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Karolinska Institutet, Stockholm, Sweden

PATHOPHYSIOLOGICAL FACTORS AND GENETIC ASSOCIATION IN ENDOMETRIOSIS

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When it is dark enough, you can see the stars.

/Ralph Waldo Emerson
ABSTRACT

Introduction: Endometriosis is a common benign gynecological disease where endometrial tissue forms lesions outside the uterine cavity. Endometriosis is estimated to affect about 10% of women of reproductive age, rising to 20-40% in patients with infertility, with a significant impact on physical, mental and social well-being of those affected. Today there is no cure for this disease and the most common medical treatments are not suitable for long term treatment due to side-effects. Although endometriosis is a well-known disease, the pathogenesis remains unclear. The most accepted theory about the pathogenesis is Sampson’s theory about retrograde menstruation. However, different properties, such as adhesion, invasion and proliferation of the shed menstrual cells seem to play an important role in the development of endometriosis. Also, altered immune surveillance, stem cells and genetic predisposition could be involved in the pathogenesis.

Aims: The overall aim of this thesis was to study the aetiology and pathophysiology of endometriosis, specifically the inflammatory profile in the follicular fluid of in vitro fertilization (IVF) patients and adhesion, attachment and invasion factors in endometriosis. Furthermore, the aim was to investigate the genetic background of endometriosis and a possible genetic linkage to rheumatoid arthritis (RA) and ovarian cancer.

Results: We found that women with endometriosis undergoing IVF have lower levels of anti-mullerian hormone (AMH) and a lower fertilization rate. These women also have an increased inflammatory profile in the follicular fluid. We observed genetic association of two RA-associated SNPs in CCL21 and HLA-DRB1 in women with moderate/severe disease. However, we could not observe any association of the ovarian cancer associated SNPs in the BNC2 gene. In addition, we found expression of ApoE, ITGB2, ITGB7, LAMC1, CD24 and JAM-1, in endometrium from healthy controls, endometriosis patients and in endometriomas.

Conclusions: Our results support a strong inflammatory component in endometriosis, which may affect the ovarian reserve and lead to infertility problems. Furthermore, genetic associations in inflammatory related genes were found in women with moderate/severe disease. An aberrant expression of factors involved in adhesion, attachment and invasion may be important in the establishment of endometriotic lesions and may at least partly be regulated by inflammatory mediators.
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LIST OF ABBREVIATIONS

AMH  Anti-mullerian hormone
ApoE  Apolipoprotein E
ASRM  American Society of Reproductive Medicine
BMI  Body mass index
BNC2  Basonuclin 2
CA125  Cancer antigen 125
CCL21  Chemokine CC motif ligand 21
cDNA  Complementary deoxyribonucleic acid
CNV  Copy number variants
CT  Threshold cycles
DNA  Deoxyribonucleic acid
dNTP  Deoxynucleotide
EAOC  Endometriosis-associated ovarian cancer
ECM  Extracellular matrix
EIA  Enzyme immunoassay
FSH  Follicle stimulating hormone
GM-CSF  Granulocyte-macrophage stimulating factor
GnRH  Gonadotropin-releasing hormone
GWAS  Genome-wide association studies
hCG  Human Chorionic Gonadotrophin
HLA  Human leukocyte antigen
HWE  Hardy-Weinberg equilibrium
ICSI  Intracytoplasmic sperm injection
IFN-γ  Interferon γ
IL  Interleukin
IRF5  Interferon regulatory factor 5
IRMA  Immunoradiometric assay
ITGB2  Integrin β2
ITGB7  Integrin β7
IVF  In vitro fertilization
JAM-1  Junctional adhesion molecule-1
K-ras  Kirsten rat sarcoma viral oncogene homologue
L1CAM  L1 cell adhesion molecule
LAMC1  Laminin γ-1
LD  Linkage disequilibrium
LDL  Low density lipoprotein
LNG-IUS  Levonorgestrel-releasing intrauterine system
LOH  Loss of heterozygosity
MAF  Minor allele frequency
MCP-1  Monocyte chemotactic protein-1
MHC  Major histocompatibility complex
MIF  Macrophage migration inhibitory factor
MMPs  Matrix metalloproteinases
MRI  Magnetic resonance imaging
mRNA  Messenger ribonucleic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatidylinositol-3, 3’, 5’-triphosphate 3-phosphate</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T-cell Expressed and Secreted</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SSRs</td>
<td>Simple sequence repeats</td>
</tr>
<tr>
<td>STAT4</td>
<td>Signal transducer and activator of transcription 4</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of matrix metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TRAF1-C5</td>
<td>TNF receptor-associated factor 1-complement component 5</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1 POPULÄRVETENSKAPLIG SAMMANFATTNING


Vi har studerat resultat av assisterad befruktning (IVF) hos kvinnor med endometrios i jämförelse med friska kvinnor med infertilitet på grund av skadade äggledare. Dessutom har vi tittat på inflammatoriska markörer i follikelvätskan, dvs. den vätska som omger de mognade äggen i äggstocken. Vi såg att kvinnorna med endometrios hade ökade nivåer av pro-inflammatoriska markörer (TNF-α, IL-15 and GM-CSF) och sänkta nivåer av en anti-inflammatorisk markör (IL-10). Dessa kvinnor hade även lägre nivåer av AMH, en markör för äggreserven i äggstocken, i follikelvätskan och serum, vilket tyder på att kvinnor med endometrios har färre ägg som kan mognas och bli befruktade. Vi kunde även se att kvinnor med endometrios svarade bra på IVF behandlingen, men att befruktning av deras ägg var sämre än i kontrollgruppen.

Kvinnor med endometrios har en ökad inflammatorisk aktivitet och man har sett att dessa kvinnor i en högre utsträckning drabbas av autoimmuna sjukdomar, bl.a. reumatism. Samband har hittats mellan olika genetiska variationer och olika autoimmuna sjukdomar. Vi ville därför undersöka om de genetiska variationer som är kopplade till reumatism även är kopplade till endometrios. Vi valde att titta på variationer i gener som är involverade i inflammation, men inget samband kunde hittas mellan endometrios och dessa variationer. Däremot, när vi delade upp kvinnorna med endometrios i två grupper beroende på sjukdomens svårighetsgrad, såg vi en koppling mellan variationer i två olika gener (CCL21 och HLA-DRB1) och moderat/svår endometrios.

Ett samband mellan endometrios och ovarialcancer har också hittats. Det är möjligt att endometrios på äggstockarna skulle kunna omvandlas direkt till cancer. En annan teori är att endometrios och ovarialcancer delar samma mekanismer eller genetiska variationer som gör att drabbade kvinnor lättare utvecklar sjukdomarna. Genetiska variationer i genen BNC2 har tidigare kopplats till ovarialcancer och dessa variationer
skulle även kunna vara kopplade till endometrios. I vår studie kunde vi inte hitta någon koppling till sjukdomen.

Vi har även studerat olika faktorer som påverkar cellernas förmåga att fästa och invadera annan vävnad. Vi såg att alla dessa faktorer (ApoE, ITGB2, ITGB7, LAMC1, CD24 and JAM-1) fanns i endometrium från friska kvinnor, i endometrium från kvinnor med endometrios och i endometriosvävnad. Olika uttryck av dessa faktorer hos friska kvinnor och kvinnor med endometrios skulle kunna göra att den vävnad som hamnar i buken under mensen kan fästa och invadera och därmed leda till att endometrioshärdar bildas.

2 INTRODUCTION

2.1 ENDOMETRIOSIS

Endometriosis is a benign gynecological disease, characterized by the presence of endometrial stroma and glands outside the uterine cavity. It occurs mostly as peritoneal surface lesions, ovarian lesions and deeply infiltrating lesions of the rectovaginal septum or gut. Endometriosis located within the muscular wall of the uterus is called adenomyosis. Endometriosis is associated with chronic pelvic pain, dysmenorrhea, dyspareunia, infertility and inflammation, although not all women with endometriosis are symptomatic.

Endometriosis affects about 10% of women of reproductive age (reviewed in (Eskenazi and Warner, 1997)), from all ethnic and social groups. About 20-40% (1994, Strathy, et al., 1982, Verkauf, 1987) of women with infertility have endometriosis. Thus, the disease also has a major impact on general physical, mental and social well-being (Jones, et al., 2004, Lorencatto, et al., 2006). In addition, endometriosis is a large healthcare problem and imposes a substantial economic burden on society. Simoens et al. estimated that the annual cost of endometriosis in the USA in 2002 was about 22 billion USD (Simoens, et al., 2007). However, not only hospital visits are costly, but also indirect cost, such as time lost from work due to the disease. Hummelshoj et al. reported that 78% of women with endometriosis in UK lose a mean of 5.3 days of work per month, due to their disease (Hummelshoj, et al., 2006).

