo-oxygenase inhibitor Rofecoxib[®] and calcitriol for 21 days followed by a 7-day break cycle for a total of 6 treatment cycles clearly reduced symptoms in two patients and eliminated the endometriotic lesions in one of the patients 15 months after the end of therapy. Both women became pregnant after 24 months (Shippen et al, 2004). These two cases point to an experimental and clinical hypothesis that is inseparably related to the aromatase concept-the study of expression and regulation of cyclo-oxygenase-2 (COX-2) and the evaluation of the clinical value of selective COX-2 inhibitors (Hayes et al, 2002). It is thus reasonable to conclude that aromatase inhibitors can effectively treat pelvic pain associated with endometriosis, which is resistant to current therapeutic modalities

1.7.2.4. Angiogenesis inhibitors

The use of angiogenesis inhibitors in the treatment of symptoms of endometriosis is based on the fact that several angiogenic factors are involved in neovascularisation which is essential for the survival and propagation of implanted viable endometrial cells; a process thought to occur within 5-8 days after implantation (Eggermont et al, 2005).

Ectopic endometrial cells induce the development of new vessels with no pericytic layers (Hull et al, 2003). The new vessels are immature and regress when exposed to hyperoxia, but the presence of vascular endothelial growth factor-A (VEGF-A) prevents this regression and promotes their survival and growth (Benjamin et al, 1998). Park et al (2004) showed that there was a significant inhibition of endometriosis explant formation in rhesus monkeys following the administration of the immunopurified anti-Flk1 antibody which blocks the VEGF receptor.

Unfortunately, drugs with anti-angiogenic potential have been shown to cause detrimental effects such as microphthalmia, fetal growth restriction and/or placental abnormalities on reproductive functions both in animal models and in patients, limiting their use in patients of reproductive age (McBride, 1978; Damato et al, 1994; Klauber et al, 1997). The inhibition of new blood vessel development may not only affect the vascularisation of endometriotic lesions, but may also affect the normal physiological angiogenesis in the female reproductive organs (Reynolds et al, 1992). As an understanding of the angiogenic mechanisms involved both in normal and ectopic endometrium is improved and the anti-

angiogenic drugs are refined, it should soon be possible for specific clinical studies to be commenced in women with endometriosis.

1.7.2.5. Selective estrogen-receptor modulators (SERMs).

Estrogens play an important role in the growth, regulation, development, and differentiation of the reproductive tract, mammary glands and the central nervous system (Klinge, 2000). Selective estrogen-receptor modulators (SERMs) such as tamoxifen and raloxifene are synthetic compounds which were developed to bind to the estrogen receptor (ER) and act as estrogen (E_2)-antagonists in selective tissues such as mammary glands but were, subsequently shown to bind to endometrial tissue and bone (Ribot et al, 1995; Jordan et al, 1999).

SERMs exert beneficial estrogen agonist effects on some tissues without undesirable effects in others (Fuchsyoung et al, 1995; Riggs et al, 2003). Raloxifene a second generation SERM was primarily developed for the treatment of postmenopausal osteoporosis but has been shown to be effective in the treatment of endometriosis in animal models (Yao et al, 2005).

In a recent randomized, placebo-controlled double-blind prospective trial of 93 patients with histologically confirmed endometriosis Stratton et al. (2008) showed that treatment with high doses (180mg) of raloxifene resulted in a significantly longer “time-to-return” of

the chronic pelvic pain as compared to a placebo. Interestingly biopsy-proven endometriosis in this group and the recurrence of endometriotic lesions was not associated with return of pain. Other factors may play a role in the pelvic pain associated with this pathology. Pain was not also well correlated with the return of the endometriosis as the effect of raloxifene on the central nervous system is yet to be fully elucidated (Bernardi et al, 2003). Further studies are required to investigate this potential therapeutic option.

1.7.2.6. Selective progesterone-receptor modulators (SPRMs)

Selective progesterone receptor modulators (SPRMs) are molecules that bind to the ligand-binding domain of the progesterone receptor (PR) and have mixed agonist-antagonist properties (Chabbert-Buffet et al, 2005). SPRMs display direct antiproliferative effects on the endometrium, although with variable effects which seem product- and dose-dependent and this may justify their use in the treatment of endometriosis. SPRMs offer the potential of greater efficacy and flexibility than traditional treatments for endometriosis based on;

- (a) selective inhibition of endometrial proliferation without the systemic effects of estrogen deprivation,
- (b) reversible suppression of endometrial bleeding via a direct effect on endometrial blood vessels, and
- (c) the potential to suppress endometrial prostaglandin production in a tissue-specific manner (Chwalisz et al, 2002).

Asoprisnil was the first SPRM to reach an advanced stage of clinical development and belongs to the class of 11 β -benzaloxime-substituted estratrienes that exhibit partial progesterone agonist/antagonist effect with high progesterone receptor specificity in animals and humans. Asoprisnil and other structurally related SPRMs suppress uterine prostaglandins activity in the guinea pig model (Elger et al, 2000 & 2004) . Asoprisnil has also been shown to induce reversible amenorrhoea through selective inhibition of endometrial proliferation and a direct effect on endometrial blood vessels without the systemic effects of estrogen deprivation. The molecular mechanism of its action on the endometrium remains to be determined.

1.7.2.7. Anti-progestogen

Mifepristone (RU 38486) [11 β -(4-dimethylaminophenyl)-17 β -hydroxy-17 α -propinyl-4, 9-estradiene-3-one] is a beta-aryl-substituted, 19-nortestosterone-derived compound (Schubert et al, 2005). This progesterone and glucocorticoid receptor antagonist is used in medical termination of pregnancies, and as such is commercially available in many countries. The effect of mifepristone on endometriosis may be related to its antiproliferative effect (endometriosis is an estrogen-dependent condition) as well as its apoptosis-promoting effect (Han et al, 2003). Three small clinical trials have been reported using three different dose schedules of mifepristone (5mg or 50mg per day for 6 months or 100mg per day for 3 months) (Kettel et al, 1993; 1996 & 1998). Improvements in symptoms were reported in all treated patients independent of the dose with a 55% mean regression of visible endometriotic lesions after 6 months of treatment with the 50mg dose (Kettel et al, 1993;

1996 & 1998). Mifepristone therefore appears to be very promising for the treatment of endometriosis. Since it does not induce the estrogen deficiency state associated with aromatase inhibitors, the side effects are likely to be more tolerable.

1.7.2.8. Immunomodulatory agents

There is evidence to suggest that endometriosis is associated with a state of subclinical peritoneal inflammation, marked by an increased peritoneal fluid volume, increased peritoneal fluid white blood cell concentration, especially of macrophages with increased activation status, and increased inflammatory cytokines, growth factors and angiogenesis promoting substances (D'Hooghe et al, 1996). While it is uncertain if the elevated cytokine levels and inflammation are a cause or a result of the disease, it is clear that cytokines may have profound effects which can lead to the establishment and further progression of the disease.

Since endometriosis is considered a local pelvic “inflammatory process”, with an altered function of peritoneal immune-related cells which are unable to eliminate the ectopic tissue, a therapeutic manipulation of the immune system may therefore result in some beneficial effects in women with endometriosis (Wilson et al, 1994). Two different types of immunomodulators have been suggested.

The first are agents that enhance the cytolytic arm of the immune response such as interleukin-12, loxorubine, levamisole and interferon (IFN)- γ -2b. These compounds are well known to be pleiotropic stimulators of the components of the immune system and have the beneficial effect of reducing serum cytokine production with a significantly longer time to recurrence of endometriosis. Combined treatment with intracystic recombinant IL-2 and GnRH agonists have also been used with great success in women with endometriosis (Acien et al, 2003; Velasco et al, 2005).

The second type are agents that reduce the inflammatory components of the immune process such as cyclo-oxygenase inhibitors (Cox) and tumour necrosis factor- α (TNF- α). TNF- α plays a critical role in the protection against microbial infection and the pathogenesis of a wide range of inflammatory and autoimmune diseases. It is one of the most studied cytokines in endometriosis with conflicting results on levels in the peritoneal fluid of sufferers. Some studies have shown an increase in the serum and peritoneal fluid levels (Rana et al, 1996; Bedaiwy et al, 2002; Richter et al, 2005), while others reported no difference when compared to controls (Vercellini et al, 1993; Kalu et al, 2007). Blocking TNF- α activity might therefore be expected to enhance programmed endometrial cell death and decrease monocyte number and activity, and thus negatively influence the establishment of endometriosis. In a study of fourteen baboons with spontaneously induced endometriosis which were randomly treated with either placebo, GnRH antagonists or subcutaneous injection of recombinant TNF-binding protein-1 (TBP-1) a reduction in the stages of endometriosis was demonstrated at laparoscopy in those treated with either GnRH antagonist or TBP-1 compared to controls (D'Hooghe et al, 2006).

Another drug which has been investigated in this group is pentoxifylline an anti-TNF and phosphodiesterase inhibitor which inhibits phagocytosis, and the generation of toxic oxygen species and proteolytic enzymes by macrophages and granulocytes *in-vitro* and *in-vivo* (Creus et al, 2008). It reduces the inflammatory action of tumour necrosis factor and interleukin-1 on granulocytes *in-vitro* as well as inhibiting tumour necrosis factor production *in-vitro*. Kamencic et al. (2008) undertook a randomized control trial in which 800mg of oral pentoxifylline was given daily for 3 months to 15 patients 24 hours following conservative surgery for endometriosis (CSE) and compared it to 19 controls that had CSE alone. The pentoxifylline group had a significantly better visual analogue scale score (VAS) at 2 and 3 months after surgery. At three months after surgery, 60% of those in the pentoxifylline group did not require analgesia, compared with 42% in the control group. Though encouraging, these results need to be replicated in larger studies.

1.7.2.9. Estrogen receptor β -agonists

Invasion and proliferation of endometriosis is estrogen dependent. Estrogen has two types of receptors, namely α and β . Estrogen receptor- α (ER- α), cloned by Green et al, (1986) is thought to be responsible for mediating many of the estrogen actions such as its trophic effects on the uterus and feedback on the hypothalamic-pituitary-ovarian axis (Green et al, 1986; Couse et al, 1999). ER- β was cloned by Kuiper et al, (1996). Stimulation of ER- β is thought to lead to modulation of the immune system and regulation of apoptosis but with no cell proliferation (Kuiper et al, 1996; Leung et al, 2006) . Both natural killer (NK) cells and macrophages are known to be involved in the pathogenesis of endometriosis and express ER- β (Cutolo et al, 1995; Curran et al, 2001).

ER β -041 is a potent and highly selective ER- β agonist that has comparable binding affinity for ER- β as the natural ligand (17 β -estradiol), and its binding selectivity to this ligand is 200 times or more than that for ER- α . Ovariectomized and gonad intact mice with established endometriosis were treated by Harris et al (2005) using ER β -041 and lesion regression was demonstrated in 40-75% of cases. This led to the suggestion that endogenous levels of 17 β -estradiol do not block the activity of an exogenous selective ER- β agonist. ER β -041 lacks the classic estrogenic activity in several *in-vivo* model systems (Harris et al, 2003), explaining the effectiveness associated with non-receptor subtype selective estrogens. Further studies evaluating ER β -041 are being awaited.

1.7.2.10. GnRH antagonists

GnRH antagonists are analogues of the native GnRH molecule which act by blocking the GnRH receptor directly and thus preventing the action of endogenous GnRH pulses on the pituitary (Reissmann et al, 1995). The secretion of gonadotropins is decreased within hours of antagonist administration and no flare-up effects occur. Moreover, discontinuation of GnRH antagonist treatment results in rapid, predictable recovery of the pituitary-gonadal axis as the pituitary receptor system remains intact (Kenigsberg et al, 1984).

GnRH antagonists inhibit dimerisation of receptors and, thus prevent secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Guo, 2008). The resulting hypoestrogenic environment without the preceding flares provides theoretical

potential advantages of faster onset of action and more effectiveness than GnRH agonists in women with endometriosis (Guo, 2008).

Rat models with endometriosis given subcutaneous injections of GnRH antagonists demonstrated a significant reduction in the size of endometriotic lesion (Jones, 1987). The degree of inhibition of growth was directly correlated with the dose of antagonist administered. A clinical trial in humans reported good results and minimal side effects but further randomized clinical trials are required (Kupker et al, 2002)

1.7.2.11. The pitfalls of medical treatment

The chronic nature of endometriosis means recurrence of symptoms following cessation of treatment is common (Valle et al, 2003). These rates could be as high as 50% 12-24 months following cessation of medical therapy (Dlugi et al, 1990; Fedele et al, 1993; Stratton et al, 2008). For this reason, medical therapies that can be administered for only a few months due to safety concerns or poor tolerability are not ideal for women with symptomatic endometriosis (Riggs et al, 2003). This has led to an increasing number of innovative medical treatment modalities. Most of these follow a better understanding of the pathogenesis of endometriosis and interrupting some of the stages in the pathogenic pathway. The high rates of recurrence following medical therapies have led to a greater role for surgery. Surgery may be performed laparoscopically or as an open procedure.

1.7.3 Surgical Methods

Surgery for the treatment of endometriosis should ideally aim to remove all the lesions and associated sequelae such as adhesions and improve symptoms. Unfortunately no single surgical approach is able to achieve these aims partly because of difficulties excising all lesions (not all lesions are visible) and the lack of a relationship between the severity/extent of endometriosis and symptoms. Consequently various surgical approaches have been tried depending on the location and extent of the lesions and the symptoms. Most of these are performed laparoscopically and include:

- (a). ablation
- (b). excision
- (c). resection and
- (d). radical surgery

Laparoscopy apart from being the current gold standard method of diagnosing endometriosis also enables an assessment of the extent of the disease. Surgery is considered the first treatment option in some women with pain symptoms desiring pregnancy (Berkes et al, 2010).

The decision by the clinician to offer surgery, choose one form of treatment over another, and or to combine surgery with adjunctive procedures appears to be based more on personal experience/preference and the patient's fecundity rather than evidence derived from prospective randomised control trials (Vercellini et al, 2006). It is not clear whether lesions should be ablated (vaporized, cauterized, or desiccated) or excised (Yeung et al, 2009). It is also unclear whether adjunctive surgical procedures, such as presacral neurectomy or uterosacral nerve ablation-transection, improve the outcomes of surgical therapy in terms of pain relief (Daniels et al, 2009).

A variety of conservative and surgical options are available depending on the extend of the disease and the surgical expertise and facilities available. They include ablation, excision or resection with laser, scissors, monopolar electrocautery or bipolar coagulation, CO₂-laser and the harmonic scalpel. Although these techniques are widely available, few studies have investigated their effectiveness in randomised trials. Hasson (1979) showed that electrocoagulation of pelvic endometriotic lesions can be an adequate therapy for chronic pelvic pain or infertility caused by endometriosis. While laparoscopic excision has been shown from randomised trials to be an effective treatment of endometriosis (Redwine et al, 1991; Sutton et al, 1997), there are no such studies for the ablative techniques. Sharp excision, bipolar diathermy and ablation by CO₂-laser are the most common techniques in laparoscopic surgery for endometriosis. Which of these techniques offers better results is a subject of extreme debate and discussion (Hart et al, 2008).

Some surgeons are reluctant to use electrocoagulation, speculating that residual necrosis due to coagulation could induce increased inflammatory activity (Gürkan et al, 1991;

Moghadami-Tabrizi et al, 1996). In addition, the risk of thermal tissue damage of adjacent tissues/structures with the electrocoagulation is thus a limiting factor in its use (Sparmann et al., 1992). However, several animal studies have demonstrated less postoperative adhesions after bipolar coagulation (Mecke et al, 1991; Wallwiener et al, 2009). This seems to be a distinctive advantage, considering the fact that postoperative adhesions are often identified as a cause of persistent pain in patients with endometriosis (Exacoustos et al, 2006).

In the treatment of deep infiltrating endometriosis as well as endometriomas excision is considered the surgical approach of choice (Bianchi et al, 2009). In cases of deep infiltrating endometriosis; complete excision of all lesions yields convincing results with respect to pain symptoms and improved fertility (Chapron, 2001; Camara et al, 2009). For endometriotic cysts, two recent studies report a better outcome in postoperative morphology and function of the ovary if coagulation is avoided and excision is performed followed by reconstruction using sutures (Hemmings et al, 1998). However, it is not clear whether this outcome is a result of the absence of coagulation or the use of reconstructive sutures (Pellicano et al, 2008). In an animal study, bipolar coagulation was found to result in extensive destruction of the ovarian parenchyma compared to other ablation techniques (Hendriks et al, 2010). As an alternative to classical sharp excision, the intra-operative use of CO₂-laser has received more attention (Sutton et al, 2002). Despite initial promising results its widespread use is still limited by high technical and financial constraints (Meuleman et al, 2009).

For the treatment of superficial forms of endometriosis, surgical techniques and their postoperative outcome have rarely been analysed for effectiveness. Wright et al. (2005) could not find a significant difference between excision and ablation in the improvement of endometriotic symptoms in cases of mild endometriosis. Similar results have been published by Jakiel et al. (2001) where success rates for both methods were equal.

Recurrence of endometriosis following laparoscopic surgery is common even in the hands of very experienced laparoscopic surgeons; for example the cumulative recurrence rate after 5 years is nearly 20% (Redwine, 1991 & 2001).

Radical surgery for the treatment of endometriosis refers to total hysterectomy and bilateral salpingo-oophorectomy. This surgical approach is often considered as “definitive” therapy despite the fact that endometriosis may recur in 5-10% of women who have undergone hysterectomy and bilateral salpingo-oophorectomy (Namnoum et al, 1995; Clayton et al, 1999).

Endometriosis associated pain can be reduced by removing the entire lesions in severe and deep infiltrating disease. If a hysterectomy is performed, all visible endometriotic tissue should be removed at the same time (Lefebvre et al, 2002).

The outcome of surgery in patients with endometriosis and associated pain is influenced by many psychological factors including their personality, marital status and sexual problems

(Schover et al, 1993). It is difficult objectively to evaluate the effect of different surgical approaches as factors such as the surgery itself, the doctor-patient relationship and complications may influence outcome. For example diagnostic laparoscopy without complete removal of endometriosis has been shown to alleviate pain in approximately 50% of patients (Sutton et al, 1994). There is a significant placebo response with regards to surgery for endometriosis. Similar results have been reported using oral placebo (Overton et al, 1994). The rate of recurrence is significantly correlated with the age of the patient. The younger the patients are at the time of the diagnosis the higher the rate of recurrence. Higher recurrence rates in younger patients seem to justify a more radical treatment in this group (Fedele & Bianchi, 2004). However, this is fraught with several problems as it is in this younger age group that several of the other factors potentially influencing symptoms prevail.

The major shortcomings of the surgical treatment of endometriosis related pain is the lack of prospective RCTs with a long enough follow-up to allow for the drawing of clear clinical conclusions. In a prospective, randomized, double-blind controlled study, surgical therapy was shown to be superior to expectant management after six months of treatment of mild and moderate endometriosis (Sutton et al, 1994). Treatment was least effective in women with minimal disease. One year later, symptom relief was still present in 90% of those who initially responded (Sutton et al, 1997).

In a randomized blinded crossover study it was confirmed that laparoscopic excision of endometriosis was more effective than placebo in reducing pain and improving quality of

life (Sutton and Hill, 1990). Surgery resulted in pain relief in 80% of patients with severe disease who did not respond to medical therapy (Sutton and Hill, 1990).

Laparoscopic cystectomy for ovarian endometriomas > 4 cm in diameter improves fertility compared to drainage and coagulation (Beretta et al, 1998; Chapron et al, 2002). Coagulation or laser vaporization of endometriomas without excision of the pseudo-capsule is associated with a significantly increased risk of cyst recurrence (Vercellini et al, 2003b; Hart et al, 2005).

Ablation of endometriotic lesions plus laparoscopic uterine nerve ablation (LUNA) in minimal to moderate disease reduces endometriosis associated pain at 6 months compared to diagnostic laparoscopy alone; the smallest effect seen in patients with minimal disease (Jacobson et al., 2001). However, there is no evidence that LUNA is a necessary component of adjunctive surgery (Sutton et al, 2001), and LUNA by itself has no effect on dysmenorrhoea associated with endometriosis (Vercellini et al, 2003a; Daniels et al, 2009).

1.7.3.1. Post-operative treatment

Postoperative hormonal treatment with the GnRHa does not produce a significant reduction in pain recurrence, but has a tendency to delay recurrence of endometriosis (Hornstein et al, 1997). Postoperative GnRHa treatment has been shown to reduced pain scores, and delay pain recurrence for more than 12 months, where the agonists were given for 6 months

(Hornstein et al, 1997; Vercellini et al, 1999b) but not if they were administered for 3 months only (Parazzini et al, 1994). Similarly, postoperative hormonal treatment with danazol 100 mg/day (low dose) during 12 months after surgery for moderate to severe endometriosis resulted in a significantly lower pain score in the treated group when compared to a placebo group. In contrast, high dose danazol (600 mg/day) for 3 months was not superior to expectant management with respect to pain recurrence in an identical patient population (Bianchi et al, 1999). In an RCT, postoperative administration of low-dose cyclic oral contraceptives did not significantly affect the long-term recurrence rate of endometriosis after surgical treatment. A delay in recurrence was evident from a life-table analysis (Muzii et al, 2000).

1.7.4. Levonorgestrel-releasing intrauterine system [Mirena[®] (LNG-IUS)].

Levonorgestrel (LNG) is a potent 19-nortestosterone derivative with androgenic and anti-estrogenic effects on the endometrium (Salmi et al, 1998; Shulman et al, 2004). The levonorgestrel-releasing intrauterine system LNG-IUS consists of a 32mm T-shaped polyethylene frame with a cylinder containing 52mg of LNG wrapped around its stem (Shulman et al, 2004). It releases 20µg/day of LNG over a 5 year period (Nilsson et al, 1986) which suppresses estrogen-induced changes in the endometrium by directly decreasing the number of estrogen receptors, leading to an inactive epithelium (Haukkamaa, 1984), glandular atrophy and pseudo-decidualisation of the stroma within one to three months of insertion (Nilsson et al, 1978; Silverberg et al, 1986). The LNG-IUS is licensed in the United Kingdom as a contraceptive and for the treatment of menorrhagia (Andersson et al, 1990). The induction of endometrial glandular atrophy and extensive

decidual transformation of the stromal, the down regulation of endometrial cell proliferation and increased apoptotic activity combined with the anti-inflammatory and immunomodulatory effects of LNG make a convincing theoretical basis for its use in the treatment of symptomatic endometriosis.

Few studies have investigated the effect of the LNG-IUS on the amelioration of endometriosis related symptoms (Vercellini et al, 1999; Lockhat et al, 2004; Lockhat et al, 2005a; Lockhat et al, 2005b; Petta et al, 2005; Varma et al, 2006). Symptom relief varies from 60-96% after 6-36 months of treatment (Lockhat et al, 2005b, Petta et al, 2005). A second look laparoscopy after six months of treatment in one of the study demonstrated an improvement in the staging of endometriosis (Lockhat et al, 2004).

Although the local effects of levonorgestrel on the endometrium are partly responsible for symptom relief, suppression of ovulation and the local action of peritoneal fluid levonorgestrel on the endometriotic lesions maybe other mechanisms by which it is effective (Lockhat et al, 2005b). Whether this affects the macrophages and the cytokines content and concentration of peritoneal fluid is uncertain.

Since endometriosis elicits a local inflammatory effect on the peritoneum (around the lesions) and significantly alters cytokines and macrophages in the peritoneal fluids, it is hypothesized that LNG-IUS improves symptoms in women with endometriosis via other mechanisms which include

- 1). Modification of estrogen and progesterone receptors in endometriotic lesions.
- 2). Changes in mast cell numbers
- 3). Alterations in some of the cytokines which are modified as a result of endometriosis.
- 4). In addition, it is hypothesized that the LNG-IUS is as effective as the gold standard treatment the GnRH agonists but with fewer side effects. The studies in this thesis were designed to investigate these hypotheses.

Chapter II

Estrogen (ER) and progesterone (PR) receptor expression in eutopic and ectopic endometrium following treatment.

2.1 Background

The levonorgestrel intrauterine system (LNG-IUS), which delivers the progestin levonorgestrel (LNG) into the uterine cavity at a steady rate of 20µg/day, has been shown in several pilot studies to significantly improve symptoms of endometriosis (Luukkainen et al., 1990; Lockhat et al, 2005b; Petta et al, 2005). The released levonorgestrel has been shown to suppress estrogen-induced changes in eutopic endometrium by directly decreasing the number of estrogen receptors (ER), leading to an inactive epithelium (Haukkamaa, 1984), glandular atrophy and pseudo-decidualisation of the stromal within 1–3 months of insertion (Nilsson et al, 1986; Silverberg, 1986). Whether this mechanism of action is similar in the ectopic endometrial tissue in women with endometriosis is uncertain. Eutopic and ectopic endometrium are known to contain ER and progesterone receptors (PR), and significant levels of LNG have been demonstrated in the serum and peritoneal fluid of women on the LNG-IUS used as treatment for symptomatic endometriosis (Lockhat et al, 2005a).

It was hypothesized that the LNG from this device is effective in women with endometriosis by its action on these receptors and that this mechanism is similar to that of the standard GnRH agonist. These hypotheses were tested in a two part study- the first investigated the effect of the LNG-IUS on these receptors and the second was a comparative study of the effect of the LNG-IUS and the GnRH agonist (decapetylTM) on these receptors.

2.2. Chemical Structure of steroid hormone.

Steroid hormones are all derived from the 27 carbon substrate cholesterol and so share the same cyclohexaphenanthrene ring structure (**Fig. 2.1**).

