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MERIT LAMP

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Genetic susceptibility factors in endometriosis

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Department of Obstetrics and Gynaecology, University of Tartu, Estonia

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Merit Lamp performed the recruitment and interviewing of gynaecological patients and was involved in the collection of their blood and serum samples.

Paper I: Participation in the study design, performing the experiments, statistical data analysis, writing the paper.

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Paper IV: Participation in the study design and in performing the experiments, statistical data analysis, writing the paper.

ABBREVIATIONS

17β-HSD1	17β-hydroxysteroid dehydrogenase type 1
ACE	Angiotensin-I converting enzyme
ACE	Angiotensin-I converting enzyme gene
ART	Assisted reproductive technology
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing protein 5 (survivin) gene
BMI	Body mass index
CDE	Cell cycle-dependent element
CDKN2BAS	Cyclin-dependent kinase inhibitor 2B antisense RNA gene
CHR	Cell cycle homology region
CI	Confidence interval
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1 (aromatase) gene
CYP2C19	Cytochrome P450, family 2, subfamily C, polypeptide 19 gene
Del	Deletion
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
ERE	Oestrogen response element
ESR1	Oestrogen receptor 1 (α) gene
ESR2	Oestrogen receptor 2 (β) gene
FAM	Carboxyfluorescein
GnRH	Gonadotrophin-releasing hormone
HOXA10	Homeobox protein A10 gene
HOXA11	Homeobox protein A11 gene
HWE	Hardy-Weinberg equilibrium
HSD17B1	17β-hydroxysteroid dehydrogenase type 1 gene
Ins	Insertion
JOE	Carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein
L	Long microsatellite allele
LD	Linkage disequilibrium
MMP	Matrix metalloproteinase
MMP2	Matrix metalloproteinase 2 gene
MMP9	Matrix metalloproteinase 9 gene
mRNA	Messenger ribonucleic acid
OD	Optical density
OR	Odds ratio
PCR	Polymerase chain reaction
PGR	Progesterone receptor gene
PR	Progesterone receptor
PRE	Progesterone response element

RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROX	Carboxy-X-rhodamine
S	Short microsatellite allele
SNP	Single nucleotide polymorphism
TAMRA	Carboxy-tetramethyl-rhodamine
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A gene
WNT4	Wingless-type mouse mammary tumour virus integration site family, member 4 gene
ZnT8	Zinc transporter 8

1. INTRODUCTION

Endometriosis is a chronic gynaecological disease affecting millions of women worldwide. It is defined as the presence of endometrial-like tissue outside the uterus and, although in some cases it may remain asymptomatic, it is often associated with severe pelvic pain and infertility, thus having a major impact both on women suffering from it as well as on their families.

The 'gold standard' for the diagnosis of endometriosis is laparoscopic visualization of endometriotic lesions. Therefore, due to the lack of an effective non-invasive diagnostic technique and the large variability in disease symptoms, the right diagnosis is often delayed for years.

Despite its high prevalence, which is about 10% of women of reproductive age, the exact cause of endometriosis is still unknown. Several theories about its pathogenesis have been proposed, but to date none of them has been fully confirmed, nor do they entirely explain all the mechanisms associated with the disease development. In addition, it has been hypothesized that different types of endometriotic lesions may have diverse origins. Therefore, it is likely that endometriosis is a multifactorial disease caused by the interaction of several different factors.

Since familial aggregation of endometriosis has been observed, genetic factors are thought to contribute to disease development. Identifying the genes involved in susceptibility to endometriosis could help us understand better various disease mechanisms and thus would aid in the development of new therapeutic methods. Given that endometriosis is considered to be an oestrogen-dependent disease, genes involved in sex steroid biosynthesis and signalling are good candidates for a genetic association study. For the present thesis we selected three genes encoding for oestrogen and progesterone receptors and two encoding for the key-enzymes involved in oestrogen production. Additional five genes were included in the study based on the most common disease theory, Sampson's implantation hypothesis, which suggests the growth of endometriotic lesions from retrogradely transported endometrial cells and implies the importance of tissue remodelling, neoangiogenesis and reduced apoptotic activity in disease development.