2.1.1 Diagnosis and staging of endometriosis

Establishing a diagnosis of endometriosis is difficult. Therefore, the time between onset of symptoms and diagnosis can take several years. In USA and UK, the delay of diagnosis is about 7-12 years (Hadfield, et al., 1996). Today, laparoscopy is the gold standard for diagnosis. Noninvasive techniques, such as ultrasound and magnetic resonance imaging (MRI), could also be used to diagnose some types of endometriosis (reviewed in (Brosens, et al., 2004)). Currently, there are no reliable biomarkers for the diagnosis or prognosis of endometriosis. Different markers in serum and peritoneal fluid, including cancer antigen 125 (CA125) (Colacurci, et al., 1996) and cytokines such as Interleukin 6 (IL-6) and tumor necrosis factor (TNF)-α (Bedaiwy, et al., 2002), have been suggested. Although promising results have been shown (Ceyhan, et al., 2010, Seeber, et al., 2010), further studies are needed to evaluate the relevance of these biomarkers for diagnosis. Interestingly, a recent study by Tokushige et al. demonstrated that Cytokeratin-19 in urine could be a possible biomarker (Tokushige, et al., 2011).

The severity of endometriosis is assessed by the revised classification system developed by the American Society of Reproductive Medicine (ASRM) (1997), using four stages (I-IV or minimal to severe disease), on the basis of type, location, appearance, depth of invasion and extent of disease. However, there is no correlation between stage and severity of pain symptoms or prediction of infertility treatment.
The extent of endometriosis varies from small lesions to large ovarian endometriotic cysts (endometriomas or chocolate cysts) with extensive fibrosis and adhesions leading to distortion of the pelvic anatomy. Endometrial lesions are mainly divided into three types; 1) peritoneal lesions on the peritoneal surface, 2) deep infiltrating lesions located at least five mm under the peritoneum and 3) ovarian endometriotic cysts. The lesions typically appear as red, black/blue or white, representing different steps in the evolutionary process of endometriosis (reviewed in (Nisolle and Donnez, 1997)). Red lesions are considered to be the first stage, as they are most active and highly vascularized. When these lesions bleed and accumulate blood, they turn into black/blue lesions, a characteristic for advanced endometriosis. White lesions are considered healed and latent, but may remodel into more active lesions (D’Hooghe, et al., 1992).

Infiltration of nerve fibers (Tokushige, et al., 2006, Zhang, et al., 2010) and neuroendocrine cells (Wang, et al., 2010) have been observed in endometriotic lesions. Over-expression of nerve growth factor (NGF) was found in peritoneal fluid from women with endometriosis, which also promoted neurite outgrowth in vitro (Barcena de Arellano, et al., 2010). Interestingly, Al-Jefout et al. observed that detection of nerve fibers in endometrial biopsies provided a reliable diagnosis of endometriosis (Al-Jefout, et al., 2009).

2.1.2 Treatment of endometriosis

Endometriosis can be treated by surgery and/or pharmacologically, with three aims: to reduce pain, increase fertility/pregnancy and delay recurrence as long as possible. Most pharmacological treatments are based on hormonal suppression, leading to a hypo-estrogenic state and the elimination or reduction in size of the endometriotic lesions. Pharmacological treatment includes progestins, danazol, gonadotropin-releasing hormone (GnRH) analogues, the levonorgestrel-releasing intrauterine system (lng-IUS) and oral contraceptives. Some treatment, like the lng-IUS (Mirena) only alleviates pain, but does not affect the disease (reviewed in (Bahamondes, et al., 2007)). Hormonal suppression with certain drugs, such as GnRH, for a longer time period, leads to undesirable side effects due to a systemic estrogen deficiency.

Other pharmacological drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat chronic pain in endometriosis patients. Regression of lesion size in rats has been observed during treatment with an aromatase inhibitor (letrozole) and reduction of pain symptoms in humans has been observed in the clinic with treatment of either aromatase inhibitors alone or in combination with oral contraceptives (Seal, et al., 2011, Verma and Konje, 2009).

Alternative non-hormonal targets for treatment of endometriosis have been suggested, such as immune-modulators, anti-angiogenic agents and anti-inflammatory drugs. For example, treatment with anti-TNF antibody has been shown to reduce the extent of endometriosis in baboons (Falconer, et al., 2006).

Surgical removal of endometriotic lesions aims at reducing pain by removing the endometriotic tissue and to restore normal pelvic anatomy. Although surgery
effectively alleviates pain (reviewed in (Jacobson, et al., 2009)), endometriotic lesions usually recur within a few years. Also, surgery of ovarian endometriosis is associated with decreased ovarian reserve (Ragni, et al., 2005).

2.1.3 Is endometriosis one disease or several?

Different types of endometriosis can be observed in the pelvis. It is not clear if peritoneal endometriosis is an early stage of the disease, later developing into ovarian and deep infiltrating endometriosis, or if these types of endometriosis could represent three different diseases with different etiologic mechanisms (reviewed in (Nisolle and Donnez, 1997)). It has also been speculated whether mild endometriosis should be regarded as a natural condition occurring in all women (Koninckx, et al., 1994). Gene expression studies have reported differences between ovarian endometriosis and deep infiltrating endometriosis, indicating that the local endocrine control and hormonal microenvironment could differ between these two types of disease (Matsuzaki, et al., 2006). This is strengthened by varied expression of aromatase between different types of endometriosis (Heilier, et al., 2006). A genetic study further supports this theory by reporting genetic association with protein tyrosine phosphatase non-receptor type 22 (PTPN22) and endometriosis, but only in women with moderate/severe disease (Gomes, et al., 2010).

2.2 PATHOGENESIS OF ENDOMETRIOSIS

The pathogenesis of endometriosis is not fully understood. Several different theories have been suggested:

- The implantation/transplantation theory indicates the development of endometriosis by the deposit of viable endometrial cells into the peritoneal cavity by retrograde menstruation through the fallopian tubes, where the endometrial cells are able to attach and grow (Sampson, 1927).

- The induction theory suggests that degenerating endometrium in the abdominal cavity releases factors and thus inducing a metaplastic process in mesenchymal cells, leading to endometriosis (Levander and Normann, 1955).

- According to the in situ development theory, ectopic endometrium develops in situ from local tissue, including remnants of the Wolffian and Mullerian ducts (Lauchlan, 1972).

The most widely accepted theory today is the implantation theory proposed by Sampson (Sampson, 1927), which is supported by several observations. Firstly, women with endometriosis have been shown to have shorter cycles (Arumugam and Lim, 1997) and heavier menstrual flow (Vercellini, et al., 1997). Secondly, viable cells in the endometrial effluent have also been observed (Keettel and Stein, 1951). In addition, Ridley and Edwards showed that endometrial tissue, collected from menstrual endometrium, could attach and proliferate in ectopic locations (Ridley and Edwards, 1958). Thirdly, patients with obstructed menstrual outflow have an increased risk of
developing endometriosis (Olive and Henderson, 1987). This has also been confirmed by D’Hooghe et al., who showed that cervical ligation in baboons leads to endometriosis (D’Hooghe, et al., 1994). Furthermore, a correlation between the amount of endometrium used for induction and the extent of endometriosis was observed in baboons (D’Hooghe, et al., 1995). These observations strongly indicate an important role of retrograde menstruation in the development of endometriosis.

It is well known that retrograde menstruation occurs in most women, with an incidence of 76-90% (Halme, et al., 1984, Liu and Hitchcock, 1986), but not all women develop endometriosis. Therefore, the development of endometriosis is likely to not only involve retrograde menstruation, but also other factors such as adhesion and invasion of endometrial cells, proliferation, angiogenesis and immune escape, seen in figure 1. Furthermore, a genetic predisposition seems to be involved.