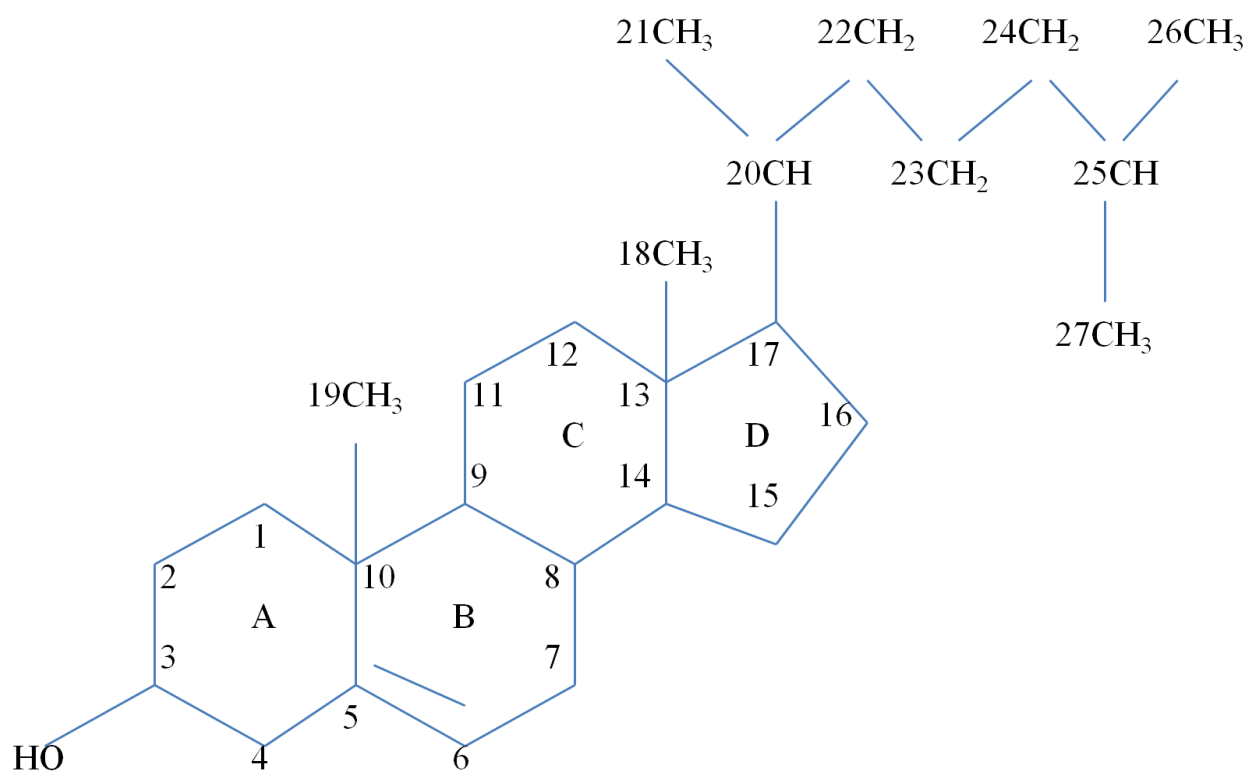


Fig. 2.1. The cyclohexaphenanthrene ring of Cholesterol-the steroidogenic substrate

2.2.1. Classification

Steroid hormones are classified into five families depending upon the number of carbon (C) atoms and the chemical groups present at key carbon residues with the classes of estrogen and progestogens illustrated in the table (**Table 2.1**). The C19 and C21 progestogens are the most widely used in clinical practice. The C19 progestogens, which include levonorgestrel, the progestogens in the LNG-IUS are androgenic while the C21 progestogens are not.

Table 2.1. Changes in the cyclohexaphenanthrene ring of two major families of steroid hormones (estrogen and the progestogens).

Steroid Family		
	Progestogens	Estrogens
No. of Carbon atoms	21	18
C21	CH ₃	Absent
C18	CH ₃	CH ₃
C17	D-ring side Chain	17 β -hydroxyl or Ketone
C11	Nil	Nil
C3	3 β -hydroxyl or Ketone	Planar hydroxyl group attached to aromatic A-ring

2.3. Estrogen and Progesterone receptors

For several years it was generally believed that only a single ER and PR existed. However, the discovery of a new ER with specificity for estrogens generated new insights into the estrogen signalling system (Kuiper et al, 1998). The PR is expressed as two major isoforms, PR-A and PR-B which arise from alternative transcriptional starting sites within the same gene. Although PR-A and PR-B were initially thought to be equally distributed, it is now clear that they are differentially expressed and thus have distinct functions in several human tissues, including the human endometrium (Graham et al, 1995; Leslie et al, 1997; Kumar et al, 1998; Graham & Clarke 2002; Shabani et al, 2007b). ER and PR expression and distribution patterns might play important roles in normal endometrial function and in pathological situations. The expression and relationship of both ER (α & β) and PR (A&B) might have essential and clinical implications (Mendelson & Hardy, 2006; Moy & Goss, 2006; Shabani et al, 2007a).

2.3.1 The estrogen receptor (ER)

Estrogen circulates in serum bound to low affinity proteins and reach the target cells, where it then binds very tightly to the ER. The intracellular location of the ER has been the subject of debate. In the absence of a ligand, the ER is thought to reside primarily in the nucleus with a small proportion of it in the cytoplasm; increased nuclear localization of the ER occurs in the presence of the ligand (McEwen et al, 1999). There are two closely related forms of estrogen receptors, ER- α and ER- β . ERs act as dimers binding to DNA at specific

target sequences called estrogen response elements (EREs), which are present in the promoter regions of responsive genes (Mangelsdorf et al, 1995)

ER knock-out studies have demonstrated the predominance of ER- α expression in the uterus and hence the main mediator of estrogen action (Couse et al, 2000). For ER- α , the presence of variant proteins has been reported in normal endometrium, endometrial hyperplasias and carcinomas (Saegusa and Okayasu, 2000). Sequence analysis of these mutated ERs proteins showed that each variant corresponds to the specific deletion of one exon, suggesting transcriptional splicing errors.

ER expression levels are maximal in the proliferative phase and decline in both the glandular and stromal compartment in the secretory phase of the menstrual cycle (Fujishita et al, 1997). Several reports suggest that ER- β expression in endometrial epithelial cells is predominantly during the proliferative phase and declines as the menstrual phase continues (Rey et al, 1998; Matsuzaki et al, 1999; Lecce et al, 2001; Mylonas et al, 2004; Mylonas et al, 2005).

Differences in the tissue distribution of ER- α and ER- β during the menstrual cycle suggest that they play a substantial role in the modulation and function of estrogen activity in human endometrial tissue. ER- β could have a different action and activity compared to that of ER- α , exerting opposite transcriptional effects after binding to estrogens and anti-estrogens (Paech et al, 1997). Therefore, a synchronized expression of ER- α and ER- β seems to be essential for estrogen-related transcriptional activity in target organs.

2.3.2 The progesterone receptor (PR).

The progesterone receptor (PR) also known as NR3C3 (nuclear receptor subfamily 3, group C, member 3), is an intracellular steroid receptor that specifically binds progesterone. PR is encoded by the single gene *PGR* residing on chromosome 11q22 (Kase et al, 1999; Gadkar-Sable et al, 2005; Fritz et al, 2005). PR has two main different isoforms, PR-A and PR-B which are differentially expressed in the human endometrium (Mangal et al, 1997).

The PR like all other steroid receptors has an amino and a carboxyl terminal and between them the regulatory domain, a DNA binding domain, the hinge section, and the hormone binding domain. A special transcription activation function (TAF), called TAF-3, is present in PR-B, in a B-upstream segment (BUS) at the amino acid terminal which is not present in PR-A.

PR expression levels in the glandular epithelium are high in the proliferative phase (under the influence of estrogen) and decrease after ovulation. There are no significant variations in endometrial stromal PR expression throughout the menstrual cycle (Garcia et al, 1988; Snijders et al, 1992a). Both PR isoforms have been demonstrated in the human endometrium and relative variations of PR-A/PR-B expression level during the menstrual cycle have been reported (Mangal et al, 1997; Wang et al, 1998). The ratio of PR-A to PR-B changes during the menstrual cycle, although the concentrations of PR-A remains constant and are higher compared to PR-B (Mangal et al, 1997). The relative expression of PR-B increases during the proliferative phase, reaching the highest levels during the periovulatory

phase. Thereafter, PR-B expressions decreases rapidly and are almost undetectable by the end of the cycle (Mangal et al, 1997). A higher PR-B expression during the proliferative and the early secretory phase, decreasing significantly in the late secretory phase, has been demonstrated recently by using a monoclonal antibody against PR-B (Mylonas et al, 2007).

2.3.3 Mechanisms of ER and PR action

The binding of a ligand to either ER or PR leads to a conformational change in the structure of the receptor with a subsequent dimerization. The receptor- dimer complex can then translocate into the nucleus where it interacts with specific palindromic sequences (DNA locus whose 5'-to-3' sequence is identical on each DNA strand): the estrogen response elements (ERE) for ER or the progesterone response elements (PRE) for PR. This interaction can modulate the synthesis of specific mRNAs and proteins with subsequent functional alterations (Gronemeyer, 1991; Kampa & Castanias, 2006). In addition to the action through classical EREs, ERs also mediate gene transcription from the activator protein 1 (AP1) enhancer element that requires ligand and the transcription factors *fos* and *jun* for transcriptional activation (Shemshedini et al, 1991; Matthews et al, 2006; Moriarty et al, 2006).

The effects of progesterone are historically thought to be mediated through PR-A or PR-B-induced gene transcription (Conneely et al, 1987; Allan et al, 1992; Leonhardt et al, 2003). Available evidence suggests that PR-A and PR-B shuttle between the nucleus and the cytoplasm, with ligand binding inducing interactions between the receptor and nuclear co-

activators (Allan et al, 1992; Leonhardt et al, 2003). PR-A and PR-B differentially regulate gene transcription, increasing the complexity of this regulatory system (Brayman et al, 2006; Aupperlee et al, 2007). For example, PR-A is a less potent transactivator than PR-B. PR-A also exerts transrepressional activity on PR-B in a promoter- and cell type-dependent manner (Brayman et al, 2006; Aupperlee et al, 2007). The progesterone-inducible genetic network is further refined by the expression of PR splice variants with variable ligand affinities and transactivational activities (Marshburn et al, 2005).

2.4. Regulation of estrogen and progesterone synthesis.

In the ovaries the synthesis of estrogen and progesterone is regulated primarily by trophic hormones secreted from the anterior lobe of the pituitary gland, acting in conjunction with endocrine and paracrine modulators of steroidogenesis. The anterior pituitary hormones, being hydrophilic, act via cell surface receptors coupled to signal transduction pathways that increase the expression and activities of steroidogenic enzymes. While several second messengers are generated in response to trophic hormones, the steroidogenic responses to various interleukins, a lipophilic factor from macrophages, calmidazolium an imidazole compound are mediated primarily through the generation of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA) (Cooke, 1999).

In this chapter the first section describe investigation of the changes in ER and PR in both eutopic and ectopic endometrium in patients with endometriosis placed on the LNG-IUS for its symptomatic treatment before and after six months. While the second section describes

investigation comparing the changes in ER and PR in both eutopic and ectopic endometrium in women with endometriosis treated with either the LNG-IUS or the gold standard the GnRH agonist (decaproylTM) in its symptomatic treatment before and after six months. Both were to test the hypotheses that (a) the LNG is effective in these women through the modulation of ER and PR and (b) that these mechanism are comparable to those of the GnRH agonist (decaproylTM).

2.5. Material and methods

2.5.1. Subjects

These were women aged between 18 to 42 years who attended the gynaecology outpatient department of the University Hospitals of Leicester NHS Trust, between April 2000 and March 2002 and also between January 2008 to December 2009, with a diagnosis of endometriosis treated with either the LNG-IUS or the GnRH agonist. Thirty four women were recruited by Ms Farhana Lockhat the clinical research between April 2000 and March 2002, 29 (85%) completed the study; five discontinued, for personal reasons (one), side effects of worsening of acne (one) and lower abdominal/pelvic pain (three). The inclusion and exclusion criteria used by both studies were inherited from the original or the study by Dr Lockhat.

2.5.2. Inclusion Criteria

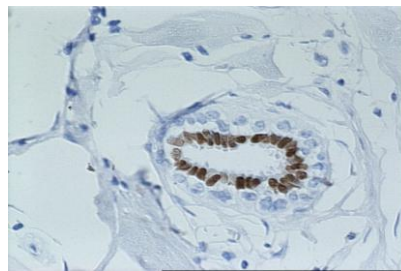
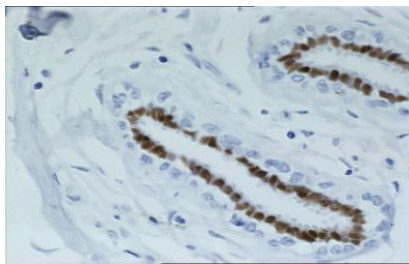
Those who met the inclusion criteria were recruited into the study after giving a written signed informed consent. These criteria included laparoscopically confirmed minimal to moderate endometriosis (American Fertility Society, 1985), not on any form of contraception or hormonal treatment for the preceding 3 months, not planning a pregnancy for the following 6 months, willingness to undergo a second laparoscopy after 6 months of treatment, no contra-indications to an intrauterine device, between 18 and 42 years of age and BMI<27kg/m². The BMI was the recommendation of the surgeons as a means to facilitate and reduce the failure rate during the procedure although a study performed by Canedo et al (2010) did not find any significant differences in the rates of conversion, major postoperative complications, or length of stay when they compared patients of normal and higher BMI.

2.5.3. Exclusion Criteria

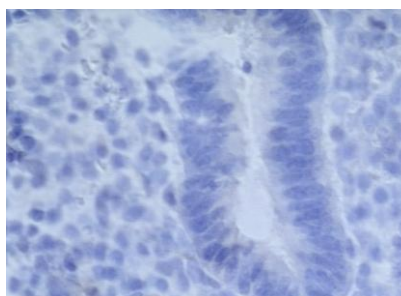
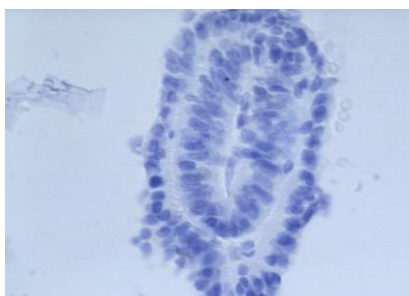
The exclusion criteria included infertility as a symptom, a clinical history of pelvic inflammatory disease (PID) and contra-indications to the use of the levonorgestrel-releasing intrauterine system (LNG-IUS or Mirena[®] Bayer, Newbury, Berkshire, United Kingdom). The study was approved by the Leicestershire, Northamptonshire and Rutland (LNR) Research Ethics Committee as seen in the ethical approval letter (**Appendix II**).

2.5.4. Tissue collection and Processing

An initial laparoscopy to confirm the diagnosis of endometriosis, biopsies of the eutopic and ectopic endometrium were collected followed by the insertion of the LNG-IUS. Six months later, a second-look laparoscopy was performed and the biopsies repeated. Thirty-four women were recruited and 28 had the second-look laparoscopy for the LNG-IUS only study, while thirty two women were recruited and nineteen had a second-look laparoscopy for the comparative LNG-IUS and the GnRH agonist study. Paraffin embedded tissue blocks (eutopic and ectopic endometrium), four in total from each woman, (two taken before and two taken 6 months after insertion of the LNG-IUS or treatment with the GnRH agonist) were studied. Breast tissues, obtained from women undergoing breast reduction surgery, were used as positive controls (2.2A). The negative controls were generated by omitting the ER- α , ER- β , PR (A&B), PR-A or PR-B antibodies in normal endometrial tissue (2.2B).



2.2A. Positive Control: Breast tissue from patient with breast reduction.



2.2B. Negative Control: Normal eutopic endometrial tissue

2.6. Immunohistochemistry

Table 2.2. Characteristics of the primary antibodies

Antibodies	Product code	Clone	FFPE suitability	Dilution
Progesterone receptor (A)	NCL-PGR-312	16	Yes	1:100
Progesterone receptor (AB)	NCL-PGR-AB	SAN27	Yes	1:40
Progesterone receptor (B)	NCL-PGR-B	SAN27	Yes	1:100
Estrogen receptor (α)	NCL-ER-6F11	6F11	Yes	1:50
Estrogen Receptor (β)	ab288	Clone14C8	Yes	1:200

This progesterone receptor mediates the actions of progesterone and can be one of two types: progesterone receptor type A (PRA) and progesterone receptor type B (PRB). The combine PR (AB), PRA and PRB were evaluated here.

This estrogen receptor mediates the actions of estradiol and can be one of two types: estrogen receptor type α (ER- α) and estrogen receptor type β (ER- β). Only ER- α and ER- β were evaluated here.

All tissues were fixed in 10% neutral-buffered formalin, and paraffin embedded blocks made using routine methods. Sections cut at 4mm thickness were mounted on silane coated slides and dried thoroughly before immunohistochemistry. Briefly, slides were deparaffinised in xylene and then hydrated through a graded series of ethanol solutions. The antigens were unmasked by immersing the sections in sodium citrate (Sigma–Aldrich, United Kingdom) buffer solution (10mM solution, pH6), and by heating them at 800W in a microwave for 30 min. The slides were then allowed to cool for 20 min and washed in running tap water for 5 min. Endogenous peroxidase activity was blocked with 6% hydrogen peroxide solution for 10 min and slides were briefly washed in running tap water for 5 min

at room temperature. Non-specific binding sites were blocked first in Tris-buffered saline supplemented with 0.1% bovine serum albumin (TBS/0.1%BSA), pH 7.6 with a stirrer. This was followed by the addition of a 1:10 dilution of normal rabbit serum [(NRS) Dako, Glostrup, Denmark] in TBS/0.1%BSA. After 30 min, slides were drained and endogenous biotin sites blocked with avidin–biotin blocking solutions according to the manufacturer’s instructions (Vector Laboratories, Burlingame, California, USA). The slides were next washed in TBS/0.1%BSA for 5 min. The sections were then incubated with PR primary monoclonal antibody NCL-PGR-AB; 1:40 dilution (Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) or PR-A primary monoclonal antibody NCL-L-PGR-312; 1:100 dilution (Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) or PR-B primary monoclonal antibody NCL-L-PGR-B; 1:100 dilution (Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) or ER- α (NCL-ER-6F11) 1:50 dilution (Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) or ER- β (clone14C8) 1:200 dilution (Abcam, Cambridge, United Kingdom) overnight at 4°C, in a humid chamber. Sections were next washed in TBS/0.1%BSA for 20 min with stirring followed by incubation with biotinylated rabbit anti- mouse immunoglobulin (Dakocytomation, Glostrup, Denmark) diluted to 1:400 in TBS/0.1%BSA at room temperature for 30 min, before being washed in TBS/0.1%BSA for 20 min. Secondary antibodies were amplified with horse-radish peroxidase conjugated avidin–biotin complexes for 30 min according to the manufacturer’s instructions (ABC Elite Vector Laboratories, Burlingame, California, USA). Antibody binding was visualised using 3,30-diaminobenzidine tetrahydrochloride (DAB) for 5 min according to the manufacturer’s instructions (Sigma, Poole, United Kingdom). Finally, sections were lightly counterstained with Mayer’s haematoxylin (Sigma–Aldrich, United Kingdom) for up to 40 seconds,

dehydrated through a series of ethanol solutions, cleared through xylene and mounted in Permount mounting medium (DPX, Fluka, Buchs, Switzerland).

2.7. Histomorphometric analysis

It is of particular interest to measure the number of receptors as they are manifested in the histological sections of the tissue. Manual counting of cells is very time consuming with the proportion of positive to the total number of nuclei receptors recorded as a percentage in 10 different fields of view of each block made up of two slides.

The automated histomorphometric computerized image analysis system has the advantages of objectivity, accuracy, repeatability, and ease of use. For the ER and PR expression, axioplan compound microscope was used to identify the positive and negative stained nuclei per field of view with the optimum red-blue threshold level chosen and the mean intensity of positive and negative nuclei recorded per sample. This was used to calculate the available range of intensities for each sample based on a six-point scoring system where 0= no, 1= minimal, 2= mild, 3= moderate, 4= marked, and 5= very marked staining. A corrected intensity (the average intensity of positive nuclei) was calculated, and from this, the Steroids Receptor Index was determined ($\text{corrected intensity} \times \text{positive nuclei receptors} / \text{total nuclei receptors}$). The kappa statistic between the histomorphometric analysis and the manual counting was 0.65, (95% CI for the difference 0.58–0.72).

Images of immunoreactive sections were captured on an Axioplan compound microscope equipped with a Sony analogue colour video camera (Model DXC-151P, Sony; Japan) and

Axiovision version 4 image capture and analysis software (Zeiss, Milton Keynes, Bucks, United Kingdom) at 40 x magnification. The possible influence of sampling error was assessed by using different concentration of the primary antibody to get the optimum staining intensity of these receptors. The concentration with the maximum staining intensity was used to process and analyse the slides, two from each block with five views taken from each slide, one from each rectangular quadrant and the fifth from the centre counting all nuclei using a 10x25 graticule. The glandular and stromal cell compartments were analysed separately. Positive cells were identified by the presence of a brown nuclear stain of ER- α , ER- β , PR (A&B), PR-A or PR-B and the negative cells by the presence of a strong blue stained nucleus. Nerve cells and blood vessels were excluded from the analyses. The number of positive cells was obtained by counting the brown nuclei and the number of negative cells by counting the blue nuclei and the percentage positivity assessed from the total. Expression level was then determined by dividing the stained nuclei by the total stained and unstained nuclei and multiplying by 100 to generate a percentage.

2.8. Statistical analysis

The results are expressed as mean and standard errors of the mean (SEM). Normality of the data was determined using the Shapiro-Wilk test and because the data were not normally distributed, non-parametric statistical analysis (Wilcoxon matched-pairs signed rank test) was performed to determine differences in the percentages of positive ER- α , ER- β , PR (A&B), PR-A and PR-B expression in eutopic and ectopic endometrium before and 6 months after treatment with LNG-IUS alone and compared with the GnRH agonist. Statistical analyses were performed with the aid of Graphpad Prism (Version 5.0) software (www.graphpad.com). Differences were considered to be significant when $P < 0.05$.

2.9. Results:

2.9. A. Effect of LNG-IUS on Steroid Receptors

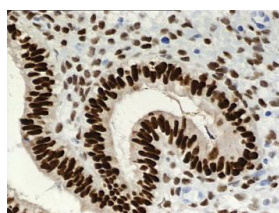
The mean age of the 28 women in the LNG only study group was 31 ± 7.2 (range 18–42) years. At the time of entering the study 2, 2 and 4 patients were in the early (day 4–6), mid (day 8–10) and late (day 11–13) proliferative phases of the menstrual cycle and 5, 6 and 9 were in the early (day 16–18), mid (day 19–21) and late (day 23–26) secretory phases, respectively (**Table 2.3 & Appendix VA**). The phases of the menstrual cycle were defined from the first day (day 1) of the last menstrual period. None was menstruating. Both glands and stromal had to be present in the eutopic and ectopic endometrium for the blocks to be considered acceptable for studying. The eutopic sample taken before treatment with LNG-IUS was dated histologically according to the criteria of Noyes and Haman (1953).

Table. 2.3. No of women in the different phases of the menstrual cycle at entry into the LNG only study.

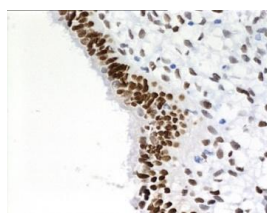
Phase of menstrual cycle	Number of women
early proliferative phase	2
mid proliferative phase	2
late proliferative phase	4
early secretory phase	5
mid secretory phase	6
late secretory phase	9

There were no discernable visible differences in the ER isoform or PR staining patterns in the eutopic and ectopic endometrium taken before treatment. Staining was observed mainly in the nuclei of both glandular and stromal cells (**Fig. 2.3**). There was some cytoplasmic staining with the ER- α and ER- β antibodies, but not with the PR antibodies (**Fig. 2.3**). A heterogeneous staining was, however, seen in the glands of eutopic and ectopic endometrium. Within the glands themselves, there were differences in the nuclear staining intensity from blue through to light brown and then to deep brown. A similar heterogeneous staining pattern was seen in the stromal cells of eutopic and ectopic endometrium.

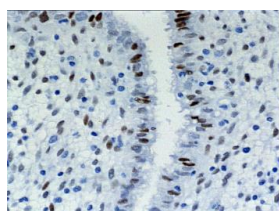
ER- α Before: A Eutopic



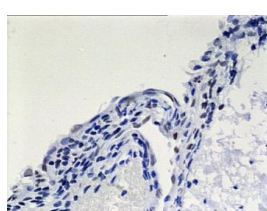
C Ectopic



ER- α After: B Eutopic

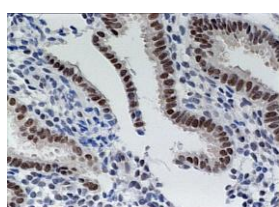


D Ectopic

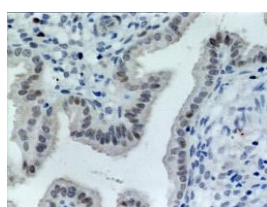


2.3A

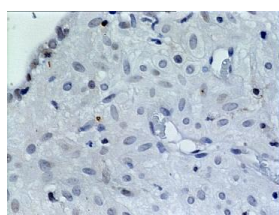
ER- β Before: A Eutopic



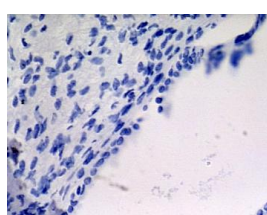
C Ectopic



ER- β After: B Eutopic

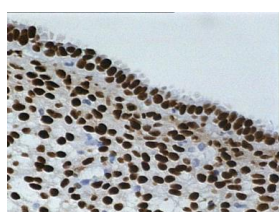


D Ectopic

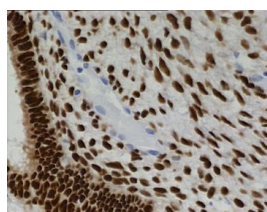


2.3B

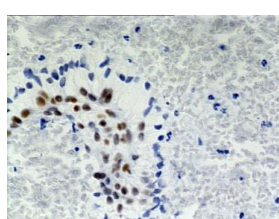
PR (A&B) Before: A Eutopic



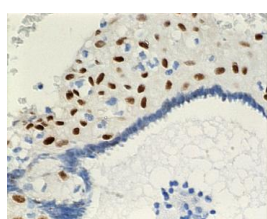
C Ectopic



PR (A&B) After: B Eutopic



D Ectopic



2.3C

Fig. 2.3 Photomicrographs of ER- α (**2.3A**), ER- β (**2.3B**) and PR (A&B) (**2.3C**) expression in eutopic and ectopic endometrium before and after 6 months treatment with the LNG-IUS.

2.9.B. Comparative effect of the LNG-IUS and GnRH agonist on receptors

Figure 2.3 show the recruitment algorithm for this study. The mean age of the women in this comparative study was 30 ± 5.7 (range 18-42) years. Similar to the LNG-IUS group alone at the time of entering this study 5, 3 and 2 women were in the early, mid and late proliferative phase of the menstrual cycle and 2, 3 and 4 were in the early, mid and late secretory phases respectively (**Table 2.4 & Appendix VB**).

Table 2.4. No of women in the different phases of the menstrual cycle at entry into the LNG and GnRH agonist groups who completed the study.