In addition to genetic factors, impaired function of the immune system and elevated levels of certain autoantibodies have been reported in association with endometriosis. Identifying disease-specific autoantibodies might be useful for the development of minimally-invasive techniques for the diagnosis of endometriosis. In view of that, autoantibodies against survivin, a protein which belongs to the family of apoptosis inhibitors and is mostly expressed in cancer tissues and in endometriotic lesions, could be a possible biomarker for endometriosis.

2. REVIEW OF LITERATURE

2.1. Endometriosis

Endometriosis is a chronic gynaecological disease defined as the presence of endometrial glands and stroma outside the uterine cavity. Endometriotic lesions are generally located on the pelvic peritoneum and viscera, as well as in the rectovaginal septum and sacrouterine ligaments. In rare cases ectopic endometrium can be found also in the abdominal wall, pleura and in the central nervous system. Endometriosis is predominantly found in women of reproductive age and its main symptoms include severe dysmenorrhoea, chronic pelvic pain, deep dyspareunia, and infertility. The diagnosis is usually made by visual inspection of the pelvis at laparoscopy. Since it is an invasive procedure, it is difficult to measure the population prevalence of endometriosis. It is estimated that around 10% of women of reproductive age suffer from endometriosis (Giudice and Kao 2004), including 6% of fertile women, and most importantly, up to 45–55% of infertile women undergoing laparoscopy have endometriosis (Mahmood and Templeton 1991; Calhaz-Jorge et al. 2004; Hemmings et al. 2004).

Endometriosis may present with three distinct forms of lesions: superficial endometriotic implants on the pelvic peritoneum and on the ovaries (peritoneal endometriosis), ovarian cysts lined by endometrial mucosa (endometriomas), and nodules comprised of endometriotic tissue, adipose and fibromuscular tissue, residing between the rectum and the vagina (rectovaginal endometriotic nodules). All three forms may occur as single or in combination. Whether these three types are variants of the same pathologic process or are caused by different mechanisms is not clear yet (Nisolle and Donnez 1997; Burney and Giudice 2012; Donnez 2012).

The common features of endometriotic lesions are the presence of endometrial cells, chronic bleeding, and inflammatory reaction which causes scarring and the formation of adhesions in the peritoneal cavity. Based on the localization, number, size and depth of invasion of ectopic lesions, and the presence of adhesions, endometriosis is classified into four different stages: I – minimal, II – mild, III – moderate, and IV – severe disease (Table 1) (ASRM 1997).

Table 1. Classification of endometriosis by points assigned based on the location, size and depth of endometriotic lesions, and the presence of adhesions (ASRM 1997).

	Endometriosis		< 1cm	1-3cm	> 3cm
Peritoneum		Superficial	1	2	4
		Deep	2	4	6
Ovary	Right	Superficial	1	2	4
		Deep	4	16	20
	Left	Superficial	1	2	4
		Deep	4	16	20
	Adhesions		< 1/3 Enclosure	1/3-2/3 Enclosure	> 2/3 Enclosure
Ovary	Right	Filmy	1	2	4
		Dense	4	8	16
	Left	Filmy	1	2	4
		Dense	4	8	16
Tube	Right	Filmy	1	2	4
		Dense	4	8	16
	Left	Filmy	1	2	4
		Dense	4	8	16
Posterior cul-de-sac obliteration			Partial 4		Complete 40
Total score		1-5	6-15	16-40	> 40
Stage		I – minimal	II – mild	III – moderate	IV – severe

2.2. Pathogenesis of endometriosis

Despite its relatively high prevalence, the aetiology and pathogenesis of endometriosis are not fully understood. Coelomic metaplasia and embryonic rest theories suggest that endometriotic lesions develop from mesothelial cells or from cells of mullerian origin, respectively, which due to a yet undetermined stimulus differentiate into endometrium-like tissue (Sasson and Taylor 2008; Burney and Giudice 2012). These mechanisms could explain the presence of ectopic endometrium in the rectovaginal septum and the rare occurrence of endometriosis before or in early puberty. However, the most common theory, thus far, is Sampson's implantation theory (Sampson 1927b), according to which during menstruation endometrial cells reflux through the fallopian tubes

into the abdominal cavity where they implant on extrauterine structures and subsequently form ectopic endometriotic lesions. Sampson also suggested that endometrial cells might spread through veins and lymphatic vessels, thus giving rise to distant ectopic lesions outside the pelvis, for example in the lungs and brain (Sampson 1927a). Implantation theory is supported by the facts that viable endometrial cells can be found in menstrual and peritoneal fluid (Koninckx et al. 1980), and that endometriosis often develops in women with vaginal obstruction of outflow (Olive and Henderson 1987).