**Figure 1.** Different factors involved in the pathogenesis of endometriosis. (Reprinted from Matarese et al. Trends Mol Med. 2003 May;9(5):223-8 with permission from Elsevier.)
2.2.1 Adhesion, invasion and proliferation of endometrial cells

Apart from only being present in the peritoneal cavity, endometrial cells are able to attach and invade the peritoneum and proliferate to create endometriotic lesions. It appears that both endometrial stromal cells and epithelial cells are able to attach to the peritoneal membrane (Witz, et al., 2001). Evidence of invasion of endometriotic lesions has also been demonstrated by Spuijbroek et al. (Spuijbroek, et al., 1992). The invasion of endometrial cells requires local degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs). In normal endometrium MMPs and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs), are needed for the control of tissue breakdown and a higher expression of these factors is seen during the menstrual phase (reviewed in (Pitsos and Kanakas, 2009)). Aberrant expression of MMPs and TIMPs has been seen in endometriosis with increased levels of MMP-9 in endometrium from women with endometriosis (Collette, et al., 2006). Also, the ratio of MMP-9/TIMP-1 protein and mRNA levels were increased. This is strengthened by other studies, which have found decreased levels of TIMP-1 in peritoneal fluid (Szamatowicz, et al., 2002) and higher mRNA levels of MMP-2, -9 and -14 in endometriotic cells (Ueda, et al., 2002). In addition, MMPs are regulated by steroid hormones and cytokines, which are important regulators of endometriosis (reviewed in (Pitsos and Kanakas, 2009)). Interleukin-1 (IL-1) increases the secretion of MMP-3 in endometrial cells from women with endometriosis (Sillem, et al., 2001). Endometrial stromal cells treated with IL-8 showed increased MMP-2 and -9 activities and invasiveness (Mulayim, et al., 2004).

Cells from endometriotic lesions have demonstrated increased capacity to proliferate compared to endometrial cells from women without endometriosis (Li, et al., 1993, Nisolle, et al., 1997). Klemmt et al. demonstrated that stromal cells from endometriotic lesions and from the endometrium of women with endometriosis had an increased adhesive and proliferative ability in response to specific ECM components (Klemmt, et al., 2007).

Cell adhesion molecules facilitate cell-cell and cell-ECM adhesion. It has been suggested that cell adhesion molecules, such as integrins and cadherins, are involved in the development of endometriosis. The adhesion of endometrial cells in endometriosis is likely to be modulated in connection with ECM molecules. Integrins, cadherins, laminin and fibronectin have been observed in the endometrium and in endometriosis (Beliard, et al., 1997, van der Linden, et al., 1994, van der Linden, et al., 1994). Beliard et al. reported a stronger expression of integrin α5 by stromal cells in endometriosis, compared to stromal cells in healthy endometrium (Beliard, et al., 1997). Furthermore, decreased expression of N-cadherin (Van Patten, et al., 2010), but also E-cadherin and CD44 (Poncelet, et al., 2002), have been observed in peritoneal endometriosis. Other factors, such as L1 cell adhesion molecule (L1CAM) may also be involved in the development of endometriosis (Finas, et al., 2008). Inhibition of L1CAM in an endometriosis epithelial cell line leads to decreased cell proliferation and invasion (Agic, et al., 2010).
2.2.2 Estrogenic effects

It has been known for some time that estrogen is involved in the pathogenesis of endometriosis. Aromatase is the key enzyme in the biosynthesis of estrogen, which is important for the establishment and growth of endometriosis. Expression of aromatase has been found in endometriotic lesions and endometrium from women with endometriosis (Noble, et al., 1996, Velasco, et al., 2006), creating an estrogenic environment. Also, stimulation of endometriotic stromal cells with IL-6 lead to a higher aromatase activity (Velasco, et al., 2006).

The survival and growth of endometriotic lesions requires an adequate blood supply, indicating an important role of angiogenesis in endometriosis. Estrogen has a function in regulating angiogenic growth factors (reviewed in (Hyder and Stancel, 1999)). Increased levels of angiogenic factors, such as vascular endothelial growth factor (VEGF) which is one of the most potent angiogenic factors, are found in the peritoneal fluid of endometriosis patients with advanced disease (Mahnke, et al., 2000). In addition, peritoneal fluid from women with endometriosis increased the expression of VEGF in endometrial cell cultures (Cosin, et al., 2010). McLaren et al. observed that VEGF production by macrophages in the peritoneal fluid was increased after stimulation with estrogen and progesterone (McLaren, et al., 1996). Macrophage migration inhibitory factor (MIF), a potential mediator of angiogenesis, is increased in the peritoneal fluid from women with endometriosis and affects the proliferation of endothelial cells (Kats, et al., 2002).

Estrogen also has a role in apoptosis. Removal of estrogen in cell cultures was associated with decreased cell viability and increased number of apoptotic cells (Song, et al., 2002). Estrogen also increases the phosphorylation of Akt, a regulator of apoptosis and cell survival (Guzeloglu Kayisi, et al., 2004). Impaired apoptosis in endometriotic cells of women with endometriosis may contribute to the pathogenesis of the disease. In healthy women, apoptosis is important to maintain the cellular homeostasis during the menstrual cycle (Kokawa, et al., 1996). In women with endometriosis, increased expression of anti-apoptotic factors and decreased expression of pro-apoptotic factors have been reported, supporting an anti-apoptotic phenotype of endometriotic cells (reviewed in (Agic, et al., 2009)). Gebel et al. demonstrated decreased apoptosis in eutopic endometrium in women with endometriosis compared to controls, which was further decreased in ectopic endometrium (Gebel, et al., 1998).

2.2.3 Inflammation and immune response

Increased inflammation and altered immune functions have been observed in women with endometriosis. A complex network of locally produced cytokines and immune cells have been proposed to modulate the growth and inflammatory behavior of the endometriotic implants (reviewed in (Osuga, et al., 2010)). An increased number of activated macrophages have been seen in the peritoneal fluid of endometriosis patients (Dunselman, et al., 1988, Keenan, et al., 1995). Oosterlynck et al. reported a decreased natural killer (NK) cell activity and cytotoxicity in peritoneal fluid (Oosterlynck, et al., 1991). A reduction of activated T-cells (Ho, et al., 1995) and mature dendritic cells (Schulke, et al., 2009) have been seen in women with endometriosis. In addition,
peritoneal fluid from endometriosis patients contained a significantly higher NK cell suppressive activity than peritoneal fluid from fertile controls (Oosterlynck, et al., 1993). This may partly explain the increased survival of endometriotic cells in the peritoneal cavity.

Inflammatory cytokines play a central role in the regulation of cell proliferation, activation, motility, adhesion, chemotaxis and morphogenesis. Several cytokines, such as IL-1, IL-5, IL-6, IL-8, IL-15, monocyte chemotactic protein-1 (MCP-1), TNF-α, transforming growth factor-β (TGF-β) and Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) have been implicated in the pathogenesis of endometriosis (reviewed in (Lebovic, et al., 2001)). It has been observed that the level of some cytokines in peritoneal fluid and serum correlates to the severity of the disease. Expression of TNF-α, IL-8 and MCP-1 was higher in early stage of endometriosis and decreased with more advanced disease, while TGF-β expression decreased with the severity of the disease (Pizzo, et al., 2002). RANTES was also increased in the peritoneal fluid in women with more severe disease (Khorram, et al., 1993).

It has been reported that endometrial cells stimulated with IL-8 showed increased survival *in vitro* (Gazvani, et al., 2002). Iwabe *et al.* demonstrated that TNF-α stimulated proliferation of endometriotic stromal cells through the induction of IL-8 (Iwabe, et al., 2000). Moreover, TNF-α increases the adherence of stromal cells to mesothelial cells in culture (Zhang, et al., 1993). Interestingly, TNF-α has a proliferative effect on endometriotic cells from endometriosis patients, but not on endometrial cells from healthy women (Braun, et al., 2002). The abnormalities seen in the peritoneal fluid from endometriosis patients may also be linked to endometriosis-associated infertility.

**2.2.4 Autoimmune features**

It has been proposed that endometriosis is associated with autoimmune disease, due to elevated autoantibody titers in women with the disease (Gleicher, et al., 1987). Sinaii *et al.* have observed higher rates of autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Sjögren’s syndrome, in endometriosis patients (Sinaii, et al., 2002). Several mechanisms, such as familial occurrence, T- and B-cell abnormalities, tissue damage and altered apoptosis, are common in both endometriosis and autoimmune diseases (reviewed in (Matarese, et al., 2003, Nothnick, 2001)).

**2.2.5 Endometrial stem cells**

Recently, it has been suggested that endometrial stem/progenitor cells are not only involved in the regeneration of endometrium, but also the pathogenesis of endometriosis (reviewed in (Gargett, 2004)). It may be possible that in women developing endometriosis, inappropriately shed stem/progenitor cells reach the peritoneal cavity by retrograde menstruation, and thereby develop endometriotic implants (reviewed in (Sasson and Taylor, 2008)). The first evidence of stem/progenitor cells in the endometrium came from cloning studies, when Chan *et al.*
observed that a small population of human endometrial epithelial and stromal cells possessed clonogenic activity \textit{in vitro} (Chan, et al., 2004). It has also been shown that cells from the endometrium can differentiate into mesodermal lineages \textit{in vitro}, such as chondrogenic (Wolff, et al., 2007), adipose (Dimitrov, et al., 2008), myogenic, and osteogenic (Schwab and Gargett, 2007) cell types, suggesting that these cells are multipotent adult stem cells with an activity similar to mesenchymal stem cells. Another possibility is that bone marrow-derived stem cells could have a role in both the regeneration of the endometrium and in endometriosis (Du and Taylor, 2007).