Phase of menstrual cycle	Number of women
early proliferative phase	5
mid proliferative phase	3
late proliferative phase	2
early secretory phase	2
mid secretory phase	3
late secretory phase	4

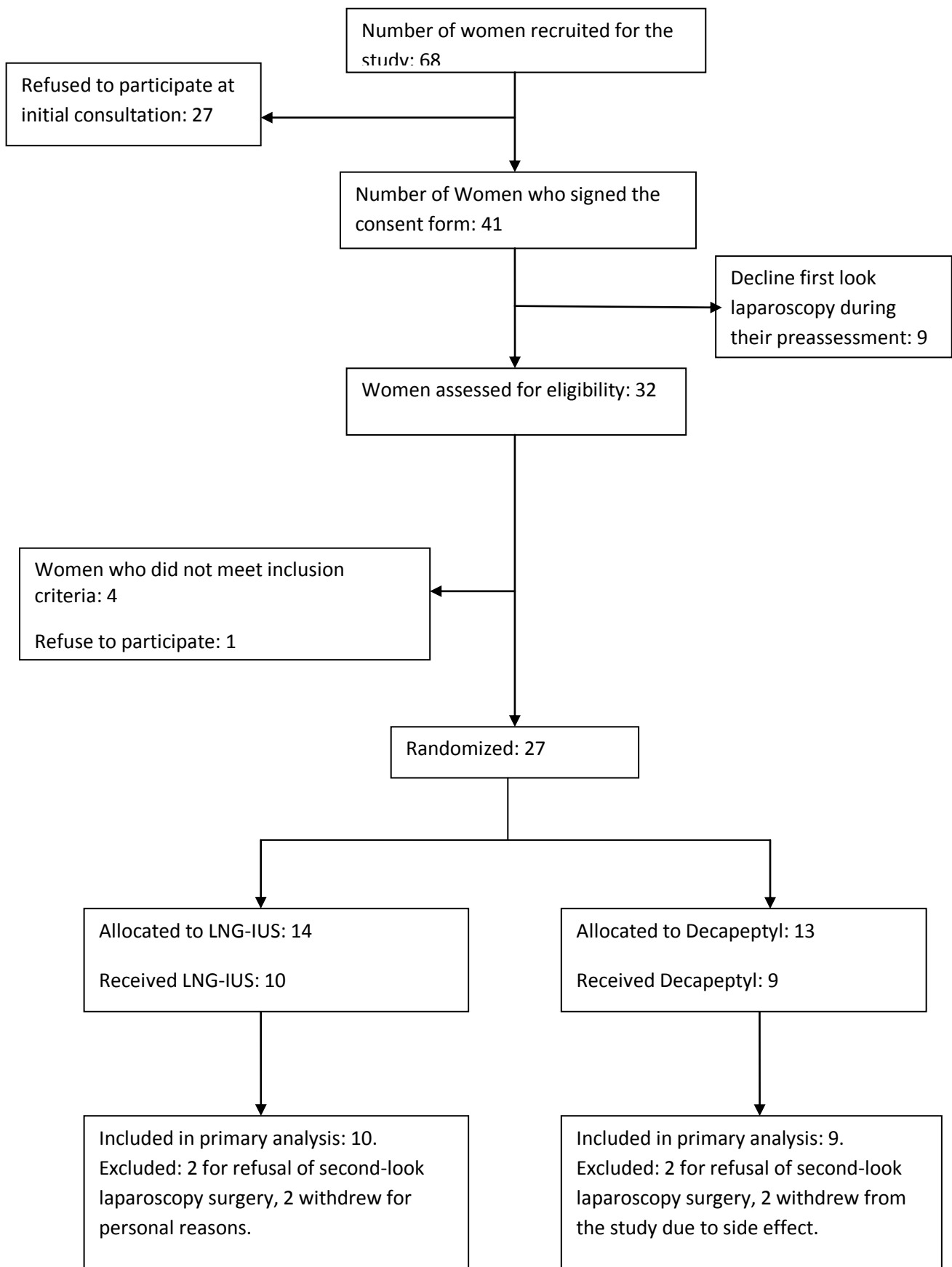


Fig. 2.4 Study flow chart from January 2008 to December 2009

Table 2.5. LNG-IUS (2000-2002): The effect of the LNG-IUS on eutopic and ectopic endometrium before and six months after treatment.

P-values				
	ER-α	Median	Ranges	*NPSS (95% CI)
*EUG	<0.0001	(98.32; 57.7)	(0.00-100.0; 0.00-97.83)	28.56-62.68
*EUS	<0.0001	(91.79; 63.67)	(0.00-99.08; 0.00-86.81)	25-74-55.14
*ECG	0.0004	(85.85; 0.00)	(0.00-100.00; 0.00-89.95)	25.40-63.95
*ECS	<0.0001	(60,47; 3.48)	(0.00-96.33; 0.00-93.07)	21.91-53.24
ER-β				
*EUG	<0.0001	(98.37; 46.35)	(0.00-100.00; 0.00-93.64)	30.22-64.86
*EUS	<0.0001	(91.38; 41.45)	(0.00-98.34; 0.00-88.25)	28.43-59.85
*ECG	<0.0001	(84.27; 0.00)	(0.00-100.00; 0.00-83.94)	20.28-59.56
*ECS	<0.0001	(73.22; 4.77)	(0.00-97.74; 0.00-55.71)	31.95-59-37
PR (A&B)				
*EUG	<0.0001	(99.55; 68.97)	(0.00-100.00; 0.00-96.70)	28.66-62.26
*EUS	<0.0001	(97.32; 57.48)	(0.00-100.00; 0.00-95.96)	29.92-60.32
*ECG	0.0023	(79.13; 0.00)	(0.00-100.00; 0.00-96.98)	9.12-53.31
*ECS	<0.0001	(69.25; 18.50)	(0.00-96.28; 0.00-89.28)	23.38-52.97

*EUG=Eutopic endometrium (gland), EUS=Eutpoic endometrium (stromal), ECG=Ectopic endometrium (gland), ECS=Ectopic endometrium (stromal), NPSS=Nonparametric statistical scores

2.9.1. Effect of the LNG-IUS on ER- α expression.

The mean level of ER- α expression (**Fig. 2.3A & 2.5 & Tab.2.5**) in the glands of the eutopic endometrium decreased from $92.7 \pm 3.6\%$ before treatment to $47.1 \pm 7.7\%$ after treatment with the LNG-IUS (95% CI for the difference 28.6 – 62.7, $P < 0.0001$) The mean stromal ER- α expression in the eutopic endometrium also reduced significantly from $85.7 \pm 3.7\%$ before to $45.3 \pm 6.3\%$ after treatment (95% CI for the difference 25.7 – 55.1; $P < 0.0001$). The mean level of ER- α expression in the glands of ectopic endometrium after treatment was lower than that in the glands before treatment. The level decreased 6 months after treatment with the LNG-IUS from $55.4 \pm 8.6\%$ before treatment to $10.7 \pm 4.3\%$ (95%CI for difference 25.4 – 64.0; $P 0.0004$). Similarly the mean stromal cell ER- α expression in the ectopic endometrium dropped from $54.0 \pm 6.2\%$ before treatment to $16.4 \pm 4.7\%$ after 6 months with the LNG-IUS (95% CI for difference 21.9 – 53.2; $P < 0.0001$).

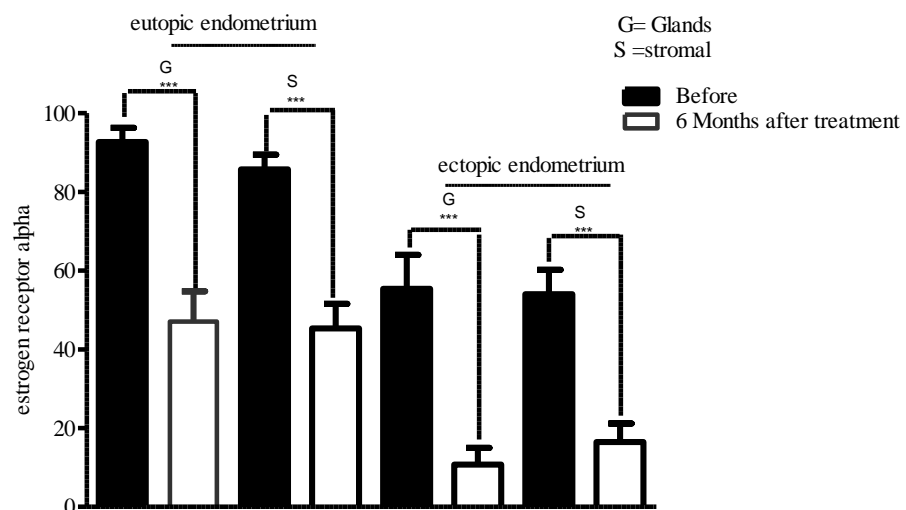


Fig. 2.5. Effect of the LNG-IUS on ER- α expression

2.9.2. Effect of the LNG-IUS on ER- β expression

The levels of ER- β expression (**Fig. 2.3B, 2.6 & Tab. 2.5**) in the glands of eutopic endometrium were significantly lower after 6 months of treatment with the LNG-IUS ($92.3 \pm 3.8\%$) versus $44.8 \pm 7.7\%$; (95% CI for the difference 30.2 – 64.9; $P < 0.0001$). The mean stromal ER- β expression in eutopic endometrium was also reduced significantly from $83.6 \pm 4.3\%$ before the LNG-IUS to $39.5 \pm 6.6\%$ six months after treatment (95% CI for the difference 28.4–59.9; $P < 0.0001$). In ectopic endometrium, the mean expression of glandular ER- β fell from $60.73 \pm 8.0\%$ before the LNG-IUS to $20.8 \pm 9.8\%$ 6 months after (95% CI for the difference 20.3 – 59.6; $P < 0.0001$). The mean stromal ER- β expression was also reduced significantly from $61.9 \pm 5.7\%$ to $16.3 \pm 3.8\%$ (95% CI for the difference 32.0 – 59.4; $P < 0.0001$).

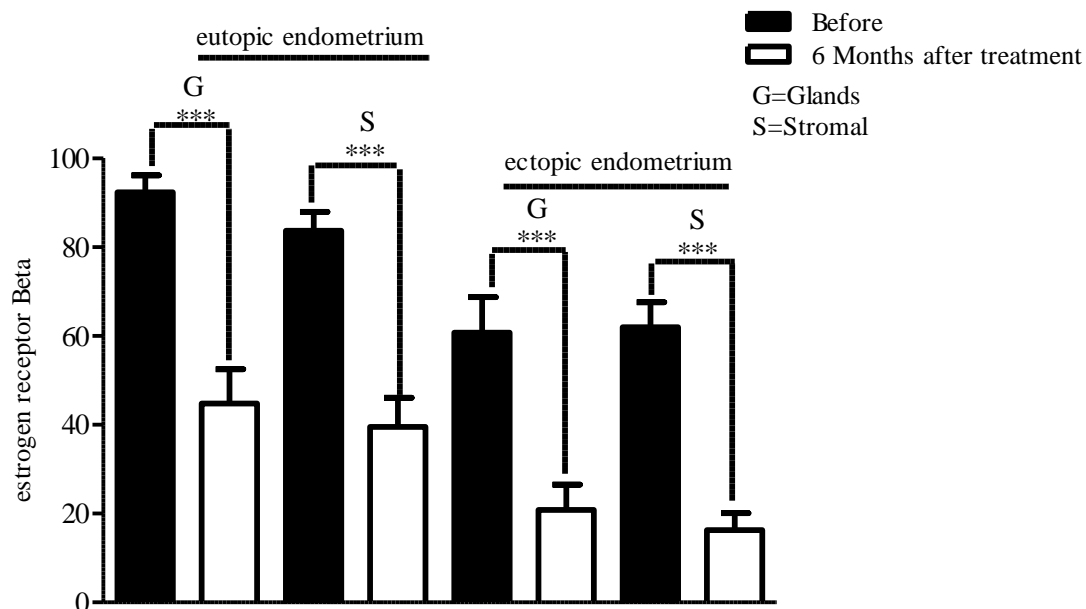


Fig. 2.6. Effect of the LNG-IUS on ER- β expression

2.9.3. Effect of the LNG-IUS on PR expression

The mean level of PR expression (**Fig. 2.3C, 2.7 & Tab. 2.5**) in the glands of the eutopic endometrium reduced significantly from $94.2 \pm 3.6\%$ before the LNG-IUS to $48.8 \pm 7.6\%$ six months after treatment (95% CI for the difference 28.7 – 62.3; $P < 0.0001$). The mean expression of stromal PR in eutopic endometrium was also significantly reduced from a baseline level of $91.5 \pm 3.6\%$ before treatment to $46.4 \pm 6.7\%$ six months after the use of the LNG-IUS (95% CI for the difference 29.9 – 60.3; $P < 0.0001$). In ectopic endometrium, the mean expression in the glands was also significantly reduced from $53.0 \pm 8.9\%$ before to $21.8 \pm 6.4\%$ after 6 months of treatment with the LNG-IUS (95% CI for the difference 9.1 – 53.3, $P = 0.0023$). The mean PR level of the stromal before treatment was $58.8 \pm 6.1\%$ and fell after 6 months on LNG-IUS to $20.6 \pm 4.2\%$ (95% CI for the difference 23.4 – 53.0, $P < 0.0001$).

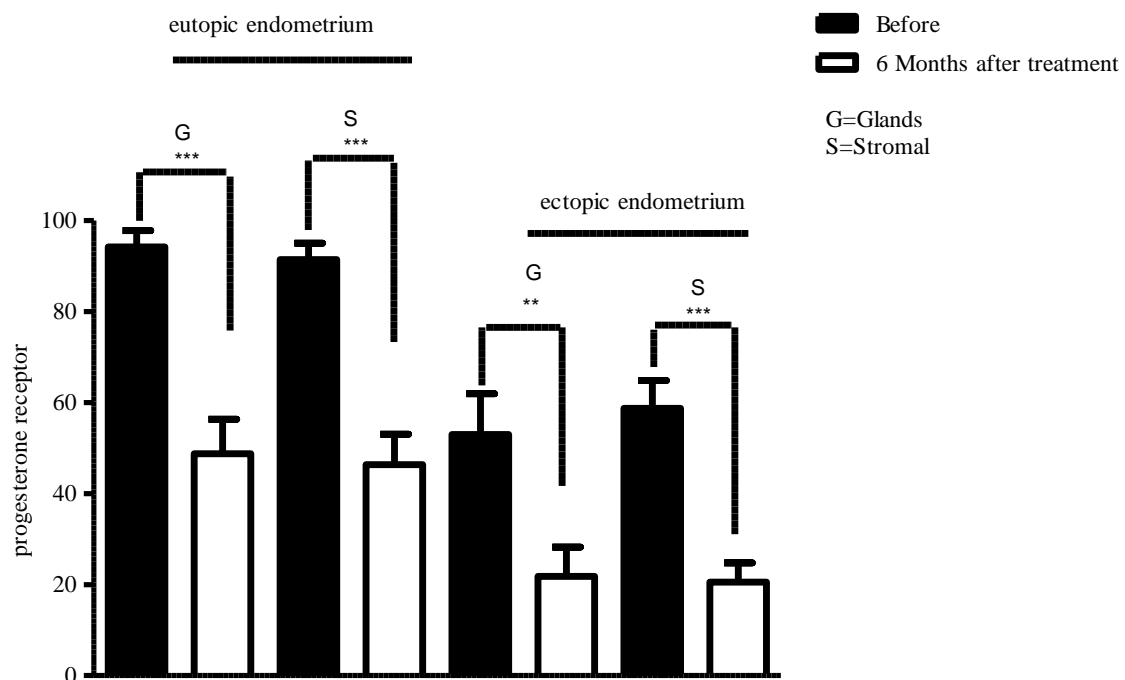


Fig.2.7. Effect of LNG-IUS on PR-(A&B) expression.

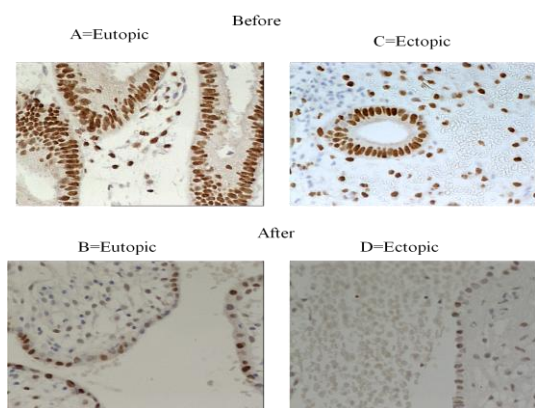


Fig. 2.8A

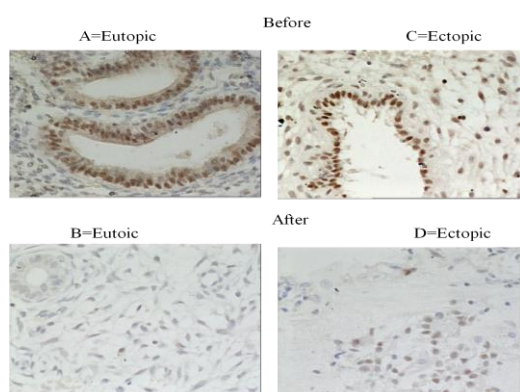


Fig 2.8B

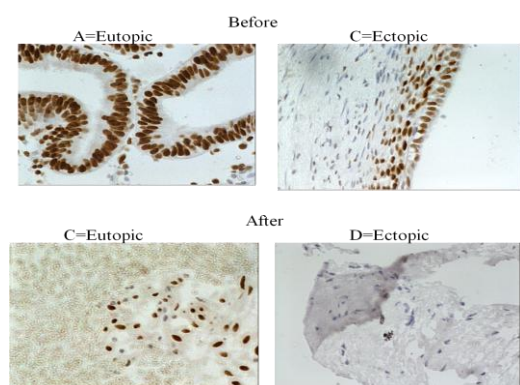


Fig 2.8C

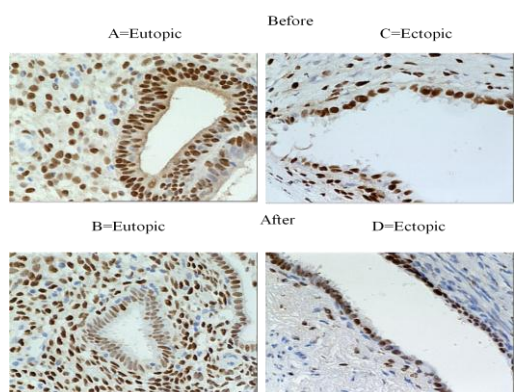


Fig. 2.8D

Fig. 2.8 Photomicrographs of ER- α (2.8A), ER- β (2.8B), PR-A (2.8C) and PR-B (2.8D) expression in eutopic and ectopic endometrium before and after 6 months treatment with the LNG-IUS.

Table 2.6. LNG-IUS (2008-2010): The effect of the LNG-IUS on eutopic and ectopic endometrium before and six months after treatment.

P-Values				
	ER-α	Median	Ranges	NPSS (95% CI)
*EUG	0.0035	(98.05; 46.80)	(0.00-100.0; 0.00-76.20)	1.01-75.07
*EUS	0.0156	(92.42; 42.66)	(0.00-96.69; 0.00-65.95)	4.45-74.01
*ECG	0.0362	(0.00; 0.00)	(0.00-85.26; 0.00-3.12)	4.82-63.42
*ECS	0.0078	(43.40; 0.00)	(0.00-84.73; 0.00-35.96)	7.30-56.90
ER-β				
*EUG	0.0078	(98.43; 44.30)	(0.00-100.00; 0.00-76.70)	1.36-76.27
*EUS	0.0156	(93.82; 42.00)	(0.00-98.48; 0.00-63.82)	3.94-74.03
*ECG	0.0313	(58.79; 0.00)	(0.00-100.00; 0.00-58.97)	0.23-69.60
*ECS	0.0313	(33.88; 11.31)	(0.00-83.20; 0.00-25.35)	0.97-50.00
PRA				
*EUG	0.0078	(97.89; 0.00)	(0.00-100.00; 0.00-87.86)	1.94-85.58
*EUS	0.0156	(94.30; 0.00)	(0.00-100.00; 0.00-78.35)	7.67-84.06
*ECG	0.0018	(82.14; 0.00)	(0.00-100.00; 0.00-48.96)	29.93-90.41
*ECS	0.0017	(83.99; 0.00)	(0.00-100.00; 0.00-47.30)	29.95-89.98
PRB				
*EUG	0.0220	(97.83; 67.43)	(0.00-100.00; 0.00-84.96)	0.88-49.77
*EUS	0.0156	(89.89; 44.99)	(0.00-99.45; 0.00-86.43)	2.16-52.29
*ECG	0.0594 ⁺	(25.05; 0.00)	(0.00-100.00; 0.00-56.08)	-8.64-62.63
*ECS	0.0078	(73.80; 22.30)	(0.00-96.28; 0.00-75.83)	7.49-70.36

*EUG=Eutopic endometrium (gland), EUS=Eutopic endometrium (stromal), ECG=Ectopic endometrium (gland), ECS=Ectopic endometrium (stromal) NPSS=Nonparametric statistical scores. ⁺Not statistical significance

2.9.4. The effect of the LNG-IUS on ER- α expression

The mean level of ER- α expression (**Fig. 2.8A, 2.9 & Tab. 2.6**) in the glands of the eutopic endometrium decreased from $75.8 \pm 14.4\%$ before treatment to $37.8 \pm 9.9\%$ after treatment with the LNG-IUS (95% CI for the difference 1.01 - 75.1, $P=0.0035$). The mean stromal ER- α expression in the eutopic endometrium also decreased significantly from $72.7 \pm 13.8\%$ before to $33.5 \pm 8.9\%$ after treatment (95% CI for the difference 4.5 - 74.0; $P=0.0156$). The mean level of ER- α expression in the glands of ectopic endometrium after treatment was lower than that in the glands before treatment. The level decreased 6 months after treatment with LNG-IUS from $34.7 \pm 13.8\%$ before treatment to $0.6 \pm 0.4\%$; (95% CI for difference 4.8 - 63.4; $P=0.0362$). Similarly the mean stromal cell ER- α expression in the ectopic endometrium dropped from $43.2 \pm 10.6\%$ before treatment to 11.1 ± 4.9 after 6 months with LNG-IUS; (95% CI for difference 7.3 - 56.9; $P=0.0078$).

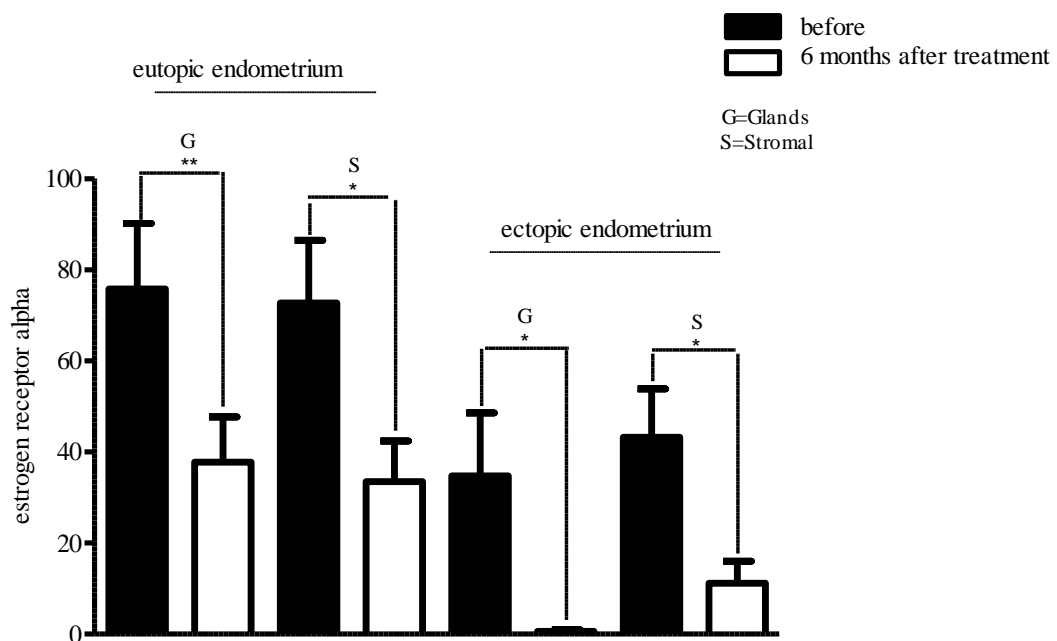


Fig. 2.9. Effect of the LNG-IUS on ER- α expression

2.9.5. The effect of the LNG-IUS on ER- β expression

The levels of ER- β expression (**Fig. 2.8B, 2.10 & Tab. 2.6**) in the glands of eutopic endometrium were significantly lower ($37.5 \pm 10.5\%$ versus $76.8 \pm 14.5\%$) after 6 months of treatment with the LNG-IUS (95% CI for the difference 1.4 - 77.3; $P=0.0078$). The mean stromal ER- β expression in eutopic endometrium also decreased significantly from $73.0 \pm 13.9\%$ before the LNG-IUS to $34.0 \pm 9.0\%$ six months after treatment; (95% CI for the difference 3.9 - 74.0; $P=0.0156$). In ectopic endometrium, the mean expression of glandular ER- β fell from $44.9 \pm 14.8\%$ before LNG-IUS to $10.0 \pm 7.0\%$ six months after; (95% CI for the difference 0.2 - 69.6; $P=0.0313$). The mean stromal ER- β expression also reduced significantly from $34.8 \pm 11.1\%$ to $9.4 \pm 3.2\%$; (95% CI for the difference 1.0 - 50.0; $P=0.0313$).

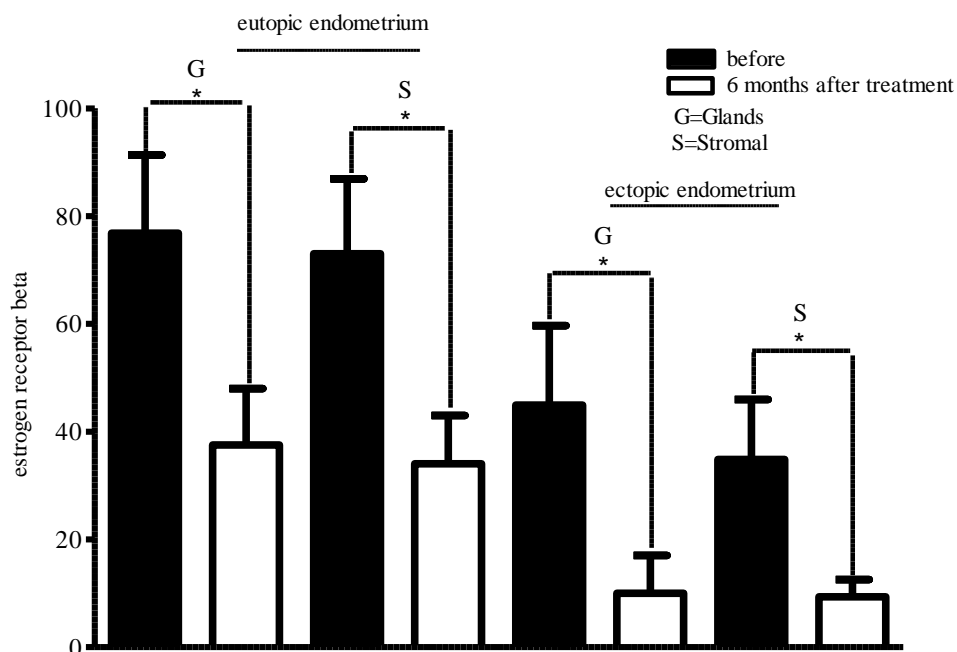


Fig. 2.10. Effect of the LNG-IUS on ER- β expression

2.9. 6. The effect of the LNG-IUS on PR-A expression

The mean level of PR-A expression (**Fig. 2.8C, 2.11 & Tab. 2.6**) in the glands of the eutopic endometrium decreased significantly from $75.7 \pm 14.4\%$ before treatment with the LNG-IUS to $31.9 \pm 13.5\%$ six months after treatment (95% CI for the difference 1.9 - 85.6; $P=0.0078$). The mean expression of stromal PR-A in eutopic endometrium also decreased from a baseline level of $72.3 \pm 13.9\%$ before treatment to $26.5 \pm 11.5\%$ six months after treatment with the LNG-IUS; (95% CI for the difference 7.7 - 84.1; $P=0.0156$). In ectopic endometrium, the mean expression in the glands also decreased from $65.6 \pm 13.8\%$ before to $5.4 \pm 5.4\%$ after six months of treatment with the LNG-IUS; (95% CI for the difference 29.9 - 90.4; $P=0.0018$). The mean PR-A level of the stromal before treatment was $67.91 \pm 13.15\%$ and fell after six months on the LNG-IUS to $7.9 \pm 5.6\%$; (95% CI for the difference 30.0 - 90.0; $P=0.0017$).