In line with Sampson's hypothesis, recent data suggest a potential role for the endometrial stem/progenitor cells in the pathogenesis of endometriosis. Stem cells are speculated to function in the cyclic regeneration of the endometrium and endometriotic implants are thought to result from the retrograde menstruation of these cells (Sasson and Taylor 2008).

Up to 90% of women with patent fallopian tubes exhibit some grade of retrograde menstruation (Halme et al. 1984), but only a minority of them develops endometriosis, considering that its prevalence is around 10%. Therefore, it is likely that also other factors are involved in the development of this disease. One possible explanation could be alterations in the eutopic endometrium, as differences in gene expression profiles in endometrium of women with and without endometriosis have been demonstrated (Kao et al. 2003; Burney et al. 2007). Also impaired function of the immune system has been implicated in the development of endometriosis (Dmowski 1995; Kyama et al. 2003; Herington et al. 2011). Increased levels of activated macrophages, cytokines, T and B cells are found in the peritoneal fluid of endometriosis patients, indicating a local inflammatory reaction which may promote the establishment of endometriotic lesions. In addition, although the ability of immune cells to produce cytokines and growth factors is retained, altered function of regulatory T cells and a decrease in natural killer (NK) cell and T cell cytotoxic activity and in macrophage phagocytic capacity has been observed, which further contributes to the survival of sloughed endometrial cells and to the formation of ectopic implants (Kyama et al. 2003; Herington et al. 2011).

2.3. Endometriosis and infertility

As mentioned above, endometriosis is often associated with reduced fertility. In normal couples, fecundity, defined as the probability of a woman achieving a live birth in a given month, ranges from 0.15 to 0.20. In untreated women with endometriosis monthly fecundity tends to be lower, being in the range of 0.02 to 0.10 (Ozkan et al. 2008; Bulletti et al. 2010). In addition, while the general prevalence of endometriosis is around 10%, it is diagnosed in about 50% of infertile women undergoing laparoscopy (Calhaz-Jorge et al. 2004; Hemmings et al. 2004). Despite intensive research, however, the precise mechanisms causing infertility in endometriosis patients are still unclear (Bulletti et al. 2010; ASRM 2012; Carvalho et al. 2012).

In case of moderate-severe endometriosis (stage III–IV), infertility is likely related to the presence of adhesions in the peritoneal cavity, which may cause anatomical distortion of pelvic organs and can thus hinder oocyte release from the ovary, ovum capture and transport (Ozkan et al. 2008; Carvalho et al. 2012). However, as women with stage I–II disease, with little anatomical effect, also suffer from infertility, additional mechanisms affecting reproductive function must be involved.

Studies on women undergoing assisted reproductive technologies (ART) have shown correlations between endometriosis and a decrease in different outcomes, like oocyte retrieval rate, fertilization rate, implantation rate and pregnancy rate (Barnhart et al. 2002). Others, however, have reported only a reduction in the number of oocytes retrieved (Suzuki et al. 2005). These observations suggest that different stages of the reproductive process may be impaired in women suffering from endometriosis. Mechanisms that reduce fertility may include endocrine and ovulatory disorders, impaired folliculogenesis and altered immunological environment in the peritoneal cavity, which may have adverse effects on the function of the oocyte, sperm, embryo and fallopian tubes. In addition, immunological and hormonal abnormalities in the eutopic endometrium may be involved, causing reduced endometrial receptivity and defective implantation. Impaired fertility may also be related to poor oocyte and embryo quality, as women with moderate to severe endometriosis who receive oocytes from disease-free women appear to have normal endometrial receptivity and pregnancy rates (ASRM 2012).

Although a firmly established causal relationship between endometriosis and infertility is still lacking, it has been shown that treatment of endometriosis may improve fertility in these women (Ozkan et al. 2008).