2.3 INFERTILITY AND ENDOMETRIOSIS

Several studies have found an association between endometriosis and infertility (reviewed in (de Ziegler, et al., 2010)). Endometriosis is present in 20-40\% of women with infertility (1994, Strathy, et al., 1982, Verkauf, 1987). A meta-analysis by Barnhart \textit{et al.} reported that women with endometriosis have $\leq 54\%$ reduction in pregnancy rate after \textit{in vitro} fertilization (IVF), compared to women with tubal factor infertility (Barnhart, et al., 2002). Moreover, decreased pregnancy rate per cycle, pregnancy per transfer and implantation rate have been observed in endometriosis patients (Anici, et al., 1996, Simon, et al., 1994). Women with endometriosis stage III/IV seem to have a worse prognosis for IVF/intracytoplasmic sperm injection (ICSI) treatment (Kuvasaas, et al., 2005).

The underlying reason for endometriosis-associated infertility is debated. Several mechanisms have been proposed, such as distorted pelvic anatomy, endocrine and ovulatory abnormalities, altered peritoneal function, poor oocyte quality and defective implantation capacity (figure 2). \textit{In vitro} studies of fertilized oocytes from endometriosis patients showed a decrease in the number of blastomeres and an increase in the percentage of arrested embryos compared with controls (Pellicer, et al., 1995), indicating a reduced quality of embryos from endometriosis patients. Oocyte donation studies report lower implantation rates in recipients who received oocytes from women with endometriosis (Simon, et al., 1994). Furthermore, cytokines in the peritoneal fluid are believed to be involved in the endometriosis-associated infertility. Since the ovaries and the fallopian tube are exposed to peritoneal fluid, different factors in the peritoneal fluid could have influence on reproductive functions. Thus, early embryos are exposed to the peritoneal fluid, which could be toxic for the preimplantation embryo (Morcos, et al., 1985). This is strengthened by Damewood \textit{et al.}, who reported that serum from endometriosis patients decreases the growth of mouse embryos (Damewood, et al., 1990). High levels of TNF-$\alpha$ and MIF have been found to reduce sperm motility \textit{in vitro} (Carli, et al., 2007, Said, et al., 2005) and high levels of RANTES have a negative effect on the sperm fertilizing ability (Barbonetti, et al., 2008). TNF-$\alpha$ also reduced maturation of porcine oocytes (Ma, et al., 2010).
2.3.1 Ovarian reserve

The ovarian reserve has been shown to decrease with increasing age, leading to a lower reproductive capacity (reviewed in (te Velde, et al., 1998)). Several different techniques have been proposed for the assessment of the ovarian reserve. Commonly used tests are the measurement of follicle stimulating hormone (FSH), estradiol and inhibin B in serum on cycle day 3 of the menstrual cycle and the number of antral ovarian follicles (antral follicle count) with ultrasound. Another marker of the ovarian reserve is the serum level of anti-mullerian hormone (AMH) (van Rooij, et al., 2002). AMH is produced by preantral and small antral follicles and may act as a paracrine regulator of early follicular growth (Weenen, et al., 2004). The concentration of AMH is not influenced by the menstrual cycle (Streuli, et al., 2009) or oral contraceptives (Streuli, et al., 2008), making it possible to measure AMH levels at any time of the menstrual cycle during hormonal treatment. Higher levels of AMH in serum have been associated with a higher number of retrieved oocytes (Seifer, et al., 2002) and a higher pregnancy rate (Hazout, et al., 2004). Reduced levels of AMH have been reported in infertile women with endometriosis (Shebl, et al., 2009).

2.4 ENDOMETRIOSIS-ASSOCIATED MALIGNANCY

Although endometriosis is a benign disease, it has been considered to have malignant characteristics. Endometriosis involves attachment and invasion of healthy tissue, loss of control of cell proliferation and is spread both locally and distantly. Furthermore, genetic, angiogenic, endocrine and immunological abnormalities have been observed in endometriosis patients. In malignancy, these properties are also altered. Epidemiological studies show association of different types of malignancy and endometriosis, such as ovarian cancer, breast cancer, colon cancer, endocrine tumours, brain tumours and non-Hodgkin’s lymphoma (Bertelsen, et al., 2007, Brinton, et al., 1997, Brinton, et al., 2005, Gennari, et al., 2010, Hornstein, et al., 1997, Melin, et al., 2006, Olson, et al., 2002). Interestingly, Melin et al. observed better survival in endometriosis patients with cancer compared to women with the same form of cancer.
but without endometriosis, especially those with breast and ovarian cancer (Melin, et al., 2010).

### 2.4.1 Ovarian cancer

Several studies have found associations between endometriosis and ovarian cancer (Brinton, et al., 1997, Brinton, et al., 2005, Melin, et al., 2006). In a Swedish population study, the risk of ovarian cancer was increased 4.2-fold in subjects with a long-standing history of ovarian endometriosis (Brinton, et al., 1997). Melin et al. reported that women with early diagnosis and long-standing endometriosis had a higher prevalence of ovarian cancer (Gemmill, et al., 2010, Melin, et al., 2006). In addition, cancer was found more often in the ovaries when endometriosis was present in the ovary (Stem, et al., 2001). Clear cell and endometrioid carcinoma were the most commonly seen types of ovarian cancers in endometriosis patients (Fukunaga, et al., 1997, Ogawa, et al., 2000, Stem, et al., 2001). The most frequent location for coexistence of endometriosis and cancer is the ovaries, which has been estimated to occur in 0.7-5.0% of all cases with ovarian cancer (Erzen and Kovacic, 1998, Ogawa, et al., 2000, Stem, et al., 2001).

### 2.4.2 Transformation of endometriosis into ovarian cancer

The mechanism by which endometriosis is transformed into malignancy is unclear. Two theories have been suggested, seen in figure 3 (reviewed in (Varma, et al., 2004)). It is possible that endometriosis is directly transformed into ovarian cancer via atypical endometriosis. Another theory is that endometriosis and ovarian cancer share common mechanisms and/or predisposing factors, such as an inflammatory environment, genetic susceptibility and hormonal factors.

![Figure 3](image-url)  

**Figure 3.** Two possible pathways of the transformation of endometriosis into ovarian cancer.
Ovarian cancer derived from the malignant transformation of endometriosis has been referred to as endometriosis-associated ovarian cancer (EAOC). Atypia has been observed in EAOC (Fukumaga, et al., 1997, Prefumo, et al., 2002) and Ogawa et al. reported evidence of transformation from endometriotic lesions to atypical endometriosis and further into carcinoma (Ogawa, et al., 2000). About 60% of cases of EAOC occur with endometriosis adjacent to the cancer or direct transformation from the endometriotic lesion (Erzen and Kovacic, 1998, Modesitt, et al., 2002). Banz et al. observed equally regulated genes in endometriosis and EAOC (Banz, et al., 2010).

A variety of different molecular changes have been suggested to be involved in the malignant transformation of endometriosis. Genetic alterations are a well-known characteristic of malignancy. Especially alterations in oncogenes and tumor suppressor genes, such as p53 alterations, Kirsten rat sarcoma viral oncogene homologue (K-ras) mutations and Phosphatidylinositol-3, 3, 5,- triphosphate 3-phosphate (PTEN) silencing, have been suggested to play a role in malignant transformation of endometriosis. In a mouse model of endometrioid ovarian cancer, PTEN deletion and K-ras activation, gave rise to endometriosis-like lesions, which further developed into endometrioid ovarian carcinoma (Dinulescu, et al., 2005). Mutations in PTEN have also been observed in endometriomas, as well as in ovarian cancer (Sato, et al., 2000) and K-ras mutations have been seen in endometrioid (Amemiya, et al., 2004) and clear cell carcinoma (Okuda, et al., 2003). Sáinz de la Cuesta et al. demonstrated an increase in expression of p53 in the transformation from atypical endometriosis to ovarian cancer (Sainz de la Cuesta, et al., 2004). Furthermore, several studies have reported loss of heterozygosity (LOH) as an event in the malignant transformation of endometriosis (Jiang, et al., 1998, Prowse, et al., 2006, Sato, et al., 2000).

Malignant transformation is also influenced by the microenvironment. The inflammatory environment in endometriosis, with increased levels of cytokines, growth factors and hormones, has been proposed to play a role in the malignant transformation of endometriosis (reviewed in (Nezhat, et al., 2008, Varma, et al., 2004)). Ness et al. reported similar alterations in inflammatory and immune response in women with endometriosis and ovarian cancer (Ness, 2003). In addition, resistance to apoptosis, angiogenesis and the ability to invade is shared between endometriosis and malignancy.