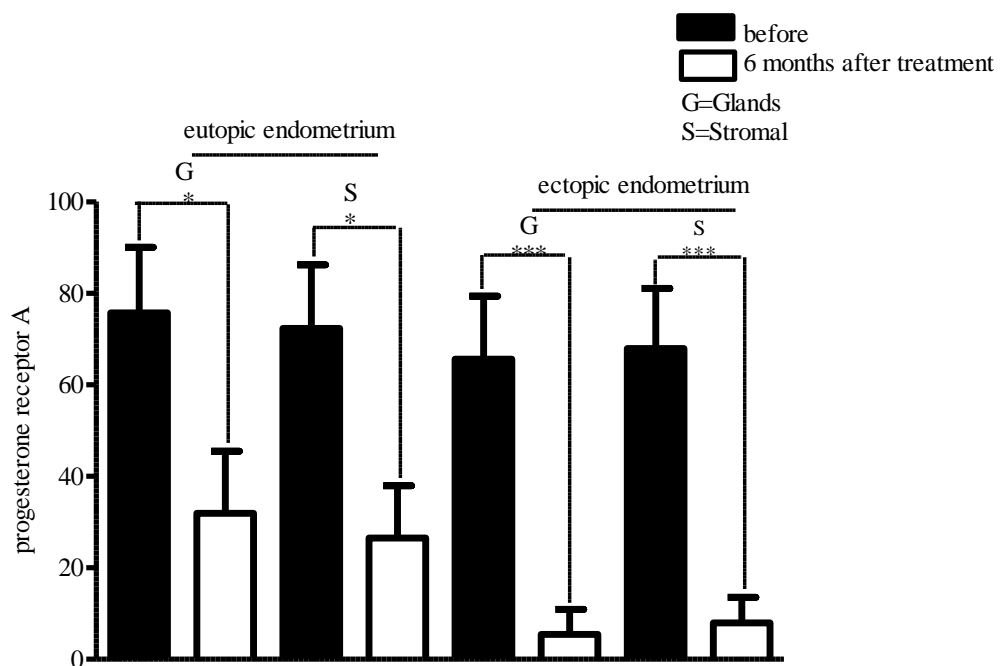


Fig.2 .11. Effect of LNG-IUS on PR-A expression

2.9.7. The effect of the LNG-IUS on PR-B expression

The mean level of PR-B expression (**Fig. 2.8D, 2.12 & Tab. 2.6**) in the glands of the eutopic endometrium decreased significantly from $75.2 \pm 14.3\%$ before to $25.3 \pm 19.3\%$ six months after treatment with LNG-IUS (95% CI for the difference 0.88 - 49.8; $P=0.0220$). The mean expression of stromal PR-B in eutopic endometrium also decreased significantly from a baseline level of $71.4 \pm 13.6\%$ before treatment to $27.2 \pm 18.6\%$ six months after treatment with LNG-IUS (95% CI for the difference 2.2-52.3; $P=0.0156$). The mean level of PR-B expression in the stromal of the ectopic endometrium decreased from $60.9 \pm 12.0\%$ to $22.0 \pm 8.8\%$ six months after treatment (95% CI for the difference 7.5 - 70.4; $P=0.0078$). There was no significant difference in the mean level of PR-B expression in the glandular compartment of ectopic endometrium before and after treatment with the LNG-IUS.

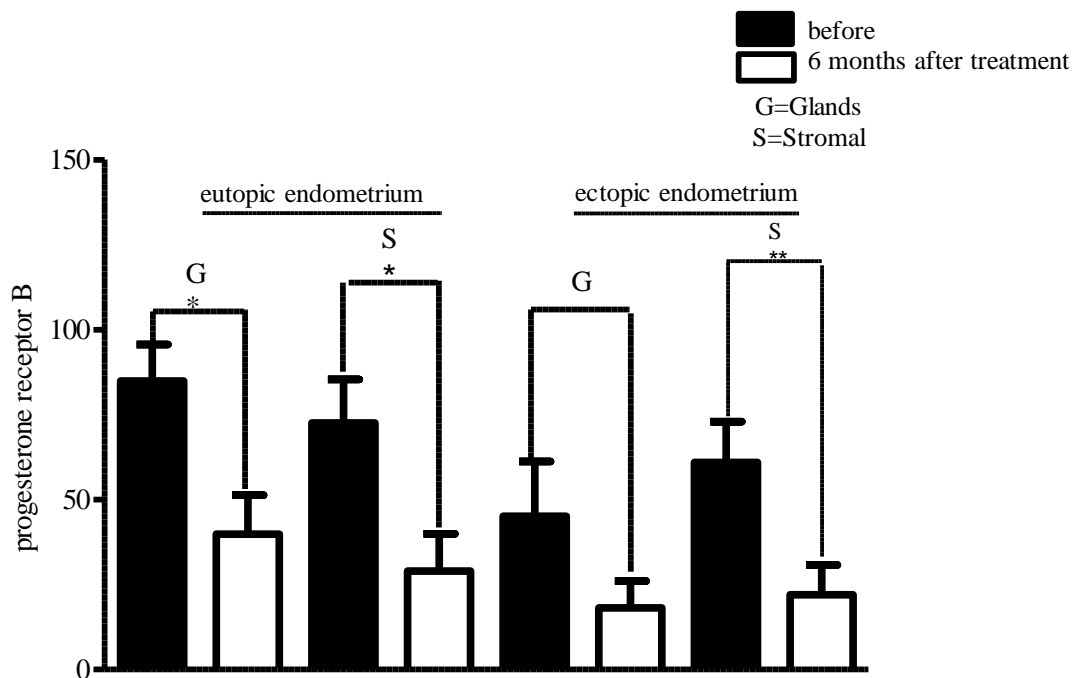


Fig. 2.12. Effect of LNG-IUS on PR-B expression

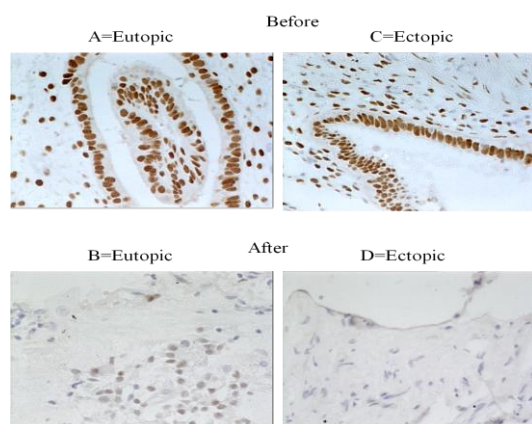


Fig. 2.13A

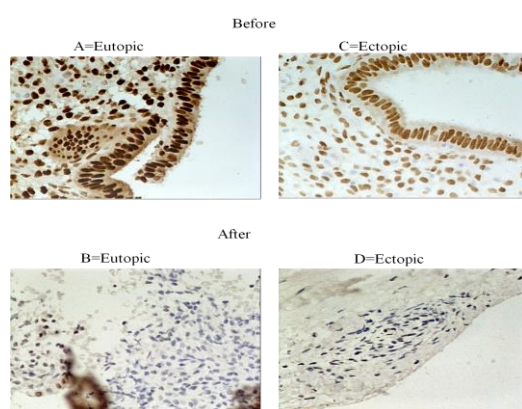


Fig 2.13B

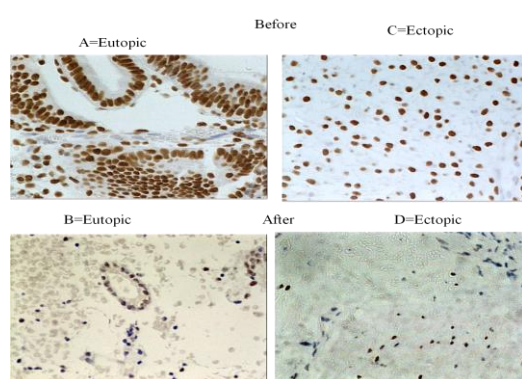


Fig.2.13C

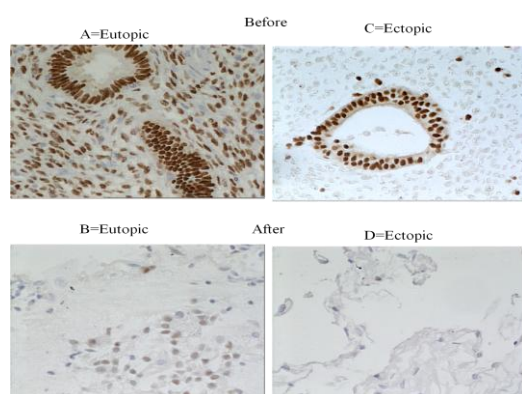


Fig.2.13D

Fig. 2.13 Photomicrographs of ER- α (2.13A), ER- β (2.13B), PR-A (2.13C) and PR-B (2.13D) expression in eutopic and ectopic endometrium before and after 6 months treatment with the GnRH agonist (decapeptyl™).

Table 2.7. GnRH agonist (decapeptyl™) [2008-2010]: The effect of the GnRH agonist on eutopic and ectopic endometrium before and six months after treatment.

P-Values				
	ER-α	Median	Ranges	NPSS (95% CI)
*EUG	0.0020	(97.83; 23.59)	(0.00-99.55; 0.00-73.78)	28.60-87.39
*EUS	0.0007	(92.52; 35.79)	(0.00-94.38; 0.00-68.08)	28.38-74.23
*ECG	0.0115	(15.52; 0.00)	(0.00-99.49; 0.00-37.78)	6.17-64.82
*ECS	0.0012	(66.05; 0.00)	(0.00-97.31; 0.00-65.96)	20.03-66.84
ER-β				
*EUG	0.0020	(98.27; 78.50)	(0.00-99.55; 0.00-91.82)	6.45-67.90
*EUS	0.0063	(86.96; 62.99)	(0.00-94.14; 0.00-71.62)	14.32-65.14
*ECG	0.0039	(75.91; 0.00)	(0.00-100.00; 0.00-13.56)	31.41-84.13
*ECS	0.0020	(73.96; 3.18)	(0.00-86.86; 0.00-73.42)	21.42-64.98
PRA				
*EUG	0.0039	(98.25; 25.00)	(0.00-100.00; 0.00-90.80)	11.71-83.25
*EUS	0.0046	(95.81; 13.51)	(0.00-99.39; 0.00-86.43)	19.33-78.18
*ECG	0.0195	(72.16; 0.00)	(0.00-100.00; 0.00-75.19)	1.64-77.38
*ECS	0.0012	(67.68; 0.00)	(0.00-96.28; 0.00-51.29)	22.73-66.19
PRB				
*EUG	0.0091	(98.96; 34.04)	(0.00-100.00; 0.00-97.13)	12.67-79.69
*EUS	0.0020	(97.07; 61.13)	(0.00-99.50; 0.00-89.72)	11.61-71.25
*ECG	0.0178	(82.30; 0.00)	(0.00-98.13; 0.00-69.23)	6.37-79.45
*ECS	0.0020	(79.38; 0.00)	(0.00-95.75; 0.00-36.82)	34.60-79.88

*EUG=Eutopic endometrium (gland), EUS=Eutopic endometrium (stromal), ECG=Ectopic endometrium (gland), ECS=Ectopic endometrium (stromal) NPSS=Nonparametric statistical scores. *Not statistical significance

2.9. 8. The effect of the GnRH-agonist (decapeptyl™) on ER- α expression

The mean level of ER- α expression (**Fig. 13A, 14 & Tab.7**) in the glands of the eutopic endometrium decreased from $87.4 \pm 9.8\%$ before treatment to $29.4 \pm 10.0\%$ six months after treatment with the decapeptyl™ (95% CI for the difference 28.6 - 87.4; $P=0.0020$). The mean stromal ER- α expression in the eutopic endometrium also significantly decreased from $81.5 \pm 9.3\%$ before to $30.2 \pm 9.0\%$ six months after treatment with the decapeptyl™ (95% CI for the difference 24.2 - 78.4; $P=0.0007$). The mean level of ER- α expression in the glands of ectopic endometrium decreased from $39.3 \pm 14.3\%$ before treatment to $3.8 \pm 3.8\%$; after treatment with the decapeptyl™ (95% CI for difference 6.2 - 64.8; $P=0.0115$). Similarly the mean stromal cell ER- α expression in the ectopic endometrium decreased from $58.5 \pm 10.1\%$ before treatment to $15.0 \pm 7.3\%$ after six months treatment with the decapeptyl™ (95% CI for difference 20.0 - 66.8; $P=0.0012$).

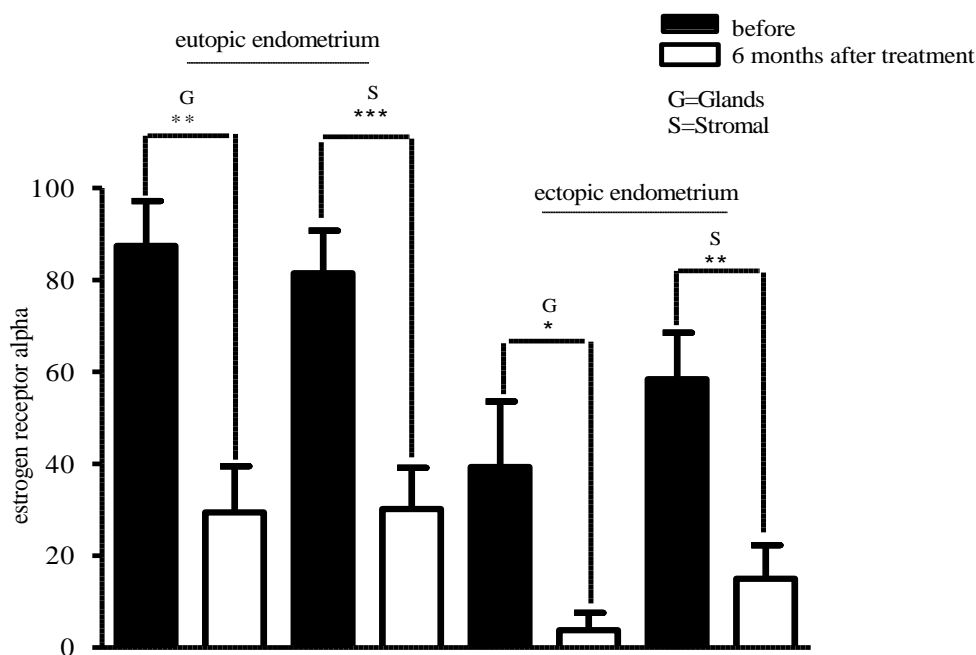


Fig. 2.14 Effect of the GnRH-agonist (decapeptyl™) on ER- α expression

2.9.9. The effect of the GnRH-agonist (decapetylTM) on ER-β expression

The levels of ER-β expression (**Fig. 13B, 15 & Tab.7**) in the glands of eutopic endometrium decreased from $50.6 \pm 13.9\%$ before treatment to $8.8 \pm 9.8\%$ after six months of treatment with the decapetylTM (95% CI for the difference 6.5 - 67.9; $P=0.0020$). The mean stromal ER-β expression in eutopic endometrium decreased significantly from $79.4 \pm 9.0\%$ before to $39.6 \pm 10.8\%$ six months after treatment with the decapetylTM (95% CI for the difference 14.3 - 65.1; $P=0.0063$). In ectopic endometrium, the mean expression of glandular ER-β decreased significantly from $59.1 \pm 12.5\%$ before to $1.4 \pm 1.4\%$ six months after treatment with the decapetylTM (95% CI for the difference 31.4 - 84.1; $P=0.0039$). The mean stromal ER-β expression also decreased significantly from $60.4 \pm 8.6\%$ to $17.3 \pm 8.3\%$ (95% CI for the difference 21.4 - 65.0; $P=0.0020$).

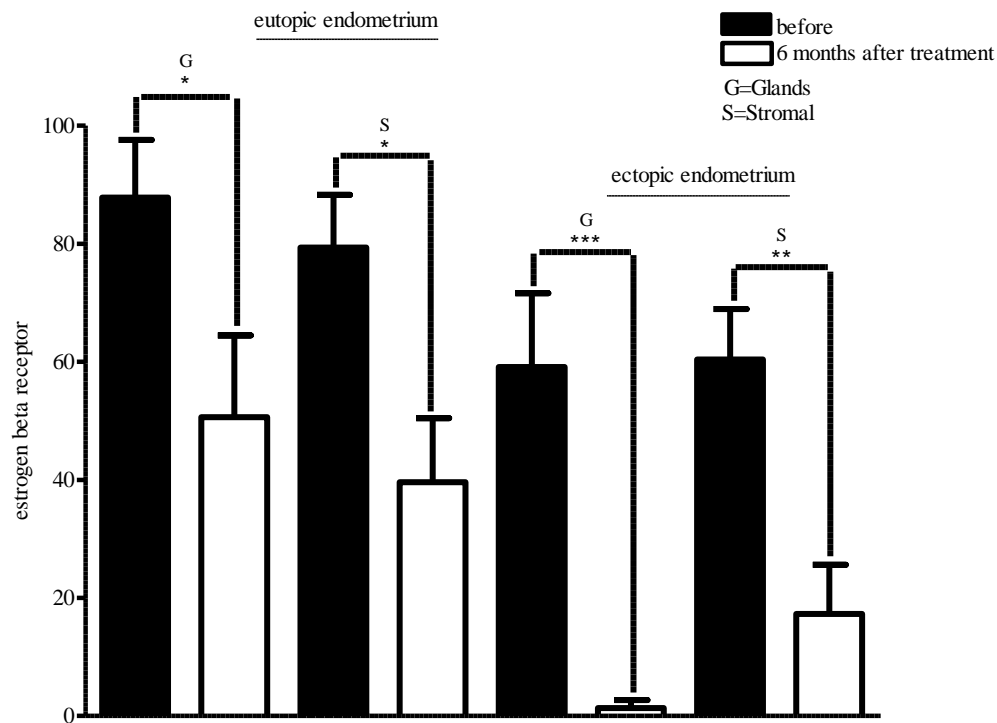


Fig. 2.15. Effect of GnRH-agonist (decapetylTM) on ER-β expression

2.9.10. The effect of the GnRH-agonist (decapeptyl™) on PR-A expression

The mean level of PR-A expression (**Fig. 13C, 16 & Tab.7**) in the glands of the eutopic endometrium decreased significantly from $87.78 \pm 9.80\%$ before treatment to $40.3 \pm 13.9\%$ six months after treatment with decapeptyl™ (95% CI for the difference 11.7 - 83.3; $P=0.0039$). The mean expression of stromal PR-A in eutopic endometrium also decreased significantly from a baseline level of $84.3 \pm 9.5\%$ before treatment to $35.5 \pm 13.0\%$ six months after the treatment with decapeptyl™ (95% CI for the difference 19.3 - 78.2; $P=0.0046$). In ectopic endometrium, the mean expression in the glands also fell significantly from $53.1 \pm 12.8\%$ before treatment to $13.6 \pm 9.1\%$ after six months of treatment with decapeptyl™ (95% CI for the difference 1.6 - 77.4; $P=0.0195$). The mean PR-A level of the stromal before treatment was $55.8 \pm 10.9\%$ and decreased significantly to $11.3 \pm 5.6\%$ after six months treatment with decapeptyl™ (95% CI for the difference 22.7 - 66.2; $P=0.0012$).

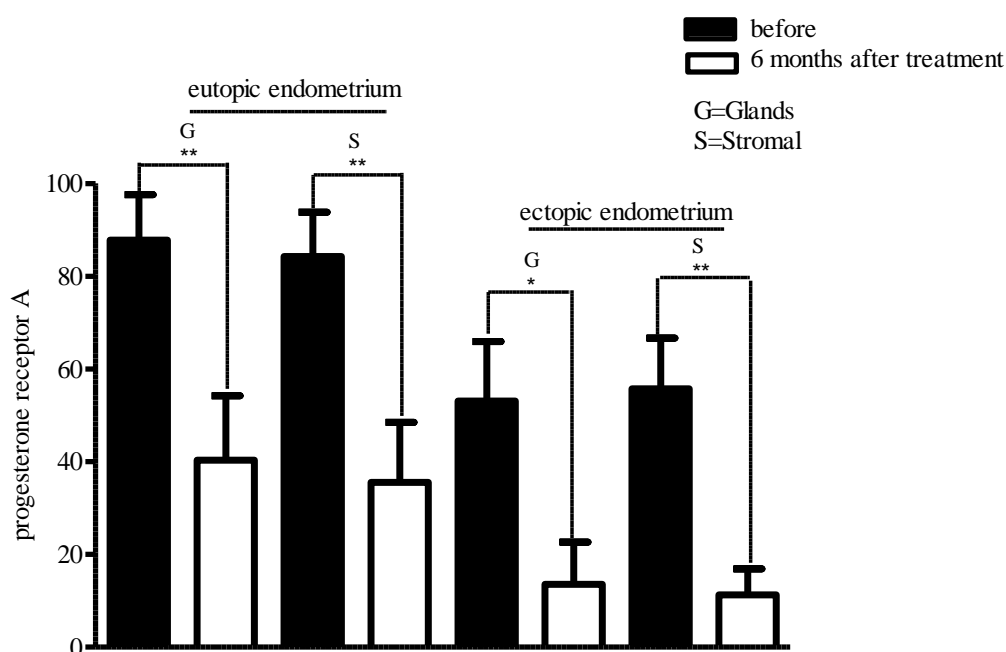


Fig. 2.16. Effect of GnRH-agonist (decapeptyl™) on PR-A expression

2.9.11. The effect of the GnRH-agonist (decapetylTM) on PR-B expression

The mean level of PR-B expression (**Fig. 13D, 17 & Tab.7**) in the glands of the eutopic endometrium decreased significantly from $89.1 \pm 9.9\%$ before to $42.9 \pm 14.5\%$ six months after treatment with decapetylTM (95% CI for the difference 12.7 – 79.7; $P=0.0091$). The mean expression of stromal PR-B in eutopic endometrium also decreased significantly from a baseline level of $87.3 \pm 9.7\%$ before treatment to $45.8 \pm 13.1\%$ six months after treatment with decapetylTM (95% CI for the difference 11.6-71.3; $P=0.0020$). In ectopic endometrium, the mean expression in the glands also decreased significantly from $55.4 \pm 15.2\%$ before to $12.5 \pm 8.4\%$ after six months of treatment with the decapetylTM (95% CI for the difference 6.4-79.5; $P=0.0178$). The mean PR-B level in the stromal decreased significantly from $70.0 \pm 10.6\%$ before to $12.8 \pm 5.3\%$ after six months of treatment with decapetylTM (95% CI for the difference 34.6 - 79.9; $P=0.0020$).

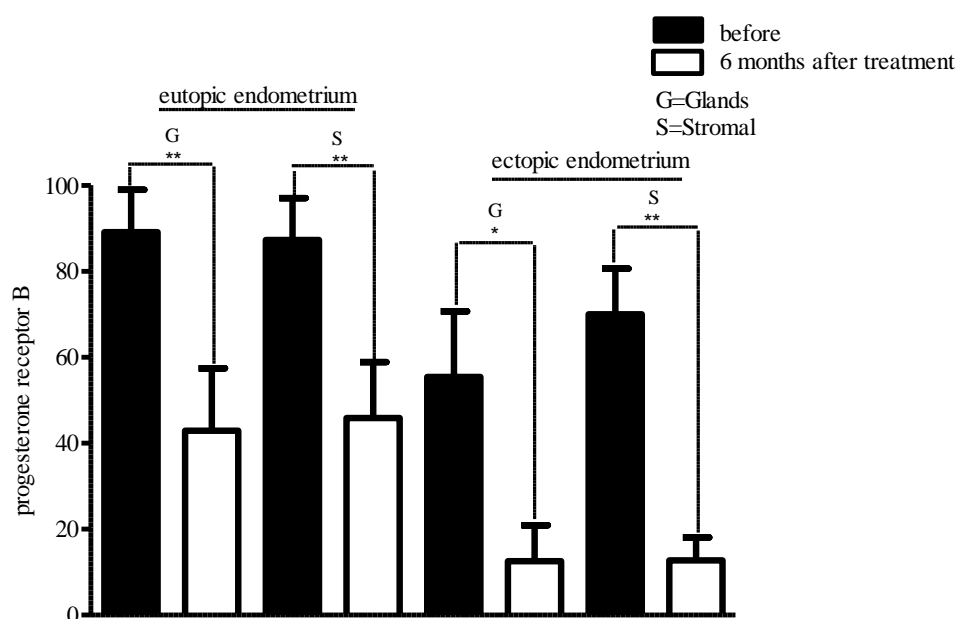


Fig 2.17. Effect of GnRH-agonist (decapetyl) on PR-B expression

2.10. Discussion

The LNG-IUS, originally designed for contraception, has now been shown to significantly improve the symptoms of minimal to moderate endometriosis (Luukkainen et al, 1990; Lockhat et al, 2005a; Petta et al, 2005). In the first study of this chapter the hypothesis that the mechanism of action of the LNG-IUS was through the action of the progestogen, LNG, on ER and PR was tested by immunohistochemistry studies of eutopic and ectopic tissues obtained from women with endometriosis before and after treatment. There was down-regulation of ER- α , ER- β and PR in the glandular and stromal compartments of eutopic endometrium after 6 months of treatment with the LNG- IUS, which was consistent with results of previous studies (Nilsson et al, 1978; Silverberg et al, 1986; Jones & Critchley, 2000). In the ectopic endometrium there was down-regulation of the glandular and stromal compartments of ER- α , ER- β , and a mixture of PR (A&B) after 6 months of treatment with the LNG-IUS.