2.4. Endometriosis and genetics

There is evidence to suggest that susceptibility to endometriosis may be influenced by genetic factors. Familial clustering of endometriosis has been described in humans and in rhesus monkeys (Kennedy 1999). A study by Simpson and colleagues showed that the incidence of endometriosis in the first-degree relatives of affected women was about seven times higher than among women without such a family history (6.9% *vs.* 1.0%) (Simpson et al. 1980). Similar findings have been reported also in other studies (Coxhead and Thomas 1993; Moen and Magnus 1993). Additionally, severe forms of endometriosis were observed markedly more often in familial than in sporadic cases (61% *vs*. 23%) (Bischoff and Simpson 2004). Evidence for genetic contributions to endometriosis risk also comes from twin studies, which show a high rate of concordance for endometriosis among monozygotic twin sisters (Moen 1994; Hadfield et al. 1997; Treloar et al. 1999).

These data suggest that endometriosis is a polygenic/multifactorial disease, meaning that many different genes contribute to disease risk and that the

contribution of each gene individually is small. Identifying which genetic factors influence susceptibility to endometriosis could help us understand better the pathogenetic mechanisms of this disease and to develop new methods for diagnosis and treatment of endometriosis.

Numerous studies on single candidate genes have been carried out in different populations, however, often the results have been inconsistent (Montgomery et al. 2008; Dun et al. 2010; Rahmioglu et al. 2012). Genome-wide linkage and genome-wide association studies (GWAS) have identified loci on chromosome 1, 2, 6, 7, 9, 10 and 12 to be associated with endometriosis (Treloar et al. 2005b; Zondervan et al. 2007; Uno et al. 2010; Painter et al. 2011a; Nyholt et al. 2012; Albertsen et al. 2013). Some of the genes within or close to these loci, like *CDKN2BAS* (cyclin-dependent kinase inhibitor 2B antisense RNA), *WNT4* (wingless-type mouse mammary tumour virus integration site family, member 4), *HOXA10* (homeobox A10), *HOXA11* (homeobox A11) and *CYP2C19* (cytochrome P450, family 2, subfamily C, polypeptide 19) are considered plausible candidate genes (Uno et al. 2010; Painter et al. 2011a; Painter et al. 2011b; Pagliardini et al. 2013), yet they explain only a small part of the variance in susceptibility to endometriosis, suggesting the additional involvement of other genetic variants.

2.5. Endometriosis and sex steroids

Endometriosis is considered to be an oestrogen-dependent disease, as it occurs mostly in women of reproductive age; symptoms usually appear after menarche and regress spontaneously after menopause (Kitawaki et al. 2002; Bulun 2009). In addition, pharmacological suppression of oestrogen by gonadotropin-releasing hormone (GnRH) analogues, oral contraceptives, progestins or aromatase inhibitors provides the regression of endometriotic lesions and reduces pain, while discontinuation of this therapy or introducing oestrogen replacement therapy in postmenopausal women may both give a relapse of the disease.

The growth of endometrial tissue is regulated by oestrogen and progesterone, with oestrogen stimulating proliferation and progesterone opposing the effects of oestrogen and inhibiting cell proliferation (Graham and Clarke 1997). In normal conditions, oestrogen is mainly produced in the ovaries, but in smaller quantities also in adipose tissue and skin, where circulating androstenedione is converted by different steroidogenic enzymes into oestrone and subsequently to the biologically more active form, oestradiol. In women with endometriosis, however, oestrogen is produced also directly in the endometriotic tissue, since differently from the eutopic endometrium, it expresses all the necessary enzymes for oestrogen biosynthesis, including the two key enzymes cytochrome P450 aromatase and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) (Noble et al. 1996; Smuc et al. 2007) (Figure 1). Thus the local level of oestradiol in endometriotic lesions might be elevated compared to normal endometrial tissue.