2.5 GENETIC STUDIES OF ENDOMETRIOSIS

Several studies have indicated a genetic contribution in endometriosis. Familial studies have shown a six to nine fold increase in risk of endometriosis among relatives (reviewed in (Kennedy, et al., 2001)). In addition, Stefansson et al. reported a relative risk of 2.20 for sisters and 1.56 for cousins to develop endometriosis (Stefansson, et al., 2002). Twin studies (Moen, 1994) and animal studies (Zondervan, et al., 2004) also support the role of genetics in the pathogenesis of endometriosis. However, not only genes seem to contribute to endometriosis, but also environmental factors, indicating that endometriosis is a complex disease (Kennedy, 1999). Treloar et al. reported that 50% of the variance of susceptibility to endometriosis in Australian twins was attributed to genetic factors (Treloar, et al., 1999).
2.5.1 Genetics of complex diseases

Diseases which do not exhibit classical Mendelian inheritance are described as complex diseases. Complex diseases are relatively common in the general population and seem to cluster within families. In addition, complex diseases are believed to be influenced by several genetic and environmental factors, as well as interactions among them. The common variant hypothesis suggests that common genetic variants with relatively high frequency, but low penetrance, are the major contributors to common complex diseases (reviewed in (Altshuler, et al., 2008)). However, rare variants, with a low frequency and high penetrance also contribute to complex diseases (Stratton and Rahman, 2008). Most likely, the genetic etiology is based on a combination of both rare and common variants.

The two most common methods used to investigate complex diseases are linkage and association studies. These two methods basically rely on the same principle, i.e. co-inheritance of adjacent DNA variants. Linkage studies identify haplotypes that are inherited for several generations within families and association studies investigate the retention of adjacent DNA versions for many generations in cases and controls (reviewed in (Cardon and Bell, 2001)).

2.5.2 Sequence variations in the genome

Even though humans are said to be 99.9% identical in respect to DNA, a lot of different variations in the human genome have been observed. Genetic variation can explain the different phenotypes among individuals, although the majority of variants are believed to be neutral with no phenotypic effect. Common variations have a minor allele frequency (MAF) of at least 1%, while rare variations (mutations) have a MAF less than 1% (reviewed in (Frazer, et al., 2009)). Genetic variations include single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs, also known as micro- and minisatellites), as well as structural variants, such as insertions and deletions (indels), inversions, block substitution and copy number variants (CNV) (reviewed in (Feuk, et al., 2006)).

SNPs are the most common genetic variation, where a single nucleotide is changed, inserted or deleted, seen in figure 4. The number of SNPs in the human genome is estimated to be around 3.3 million, with an average of 1 SNP in 1000 bases (reviewed in (Altshuler, et al., 2008, Frazer, et al., 2009)). SNPs are found both in non-coding and coding regions. Synonymous SNPs, localized in coding regions, can cause an amino-acid change, frame shift or termination of translation, thereby giving a functional effect on protein level, or the SNP does not affect the amino-acid sequence. SNPs located in non-coding regions, non-synonymous SNPs, could also have a functional effect, due to localization in regulatory elements such as gene promoters, enhancers or silencers.
Association studies investigate the correlation between a genetic variation and a trait, thereby comparing the differences in allelic frequency between cases and controls. Often the genetic variant lies within a candidate gene, which is chosen due to its relevance in the pathophysiology of the disease, or from previously found linkage regions. Association between a marker and a phenotype can be due to two reasons; either by marking the causal allele itself or by marking neighboring variants in linkage disequilibrium (LD). LD defines genetic variants positioned close, which tend to be inherited together, and the combination on those linked variants are called haplotypes (Daly, et al., 2001). Associated variants need to be replicated in several independent populations and functional studies are required to determine the effect of the variant.

2.5.3 Linkage and GWAS studies in endometriosis

Genome-wide association studies (GWAS) makes it possible to genotype a million SNPs in one individual at a time (reviewed in (McCarthy and Hirschhorn, 2008)). Until today, only a few linkage studies and GWAS have been performed in the field of endometriosis (reviewed in (Montgomery, et al., 2008)). Treloar et al. reported a susceptibility locus for endometriosis on chromosome 10q26 and a suggestive linkage on chromosome 20p13 (Treloar, et al., 2005). Another locus on chromosome 7p13-15 has been found in linkage studies (Zondervan, et al., 2007) and the locus at 7p15.2 was identified in a GWAS performed by Painter et al. (Painter, et al., 2011). Furthermore, the CDKN2BAS locus has been associated with endometriosis in Japanese in a GWAS (Uno, et al., 2010) and the region around IL1α on 2q13 has been suggested as a candidate (Adachi, et al., 2010). However, in most linkage and GWAS the candidate genes and their functional relevance remain to be identified.

2.5.4 Candidate genes in endometriosis

Several different candidate genes have been suggested to be involved in endometriosis, including genes involved in inflammation, steroid synthesis, hormone receptors, growth factors, adhesion molecules, cell cycle regulation, apoptosis and oncogenesis (reviewed in (Falconer, et al., 2007)). Association of polymorphisms in inflammatory genes, such as IL-10, IL-16, IL-18, TGF-β, Interferon γ (IFN-γ) and TNF-α, has been observed, mostly in Asian populations (Ayaz, et al., 2011, Gan, et al., 2010, Hsieh, et al., 2005, Juo, et al., 2009, Kitawaki, et al., 2004, Lakshmi, et al., 2010, Teramoto, et al., 2004). Human leukocyte antigen (HLA) genes play a key role in the immune response and are highly polymorphic. Association of different HLA variants have been reported in some Japanese studies (Ishii, et al., 2002, Kitawaki, et al., 2002), while other studies could not find any association (Roszkowski, et al., 2005, Whang, et al., 2006). Studies have also reported conflicting results regarding the association of PTPN22 and
endometriosis, with two studies showing association (Ammendola, et al., 2008, Gomes, et al., 2010) and one showing no association (Ploski, et al., 2009). Many conflicting results like these have been found among the association studies, making it difficult to conclude the genetic effects in endometriosis. Most studies have small populations with poorly-defined control populations. In order to understand more about the genetic contribution to endometriosis, larger and more controlled studies are needed (Colhoun, et al., 2003, Zondervan, et al., 2003). In addition, the results should be replicated in several populations.
3 AIMS

The overall aim of this thesis was to study the aetiology and pathophysiology of endometriosis, in order to obtain a better understanding of the disease. Furthermore, the aim was to investigate the genetic background of endometriosis and genetic linkage to other diseases.

The specific aims were:

- To study the cytokine expression profile in follicular fluid from endometriosis patients undergoing IVF. (Paper I)

- To investigate a possible shared genetic background between endometriosis and RA. (Paper II)

- To evaluate if endometriosis and ovarian cancer shares disease associated genes. (Paper III)

- To identify factors involved in adhesion, attachment and invasion in endometrium and endometriosis. (Paper IV)
4 MATERIAL AND METHODS

4.1 STUDY SUBJECTS

4.1.1 IVF patients (I)
Serum samples and follicular fluid were collected at the fertility unit (RMC) at the Karolinska University Hospital, Sweden. The study included 34 women with laparoscopically-verified endometriosis. In addition, a control group, consisting of 38 women with tubal factor infertility and with no signs of endometriosis upon laparoscopy, was used for comparison. Both groups were similar with respect to age, body mass index (BMI), smoking and duration of infertility. All women underwent IVF and embryo transfer according to standard protocols.

4.1.2 Belgian cases and controls (II, III)
In study II and III, a sample set of 1149 women (798 endometriosis patients and 351 controls) was used. All women were Caucasian and had undergone laparoscopy for subfertility with or without pain at the Leuven University Hospital, Leuven, Belgium during 1998-2007. Peripheral blood samples (n=948, 660 endometriosis patients and 288 healthy controls) were collected and peritoneal biopsies (n=229, 161 endometriosis patients and 68 controls) were taken during surgery. From some patients and controls both blood sample and tissue were obtained. The absence of endometriosis was confirmed laparoscopically in control patients (age 32 +/- 5 years; BMI 23.4 +/- 4). Women with endometriosis (age 31 +/- 4 years; BMI 23.2 +/- 4) had either minimal (stage I; n=176), mild (stage II; n=116), moderate (stage III; n=88) or severe (stage IV; n = 179) disease, staged according to the revised classification system of the American Society of Reproductive Medicine (1997).