PR has at least two isoforms; PR-A and PR-B which are differentially regulated in eutopic endometrium across the normal menstrual cycle (Clarke et al, 1987; Mote et al, 2000). PR-B appears to be preferentially down-regulated in eutopic endometrium in response to progesterone and also significantly lower in ectopic endometrium compared to eutopic endometrium (Attia et al, 2000; Bulun et al, 2006). Mote et al (2000) demonstrated that an immunoreactive form of PR-B is found primarily in the eutopic endometrial epithelial cells, whereas stromal cells contained predominantly PR-A. A mixture of PR (A & B) antibodies was used in this study and demonstrated a significant down-regulation of receptor

expression in both the glandular and stromal ectopic endometrium. Critchley et al. (1998) used separate PR-A and PR-B antibodies and demonstrated a marked down-regulation of PR (B) in both the glands and stroma following treatment of eutopic endometrium with the LNG-IUS.

Using RNase protection assay Attia et al. (2000) demonstrated that while PR-A and PR-B transcripts were detectable in eutopic endometrium, only PR-A transcripts were detectable in endometriotic tissues, suggesting the absence of PR-B protein in endometriotic tissue. They concluded that progesterone resistance in the endometriotic tissue may be accounted for by the overall reduction in PRs, and the absence of PR-B (Attia et al, 2000). This supports the result here in that only the PR-A and the stromal compartment of PR-B was down-regulated in ectopic endometrium but not in the glandular compartment after treatment with LNG-IUS.

Although it has been well documented that ER- α , ER- β and PR (A&B) expression in normal endometrium changes with the phase of the menstrual cycle (Bouchard et al, 1991; Snijders et al, 1992; Coppens et al, 1993), most studies have failed to detect any changes in receptor expression at different phases of the menstrual cycle in endometriotic lesions (Lessey et al, 1989; Bergqvist, 1991; Prentice et al, 1992; Bergqvist et al, 1993). Whether local environmental factors, such as the site of the lesions, inflammatory cytokines, or depth and degree of fibrosis of the lesions, may determine the amount of steroid hormone reaching the endometriotic foci and thereby affecting receptor expression as demonstrated by Howell et al. (1994) is still unknown.

The data here show that there are significant differences in ER- α , ER- β and PR (A&B) expression in the glandular and stromal cells of eutopic and ectopic endometrium of women with mild to moderate endometriosis 6 months after treatment with the LNG-IUS. All of these suggest differential responses by these tissues to LNG.

The second study compared the efficacy of the LNG-IUS with the gold standard medical treatment GnRH agonist (decapetylTM) used for the symptomatic treatment of minimal to moderate endometriosis. While ER- α , ER- β , PR-A and PR-B in the ectopic endometrium were down-regulated by the GnRH agonist, only ER- α , ER- β , PR-A and the stromal compartment of PR-B were down-regulated in the ectopic endometrium when the LNG-IUS was used. There was no significant difference of PR-B in the glandular compartment of the ectopic endometrium after 6 months of treatment with the LNG-IUS. This is the first time the effect of both the LNG-IUS and the GnRH agonist has been reported on both the glands and stromal of eutopic and ectopic endometrium in patients with minimal to moderate endometriosis.

The precise mechanism by which symptom improvement occurs in women with endometriosis treated with the LNG-IUS has not been thoroughly investigated. Recently, however, Gomes et al. (2009) investigating a potential mechanism, in a similar study but with different inclusion criteria (their patients had previous surgical treatment of endometriosis before going on either the LNG-IUS or the GnRH agonist and were also severe or stage IV disease) found similar changes. Gomes et al (2009) studied only ER- α and PR-A expression before and after treatment with the LNG-IUS and the gold standard

GnRH agonist (decapeptylTM). The effect of the LNG-IUS and the gold standard GnRH agonist (decapeptylTM) on ER- β and PR-B were not examined.

In conclusion, taken together, the data presented here support the following new observations i) a significant down-regulation of glandular and stromal ER- α , ER- β and PR (A&B), in ectopic endometrium in patients with endometriosis treated with the LNG-IUS for 6 months; ii) It suggest that the LNG-IUS improves symptoms in women with minimal to moderate endometriosis, via the suppression of ER- α , ER- β , PR (A&B), PR-A and the stromal compartment of PR-B expression. This is so as there is a clininal improvement in their symptoms as seen later in chapter 5.

Chapter III

The effect of the levonorgestrel-releasing intrauterine system (LNG-IUS) on mast cells numbers.

3.1. Introduction

Although the precise pathogenesis of endometriosis is unknown, it is believed that an inflammatory process is involved not only in its development and progression but also in the symptoms that sufferers experience. Studies demonstrating a characteristic autoantibody production (Lebovic et al, 2001; Nothnick, 2001) suggest that endometriosis might be an autoimmune disorder with immune system dysfunction. This is further supported by other studies demonstrating an increase in inflammatory cytokines especially interleukin-2 (IL-2), IL-6, IL-8, vascular endothelial growth factor (VEGF) and soluble intracellular adhesions molecule (sICAM) in the peritoneal fluid (PF) from women with endometriosis compared to controls (Kalu et al, 2007; Laschke et al, 2008; Kuroda et al, 2010). In addition mast cells have also been shown to be significantly increased in the peritoneal fluid (PF) (Sugamata et al, 2005) as well as the lesions (Anaf et al, 2006) in women with endometriosis.

Mast cells (MC) are multifunctional, tissue-dwelling cells that have traditionally been associated with allergic responses. Studies suggest that these cells are capable of regulating inflammation, host defence, and innate immunity (Frew, 1996; Marshall et al, 2003). Although they originate from marrow-derived progenitor cells and continue their maturation and differentiation in peripheral tissues, they undergo activation by antigen/allergen via the high-affinity receptor for immunoglobulin E, after which they express histamine, leukotrienes and prostanoids, as well as proteases and many cytokines and chemokines. These mediators may be pivotal to the genesis of inflammatory responses and play active roles in many diseases such as endometriosis by virtue of their location and mediator expressions. Progestogens which are effective in

the control of symptoms of endometriosis exert anti-inflammatory properties (Hickey & Salamonsen; 2008). LNG-IUS which delivers the progestogen levonorgestrel (LNG) into the uterine cavity at a steady rate of 20 µg/day has been shown in several pilot studies to be effective in symptom control in women with minimal to moderate endometriosis (Lockhat et al, 2004, 2005a & 2005b; Petta et al, 2005). One possible mechanism by which the LNG-IUS may be effective in symptom control in endometriosis is alteration of the inflammatory environment of the peritoneal cavity via actions on mast cells.

Since mast cells increased in number in the peritoneal fluid in women with endometriosis and play a potential role in the propagation of inflammation and pain, it is hypothesized that the LNG-IUS modulates the pain in women with endometriosis via its action on mast cells. In this chapter a study was undertaken to investigate changes in mast cells in women with endometriosis after 6 months treatment with LNG-IUS.

3.2. Materials and methods

3.2.1. Subjects

Thirty four women with symptoms suggestive of endometriosis attending the University Hospitals of Leicester NHS Trust were recruited by Ms Farhana Lockhat (clinical research fellow between 2000-2002) between April 2000 and March 2002, with 29 (85%) completing the study; five discontinued, for personal reasons (one), side effects of worsening of acne (one) and lower abdominal/pelvic pain (three). This was approved by the Leicestershire,

Northamptonshire and Rutland (LNR) Research Ethics Committee. All the volunteers who met the inclusion criteria gave a signed informed consent prior to entering into the study. Biopsies of eutopic and ectopic endometrium and normal peritoneum were collected at laparoscopy to diagnose endometriosis and at a second look laparoscopy 6 months after treatment. Paraffin embedded tissue blocks were then fixed in 10% neutral-buffered formalin using routine methods. Six tissue blocks in total were identified from each woman; three taken before and three 6 months after insertion of LNG-IUS. The phases of the menstrual cycle were classified as early, mid and late proliferative and early, mid and late secretory, at the entry into the study defined from the date of the last menstrual period. The eutopic samples taken before treatment were dated histologically according to the criteria of Noyes and Haman (1953).

3.2.2. Technique used

Sections from the tissues were cut at 4 μ m thickness, mounted on silane-coated slides and dried thoroughly before immunohistochemistry (IHC) and toluidine blue (TB) (Sigma-Aldrich, United Kingdom) staining. The IHC sections were incubate as detailed with the primary antibodies used in chapter two which compared the effect of the LNG-IUS and the GnRH agonist. Tissue sections were stained with toluidine blue (TB) (Sigma Aldrich, United Kingdom) to allow the identification of mast cells before mounting in Permount mounting medium (DPX, Fluka, Buchs, Switzerland). This stains mast cells red-purple (metachromatic staining) and the background blue (orthochromatic staining).

3.3. Histomorphometric analysis.

Images were captured on an Axioplan compound microscopic equipped with Sony analogue colour video camera (Model DXC- 151P, Sony; Japan) and Axiovision version 4 image capture and analysed using Zeiss software (Zeiss, Milton Keynes, Bucks, United Kingdom) at 40x magnification. The localization of the steroid receptors and the mast cells (identified by their primary monoclonal antibodies and toluidine blue (TB) staining, respectively were determined by counting the steroid nuclei and mast cells from ten random views taken from each slide using a 10x25 graticule. Only cells with visible steroid nuclei and mast cells were included in the count. Mast cells in the glandular and stromal compartments were analysed separately. Positive steroid nuclei were identified by the presence of a brown nuclear stain for ER- α , ER- β , PR-A or PR-B. The total numbers of cells (steroid receptor positive and mast cells) in the ten random views per slide were counted. The density of mast cells in these views (i.e. proportion of cells that were identified as mast cells based on TB staining) was expressed as a percentage (mast cells/total number of cells x 100).

3.4 Statistical analysis

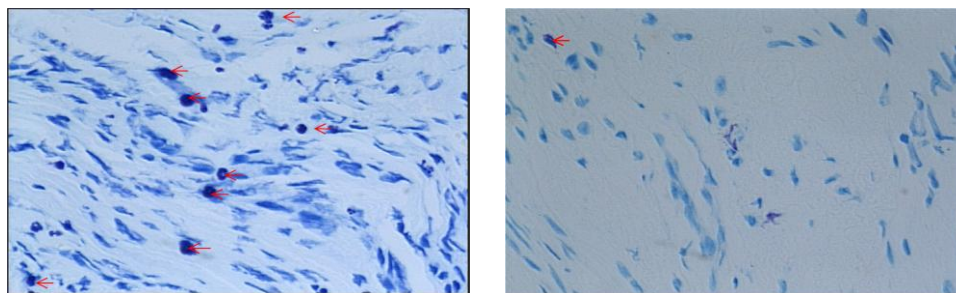
The results are expressed as mean and standard errors of the mean (SEM). Normality of the data was determined using the Wilks–Shapiro test and because the data were not normally distributed, non-parametric statistical analysis (Wilcoxon matched-pairs signed rank test) was performed to determine differences in the density of positive mast cells expression in eutopic and ectopic endometrium as well as the normal pelvic peritoneum before and 6

months after treatment with the LNG-IUS. Statistical analyses were performed with the aid of Graphpad Prism (Version 5.0) software (www.graphpad.com). Differences were considered to be significant with $P < 0.05$.

3.5. Results

Twenty-eight women with minimal to moderate endometriosis diagnosed at their first diagnostic laparoscopy were studied. Their mean (\pm SEM) age was 31 (± 7.2) (range 18–42) years. The ectopic tissues were confirmed histologically by staining positive for both estrogen and progesterone receptors and visualization of gland-like regions. No steroid receptors were found in mast cells after TB staining was preceded by IHC for ER and PR in eutopic and ectopic endometrium before and after treatment. The data on ER and PR receptor changes during treatment were presented in chapter 2.

Mast cells were present mainly in the stromal and not in the glands of ectopic and eutopic endometrium. The number of mast cells expressed in the ectopic endometrium decreased from 5.9 ± 1.6 before treatment to 1.9 ± 0.9 after treatment [95% CI for the difference 0.4 – 7.6, $P = 0.0059$] (**Fig. 3.1A**). The number of mast cells expressed in the eutopic endometrium also reduced significantly from 1.1 ± 0.5 before treatment to 0.2 ± 0.2 after [95% CI for the difference $-0.02 - 1.9$; $P = 0.0324$] (**Fig. 3.1B**). This decrease was associated with a significant decrease in symptoms and those with a greater improvement in symptoms had the greatest decrease in mast cell numbers. No significant difference in the number of MCs was observed in normal peritoneal tissues of these women (**Fig. 3.1C**).



Before

After

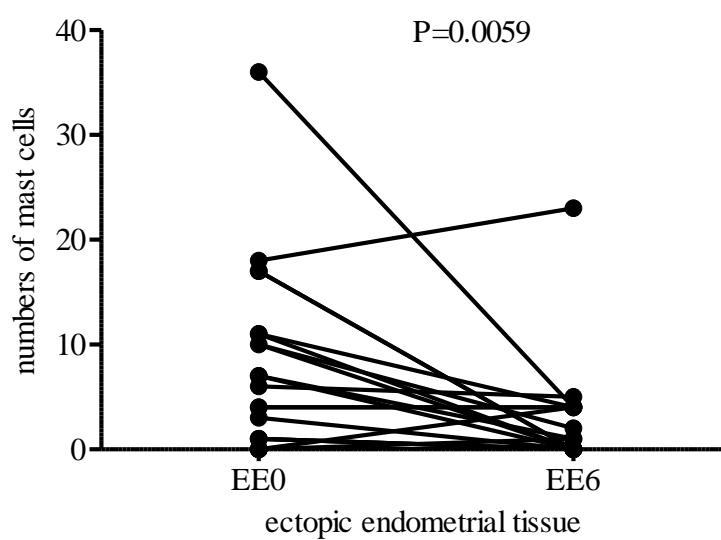
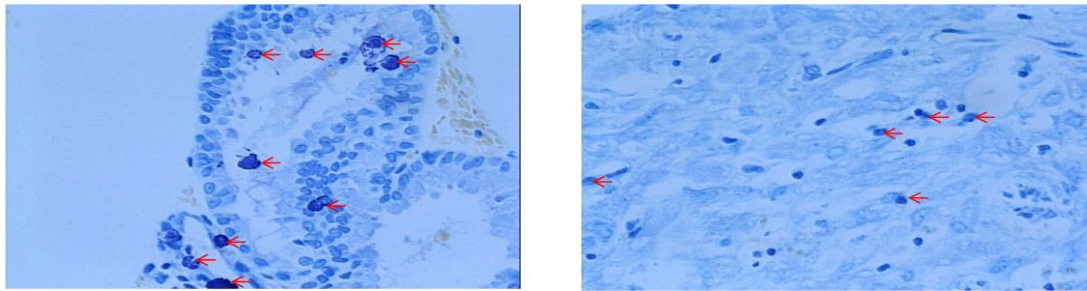


Fig 3.1A. Effect of the LNG-IUS on mast cells in ectopic endometrium on the symptomatic treatment of minimal to moderate endometriosis



Before

After

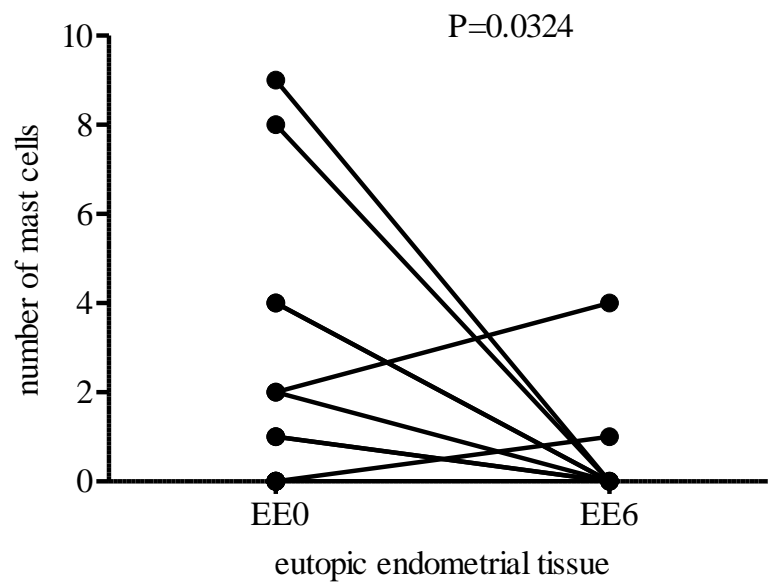


Fig. 3.1B. Effect of the LNG-IUS on mast cells in eutopic endometrium on the symptomatic treatment of minimal to moderate endometriosis.

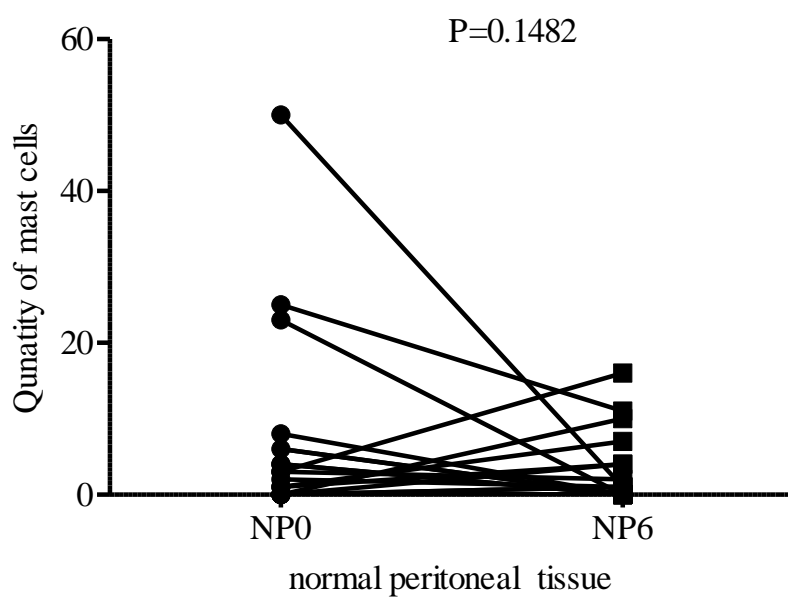
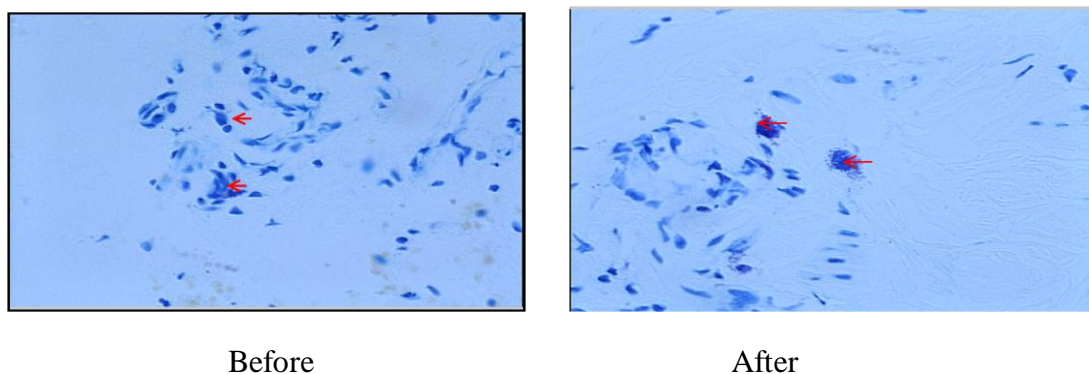


Fig. 3.1C. Effect of the LNG-IUS on mast cells in normal peritoneum on the symptomatic treatment of minimal to moderate endometriosis.

3.6. Discussion

Endometriosis remains an enigmatic disorder in that the cause, the natural history, and the precise mechanisms by which it causes pain are not completely understood. The pain symptoms most commonly attributed to endometriosis are dysmenorrhea, dyspareunia, and chronic pelvic pain which may be due to nociceptive, inflammatory, or neuropathic mechanisms.

In the last few years, evidence has emerged pointing to a possible role of immunologic factors and the lack of adequate immune surveillance in the pathogenesis of endometriosis (Matsuzaki et al, 1998; Anaf et al, 2006). The involvement of an allergic-type inflammatory process with a pivotal role played by the mast cells has been suggested. Kempuraj et al. (2004) observed increased numbers of highly activated mast cells in the stromal of peritoneal endometriotic lesions as compared to eutopic endometrium. These mast cells were also found in deep infiltrating endometriosis near nerve fibers, leading to the suggestion they might play a pivotal role in endometriosis-related pain and inflammation (Anaf et al, 2006). This thus led to the suggestion that mast cells maybe affected by the treatment of minimal to moderate endometriosis with the LNG-IUS.

In this study it was shown that the number of mast cells significantly decreased in eutopic ($P = 0.0358$) and ectopic ($P = 0.0220$) endometrium after six months of treatment with the LNG-IUS. No significant differences in mast cell numbers were observed in the normal peritoneum after 6 months treatment with the LNG-IUS. There were no estrogen (ER) and

progesterone (PR) receptors found in MCs in this group of patients, suggesting that the reduction in mast cells number was unlikely to be via the ER or PR. These data indicate that mast cells in both the eutopic and ectopic endometrium of women with endometriosis prior to the LNG-IUS insertion are similar to that reported by Matsuzaki et al. (2004). Significantly higher numbers of mast cells in this small series were found in the stromal rather than in glands. This finding in the eutopic endometrium is similar to that reported by Yin et al. (1993).

Anaf et al. (2006) demonstrated diffuse infiltration of endometriosis with numerous activated and degranulated mast cells. These cells had a close histological relationship with nerves leading them to strongly suggest that the mast cells could contribute to the development of pain and the hyperalgesia in endometriosis and possibly by a direct effect on nerve structures. This finding of decreased mast cell numbers following treatment and associated with improvements in pain symptoms in this small study would confirm this hypothesis.

Sugamata et al. (2005) reported degranulated mast cell infiltration of endometriosis which were rarely demonstrated in eutopic and normal uterine serosa. This would support the suggestion that the pain of endometriosis may be related to the immune response generated by the mast cells. It may be that degranulation provokes the immunological changes that contribute to the pathogenesis of the symptoms of endometriosis. This proposed mechanism is based on the increase in degranulated mast cells in endometriotic lesions reported by Sugamata et al. (2005).

Failure to demonstrate ER and/or PR in the MCs found in the endometriotic lesions in this study is in contrast to several other studies that demonstrated the presence of these receptors on MCs present in other major organs of the human body [Pang et al, 1995; Zhao et al, 2001; Nicovani & Rudolph 2002) was surprising. If these findings are correct, then they would suggest that MCs behave differently in different pathological conditions and in different organs of the body. The immune system dysfunction linked with this condition might also be a possible explanation of these differing results (Lebovic et al, 2001; Nothnick, 2001). Despite recent clinical and histological studies on the symptomatic treatment of minimal to moderate endometriosis with the LNG-IUS, there have been no reports on the effect of this modality of treatment on changes in mast cells from pre to post treatment. Since mast cells express leukotrienes and prostanoids, a reduction in their numbers in women with endometriosis induced by the LNG-IUS is most likely to result in an alteration of the inflammatory milieu in favour of less reaction and consequently a reduction in the symptom of pain, as seen in women having this option for symptom relief. Peloggia et al. (2006) investigated differences in mast cell numbers and function in endometrial biopsies from women with endometriosis on the LNG-IUS who were either amenorrhoeic or were menstruating. They failed to demonstrate any differences in mast cell numbers in these two groups of patients. In the study mast cell numbers were not compared to those prior to initiation of treatment as was the case in this study. This and previous observations of a reduction in the number of ER and PR in the endometriotic cells (Engemise et al, 2011) are thus possible mechanisms by which the LNG-IUS benefits women with endometriosis.

An assumption was made that the endometriotic biopsies taken before and after treatment represented the same site of the lesions. This is unlikely to be the case and therefore could

be an important confounder in this study. However, the decrease in endometriotic mast cell numbers following treatment will suggest that despite this limitation, these observations are most likely to be the generalised effect of the LNG-IUS on mast cells in these patients. The effect of the LNG-IUS on mast cells number in both the eutopic and ectopic endometrium is a secondary inflammatory response of the levonorgestrel on the mast cells number.

In conclusion, it was demonstrated that mast cells are down regulated following six months symptomatic treatment of endometriosis with the LNG-IUS and suggest that this is not through ER and/or PR as these receptor were not found in them.

Chapter IV

Effects of the LNG-IUS and DecapeptylTM on cytokines in peritoneal fluid

4.1. Introduction

The aetiology of endometriosis is complex and multifactorial in nature involving intrinsic molecular anomalies in ectopic endometrium and several immunological alterations. A decreased cytotoxic cell activity and an increase in the synthesis of several peritoneal macrophage-derived cytokines facilitate the implantation and progression of ectopic endometrial cells in the peritoneal cavity (Wilson et al, 1994; Witz et al, 1994). The ectopic endometrium is bathed with peritoneal fluid (PF). This PF undergoes a number of biological changes including local inflammatory-reparative phenomena and consequently an increase in the number of peripheral blood mononuclear cells (PBMC) present in it. These activated cells as well as the endometriotic cells secrete various cytokines with pleiotropic biological activities.

4.1.1. Cytokines: chemistry and sources

Cytokines are a heterogeneous group of soluble regulatory polypeptides that are released from cells and bind to specific receptors to exert their effects which are autocrine and paracrine. Unlike hormones, cytokines are produced by a variety of cell types not localized in distinct glands and act on different types of target cells some of which are found in peritoneal fluid (PF). They are critical to the function of both innate and adaptive immune responses. Apart from their importance in immune system development and function, they play a major role in a variety of immunological, inflammatory and infectious diseases. They are also implicated in several developmental processes throughout embryogenesis.

Cytokines found in the peritoneal fluid of women with endometriosis are potentially produced by activated macrophages and T-lymphocytes (Rana et al, 1996; Gupta et al, 2006). They are secreted in response to inflammatory stimuli and exert a variety of effects which may be autocrine, paracrine, or endocrine (Gupta et al, 2006). Peritoneal fluid is richly colonized by macrophages, lymphocytes, eosinophils and mast cells. Normally, the peritoneal fluid contains $1/2 - 2 \times 10^6$ leucocytes per millilitre (Syrop & Hamle, 1987; Ho et al, 1997). Approximately 80% of the cells in the peritoneal fluid are macrophages. Macrophage activation is a key step in the development of endometriosis (Halme et al, 1983; D'Hooghe et al, 1996; Oral et al, 1996).

The effect of a particular cytokine on a given cell depends on the cytokine, its extracellular abundance, the presence and abundance of the complementary receptor on the cell surface, and downstream signals activated by receptor binding; these last two factors can vary by cell type. In women with endometriosis, cytokines attract and recruit more immune cells (Akoum et al, 1996; Hornung et al, 2001), promote implantation and growth of ectopic endometrial cells by inducing proliferation (Iwabe et al, 2000) and angiogenesis (Goteri et al, 2004), contribute to the attachment of endometrial cells to the peritoneal surface (Wu et al, 2004), and help the invasion of these cells into the mesothelium (Osteen et al, 2004).

4.1.2. Choice of cytokines to be measured

Interleukin 6 (IL-6) and soluble intercellular adhesion molecule-1 (sICAM-1) and their receptors are some of the most commonly associated cytokines studied in women with

endometriosis. They are either involved in implantation, inflammation or angiogenesis (Arai et al, 1990).