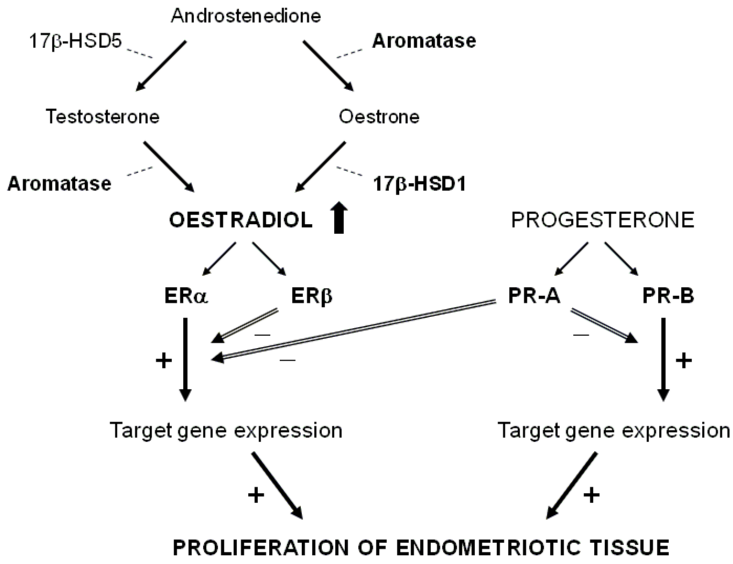

Figure 1. The role of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and aromatase in the production of oestradiol, and the function of oestrogen and progesterone in endometriotic lesions mediated through oestrogen receptors α (ERα) and β (ERβ), and through progesterone receptors A (PR-A) and B (PR-B).

The role of progesterone in the eutopic endometrium is to exert an anti-oestrogenic effect, inhibiting tissue proliferation (Graham and Clarke 1997). In case of endometriosis, however, endometriotic tissue fails to respond adequately to progesterone, which additionally contributes to the stimulating effect of the abnormally high oestrogen levels (Bulun et al. 2006).

Besides their direct effect on endometrial proliferation, oestrogen and progesterone are also involved in the regulation of the immunological micro-environment at eutopic and ectopic sites (Herington et al. 2011). Therefore, an imbalance in their function may influence the growth of endometriotic lesions also indirectly.

Considering the heavy involvement of oestrogen and progesterone in the development of endometriosis, a search for candidate genes should include those involved in biosynthesis and signalling pathways of sex steroids.

2.5.1. Oestrogen receptors

In humans, the effects of oestrogens are classically mediated via two oestrogen receptors ERα and ERβ, which are members of the nuclear receptor subfamily 3 and function as ligand-activated transcription factors. They share a common structural and functional organization, with distinct domains for ligand binding, DNA binding, and transcriptional activation (Enmark and Gustafsson 1999).

ERs modify the expression of many different genes involved in cell growth, proliferation and differentiation by binding to oestrogen response elements (ERE) located in gene promoters. Both receptors can form homodimers or heterodimers with the other subtype (Enmark and Gustafsson 1999). ERα and ERβ are encoded by two different genes, *ESR1* (6q25.1) and *ESR2* (14q23.2), respectively (Walter et al. 1985; Mosselman et al. 1996). Both genes are subject to alternative splicing and, besides the wild-type receptor, at least two other ERα isoforms and four ERβ isoforms exist (Gibson and Saunders 2012). However, the impact of these ER variants on cell function remains poorly understood thus far. ERα and ERβ show partially overlapping but also unique functions and expression patterns in human tissues (Matthews and Gustafsson 2003). In the reproductive tract ERα is primarily expressed in the uterus, whereas ERβ is more expressed in the ovary (Matthews and Gustafsson 2003). In tissues co-expressing ERα and ERβ, cellular response to estrogens depends on the ERα/ERβ ratio, since ERβ tends to inhibit ERα-mediated gene expression (Hall and McDonnell 1999) (Figure 1). Both oestrogen receptors are expressed in normal endometrium as well as in endometriotic lesions (Moutsatsou and Sekeris 2003), and genetic variants in their respective genes could alter relative expression levels of receptor subtypes, thus modifying tissue sensitivity to oestrogens.

Within the *ESR1* gene locus, more than 2800 single nucleotide polymorphisms (SNP) have been identified thus far (snpper.chip.org). One of the most frequently studied polymorphisms is the c.454 -397T/C SNP in intron 1 (rs2234693), defined by the restriction enzyme *Pvu*II. The *Pvu*II C allele creates a possible binding site for the *myb* family of transcription factors and might in certain conditions amplify *ESR1* transcription and oestrogen actions (Herrington et al. 2002). Another potentially functional polymorphism is a $(TA)_n$ microsatellite repeat situated upstream from the coding region. *ESR1* gene has at least seven different promoters, which produce different mRNA molecules with unique 5'-UTRs, but mostly with identical coding regions (Kos et al. 2001). The $(TA)_n$ locus lies between exons B and C (nomenclature by Kos et al, 2001) and therefore might influence alternative promoter usage and the expression of ERα (Becherini et al. 2000). Both, the *Pvu*II T/C SNP and the $(TA)_n$ repeat, have been found to be associated with oestrogen-dependent diseases like breast cancer (Anghel et al. 2006; Li et al. 2010) and osteoporosis (Gennari et al. 2005). Several studies have also investigated their role in endometriosis, but with conflicting results (Georgiou et al. 1999; Kitawaki et al. 2001; Wang et al. 2004; Hsieh et al. 2005b; Kim et al. 2005b; Luisi et al. 2006; Renner et al. 2006; Hsieh et al. 2007b; Xie et al. 2008; Govindan et al. 2009).