4.1.3 Healthy women and endometriosis patients (IV)
Endometrial biopsies from healthy volunteers were taken at the Karolinska University Hospital, Sweden in both the proliferative and the secretory phase of the menstrual cycle. All women had regular menstrual bleeding and had not used any hormonal medication or an IUS at least 3 months prior to recruitment. Endometrium and endometriomas from endometriosis patients were collected during both the proliferative and the secretory phase at Danderyds Hospital and Södersjukhuset, Sweden, and the University Hospital of Florence, Italy. The endometriosis patients had not used any IUS or hormonal medication 3 months prior to recruitment. Endometrial biopsies were taken with a Randall curette or a pipelle. The biopsies were divided and placed in RNA later for RNA isolation and formaldehyde/paraformaldehyde for immunohistochemical studies. Endometrial dating was performed histopathologically, to verify the phase of the menstrual cycle, according to Noyes criteria (Noyes, et al., 1975).
4.2 OVARIAN STIMULATION PROTOCOL (I)

All patients followed the long Gonadotrophin releasing hormone agonist (GnRHa) protocol (Fried, et al., 1996). GnRHa was administered (6x200 μg/day) (Suprefact®, Hoechst AB, Stockholm, Sweden), starting on day 21 of the menstrual cycle. It was reduced to half of the dose after the start of FSH injections. Down regulation was verified by vaginal ultrasound scanning, followed by administration of recombinant FSH (75-300 IU/day) (Gonal-F®, Serono Nordic AB, Sollentuna, Sweden). Starting dose of FSH was generally 150 IU up to age 35 and 225 IU above age 35, unless previous FSH stimulation during IVF indicated otherwise. Endometrial growth and follicular development were followed by vaginal ultrasonography, together with serum estradiol levels. After adequate stimulation, i.e. a controlled rise of serum estradiol and a leading follicle diameter of at least 17 mm, human Chorionic Gonadotrophin (hCG) (Profasi®, Serono Nordic AB, Sollentuna, Sweden) was administered. Oocyte retrieval was performed approximately 35 hours later by transvaginal ultrasound-guided follicle aspiration. Progesterone (3x400 mg/day) (Progesteron MIC APL, Sweden) was given until pregnancy test, and with a positive test, continued for 8 weeks after embryo transfer.

4.3 EXPRESSION ANALYSIS

4.3.1 Hormonal assays (I)

The concentration of AMH in serum and follicular fluid was measured by enzyme immunoassay (EIA) (A16507, Immunotech, Marseille, France). Estradiol and FSH in follicular fluid were determined by chemiluminescent enzyme immunoassay (LKE21 resp. LKFS1, Diagnostic Products Corporation, California, USA). The estradiol samples were diluted 1:1 000 before analysis. All assays were done according to manufacturer’s protocol.

4.3.2 Cytokine assays (I)

The level of TNF in follicular fluid was determined by immunoradiometric assay (IRMA), according to manufacturer’s protocol (KIC1751, Biosource, California, USA).

4.3.3 Real Time RT-PCR (IV)

RNA extraction was performed using a dismemranation apparatus (Retsch KG, Haan, Germany) together with TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA). RNA was treated with RQ1 RNase- free DNase (Promega Biotech AB, Stockholm, Sweden) before cDNA preparation with Superscript II RNase H’ Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). RNA levels were quantified with RealTime RT-PCR, using the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers and probes were purchased from available Taqman gene expression assays (Applied Biosystems).
The threshold cycles (CT), where an increase in reporter fluorescence above the baseline signal could first be detected, were determined. 18S was used as endogenous control. The mean CT value of 18S was used for normalization and was subtracted from the mean CT value of the respective gene, to obtain ∆CT values.

4.3.4 Immunohistochemistry (IV)

For immunohistochemical analysis a panel of antibodies has been used (for detailed information, see paper IV). Biopsies were fixed, paraffin-embedded and sectioned. All staining was performed with the MACH 3™ Mouse-Probe HRP-polymer kit or MACH 3™ Rabbit-Probe HRP-polymer kit (Biocare Medical, CA, USA). Stainings were developed using DAB and counterstained with Mayer’s hematoxylin. Negative controls were performed by omitting the primary antibody or by using primary isotype-matched immunoglobulins; Dako Universal Negative Control Mouse (N1698, Dako, Carpinteria, CA, USA) and ChromPure Rabbit IgG (011-000-003, Jackson Immunoresearch, West Grove, PA, USA). Sections were analysed in a Zeiss Axiovert 200M microscope (Zeiss, Göttingen, Germany) and images were captured with Qcapture, version 3.1.1. (QImagin, Surrey, BC, Canada).

For all immunohistochemical stainings, the intensity of staining and the number of positive cells were observed. Scoring was done blindly by 2 independent observers. The intensity of staining was graded as 0= no staining, 1= weak staining, 2= moderate staining and 3= strong staining. The percentage of stained cells was graded as following; 0= no staining, 1= <10%, 2=11-50%, 3= 51-80% and 4= >81%. The final score was calculated by multiplying the two scores.

4.4 GENETIC ANALYSIS

4.4.1 Genotyping (II, III)

Taqman allelic discrimination assays were performed according to manufacturer’s protocol (Applied Biosystems, Carlsbad, USA) with fluorescent-labeled allele-specific probes. After PCR the plates were read in a 7900 HT Fast-Real Time PCR system (Applied Biosystems) and analyzed with the SDS 2.2 software (Applied Biosystems).

4.4.2 Association analysis (II, III)

Case-control based associations were performed using three different models; the co-dominant model, the dominant-recessive model and allelic model. In paper II and III, the allelic model is presented.

4.4.3 Haploview (II, III)

The Haploview software 4.2 (Cambridge, MA, USA) was used to investigate linkage disequilibrium (LD) between SNPs. Permutation test (10 000 permutations) were used to correct for multiple testing.
4.5 STATISTICAL ANALYSIS

All statistical analyses were performed with the statistical package Graphpad Prism (Graphpad Software Inc., San Diego, USA). In paper I, the Mann-Whitney U-test and Fisher’s exact test was used for comparisons between women with and without endometriosis. Outliers were identified with Grubb’s outlier detection test and correlations were assessed by Spearman’s rank correlation test. In paper II and III, Bonferroni correction was used for multiple testing of SNPs. The Kruskal-Wallis test was applied in paper IV, to compare healthy endometrium, endometrium from endometriosis patients and endometriomas during the menstrual cycle. P-values less than 0.05 were considered significant.

4.6 ETHICAL PERMISSION

Permission from local ethics committees in Sweden, Italy and Belgium was obtained for all studies.
5 RESULTS AND DISCUSSION

5.1 PAPER I- IVF OUTCOME IN WOMEN WITH ENDOMETRIOSIS IN RELATION TO AMH AND INFLAMMATORY MARKERS

Many women with endometriosis suffer from infertility. It has been suggested that the increased inflammatory activity seen in endometriosis contributes to the reduced reproductive potential (reviewed in (Halás and Arici, 2004)). The IVF outcome was investigated in correlation to levels of AMH and inflammatory markers in IVF patients with endometriosis compared to a control group with tubal factor infertility. It was observed that women with endometriosis had significantly lower levels of AMH in both serum and follicular fluid. In addition, levels of the pro-inflammatory markers TNF-α, IL-15 and granulocyte-macrophage stimulating factor (GM-CSF) were increased in follicular fluid, while levels of the anti-inflammatory IL-10 were decreased. Women with endometriosis responded well to the IVF-treatment, but had lower fertilization rates.

AMH is considered to be an indicator of the ovarian reserve, and a poor response in IVF has been associated with low concentrations of serum AMH (Al-Qahtani and Groome, 2006, van Rooij, et al., 2002). The lower levels of serum AMH seen in our study, corroborates earlier finding of low levels of serum AMH at cycle day 3 in women with minimal/mild endometriosis (Lemos, et al., 2008). A positive correlation of AMH levels in follicular fluid with the total number of follicles was also observed. It seems possible that the decreased recruitment of follicles in women with endometriosis could be due to an altered hormone-cytokine balance.

In this study, increased levels of TNF-α in follicular fluid from endometriosis patients was found. These findings support a previous report of increased production of TNF-α in cultured granulosa cells from women with endometriosis (Carlberg, et al., 2000). It has been shown that TNF-α in follicular fluid correlates to oocyte quality with higher levels of TNF-α in poor quality oocytes (Lee, et al., 2000), indicating a role of TNF-α in endometriosis-associated infertility. In addition, it has been demonstrated that TNF-α regulates AMH in mouse testis (Hong, et al., 2003). It is possible that this regulation could also take place in the ovary. An earlier study by Kilic et al., could not demonstrate any differences in TNF-α levels in the follicular fluid from women with endometriosis compared to women with unexplained infertility (Kilic, et al., 2007). These contradictory results could be due to differences in control groups or heterogeneity among endometriosis patients.