The most frequently studied cytokine in endometriosis is IL-6 (Kalu et al, 2007). It is a pleiotropic cytokine that is produced by a variety of cells types, including monocytes, lymphocytes, fibroblasts, endothelial cells, keratinocytes and mesangial cells (Ray et al, 1997). This cytokine which appears to mediate numerous physiological and pathogenic processes, acts on a wide variety of cells and regulates immune responses, acute phase responses of the liver, haematopoiesis, neuronal functions and osteoclastogenesis (Gorospe et al, 1992). IL-6 is a major mediator of the host response to tissue damage and infection. It regulates inflammation and immunity and also modulates the secretion of other cytokines; promotes T-cell activation and B-cell differentiation (Lebovic et al, 2001). IL-6 secretion is increased by peritoneal macrophages in endometriosis patients and by stromal cells of eutopic and ectopic endometrium, with the ectopic stromal cells producing the highest levels (Berkkanoglu & Arici, 2003). This cytokine normally inhibits the growth of endometrial cells; however, this growth-inhibitory effect is lost in endometriotic tissues leading to high levels in patients with the disease (Dmowski & Braun, 2004; Kalu et al, 2007).

Intercellular adhesion molecule-1 (ICAM-1 or CD54) is a member of the immunoglobulin superfamily, constitutively expressed on the surface of numerous cells, including endometrial cells, endothelial cells and leukocytes, and its expression is modulated by cytokines (van de Stolpe & van der Saag, 1996). ICAM-1 plays different roles, all related to immune processes, depending on the cell type where it is expressed. A shedding form of this adhesion molecule, soluble intracellular adhesion molecule-1 (sICAM-1) has been identified

and characterized in at least three identifiable molecular forms with molecular masses of ~240, 430 and >500 kDa (Seth et al, 1991). sICAM-1 promotes adhesion between cells and is stimulated by IL-1 and TNF- α . The soluble form of ICAM-1 is regarded as a useful parameter in the diagnosis and monitoring of various inflammatory, neoplastic, and immune disorders (Seth et al, 1991). The release of a considerable quantity of sICAM-1 in cultured endometrial stromal shedding is found in patients with advanced stages of endometriosis and women with endometriosis-associated infertility have been shown to have significantly higher levels in their peritoneal fluid compared with fertile women without endometriosis ((Somigliana et al, 1996; Skrzypczak et al, 2005). sICAM-1 promotes angiogenesis and serves as an indicator of vascular endothelial cell activation or damage (Gho et al, 1999; Constans & Conri, 2006). It also functions as an inhibitor of transmembrane ICAM-1 mediated activities such as monocyte adhesion to activated endothelial cells and sensitivity of tumour cells to NK cell-mediated lysis (Fiore et al, 2002; Tsakadze et al, 2006).

Having demonstrated in chapter 2 and 3 that the LNG-IUS is effective in symptom relief possibly via alteration in the steroid receptors expression on the ectopic endometrium and also by reducing the mast cell numbers in ectopic endometrium, it was further hypothesized that another mechanism of action is through alteration in the cytokine milieu of PF. The aim of this part of the thesis was therefore to investigate the effects of the LNG-IUS and the gold standard treatment GnRH agonist (decapeptyl™) on peritoneal fluid IL-6 and sICAM-I concentrations following six months treatment of women with minimal to moderate endometriosis. The choice of IL-6 and sICAM-1 was based on their level of involvement in inflammation. sICAM-1 promotes angiogenesis and serves as an indicator of vascular endothelial cell activation, while IL-6 is a major mediator of the host response to tissue damage and inflammation.

4.2. Material and methods.

4.2.1. Subjects

This was a prospective randomised control trial in which peritoneal fluid was collected from women with confirmed minimal to moderate endometriosis at diagnostic laparoscopy prior to the initiation of treatment and then six months after treatment at a second look laparoscopy at the University Hospitals of Leicester NHS Trust, from January 2008 to December 2009. The study was approved by the Leicestershire, Northamptonshire and Rutland (LNR) Research Ethic Committee. The inclusion and exclusion criteria have already been described in the material and methods of section 2.5.

4.2.2. Sample Collection and Processing

4.2.2.1. Fluid collection

The PF was collected by aspiration from the pouch of Douglas (POD) using a 20mls syringe attached to a Veress needle. The sample was then transferred into a sterile universal bottle and kept on ice until processed within an hour of collection. The PF was then centrifuged at 1250g for 15 minutes at a regulated temperature of -5°C. The supernatant was aliquoted into 0.5-1ml eppendorfs and stored at -80°C until analysed. These stored samples were stocked in several batches of 0.5mls to avoid repeated freeze-thawing of the samples. Only straw-coloured PF was considered suitable for processing while blood stained PF was discarded. 0.5 to 1µl of one micromole (1µM) pepstatin A, a protease inhibitor was added into each 0.5ml aliquot to prevent degradation of the cytokines.

4.2.3. Enzyme-linked immunosorbent assay (ELISA).

IL-6 and sICAM-1 were measured in the PF supernatants using the commercially available standard cytokine-specific enzyme-linked immunosorbent assay (ELISA) (R&D systems Europe Ltd, Oxon, UK) kits. All measurements were performed in duplicate, according to the manufacturer's instructions. Monoclonal antibodies specific for IL-6 and sICAM-1 were pre-coated onto 96 well microplates.

The PF was then diluted and mixed with a cocktail of biotinylated detection antibodies. The standard and samples were added to the wells to allow the cytokines to bind to the fixed antibodies. After washing, enzyme-linked polyclonal antibodies for IL-6 and sICAM-1 were added onto the well. After a further wash, a substrate solution was added onto the well and a colour change was observed in proportion to the concentration of the cytokine in the sample. The results were obtained using a plate reader measuring absorbance at 450nm. A calibration curve was performed in duplicate using standards provided with the kit to allow calculation.

The relative Optical Density at 450 nanometre (nm) (OD_{450}) = the OD_{450} of each well minus (the OD_{450} of Zero well) was used to calculate the cytokine level. The standard curve was plotted as the relative OD_{450} of each standard solution (Y) versus the respective concentration of the standard solution (X) (**Fig. 4.1**).

The IL-6 and sICAM-1 concentrations in each sample were calculated by interpolation from the standard curve. If the samples measured were diluted, the dilution factor was

multiplied to the concentration from the interpolation so as to obtain the concentration before dilution.

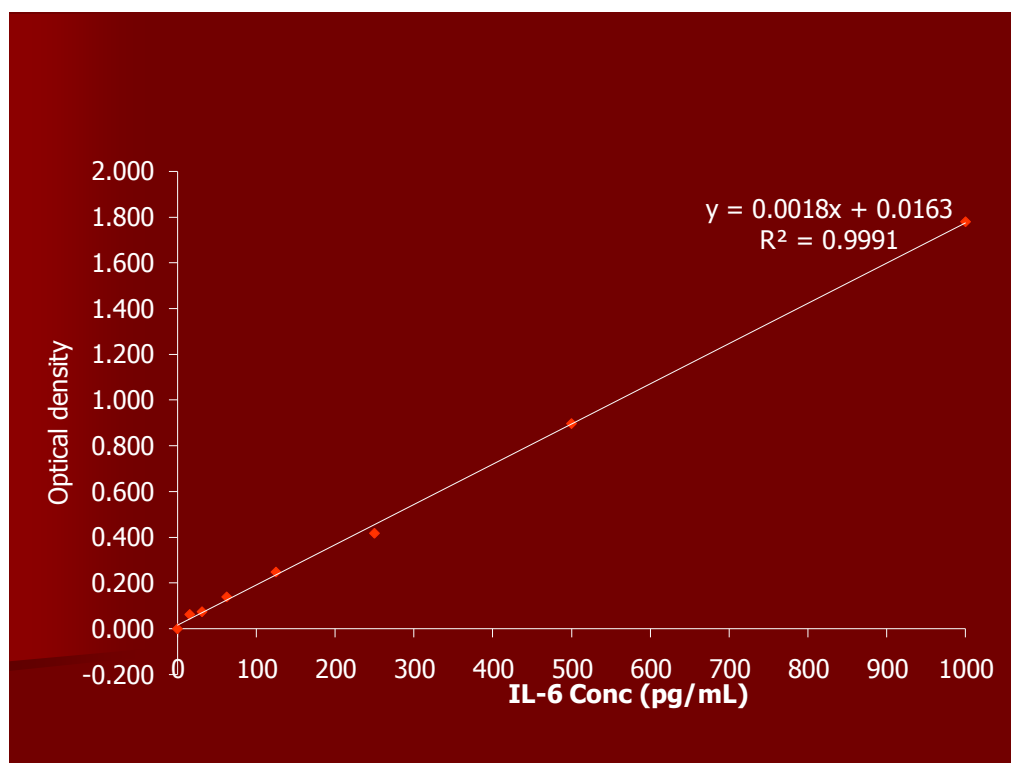


Fig. 4.1 Graphic representation of optical density versus concentration of cytokine

4.2.4. Statistical analysis

The paired t-test was used to compare cytokines levels in the PF. The results were expressed as mean and standard errors of the mean (SEM). Normality of the data was determined using the Wilks-Shapiro test. The non-parametric approach was used because cytokine levels were not normally distributed. Statistical analysis was performed using the Graphpad Prism (version 5.0) software (www.graphpad.com). Differences were considered significant for $P < 0.05$.

4.3. Results

Twenty seven women with minimal to moderate endometriosis were studied. There were no statistical difference in the age of the patients who were randomized to either the LNG-IUS or the GnRH agonist [29.5 ± 6.8 (range 18 - 40) versus 27.9 ± 7.0 (range 19 - 42) respectively]. Fourteen patients were randomized to the LNG-IUS group and thirteen to the GnRH agonist group. Six PF samples were analysed in the LNG-IUS group (eight were excluded because of the following: 2 withdrew from the study just after the initial laparoscopy, 2 for refusal of the second look laparoscopy, 1 did not have enough PF during the initial laparoscopy and 3 had heavily blood stained PF, one during the initial and two during the six month laparoscopy). Five PF samples were analysed in the GnRH agonist (decapeptyl™) group (eight were excluded because of the following reasons: 1 had no PF during the initial and second look laparoscopy, 2 for refusal of sixth month laparoscopy, 2 withdrew from the study after the initial laparoscopy due to severe side effect of the GnRH agonist, 2 did not have enough PF during the six month laparoscopy and 1 had heavily blood stained PF during the six month laparoscopy). **Tables 4.1 & 4.2 & Figs. 4.2, 4.3, 4.4 & 4.5** show the levels of IL-6 in and sICAM-I before and after treatment with either the LNG-IUS or the GnRH agonist. In this group there was a significant reduction in IL-6 ($P=0.0313$) levels in the peritoneal fluid in patients with endometriosis treated with the LNG-IUS for six months (24.65 ± 10.12 pg/ml before and 7.70 ± 2.83 pg/ml after treatment). There were no significant differences between the levels of IL-6 and sICAM-1 before and six months after treatment with the GnRH agonist and no difference in the sICAM-I who were treated with the LNG-IUS. We also compared the levels of both IL-6 and sICAM-I in women with and without endometriosis, the later admitted for routine laparoscopic sterilisation (**Tables 4.3 & Figs. 4.6 & 4.7**). A significant reduction in the sICAM-1

($P=0.0322$) levels in the peritoneal fluid was observed in patients with endometriosis compared with controls (1.83 ± 0.16 pg/ml for cases compared with 1.28 ± 0.20 pg/ml for controls). There was also a significant reduction in IL-6 ($P=0.0068$) levels in the peritoneal fluid in patients with endometriosis compared with controls (83.12 ± 16.92 pg/ml for cases compared with 32.68 ± 7.65 pg/ml for controls).

Tab. 4.1. Cytokines levels in PF before and after treatment with the GnRH agonist

Levels of Cytokines in PF				
Patients No	IL-6 (pg/dl)		sICAM-I (pg/dl)	
	Before	After	Before	After
1	4.74	5.53	4.64	3.75
2	3.05	28.73	4.45	5.19
3	82.18	3.91	3.46	2.29
4	46.04	17.38	3.15	4.22
5	25.81	0.38	0.98	0.91

Tab. 4.2. Cytokines levels in PF before and after treatment with the LNG-IUS

Levels of Cytokines in PF				
Patients No	IL-6 (pg/dl)		sICAM-I (pg/dl)	
	Before	After	Before	After
1	14.71	3.06	3.15	4.15
2	8.74	3.94	3.56	3.20
3	8.87	3.05	3.80	3.78
4	45.74	20.29	1.46	1.00
5	65.04	11.47	1.61	1.57
6	4.82	4.40	1.04	0.61

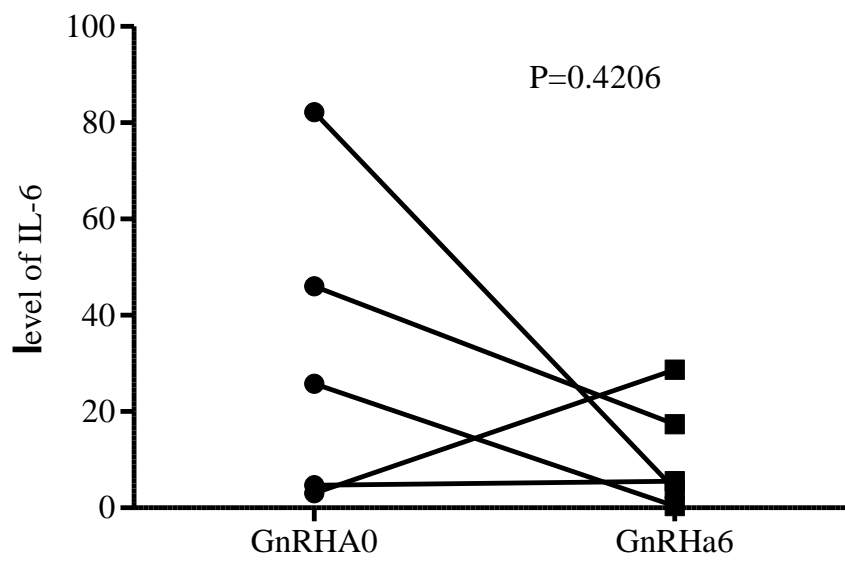


Fig. 4.2. IL-6 levels in PF of women with endometriosis before and after treatment with the GnRH agonist.

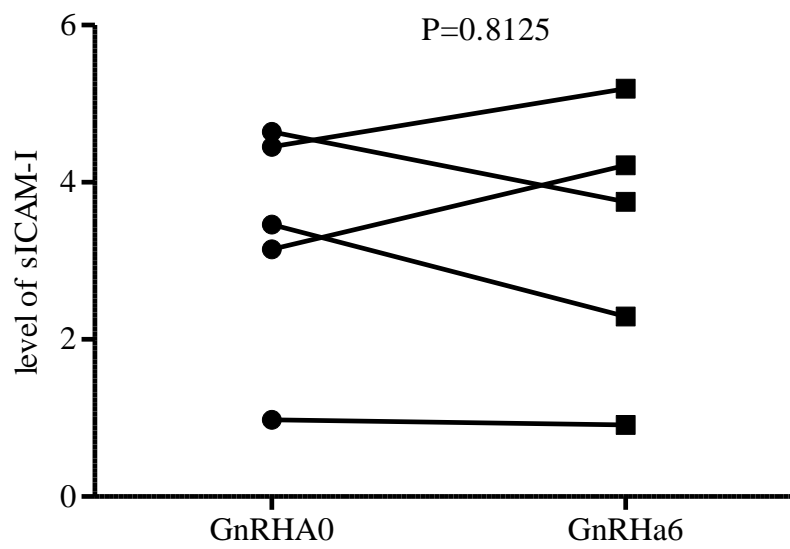


Fig. 4.3. sICAM-1 levels in PF of women with endometriosis before and after treatment with the GnRH agonist.

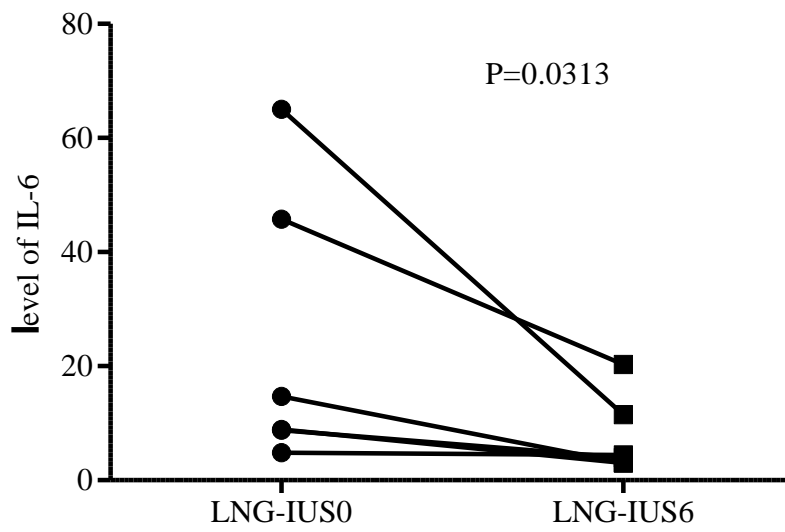


Fig. 4.4. IL-6 level in PF of women with endometriosis before and after treatment with LNG-IUS.

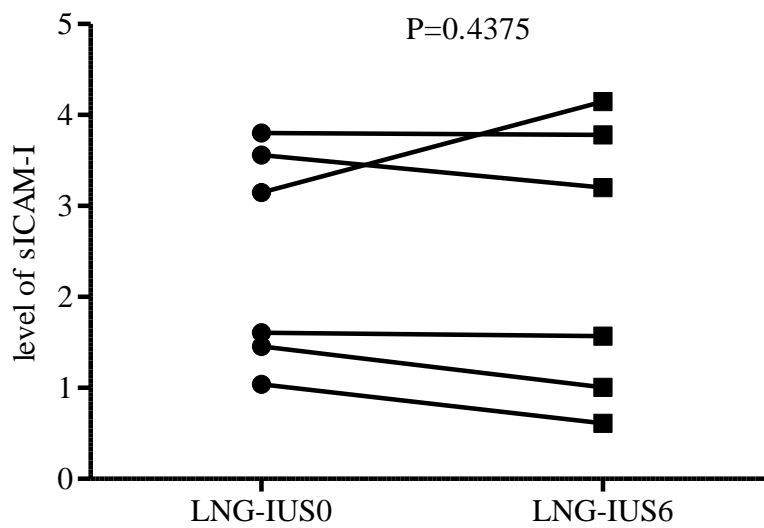


Fig. 4.5. sICAM-1 levels in PF of women with endometriosis before and after treatment with the LNG-IUS

Tab. 4.3. Cytokines levels in PF of patient with and without endometriosis

Levels of Cytokines in PF				
IL-6 (pg/dl)		sICAM-I (pg/dl)		
Control	Endo	Control	Endo	
6.32	71.77	0.77	1.34	
39.47	58.07	0.96	2.10	
16.65	173.15	1.19	2.41	
10.39	39.28	2.80	2.01	
21.61	22.25	1.73	0.93	
52.59	17.48	0.66	1.70	
45.18	61.02	1.13	2.24	
83.76	170.66	1.52	2.51	
59.05	89.41	0.91	1.41	
4.21	63.78	1.17	1.70	
20.26	147.51			

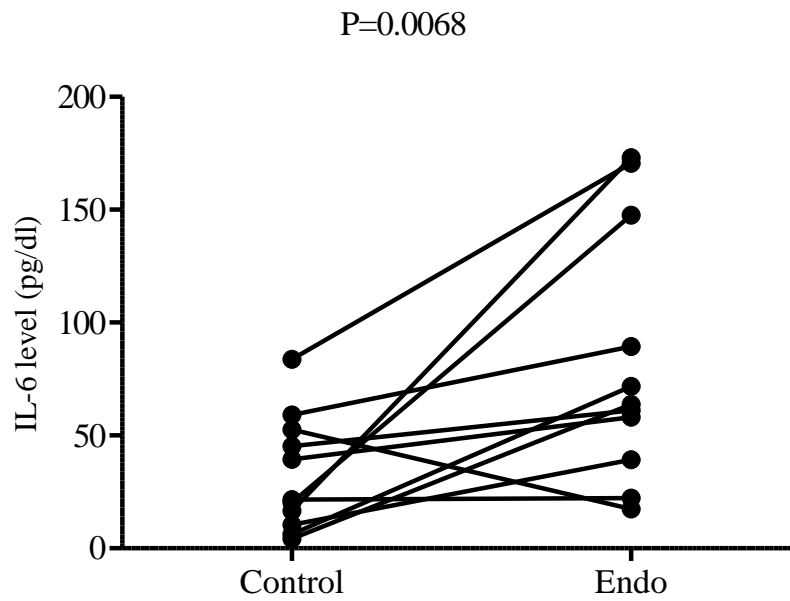


Fig. 4.6. Comparing the levels of IL-6 in PF of women with and without endometriosis.

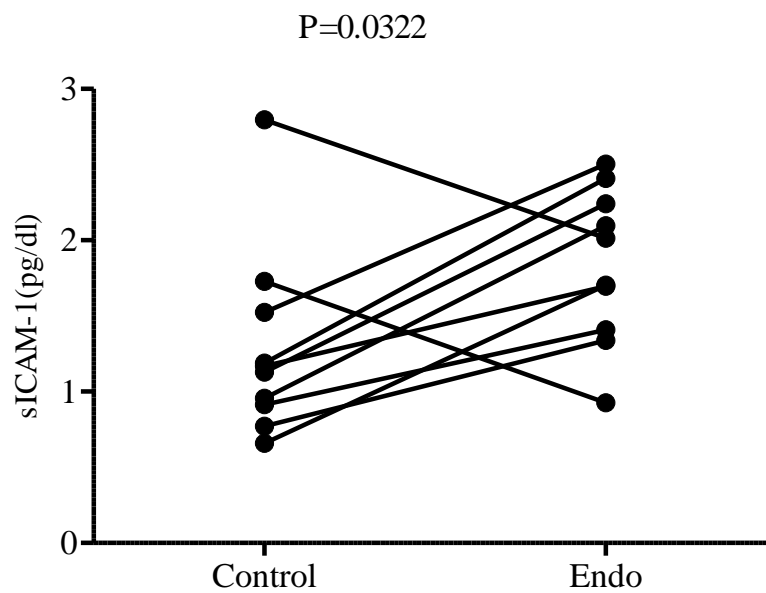


Fig. 4.7. Comparing the levels of sICAM-I in PF of women with and without endometriosis.

4.4. Discussion.

The role of PF cellular population and soluble factors has been studied extensively and these are considered to contribute to the implantation and progression of endometriosis (Calhaz-Jorge et al, 2003). Measurement of their concentrations is complicated by the fact that they have very short half-lives and are never produced in isolation, but as a mixture which may have similar or opposing effects. PF levels of IL-6 and sICAM-1 were measured in women with minimal to moderate endometriosis before and six months after treatment with the LNG-IUS and the GnRH agonist. The levels of PF of IL-6 and sICAM-1 were also measured in women without endometriosis admitted for routine laparoscopic tubal sterilization and this was compared with those with minimal to moderate disease. IL-6 and sICAM-1 were chosen as they have been implicated and are important in the pathogenesis of endometriosis.

In this study, despite an improvement in the clinical symptoms of these women as seen in chapter 5 there was only a significance difference in the PF levels of IL-6 ($P=0.0313$) following a six month treatment with the LNG-IUS. There were no differences in PF levels of IL-6 and sICAM-1 in women with minimal to moderate endometriosis after six months of treatment with the GnRH agonist and no difference in the PF levels of sICAM-I after six months of treatment of these women with the LNG-IUS. Chen Xun et al (2008) found a significance decrease in the IL-6 levels of the serum and PF of women with endometriosis before and 3 months treatment with the GnRH agonist along and GnRH agonist with estroprogestin. This is contrary to our finding were there was no difference of IL-6 levels in the PF after a six months treatment with the GnRH agonist. This is the first study carried out looking at the six months effect of the LNG-IUS on the PF levels of sICAM-1 on

women with minimal endometriosis. Similar other studies have been carried out on the serum levels of sICAM-1 and/or IL-6 with either the LNG-IUS or the GnRH agonist such as Ferreira et al (2010) who compared the serum of the IL-6 following a for six month treatment of the LNG-IUS and the GnRH agonist in women with endometriosis. They found a significant change in the IL-6 level in the GnRH agonist group but not with the LNG-IUS group. Similarly, Iwabe et al (2003) also found a significant change in the serum level of IL-6 in women with endometrioma following three month treatment with a GnRH agonist. Matalliotakis et al (2001) on the other hand, found no significant difference in the serum concentration levels of the sICAM-1 of women with endometriosis who were treated either with the GnRH agonist (Leuprorelin acetate) or danazol in six months.

Although the LNG-IUS and the GnRH agonist do not have an effect on the PF levels of the sICAM-1 and the GnRH agonist an effect on the IL-6 after treatment, they improved the symptoms of these women. It would seem from these findings that there is a complex regulatory network of reactions at multiple levels not only involving cell-to-cell interactions, production of growth factors and hormones, but also the molecular events dictating the type of mediator secreted. It is crucial to know that sICAM-1 exists in multiple molecular forms (Inazuka et al, 1995) that are perhaps activated by different triggering mechanisms. This may also be true of IL-6. The fact that the ICAM-1 promoter contains several enhancer elements, each specific to a certain stimulus, explains the complexity of the system, which becomes evident from the observations of Kupker et al. (1998). They reported a significant decrease in the mean concentration of ICAM-1 in the PF of their study group after a 4 month treatment with a GnRH agonist which is contrary to the findings here.

When the PF levels of IL-6 and sICAM-I of women with minimal to moderate endometriosis were also compared with that of the control group, we found that there were significantly higher levels of IL-6 ($P=0.0068$) and sICAM-I ($P=0.0322$) in the disease women than those of controls. This is similar to Yong-lai et al (2007) who found a similar result following the study of sICAM-1 in a similar group of patients. This was not the case with the level of IL-6 as previous investigators did not find any difference in disease patients compared with their control group (Buyalos et al, 1992; Bedaiwy et al, 2002).

Whether the PF levels of IL-6 and sICAM-1 changes with the phase of menstrual cycle is still unknown as this has not been previously investigated. The discrepancy and differences between the serum levels of IL-6 and sICAM-1 and those detected in the PF by different investigators signify that a not so simple, as anticipated, cellular machinery governs the regulation of cytokines in endometriosis. Another reason for this discrepancy may be related to sample size and processing. The fact that some of the samples were stored for a longer time before processing may also explain the differences.

Before definite conclusions can be made on the effect of these treatment modalities on PF cytokine levels, it would be useful to repeat the studies with larger numbers and reduce the storage time from collection to processing of samples. Such a study may provide evidence to support a possible mechanism through which the LNG-IUS modulates symptoms of endometriosis.

Chapter V

Clinical outcome after treatment with either the LNG-IUS or the GnRH agonist (decapeptylTM).