Increased levels of inflammatory cytokines are seen in endometriosis (Matarese, et al., 2003). Our findings regarding increased levels of TNF-α, IL-15 and GM-CSF support previous results. This inflammatory environment is further supported by decreased levels of the anti-inflammatory cytokine IL-10 seen in our study. This altered cytokine balance may not only affect the folliculogenesis and oocyte development, but also the fertilization process. For example, TNF-α has been reported to influence sperm motility.
in vitro and to reduce the maturation of porcine oocytes (Ma, et al., 2010, Said, et al., 2005). Thus, cytokine levels may be important in endometriosis-associated infertility.

Our results indicate that women with endometriosis have a poor IVF outcome compared to controls. The diminished ovarian reserve may be due to an increased inflammatory activity in the ovarian follicles. However, the mechanism of this action needs to be evaluated.

5.2 PAPER II- RA SUSCEPTIBILITY GENES AND ENDOMETRIOSIS

The pathogenesis of endometriosis is still unclear, although evidence suggests that inflammation plays an important role in the disease. Several inflammatory factors, such as TNF-α, RANTES and IL-8 are up regulated in peritoneal fluid from women with endometriosis (Khorram, et al., 1993, Pizzo, et al., 2002) and abnormalities in the immune system have been observed (reviewed in (Osuga, et al., 2010)). Several of these alterations, such as B- and T-cell abnormalities and altered apoptosis are also seen in patients with autoimmune diseases, such as RA. In addition, reports indicate that endometriosis patients have an increased incidence of autoimmune diseases (Sinaii, et al., 2002). Lately, it has been indicated that genetics also could contribute to development of endometriosis. Therefore, we wanted to investigate genetic similarities between molecular and cellular pathways of endometriosis and RA.

Our material consisted of Caucasian women undergoing laparoscopy for subfertility at the Leuven University hospital in Leuven, Belgium. Samples were obtained from 798 patients with endometriosis and 351 healthy controls. We chose to investigate six RA associated SNPs, i.e. PTPN22 rs2476601, STAT4 rs10181656, TRAF1-C5 rs3761847, CCL21 rs2812378, CD40 rs4810485 and IRF5 rs3807306. No association was found between the selected SNPs and endometriosis.

Due to the heterogeneity of endometriosis, the genetic differences between stage I-II (minimal-mild) and III-IV (moderate-severe) was explored. Previous studies have found different gene expression between different types of endometriosis (Matsuzaki, et al., 2006) and differences in cytokine expression have been observed in patients with different stages of the disease (Khorram, et al., 1993, Pizzo, et al., 2002). In our study, an association between CCL21 rs2812378 and moderate/severe endometriosis was found. However, in RA patients an increased frequency was observed with the G allele (Orozco, et al., 2010, Raychaudhuri, et al., 2008), while in endometriosis, an increase in the A allele was found. This suggests a different role of this SNP in the two diseases. Unfortunately, we do not have any record of autoimmune diseases in our material, but it would have been interesting to see if endometriosis patients with increased A allele had a lower frequency of autoimmune diseases.

In RA, genetic associations have been found with HLA-DRB1 variants. Therefore, two RA associated HLA-DRB1 SNPs, rs660895 and rs2395175, were also investigated in our material. No association of these SNPs was found in endometriosis as one group; although association of rs660895 was observed in patients with moderate/sever disease. Previous genetic studies of endometriosis have shown conflicting results regarding
HLA-DRB1. Some studies have found associations (Ishii, et al., 2002, Kitawaki, et al., 2002) while others could not observe any association (Roszkowska, et al., 2005, Whang, et al., 2006). These differences may be due to small study populations, poorly defined controls or differences in ethnicity. Most genetic studies of endometriosis include very few individuals, therefore making it difficult to establish associations, especially associations with low genetic effect. Also, most associations have only been observed in one study population and not replicated in other study populations. Our study is one of the largest on endometriosis, although compared to other diseases, it is relatively small. However, we have a better power to detect associations than previous candidate gene studies. One drawback with our study is that we do not have access to another population to replicate our results, but we hope that other research groups with genetic material from Caucasian populations will find our studies interesting and perform replication of our results. While performing genetic studies, collaborations will be needed to obtain larger populations and replication of results.

In this study, association between SNPs in CCL21 and HLA-DRB1 was observed. However, we do not know the functional effect of these alleles. We can only speculate about the function, relying on previous studies of these genes. Since inflammation is an important event in endometriosis, we could assume that CCL21 and HLA-molecules are involved in the disease. CCL21 is a chemokine, responsible for recruitment of lymphocytes and dendritic cells. In endometriosis, aberrant expression of lymphocytes (reviewed in (Osuga, et al., 2010)) and a reduction of mature dendritic cells have been observed (Schulke, et al., 2009). Furthermore, CCL21 co-stimulates the expansion of naïve T-cells and increases the expression of inflammatory cytokines, such as TNF-α and INF-γ (Flanagan, et al., 2004), which are also increased in endometriosis. Increased expression of CCL21 has been seen in endometriosis patients and could be a reason for this increased inflammatory profile (Chand, et al., 2007). Also, HLA-molecules are important in the immune system, by differentiating T-cells and inhibiting the killing of NK cells. The expression of major histocompatibility complex (MHC) class I on cells protects against NK cell lysis. In endometriosis, a reduction of activated T-cells and defective NK cell activity has been observed (Ho, et al., 1995, Oosterlynck, et al., 1991). In addition, increased expression of HLA class I and II molecules have been reported in the endometrium from women with endometriosis compared to controls (Baka, et al., 2010, Vernet-Tomas Mdel, et al., 2006). An increased proportion of HLA-DR positive stromal and glandular epithelial cells have also been reported (Chiang and Hill, 1997, Nisolle and Donnez, 1997). This indicates an increased resistance against NK cell lysis in endometrial cells in women with endometriosis.

Our results indicate a potential role of CCL21 and HLA in endometriosis. Since the HLA region has a high degree of LD, it is difficult to establish the SNP of functional value. It is possible that it is not our associated SNPs that give a functional effect, but it is in LD with another SNP, causing the effect. Therefore, sequencing of the entire region and functional studies are needed to further investigate our findings.
5.3 PAPER III- REPLICATION OF OVARIAN CANCER SUSCEPTIBILITY GENES IN ENDOMETRIOSIS

Endometriosis is a benign disease, although it is believed to have malignant characteristics. Several epidemiological studies have found an association between endometriosis and malignancy, such as ovarian cancer (Brinton, et al., 1997, Melin, et al., 2006). However, it is not clear how endometriosis may be transformed into ovarian cancer. It has been suggested that endometriosis could transform to cancer via atypical endometriosis, or that both diseases could share common mechanisms and/or predisposing factors, such as genetic susceptibility. A recent GWAS has reported association of several SNPs in the BNC2 gene and ovarian cancer (Song, et al., 2009). We therefore wanted to investigate if the same SNPs would be associated to endometriosis in our Belgian cases and control samples, to find out if BNC2 could be a possible link between endometriosis and ovarian cancer.

In our material, association of the selected SNPs in the BNC2 gene with endometriosis could not be observed, either as one group or divided into minimal/mild and moderate/severe disease. These results indicate that BNC2 is not associated with endometriosis. However, it is possible that we do not have enough power to detect an association and replication of our data is needed in another, preferably larger, study population.

The function of BNC2 is not clear, but it is believed to participate in mRNA processing (Vanhoutteghem and Djian, 2006). Expression of BNC2 has been found in the ovary, uterus and testis (Romano, et al., 2004, Vanhoutteghem and Djian, 2004), indicating an possible role in reproduction. Perhaps BNC2 is affecting mRNA levels of proteins involved in reproduction and thus has a role in endometriosis-associated infertility. To explore this possibility, it would be of interest to investigate the expression of BNC2 in endometriosis patients and especially in infertile women with endometriosis. It would also be interesting to follow the development of ovarian cancer in women with endometriosis, to investigate if women developing ovarian cancer have an aberrant genetic profile compared to the women who do not develop ovarian cancer. Then we can be more certain that BNC2 is not involved in endometriosis.