5.1. Background

Treatment for endometriosis as detailed in chapter one can either be medical, surgical or a combination of both. Medical therapy alone is used mostly for minimal to moderate endometriosis (Revised AFS I & II). Such treatment is usually in the form of either anti-estrogens, which induce a pseudopregnancy or pseudomenopause state. The gold standard for medical treatment is the GnRH agonist (Prentice et al, 2000). This alleviates symptoms in approximately 70% of women after 6 months of therapy (Fidele & Berlanda, 2004). It is however, associated with severe side effect notably menopausal symptoms with increase risk of osteopenia. Compliance and efficacy are affected by these symptoms and although addback therapy maybe given to minimise them, a significant number of patients still discontinue therapy. Additionally, between 30 and 40% of patients become significantly symptomatic 6-12 months after completing a six months course of therapy (Lockhat & Konje, 2001).

Progestogens are an effective alternative to GnRH agonists but because of their high systemic levels following oral or depot administration, tolerance is poor. Lockhat et al (2005a) demonstrated in a pilot observational trial that the progestogen, levonorgestrel delivered locally by means of an intrauterine device directly into the uterine cavity is effective in 70% of cases after 6 months. This high efficacy rate persisted for up to 3 years after insertion (Lockhat et al, 2005b). Similar results have also been obtained from much smaller studies (Vercellini et al, 1999; Fedele et al, 2001). The LNG-IUS confers several advantages over others forms of medical options including cost effectiveness, single administration, effective contraception and reduction in menstrual loss. The reported efficacy rate of the LNG-IUS in the above studies is very similar to that of the gold standard treatment. No comparative studies have been reported in patients with minimal to moderate

endometriosis although Petta et al, (2005) reported on a comparative study but on patients with minimal to severe disease. In this chapter a randomised controlled trial comparing the clinical outcome of the LNG-IUS and the gold standard GnRH agonist (Decapeptyl-Triptorelin acetate) in the symptomatic treatment of women with minimal to moderate endometriosis is presented. It was undertaken not only to compare efficacy but also their side effect profiles.

5.2. Materials and Methods

5.2.1 Study Population

These were women attending the gynaecology outpatients' clinic of the University Hospitals of Leicester NHS Trust at the Leicester Royal Infirmary with symptoms suggestive of endometriosis from January 2008 to December 2009 respectively. The study was approved by the Leicestershire, Northamptonshire and Rutland (LNR) research ethic committee. All the volunteers recruited into the study gave written signed informed consent (**Appendix III**). The inclusion and exclusion criteria have already been discussed in section **2.5.2** and **2.5.3** respectively. Following recruitment, the volunteers were listed for a diagnostic laparoscopy performed by one of two consultant gynaecologists (Mr JO Emembolu & Prof. JC Konje) who had standardized the procedure, documentation and sample collection.

5.2.2. Randomization

This was by means of two randomly computer-generated alphabetical letters each representing either the LNG-IUS or the GnRH agonist (decapetylTM) placed in sealed brown envelopes labelled with ascending order of numerical numbers prepared by an independent statistician not involved in the investigation. These numbered brown envelopes starting from number '1' in batches of fifty's with the smallest number allocated to a patient who has agreed to the study by signing the informed consent form. The envelope was then opened by the pharmacist dispensing the medication with the clinical research fellow in attendance. The patients were randomized into either the GnRH agonist (decapetyl) or the LNG-IUS treatment.

5.2.3 Menstrual Diary

For the month preceding the LNG-IUS insertion or the administration of the first dose of the GnRH agonist (decapetylTM), each volunteer completed a diary for the generation of baseline variables, which were used for the assessment of response to treatment.

Quantified menstrual loss was estimated from the pictorial menstrual blood loss chart of Higham et al (1990). Each volunteer was given a diary with these charts and instructed to shed it depending on the heaviness of the bleeding (**Appendix IV**). The chart consisted of a series of diagrams representing lightly, moderately and heavily soiled tampons or towels, a

mark being made in the appropriate box at the time and were counted in groups of five. In addition passage of clots (size equated with that of coin) and episodes of flooding were recorded. The total loss was then determined from these chart and scores assigned were 1 for each lightly stained tampon, 5 if moderately soiled and 10 if completely saturated with blood. The sanitary towels were also given scores of 1, 5, and 20 respectively. Small and large clots were given scores of 1 and 5 respectively. The total numerical score was then determined from these scores. A score of >100 was used as menorrhagia. Only the loss in the month prior to a follow-up visit was quantified.

Since bleeding abnormalities are common with the LNG-IUS, the WHO definition of various bleeding pattern were used to characterise the abnormalities reported by the volunteer. Bleeding patterns were assessed as: 0 = no bleeding; 1 = spotting (light bleeding not requiring sanitary protection); 2 = light bleeding (light bleeding requiring sanitary protection); 3 = normal bleeding (bleeding similar to normal menstrual blood flow); and 4 = heavy bleeding (bleeding exceeding normal menstrual blood flow). No bleeding was defined as 30 consecutive days with a bleeding score of 0 (Rodriguez et al, 1976; World Health Organization, 1986). The mean bleeding score was calculated by dividing the sum of the daily scores by the number of days in each observation period.

Response to treatment was assessed by changes in the variables, which included the patient's perception of pelvic pain severity using a visual analogue scale (VAS) and her rating of both types of pelvic pain (dysmenorrhoea and/or non-cyclical pelvic pain) on a verbal rating scale (VRS). The VAS was a subjective assessment of pain on a scale of 0 (no pain) to 10 (most severe pain). It was recorded on a 10-cm ruler in the diary at each follow-

up visit and reflected the severity of this symptom as perceived by the patient in the preceding month. The 10-cm ruler VAS used in this study is cumbersome to administer because it requires adequate levels of visual acuity, motor function, and the cognitive ability to translate a sensation of pain into a distance measure. The scale gaining popularity for use in clinical settings is the 10-cm (100 mm) ungraduated horizontal VAS due to its advantage for measuring pain on a continuous line (Marquie, et al., 2003). This has eliminated the difficulty and bias of the graduated VAS scale. A 4-point scale (0-3; where 0 = no pain, 1 = mild pain, 2 = moderate pain, 3 = severe pain) was used to rate dysmenorrhoea and/or non-cyclical pain on a daily basis. A monthly score was then generated from the summation of the daily VRS over a 28-day period (0 = no pain, 96 = maximum pain). Once again this $VRS_{monthly}$ was only determined for the 28 days prior to the follow-up visit with well recognized advantages and drawbacks (von Korff et al, 2000).

Follow-up visits were scheduled every 28 ± 3 days after initiation of treatment until six completed visits. Women randomized to the LNG-IUS were allowed to retain the device if they wanted to after completing the study. Changes in the patient's daily perception of pelvic pain, daily bleeding score and the occurrence of side effects were recorded. The VAS and VRS scores were compared at different points in time during the treatment.

5.3. Sample size

On the basis that the LNG-IUS improves symptoms in 70% of treated cases after 6 months (Lockhat et al, 2004a) it was estimated that for a difference of 30% in the satisfaction rate

between GnRH and the LNG-IUS with an 80% chance of detecting such a difference at an overall significant level of 5%, twenty three patients were required in each arm. Allowing for dropouts, the plan was to recruit a total of 60 women.

5.4. Statical Analysis

The baseline characteristics of the study population were compared using the student t test or Mann-Whitney test. The student t test was used to compare differences in pain symptoms measured on the VAS scale between the two study groups. The Mann-Whitney rank sum test was used to compare differences in pain symptoms measured on the multidimensional categorical rating scale between the two study groups. $P < 0.05$ was considered statistically significant. Data were analyzed using the Graphpad Prism (version 5.0) software (www.graphpad.com).

5.5. Results

Thirty two women were consented for the study, twenty seven had a diagnostic laparoscopy and nineteen completed the trial; five withdrew before the diagnostic laparoscopy; three in the GnRH agonist group and two from the LNG-IUS group. Four in the LNG-IUS group (28.6%) and four in the GnRH agonist (30.8%) withdrew from the study for various reasons after the diagnostic laparoscopy. In the GnRH group, one failed to attend the second look laparoscopy, two withdrew on the second and third month after the diagnostic laparoscopy and the other had a positive pregnancy test two weeks after receiving the 1st injection of the

decapeptylTM. In the LNG-IUS group, two declined a second-look laparoscopy and the other two withdrew for personal reasons (**Fig. 2.4**).

There were no statistically significant differences in the mean age and BMI (kg/m²) in both groups ($P < 0.5$). The Mean (SD) age of subjects were 29.5 (± 6.8) years in the LNG-IUS group and 27.9 (± 7.0) years in the GnRH agonist group. The mean (SD) BMI in the LNG-IUS was 25.8 (± 4.7) and 23.9 (± 6.4) in the GnRH agonist group.

There were no statistically significant differences between the two groups with respect to baseline data including the stage of endometriosis, smoking habits, parity, and use of medication prior to entering the study.

In the month preceding treatment, 10 women in the LNG-IUS group and 9 women in the GnRH group had VAS pain scores ≥ 3 and ≤ 7 , while 4 women in each of the LNG-IUS and GnRH agonist group had VAS pain scores > 7 .

Data analysis included only those who had completed at least one month after initiation of treatment and their VAS pain diary was labelled correctly throughout their study period ($n=27$). The mean pre-treatment VAS pain score was (6.7 ± 0.6) in the LNG-IUS group and (7.5 ± 0.4) in the GnRH agonist group; and no difference between the two groups ($P=0.2174$). By the end of the first month of treatment a significant reduction in score had already been achieved in both groups (4.1 ± 0.3) in the LNG-IUS group and (4.3 ± 0.4) in the GnRH agonist. There was no differences between the two groups ($P = 0.5518$). This

therapeutic effect persisted throughout the six months of treatment and there was no inter-group difference after the six months period ($P=0.9848$) (**Fig.5.1.**).

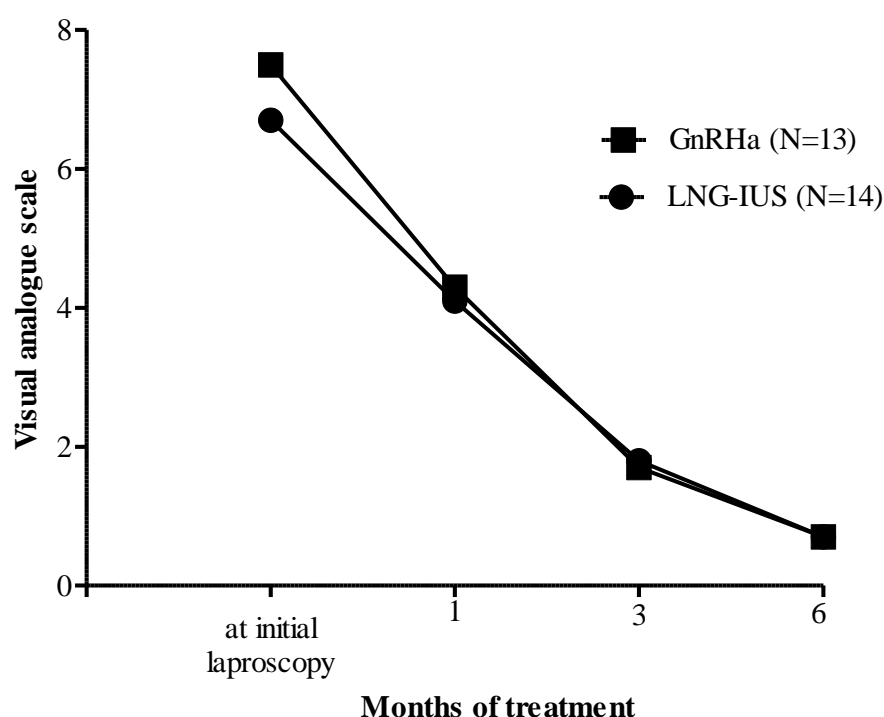


Fig.5.1. Changes in the visual analogue scale score of pain between the two treatment groups. Values are mean (\pm SEM). P-value between the two groups over six month: ($P=NS$)

From entry into the study to completion (between visits 1 and 6), there was a 6.0 ± 0.2 point decrease in VAS pain score in the LNG-IUS group and a 6.9 ± 0.1 point decrease in the GnRH agonist group.

In 4 of the 14 women on the LNG-IUS and 4 of the 13 on the GnRH agonist, the VAS pain score remained >3 at the end of the first month of evaluation, whereas at the end of the 6th month of treatment only two and one woman respectively in the LNG-IUS and GnRH agonist group failed to report a VAS pain score < 3 (**Tab. 5.1 & 5.2**). The reduction in the pain score at each month of follow-up was unrelated to the VAS pain score at baseline in either treatment groups ($P=0.3830$).

Tab. 5.1. Six months changes in the visual analogue scale (VAS) score in women with endometriosis treated with the GnRH agonist (decapeptyl™)

Changes in VAS of individual patient during the 6 months period				
No of Patient	VAS₀	VAS₁	VAS₃	VAS₆
1	10.0	4.3	-	-
2	7.0	2.7	1.9	0.5
3	7.0	2.8	0.9	-
4	6.8	4.1	3.8	1.0
5	6.9	3.0	0.0	0.0
6	9.0	6.2	4.1	3.2
7	7.0	-	-	-
8	6.7	4.0	1.2	0.3
9	7.0	2.7	1.6	1.1
10	8.0	2.1	-	-
11	6.7	2.2	0.7	0.9
12	10.0	2.1	0.4	0.0
13	5.8	2.9	2.9	2.3

Tab.5.2. Six months changes in the visual analogue scale (VAS) score in women with endometriosis treated with the LNG-IUS.

Changes in VAS of individual patient during the 6 months period.				
No of Patient	VAS₀	VAS₁	VAS₃	VAS₆
1	6.0	4.2	-	-
2	6.5	3.2	2.5	0.0
3	5.5	2.8	1.7	1.6
4	10.0	5.9	4.1	3.2
5	5.3	2.4	1.8	-
6	9.0	2.7	0.5	0.0
7	4.9	1.6	1.0	0.2
8	6.2	2.8	0.0	0.0
9	4.9	2.7	2.1	-
10	9.0	-	-	-
11	7.0	2.9	1.0	0.0
12	10.0	6.0	4.5	3.7
13	5.8	2.0	1.3	0.4
14	3.5	2.0	1.3	0.5

Bleeding scores were higher for LNG-IUS users than for GnRH agonist's users at all periods of observation although there was no significant difference between the two groups (Fig.5.2.).

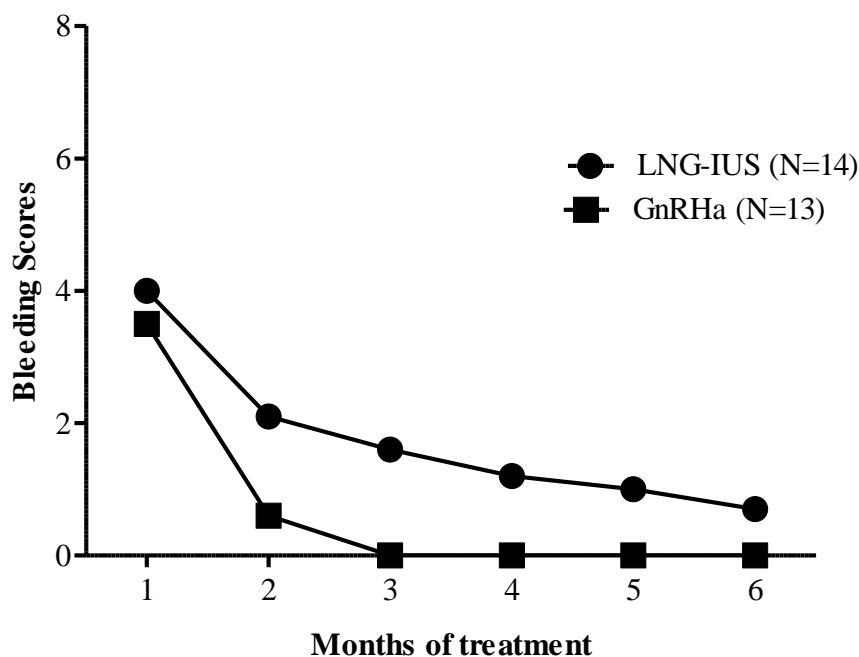


Fig.5.2. Changes in the bleeding scores between the two treatment groups. Values are mean (\pm SEM). P-value between the two groups over six month (P=NS).

Twenty-one per cent of those on the LNG-IUS and fifty-four per cent of those on the GnRH agonists reported no bleeding between the first and second visit, while 50 and 100% and 79 and 100% reported no bleeding between the 3rd and 4th visit and 5th and 6th visit respectively.

Thirteen women who had been taking either paracetamol, Ibuprofen or both at the start of the study did not require any analgesia by the sixth visit. Seven were from the decapeptylTM group and six from the LNG-IUS group. One of the seven patients from the decapeptylTM

group was also on citalopram for depression. She stopped this completely after the 4th injection of decapeptyl and never used it again during the follow-up period.

Eight women in the LNG-IUS group (57.1%) and all the thirteen women in the GnRH agonists group (100.0%) experienced adverse effects of treatment (P=0.0219). **Table 5.3.**

Shows the adverse effects experienced.

Table 5.3. Adverse effect of treatment reported during the 6 months period by the GnRH agonist and the LNG-IUS.

Adverse Effects	GnRH agonist	LNG-IUS
Hot flushes	13(100.00%)	1(7.14%)
Night sweats	13(100.00%)	0(00.00%)
Vaginal dryness	13(100.00%)	0(00.00%)
Spotting/irregular bleeding	0(00.00%)	6(42.86%)
Depression	4(30.77%)	1(7.14%)
Loose of Libido	3(23.08%)	0(00.00%)
Mood change	3(23.08%)	0(00.00%)
Loose bowel motions	0(00.00%)	1(7.14%)
Breast tenderness	0(00.00%)	1(7.14%)
Crampy lower abdominal pain	0(00.00%)	1(7.14%)
Bloatedness	1(7.69%)	0(00.00%)
Persistent breakthrough bleeding	0(00.00%)	1(7.14%)
At least one adverse effect	4(30.77%)	2(14.29%)

These included; hot flushes, night sweat and vaginal dryness which were significantly more frequent in the GnRH agonist group than in the LNG-IUS group ($P=0.0127$). In the GnRH group, 100.00% of the women complaint of hot flushes, vaginal dryness and night sweats, 30.8% of depression, hot flushes, vaginal dryness and night sweat, 23.1% of decreased libido hot flushes, vaginal dryness and night sweat, 23.1% of mood change, hot flushes, vaginal dryness and night sweat and 7.7% of constipation and hot flushes. In the LNG-IUS group, 42.9% of the women complaint of irregular bleeding, 7.1% of breast tenderness and irregular bleeding, 7.1% of persistent breakthrough bleeding and irregular bleeding, 7.1% of crampy abdominal pain and irregular bleeding, 7.1% of loose stool and 7.1% of bloatedness in one woman.

The mean quantified menstrual blood loss from the PBAC chart was 149.7 ± 34.3 for the LNG-IUS before the diagnostic laparoscopy and 8.9 ± 3.1 six months after treatment ($P=0.0009$) (**Table 5.4**) and 119.1 ± 31.3 for the GnRH agonist before the diagnostic laparoscopy and 1.3 ± 1.2 six months after treatment ($P= 0.0027$) (**Table 5.5**). The mean number of the bleeding days per month by the end of the sixth months on the LNG-IUS was not significantly different from that at commencement of the treatment (8.1 ± 2.6 days versus 5.5 ± 1.5 , $P=0.689$), but this was significantly different in the GnRH agonist group 9.2 ± 1.3 days versus 2.6 ± 0.3 , $P=0.0023$).

Table 5.4. Quantified monthly blood loss (mls) of the patients over the 6 month with the LNG-IUS

Pre-insertion	3 months	6 months
226	56	22
52	6	0
278	65	12
159	243	15
72	9	0
179	37	0
295	168	16
44	23	19
38	7	0

Table 5.5. Quantified monthly blood loss (mls) of the patients over the 6 month with the GnRH agonist

Pre-insertion	3 months	6 months
120	0	0
50	0	0
282	70	8
127	21	1
42	0	0
148	9	0
65	0	0

Figs. 5.3 & 5.4 show the changes in the stages and scores of endometriosis before and after treatment with the GnRH agonist and the LNG-IUS. There was a statistically significant change in both the stage ($P<0.05$) and Score ($P<0.05$) with both treatment options.

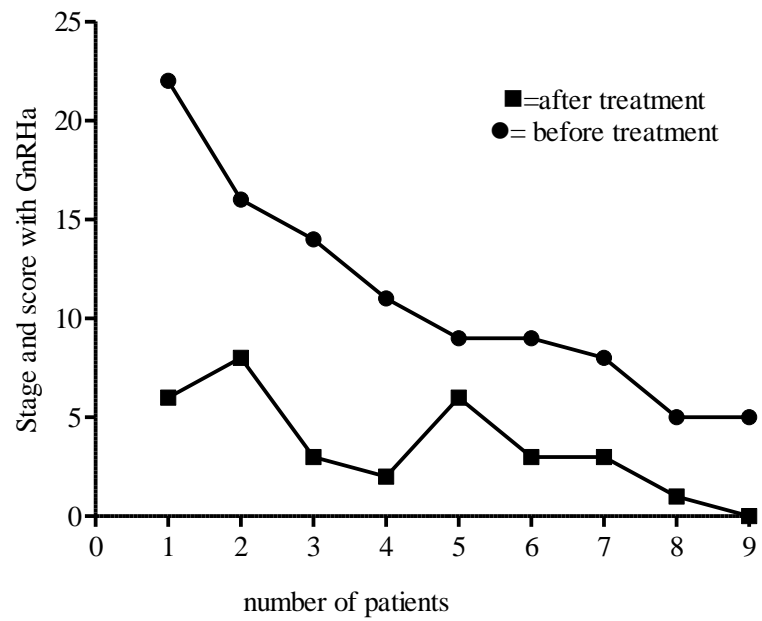


Fig.5.3. Stages and scores of endometriosis before and after treatment with the GnRH agonist

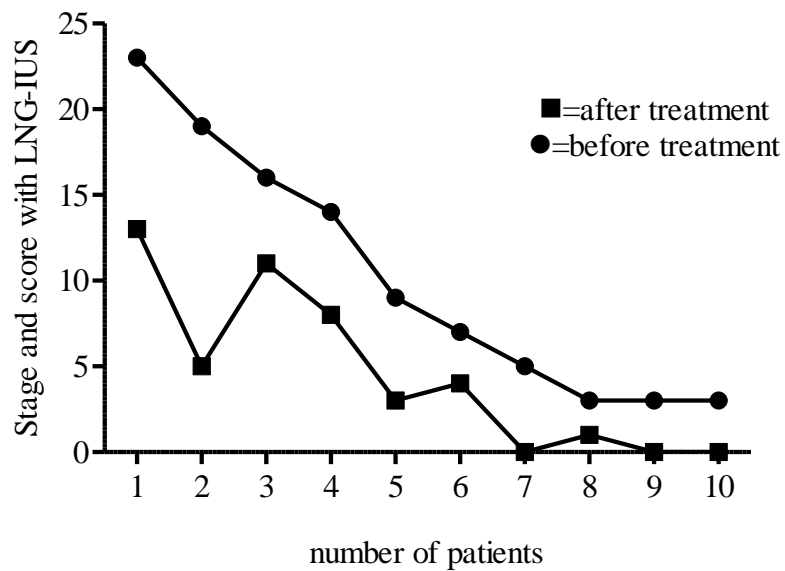


Fig. 5.4. Stages and scores of endometriosis before and after treatment with the LNG-IUS.

5.6 Discussion

Although this series is small, it is the first randomised controlled trial comparing the LNG-IUS with a GnRH agonist in the symptomatic treatment of minimal to moderate endometriosis. Two previous randomised controlled trials, one studied patients with severe endometriosis (stage III–IV) comparing the LNG-IUS after surgery with expectant management (Vercellini et al, 2003) and the other studied patients with minimal to severe disease comparing the LNG-IUS with a GnRH agonist therapy for six months (Petta et al, 2005). Due to the high prevalence of endometriosis in the reproductive age group, there has been an increased demand for long-term therapeutic options for the control of endometriosis related pelvic pain, dysmenorrhoea and dyspareunia, as well as controlling bleeding at an affordable cost and with minimal side effects.

Improvement in pelvic pain due to endometriosis was achieved by the second visit following the commencement of treatment in the GnRH agonist group and the second to the third visit in the LNG-IUS group with the pain scores decreasing significantly from a high score (≥ 7) to a lower score (≤ 3) in both groups. These findings are in agreement with previous studies that showed pain relief in women with endometriosis during the use of the GnRH agonist (Prentice et al, 2000; Schroder et al, 2004; Petta et al, 2005) and on the LNG-IUS (Fedele et al, 2001; Vercellini et al, 2003; Lockhat et al, 2004; Petta et al, 2005).

GnRH agonists have long been used to treat endometriosis associated pain, principally because they induce anovulation and hypoestrogenism with amenorrhoea by down

regulating the GnRH receptors in the hypothalamus. The stopping of the production of luteinising (LH) and follicle stimulating hormones (FSH) from the pituitary eventually results in the cessation of estrogen and progesterone production from the ovaries with a subsequent reduction in endometriotic lesions (Prentice et al, 2000; Schroder et al, 2004). However, the main concerns with respect to hypoestrogenism are the induction of vasomotor symptoms and the effect on bone mineral density, including the risk of osteoporosis. For this reason, treatment with the GnRH agonist alone is usually limited to a period of 6 months, although longer treatment with add-back hormone therapy is now common (Prentice et al, 2000). GnRH agonists are in addition to being very expensive and not readily available worldwide, especially in developing countries. There is therefore, the need for better treatment options than the ones currently available, offering therapeutic efficacy over a longer period of time at an affordable cost, and with ease of administration.

This study provides evidence that the LNG-IUS is as effective as the GnRH agonist in the control of pain in women with minimal to moderate endometriosis. The duration of treatment was limited to 6 months although the therapeutic effect of the LNG-IUS is known to last longer since the device has been approved for 5 years of use. During this period of use the LNG-IUS down-regulate ER and PR, reduces the number of mast cells in ectopic endometrium and induces endometrial atrophy (chapter 2 & 3).

The adverse effects of the LNG-IUS and the GnRH agonist reported here were as expected. Some of the adverse effects occurred in both groups, with no woman in either treatment group discontinuing treatment because of these side effects. These women have a chronic condition that has a great impact on their quality of life and fertility (Marques et al, 2004),

therefore they are presumably highly motivated to continue using the GnRH agonist or the LNG-IUS for the control of pain despite the occurrence of side-effects.

Higher bleeding scores were recorded in the LNG-IUS patients than in the GnRH agonist group. As observed in clinical trials on the use of the LNG-IUS for contraception (Hidalgo et al, 2002; Baldaszti et al, 2003), LNG-IUS patients experienced light, irregular bleeding during the initial months of use, which decreased after the 3rd to the 6th month. By the end of the study, 60% of the LNG-IUS patients were amenorrhoeic while all the GnRH agonist women had become amenorrhoeic by the second to third month of use.