Other candidate genes have been identified in ovarian cancer, such as genes encoding cell cycle proteins, oncogenes and tumor suppressor genes (Goode, et al., 2010, Obata, et al., 1998, Quaye, et al., 2009). Pathways regulating processes such as apoptosis, angiogenesis and invasion have also been suggested. Similar alteration has been observed in the inflammatory and immune response between ovarian cancer and endometriosis (Ness, 2003), indicating a shared inflammatory pathway. There are several pathways to investigate and the difficulty is to know where to start. Finding proteins with aberrant expression in both diseases may give a clue about which pathways to investigate further. It would also be interesting to collect DNA and biopsies from patients with endometriosis or ovarian cancer and from patients with both diseases, and investigate the possible link between the diseases.
5.4 PAPER IV- EXPRESSION OF ADHESION, ATTACHMENT AND INVASION FACTORS IN ENDOMETRIOSIS

To obtain the normal function of the endometrium, different cell properties such as adhesion, attachment and invasion are important. However, the same properties may also be involved in the establishment of endometriotic lesions. According to Sampson’s theory about retrograde menstruation, viable cells are shed into the peritoneal cavity (Sampson, 1927). These cells need to be able to adhere to, attach to and invade at ectopic sites in order to cause endometriosis. It is possible that factors involved in these abilities could be expressed differently in women with endometriosis. Therefore, we investigated the expression and localization of six factors involved in the above functions, i.e. ApoE, ITGB2, ITGB7, LAMC1, CD24 and JAM-1, in endometrium from healthy controls and endometriosis patients as well as in endometriomas. In order to investigate if these factors were expressed in a cyclic pattern, we observed mRNA and protein expression in both the proliferative and secretory phases of the menstrual cycle.

In this study, we found endometrial expression of ApoE, ITGB2, ITGB7 and LAMC1 in stromal cells and ITGB7, LAMC1, CD24 and JAM-1 in epithelial cells. In endometriomas, we could observe expression of ApoE, ITGB2, ITGB7 and LAMC1 in sub epithelial regions and CD24 and JAM-1 in epithelial cells. The mRNA levels of CD24 and JAM-1 were significantly lower in endometriomas compared with healthy endometrium. Also, significantly lower ApoE mRNA levels were seen in the proliferative phase in endometriosis patients compared to controls. At protein level, a cyclic pattern of CD24 expression was found in epithelial cells from healthy endometrium. Protein levels in endometriomas were not quantified due to heterogeneity in the staining pattern and low protein expression for all studied factors. The lower mRNA and protein levels in endometriomas were surprising, since a higher expression of factors with adhesive, attaching and invasive properties was expected. It could be possible that the studied factors have other properties that promote the establishment of endometriosis when they are down regulated.

ApoE associate with cell surface receptors of plasma lipoprotein particles. It has been reported that women with endometriosis have an unfavourable lipid profile with increased levels of low density lipoproteins (LDL) (Melo, et al., 2010). Oxidised LDL induces MCP-1 production by mesothelial and endometrial cells (Rong, et al., 2002). In addition, oxidised LDL triggers inflammatory signalling via toll like receptors (Stewart, et al., 2010). Thus, ApoE may play a role in the inflammatory profile seen in women with endometriosis. Furthermore, ApoE has been reported to be a marker for cell survival and proliferation (Chen, et al., 2005), events that are important in the establishment of endometriosis. It would be interesting to further look into the function of ApoE in endometriosis, related to inflammation, cell survival and proliferation.

Integrin expression has previously been shown in the endometrium. However, there are many different integrins with different functions. Integrins have been observed in menstrual endometrium (Koks, et al., 2000, van der Linden, et al., 1994) and TNF-α has been shown to increase its adherence (Sillem, et al., 1999). Since TNF-α is an important inflammatory mediator in endometriosis, the expression of integrins may also
be important in the development of the disease. Stronger expression of integrin α5 has been observed in stromal cells from endometriosis patients compared to controls (Beliard, et al., 1997). Endometriotic stromal cells are able to adhere to laminin in the ECM and an aberrant integrin expression may be involved in this adhesion (Adachi, et al., 2010). Thus, several observations indicate a possible role of integrins in endometriosis.

An increased adhesive capacity has been observed in stromal cells from endometriotic lesions and endometrium from women with endometriosis in response to specific ECM components (Klemmt, et al., 2007). Laminins are located in the basement membrane of the ECM. LAMC1 have been reported to promote adhesion and migration of monocytes (Pedraza, et al., 2000). An altered expression of LAMC1 could contribute to the aberrant profile of immune cells seen in endometriosis patients. In addition, anti-Laminin antibodies have been associated with endometriosis-associated infertility (Inagaki, et al., 2003) and laminin has been suggested to be involved in the regulation of proliferation and differentiation of trophoblast cells during implantation (Klaffky, et al., 2001).

Previous studies have observed expression of CD24 and JAM-1 in the endometrium (Kim, et al., 2009, Koshiba, et al., 2009). Our findings support these reports. CD24 has been suggested to play a role in tumor progression in the ovaries (Choi, et al., 2005). In addition, aberrant expression of CD24 and JAM-1 have been found in reproductive cancers (Koshiba, et al., 2009, Kristiansen, et al., 2002), indicating that these proteins are involved in tumorgenesis. The establishment of endometriosis has similarities to malignant processes and therefore CD24 and JAM-1 may also be involved in the establishment of the disease, by allowing cells that are shed during menstruation to attach to the peritoneal surface and form lesions.

Our study has shown expression of ApoE, ITGB2, ITGB7, LAMC1, CD24 and JAM-1 in endometrium and endometriomas. However, the function of these proteins in the initiation of endometriosis and lesion needs to be investigated further. In addition, it would be interesting to evaluate the hormonal regulation of these proteins. Furthermore, several of these proteins are involved in malignancy and it would be interesting to evaluate their potential role in the transformation of endometriosis to ovarian cancer.
6 CONCLUSIONS AND FUTURE PERSPECTIVES

The following conclusions can be drawn based on the findings of this thesis:

- Women with endometriosis seem to have a diminished ovarian reserve, indicated by low levels of AMH and a poor IVF outcome. Furthermore, an aberrant inflammatory profile was seen with high levels of TNF-α, IL-15 and GM-CSF and low levels of IL-10. Thus, the diminished ovarian reserve could be due to the increased inflammatory activity in the ovarian follicles.

- We found an association of CCL21 rs2812378 and HLA rs660895 with moderate/severe endometriosis, but not with endometriosis as one group, in a Caucasian population. These findings indicate that the genetic background in endometriosis might differ depending on type and severity of disease. Our results do not indicate shared pathways of endometriosis and RA.

- No association of polymorphisms in the BNC2 gene was observed in endometriosis patients, suggesting that SNPs in BNC2 are unlikely to contribute to the reported risk of ovarian cancer in endometriosis.

- Endometrium from healthy women and endometriosis patients, together with endometriomas, express factors involved in adhesion, attachment and invasion, such as ApoE, ITGB2, ITGB7, LAMC1 CD24 and JAM-1. However, their role in the development of endometriosis needs to be studied further.

Altogether, these results indicate that the increased inflammatory profile seen in women with endometriosis may affect the ovarian reserve and thus lead to infertility problems in these women. Changes in the follicular fluid might influence the maturity and quality of the oocyte, fertilization, early embryonic development and pregnancy outcome. Genetic associations in inflammatory related genes were also found in women with moderate/severe disease, further indicating the importance of inflammation in endometriosis. In addition, the expression of factors involved in adhesion, attachment and invasion may be important in the establishment of endometriotic lesions. These factors may also be regulated by inflammatory mediators.

For future studies, it would be interesting to investigate the function of CCL21 in endometriosis and to find out if the CCL21 rs2812378 has a functional role in the disease. To start with, the expression of CCL21 could be compared to the rs2812378 alleles, to see the impact of the endometriosis-associated A allele. It would also be interesting to investigate other SNPs that are in LD with rs2812378, to see if the endometriosis associated SNP(s) could lie elsewhere.

In general, I would like to collect a new sample set of Swedish cases and controls, which is larger than our previous material and with more information about the patients. It would be interesting to collect data of type and stage of endometriosis, other diseases, such as autoimmune diseases and cancer, and environmental factors. From
these patients, biopsies and serum samples could also be collected to do follow-up functional studies and expression analysis. A GWAS could be used to localize candidate genes and our samples from Belgium could be used for replication.

Since we have found expression of different factors involved in adhesion, attachment and invasion (ApoE, ITGB2, ITGB7, LAMC1 CD24 and JAM-1), it would be interesting to evaluate the possible role of these factors in endometriosis. I would like to perform invasion and proliferation assays with cells expressing these markers and compare between cells from endometrium from healthy controls and endometriosis patients, to see if the function is changed in endometriosis patients. I would also like to investigate the hormonal regulation of these factors, especially the effect of estrogen. Furthermore, it would be interesting to see if there is any connection between stem/progenitor cells and the expression of factors involved in adhesion, attachment and invasion. Is it possible that endometrial stem/progenitor cells with increased adhesive and invasive properties are shed during menstruation in endometriosis patients, but not in healthy women?
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8 REFERENCES


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