Most of the women in this study were young and had not completed their family. These results suggest that the LNG-IUS is a possible treatment option for these women since it offers the advantage of retaining it for up to 5 years and providing effective contraception at the same time.

In conclusion, the results of the present study, although relatively from a small sample size, confirm that the LNG-IUS is as effective as the GnRH agonist in the treatment of minimal to moderate endometriosis as measured by VAS score and the stage and score of the disease before and after treatment. These were correlated with the clinical symptoms of endometriosis. However, there was no statistically significant difference in the efficacy between the medications. This randomized pilot study was only of small number and a larger number of women are needed to confirm that the LNG-IUS is as effective as the GnRH agonist in the treatment of women with minimal to moderate endometriosis.

Chapter VI

General Discussion and Future Directions

6.1 Discussions

This thesis was design into two parts:

- (a). an investigation of the potential mechanisms by which the LNG-IUS may act in alleviating symptoms in women with endometriosis and
- (b). a randomised controlled trial to compare the efficacy of the LNG-IUS and that of the gold standard GnRH agonist (decapetylTM) in the symptomatic treatment of minimal to moderate endometriosis.

In the first part of the study it was found that the ER- α , ER- β and PR (A&B) are down regulated by the LNG-IUS. Similar effect by the GnRH agonist (decapetylTM) were found in the comparative trial. PR-B was down regulated by the GnRH agonists but there was no significant difference in the PR-B expression in the glandular compartment of ectopic endometrium before and after treatment with the LNG-IUS. The absence of an effect in the glandular compartment of PR-B by the LNG-IUS needs further investigations as it maybe of importance in solving the frequent irregular spotting associated with it and the hypoestrogenic effects reported by patients who are treated with GnRH agonists.

When we also looked at the ER and PR expression on the phase of menstrual cycle, there was no change in their expression which was similar to other studies (Lessey et al, 1989;

Bergqvist, 1991; Prectice et al, 1992) but contrary to others (Bouchard et al, 1991; Snijders et al, 1992; Coppens et al, 1993).

In chapter three of this thesis, we found a decrease in the number of mast cells from the ectopic and eutopic endometrium following six months of treatment with the LNG-IUS. This might be an indirect response of the LNG-IUS to the inflammatory process which will subsequently affect the mast cells number. This was found not to be through the ER and PR. In this part of the study an assumption was made that the endometriotic biopsies taken before and after treatment represented the same site of the lesion which was very unlikely.

The changes in the ER and PR as well as a reduction in mast cell numbers in the eutopic and ectopic endometrial tissue are considered possible mechanisms of action of the LNG-IUS. The changes in ER and PR are consistent with those reported in the literature (Critchley et al, 1998; Petta et al, 2009), while the changes in mast cell numbers had not been reported before. ER and/or PR were not demonstrated in the mast cells found in the endometriotic lesions in contrast to several other studies that had demonstrated the presence of these receptors in mast cells present in other major organs of the human body [Pang et al, 1995; Zhao et al, 2001; Nicovani & Rudolph 2002). This would suggest that mast cells behave differently in various pathological conditions and in different organs of the body. The immune system dysfunction linked with this condition might also be a possible explanation of these differing results (Lebovic et al, 2001; Nothnick, 2001).

In chapter 5 of the study, despite an improvement in the clinical symptoms of the women with minimal to moderate endometriosis there was only a significance difference in the PF levels of IL-6 following a six month treatment with the LNG-IUS. There were no differences in PF levels of IL-6 and sICAM-1 with the GnRH agonist and no difference in the PF levels of sICAM-I with the LNG-IUS. The effect of the PF levels of IL-6 and sICAM-1 might be related to the phase of menstrual cycle although this has not been previously investigated. The effect of phase of menstrual cycle on cytokines needs to be investigated in future studies. Another reason for this discrepancy may be related to sample size and processing as some of the samples were stored for a longer time.

The clinical outcome results of this study, although relatively from a small sample size, confirm that the LNG-IUS is as effective as the GnRH agonist in the treatment of minimal to moderate endometriosis as measured by VAS score and the stage and score of the disease before and after treatment. These were correlated with the clinical symptoms of endometriosis. The adverse effects of the LNG-IUS and the GnRH agonist reported were as expected; however, there was no statistically significant difference in the efficacy between the medications.

6.2. Future directions

The results of the studies within this thesis have highlighted some of the possible mechanisms of action of the LNG-IUS in the symptomatic treatment of minimal to moderate endometriosis. They have also shown that the LNG-IUS is as effective as the gold

standard GnRH agonist in these women. These findings are clinically important and provide the basis for future research to improve both the understanding of these treatment modalities and bases for including the LNG-IUS as an effective long term treatment option.

The potential areas for future reseach include;

(1). investigating the changes in gene receptor (estrogen and progesterone) expression in endometriotic and endometrial tissues before and after symptomatic treatment of minimal to moderate endometriosis with the gold standard GnRH agonist and the LNG-IUS. This might be of benefit as gene therapy offers exciting promise in the treatment of many disorders.

(2). further analysis of the changes in gene expression patterns of endometriotic stroma and epithelial glandular cells before and after the LNG treatment may reveal other mechanisms of action and lead to a better and more focused treatment for peritoneal endometriosis.

(3). quantification and comparison of the changes and effects of various treatment options on macrophages before and after symptomatic treatment. Macrophages mediate the destruction of retrograde endometrial cell debris and are increased in the PF of patients with endometriosis (Badawy et al, 1984; Wu et al, 2002). Peritoneal macrophages isolated from patients with endometriosis tend to produce inflammatory cytokines and a poor cytolytic capability with their cytotoxic function affected by the stage of the disease (Steele et al, 1984; Braun et al, 1992; Dmowski et al, 1998; Chuang et al, 2009). As shown in the studies here, the LNG-IUS and the GnRH agonist improved the stage and score of endometriosis.

Hence the cytotoxic and cytolytic functions of the macrophages might have been modified in patients treated with these agents.

(4). a large study comparing the efficacy of the LNG-IUS and the GnRH agonist in women with minimal to moderate endometriosis to confirm the findings here.

(5). in normal endometrium, progesterone acts via PR on stromal cells to induce the secretion of paracrine factor(s) that in turn stimulate neighbouring epithelial cells to express the enzyme 17 β -hydroxysteroid dehydrogenase type 2 (17 β -HSD) type 2. 17 β -HSD type 2 is an extremely efficient enzyme and rapidly metabolizes the biologically potent estradiol to weakly estrogenic estrone. In endometriotic tissue, progesterone is incapable of inducing epithelial 17 β -HSD type 2 expression due to a defect in stromal cells. It would be useful to study the molecular effect of the LNG-IUS on 17 β -HSD type 2 and to determine if LNG ameliorates the induction of paracrine factors which increase the production of the enzyme 17 β -HSD type 2 and the metabolism of estrogen.

(6). Before definite conclusions can be made on the effect of these treatment modalities on PF cytokine levels, it would be useful to repeat the studies with larger numbers and reduced the interval from collection to processing of samples. Such a study may provide evidence to support a possible mechanism through which the LNG-IUS modulates symptoms of endometriosis.

Appendices

Appendix I

AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE.

REVISED CLASSIFICATION OF ENDOMETRIOSIS.

Patient Name: _____ Date _____

Stage I (Minimal) 1-5 Laparoscopy _____ Laparotomy _____ Photography _____

Stage II (Mild) 6-15 Recommended Treatment _____

Stage III (Moderate) 16-40 _____

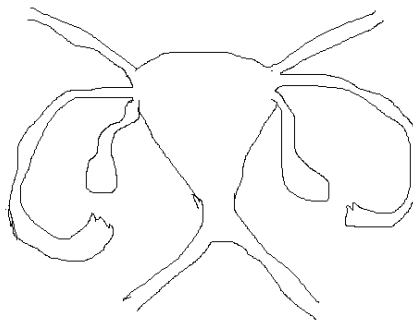
Stage IV (Severe) >40 Prognosis _____

Total _____

	ENDOMETRIOSIS	<1CM	1-3CM	>3CM
PERITONEUM	Superficial	1	2	4
	Deep	2	4	6
OVARY	R Superficial	1	2	4
	Deep	4	16	20
	L Superficial	1	2	4
	Deep	4	16	20
	POSTERIOR CUL DE SAC OBLITERATION	Partial	Complete	
		4	40	
OVARY	ADHESIONS	<1/3 Enclosure	1/3-2/3-Enclosure	>2/3 Enclosure
	R Filmy	1	2	4
	Dense	4	8	16
	L Filmy	1	2	4
	Dense	4	8	16
TUBE	R Filmy	1	2	4
	Dense	4	8	16
	L Filmy	1	2	4
	Dense	4	8	16

.If fimbriated end of the fallopian tube is completely enclosed; change the point assignment to 16.

Denote appearance of superficial implant types as red (R), red, red-pink, flamelike, vesicular blobs, clear vesicles, white [(W), opacifications, peritoneal defects, yellow-brown], or black [(B) black, hemosiderin deposits, blue]. Denote percent of total described as R — %, W— % and B —%. Total should equal 100%.

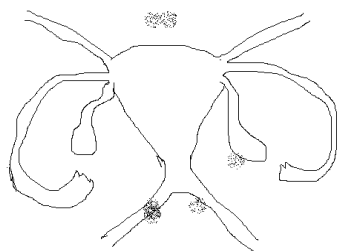


L

R

Normal uterus, tubes and ovaries.

STAGE I (MINIMAL)



PERITONEUM

Superficial Endo - 1-3cm -2

R. OVARY

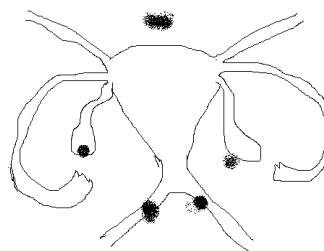
Superficial Endo - <1cm -1

Filmy Adhesions - <1/3 -1

TOTAL POINTS ---

4

STAGE II (MILD)



PERITONEUM

Deep Endo - >3cm -6

R.OVARY

Superficial Endo - <1cm -1

Filmy Adhesions - <1/3 -1

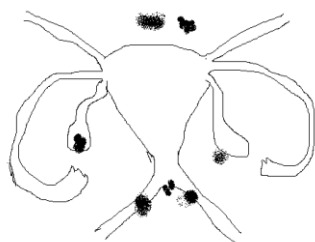
L. OVARY

Superficial Endo. - <1cm -1

TOTAL POINTS ---

9

STAGE III (MODERATE)



PERITONEUM

Deep Endo - >3cm -6

CUL DE SAC

Partial Obliteration -4

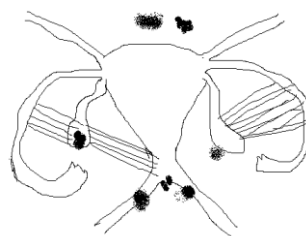
L. OVARY

Deep Endo - 1-3cm -16

TOTAL POINTS ---

26

STAGE IV (SEVERE)



PERITONEUM

Superficial Endo. - >3cm -4

L. OVARY

Deep Endo 1-3cm -32

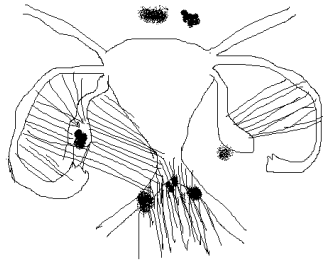
Dense Adhesions - <1/3 -8

L. TUBE

Dense Adhesions - <1/3 -8

TOTAL POINTS 52

STAGE IV (SEVERE)



PERITONEUM

Deep Endo - >3cm -6

Point assignment changed to 16

UL DE SAC

Point assignment doubled

Complete Obliteration -40

R. OVARY

Deep Endo - 1-3cm -16

Dense Adhesions - <1/3 -4

L. TUBE

Dense Adhesions - >2/3 -16

L. OVARY

Deep Endo - 1-3cm -16

Dense Adhesions - >2/3 -16

TOTAL POINTS 114

Determination of the stage or degree of endometrial involvement is based on a weighted point system. Distribution of points has been arbitrarily determined and may require further revision or refinement as knowledge of the disease increases.

To ensure complete evaluation, inspection of the pelvis in a clockwise or counterclockwise fashion is encouraged. Number, size and location of endometrial implants, plaques, endometriomas and/or adhesions are noted. For example, five separate 0.5cm superficial implants on the peritoneum (2.5cm total) would be assigned 2 points. (The surface of the uterus should be considered peritoneum). The severity of the endometriosis or adhesions should be assigned the highest score only for peritoneum, ovary, tube or cul de sac. For example, a 4cm superficial and a 2cm deep implant of the peritoneum should be given a score of 6 (not 8). A 4cm deep endometrioma of the ovary associated with more than 3cm of superficial disease should be scored 20 (not 24).

In those patients with only one adnexa, points applied to disease of the remaining tube and ovary should be multiplied by two. " Points assigned may be circled and totalled. Aggregation of points indicates stage of disease (minimal, Mild, Moderate, or severe).

The presence of endometriosis of the bowel, urinary tract, fallopian tube, vagina, cervix, skin etc., should be documented under "additional endometriosis." Other pathology such as tubal occlusion leiomyomata, uterine anomaly, etc. should be documented under "associated pathology." All pathology should be depicted as specifically as possible on the sketch of pelvic organs, and means of observation (laparoscopy or Laparotomy) should be noted.

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REPRODUCE BY DR SAMUEL ENGEMISE

Appendix II

DIRECTORATE OF RESEARCH AND DEVELOPMENT

Leicester Royal Infirmary

Leicester
LE1 5WW

Direct Dial: (0116) 250 2304
Fax No: (0116) 250 2303
e-mail : aimee.geary@uhl-tr.nhs.uk


Tel: 0116 2541414
Fax: 0116 2585631
Minicom: 0116 2586878

27 September 2000

Dr J.C. Konje
Senior Lecturer
Obstetrics Gynaecology
LRI

Dear Dr Konje

RE: Project Number: 1426 *[Please quote this number in all correspondence]*
A pilot observational trial of the efficacy of the levonorgestrel containing intrauterine contraceptive device (Mirena) in the treatment of endometriosis.

We have now been notified by the Ethical Committee that this project has been given ethical approval (~~please see the attached letter from the Ethical Committee~~). 

Since all other aspects of your LRI R&D notification are complete, I now have pleasure in confirming full approval of the project on behalf of the University Hospitals of Leicester NHS Trust.

This approval means that you are fully authorised to proceed with the project, using all the resources which you have declared in your notification form.

The project is also now covered by Trust Indemnity, except for those aspects already covered by external indemnity (e.g. ABPI in the case of most drug studies).

We will be requesting annual and final reports on the progress of this project, both on behalf of the Trust and on behalf of the Ethical Committee.

In the meantime, in order to keep our records up to date, could you please notify the Research Office if there are any significant changes to the start or end dates, protocol, funding or costs of the project.

I look forward to the opportunity of reading the published results of your study in due course.

Yours sincerely



Dr Nichola Seare
Research and Development Business Manager

cc Sharon Oliver

Appendix III

Study Number: _____
Patient Identification Number for this trial: _____

CONSENT FORM

Title of Project: *Randomised controlled trial of the levonorgestrel intrauterine system (Mirena®) versus a Gonadotrophin releasing hormone (GnRH) agonist in the treatment of symptoms of endometriosis*

Name of Researcher / Principal Investigator: Professor Konje

- Please initial box
1. I confirm that I have read and understand the information sheet dated 20/02/06 version 1 for the above study and have had the opportunity to ask questions. ☐
 2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
 3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the research team, or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records ☐
 4. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐
 5. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐

Participant Consent Form

Version 1 dated 20/02/06

Ref: 06-Q2502-28cf-p060220
Issued 15th December 2005

6. I understand that tissue samples and associated clinical data may be transferred to non-commercial research partners of the University Hospitals of Leicester NHS Trust and Leicester University, but that the information will be anonymised prior to transfer. ☐
7. I understand that if research using my tissues produces information, which has immediate clinical relevance to me, I will be informed by my hospital consultant or GP and be given an opportunity to discuss the results. ☐
8. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐
9. The samples which I hereby consent to donate are (delete from the list):
a biopsy from the lining of my womb, a biopsy from the endometriosis and a biopsy from the lining of the inside of my tummy (which is free of endometriosis), blood and fluid which is collected from my tummy ☐
10. I agree to take part in the above study. ☐

_____ Name of Patient	_____ Date	_____ Signature
_____ Name of Person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Researcher	_____ Date	_____ Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Participant Consent Form

Version 1 dated 20/02/06

Ref: 06-Q2502-28cf-p060220
Issued 15th November 2005

Appendix IV

Mirena in Endometriosis Endometriosis Study Diary

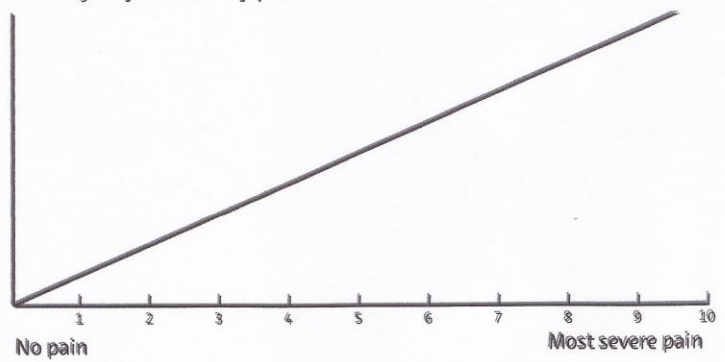
**Leicester Royal Infirmary
Department of Obstetrics & Gynaecology**

Study Number

--	--	--

Study Number: Date: 1st day of last menstrual period

Mark a cross along this line to indicate how you gauge the severity of your tummy pain.



Patient: Diary: Study Number:

NOTES

(Please note down anything you think may be relevant)

Patient: ☐☐☐

Diary: ☐☐

Keeping a Diary

Thank you for agreeing to take part in our study.
We would be very grateful if you could fill in one column of the diary each day—as shown in the following (Example) page.

We would like to know each day if you have :

- had any abdominal, pelvic or back pain and how severe the pain is
- had any menstrual bleeding and how heavy it is
- used any sanitary towels or tampons

The doctor you have seen at the clinic, Dr _____, will be happy to answer any questions you may have about filling in the diary.

Alternatively, Dr can be contacted at Leicester Royal Infirmary concerning the diary or any other aspect of your current gynaecological treatment.

The contact telephone number is: **0116 2541414** Bleep No:




Example




Week Beginning 5th April

Study Number: Patient: Diary:

Monday Tuesday Wednesday Thursday Friday Saturday Sunday
5 6 7 8 9 10 11

Abdominal / Pelvic / Back Pain							
None					✓	✓	✓
Mild				✓			
Moderate		✓	✓				
Severe	✓						

Towels Used							
 Include Spotting							
							
							

Tampons Used							
 Include Spotting							
							
							

Clots/ Flooding							
--------------------	--	--	--	--	--	--	--

Additional Medication for pain							
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	




Week Beginning *5th April*




Study Number: Patient:

Diary:

Monday Tuesday Wednesday Thursday Friday Saturday Sunday
5 6 7 8 9 10 11

Abdominal / Pelvic / Back Pain							
None							
Mild							
Moderate							
Severe							

Towels Used							
							
Include Spotting							
							
							

Tampons Used							
							
Include Spotting							
							
							

Clots/ Flooding		10% size dot and Flooding	20% size dot and Flooding	Rein size dot			




Additional Medication for pain							
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	

Week Beginning Sat. 1st Oct Study Number: Patient: Diary: ☐☐

Monday Tuesday Wednesday Thursday Friday Saturday Sunday
2 3 4 5 6 7 8

Abdominal / Pelvic / Back Pain							
None							
Mild							
Moderate							
Severe							

Towels Used							
 Include Spotting							
							
							

Tampons Used							
 Include Spotting							
							
							

Clots/ Flooding							
--------------------	--	--	--	--	--	--	--

Additional Medication for pain							
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	




Week Beginning *6/6/2012*

Study Number: Patient:

Diary:

Monday Tuesday Wednesday Thursday Friday Saturday Sunday
5 6 7 8 9 10 11

Abdominal / Pelvic / Back Pain							
None							
Mild							
Moderate							
Severe							

Towels Used							
 Include Spotting							
							
							

Tampons Used							
 Include Spotting							
							
							

Clots/ Flooding							
--------------------	--	--	--	--	--	--	--




Additional Medication for pain							
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	

Week Beginning Sat. 1st

Study Number: Patient: Diary:

Monday Tuesday Wednesday Thursday Friday Saturday Sunday
1 2 3 4 5 6 7

Abdominal / Pelvic / Back Pain							
None							
Mild							
Moderate							
Severe							

Towels Used							
 Include Spotting							
							
							

Tampons Used							
 Include Spotting							
							
							

Clots/ Flooding							
--------------------	--	--	--	--	--	--	--

Additional Medication for pain							
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	

APPENDIX V

VA. Patient Characteristics and samples collected (2000-2002) in the LNG-IUS group

No	MP	SE	S ₀	S ₆	PF ₀	PF ₆	T ₀	T ₆
002	MS	mild	6	4	24	23	Eu, Ec	Eu, Ec
003	LP	mild	7	3	1.25	2.20	Eu, Ec	Eu, Ec
004	ES	moderate	37	16	27	BS	Eu, Ec	Eu, Ec
005	LS	mild	13	6	10	11.40	Eu, Ec	Eu, Ec
006	ES	mild	7	10	20	23.25	Eu, Ec	Eu, Ec
007	MS	mild	10	-	21	-	Eu, Ec	Eu
008	MS	moderate	29	11	7.50	18.50	Eu, Ec	Eu, Ec
009	LP	minimal	6	10	4	BS	Eu, Ec	Eu, Ec
010	EP	minimal	4	-	3	-	Eu, Ec	Eu
011	LS	mild	18	8	1	0.30	Eu, Ec	Eu, Ec
012	LS	mild	11	12	15.50	10.55	Eu, Ec	Eu, Ec
013	MS	moderate	27	33	8	1.70	Eu, Ec	Eu, Ec
014	EP	minimal	3	5	1.90	8.35	Eu, Ec	Eu, Ec
015	ES	mild	15	-	15.50	4.95	Eu, Ec	Eu, Ec
016	LS	mild	6	-	9	-	Eu, Ec	Eu
017	LS	mild	14	3	4.25	6	Eu, Ec	Eu, Ec
018	ES	moderate	36	14	1.35	0.87	Eu, Ec	Eu, Ec
019	MS	moderate	25	20	12.25	6.50	Eu, Ec	Eu, Ec
020	MP	mild	12	-	14.30	-	Eu, Ec	Eu
022	LS	mild	7	4	16.25	19.20	Eu, Ec	Eu, Ec
023	ES	moderate	23	-	7.10	-	Eu, Ec	Eu
025	MS	minimal	5	3	8.65	0.75	Eu, Ec	Eu, Ec
026	EP	mild	7	6	10.35	8.20	Eu, Ec	Eu, Ec
027	ES	mild	7	4	14	6.92	Eu, Ec	Eu, Ec
028	LP	moderate	21	15	20	2.95	Eu, Ec	Eu, Ec
031	LS	minimal	5	17	11.15	7.40	Eu, Ec	
033	MS	mild	6	9	36	3.50	Eu, Ec	Eu, Ec
035	LP	minimal	1	1	12	3	Eu, Ec	Eu, Ec

No=Patient Number, MP=Menstrual phase, SE=stage of endometriosis, S₀=Score before treatment, S₆=Score after 6 months of treatment, PF₀=Amount of peritoneal fluid before treatment, PF₆=Amount of peritoneal fluid after 6 months of treatment, T₀=Tissue collected before treatment, T₆=Tissue collected after 6 months of treatment, Eu=Eutopic endometrium, Ec=Ectopic endometrium, ES= Early secretory phase, MS=Mid secretory phase, LS=late secretory phase, EP=Early proliferative phase, MP=Mid proliferative phase, LP=Late proliferative phase BS= Blood stained.

VB. Patient Characteristics and samples collected (2007-2009) in the LNG-IUS and the GnRH agonist

No	MP	SE	S ₀	S ₆	PF ₀	PF ₆	T ₀	T ₆
001D	EP	minimal	5	-	16	-	Eu, Ec	-
002D	LS	mild	8	3	2	13	Eu, Ec	Eu, Ec
003C	LP	moderate	25	-	32	-	Eu, Ec	-
004C	ES	mild	14	8	4	50	Eu, Ec	Eu, Ec
005D	MS	mild	16	-	1.80	-	Eu, Ec	-
006C	MS	mild	7	4	35	12	Eu, Ec	Eu, Ec
007D	LP	moderate	18	8	3	1	Eu, Ec	Eu, Ec
008D	EP	minimal	5	0	5	3.5	Eu, Ec	Eu, Ec
009C	MS	moderate	23	17	0	1	Eu, Ec	-
010C	LP	mild	12	-	7	-	Eu, Ec	-
011D	MP	moderate	16	8	0	0	Eu, Ec	Eu, Ec
012C	EP	minimal	5	0	1	3.5	Eu, Ec	Eu, Ec
013D	MP	moderate	18	6	-	10.5	Eu, Ec	Eu, Ec
014C	LS	moderate	17	16	BS	2	Eu, Ec	Eu, Ec
015C	ES	minimal	3	0	6.5	3	Eu, Ec	Eu, Ec
016C	LP	mild	13	-	BS	-	-	-
017D	LP	mild	9	3	14	15	Eu, Ec	Eu, Ec
018D	LS	mild	11	3	14.1	1	Eu, Ec	Eu, Ec
019C	ES	moderate	19	-	-	-	Eu, Ec	-
020C	EP	minimal	3	0	23.5	10	Eu, Ec	Eu, Ec
021D	MS	mild	14	5	33	3	Eu, Ec	Eu, Ec
022D	EP	moderate	22	7	4.5	-	Eu, Ec	Eu, Ec
024C	EP	moderate	23	13	15	8.5	Eu, Ec	Eu, Ec
025C	LS	moderate	16	-	6	-	Eu, Ec	-
026D	LS	miminal	3	1	6.5	12	Eu, Ec	Eu, Ec
027C	MP	minimal	5	2	8	1	Eu, Ec	Eu, Ec
028D	MS	moderate	19	-	6	-	-	-

No=Patient Number, MP=Menstrual phase, SE=stage of endometriosis, S₀= Score before treatment, S₆=Score after 6 months of treatment, PF₀=Amount of peritoneal fluid before treatment, PF₆=Amount of peritoneal fluid after 6 months of treatment, T₀=Tissue collected before treatment, T₆=Tissue collected after 6 months of treatment, Eu=Eutopic endometrium, Ec=Ectopic endometrium, ES= Early secretory phase, MS=Mid secretory phase, LS=late secretory phase, EP=Early proliferative phase, MP=Mid proliferative phase, LP=Late proliferative phase, BS=Blood stained, D=decapeptyl or GnRH agonist, C=Coil or LNG-IUS.

Published articles removed:

Due to third party copyright restrictions the published articles have been removed from the appendix of the electronic version of this thesis. The unabridged version can be consulted, on request, at the University of Leicester's David Wilson Library.

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