The *ESR2* gene contains over 700 SNPs (snpper.chip.org). In addition, there is a highly polymorphic dinucleotide $(CA)_n$ repeat polymorphism in intron 5, which similarly to the *ESR1* gene variants, has been associated with breast cancer risk (Anghel et al. 2006; Tsezou et al. 2008) and bone mineral density (Gennari et al. 2005). However, according to our knowledge, there are no published studies investigating its role in endometriosis.

2.5.2. Progesterone receptor

The physiologic effects of progesterone are mediated via progesterone receptor (PR) which, like ERs, belongs to the steroid receptor superfamily and functions as a ligand-activated transcription factor. PR is expressed as two isoforms, PR-A and PR-B, which after ligand binding form homo- or heterodimers and regulate the expression of target genes by binding to the progesterone-response elements (PRE) in their promoter regions (Graham and Clarke 1997). Both isoforms are encoded by a single gene, *PGR* (11q22-q23), using separate promoters and translational start sites. PR-B contains an additional 165 amino acids at the N terminus and acts mostly as a transcriptional activator, while PR-A represses the transcriptional activity of PR-B and of other steroid receptors (Conneely et al. 2003) (Figure 1). As the two isoforms are functionally different, tissue responsiveness to progesterone may be modulated by alterations in the ratio of PR-A and PR-B (Graham and Clarke 1997). Both PR isoforms are expressed in normal endometrium as well as in endometriotic lesions (Mote et al, 1999; Misao et al, 1999). Therefore, genetic variants in the *PGR* capable of altering the expression levels of receptor subtypes might modify tissue sensitivity to progesterone and influence susceptibility to endometriosis.

In the *PGR* gene, over 900 SNPs have been described so far (snpper.chip.org). Two widely studied functional variants are a +331G/A SNP (rs10895068) in the *PGR* promoter region and the PROGINS haplotype, which consists of a single amino acid substitution in exon 4 (Val660Leu), a silent point mutation in exon 5 (His770His), and a 306-bp Alu-insertion (Ins) in intron 7 which are in complete LD with each other (De Vivo et al. 2002). The +331G/A SNP A allele creates a unique transcription start site that favours the production of PR-B isoform (De Vivo et al. 2002), while PROGINS (660Leu/770His/AluIns) has been shown to reduce PR-A mediated inhibition of cell proliferation (Romano et al. 2007). Both of these polymorphisms have been associated with the risk of different gynaecological tumours (De Vivo et al. 2002; De Vivo et al. 2003; Govindan et al. 2007; Pearce et al. 2008). Several studies have investigated their role also in endometriosis, but the results have been conflicting (Wieser et al. 2002; Lattuada et al. 2004; Treloar et al. 2005a; De Carvalho et al. 2007; Govindan et al. 2007; van Kaam et al. 2007; Gentilini et al. 2008b).

2.5.3. Aromatase

One of the key enzymes in oestrogen biosynthesis is cytochrome P450 aromatase, which catalyses the conversion of testosterone and androstenedione to oestradiol and oestrone, respectively (Figure). It is expressed in a number of human tissues, like gonads, placenta, adipose tissue, skin, brain etc (Bulun et al. 1997). Normal eutopic endometrium does not express aromatase, but in eutopic and ectopic endometrium of women with endometriosis, it is significantly up-

regulated (Noble et al. 1996; Smuc et al. 2007), suggesting the existence of local oestrogen production and the importance of aromatase in the pathogenesis of endometriosis.

Aromatase is encoded by the *CYP19A1* (cytochrome P450, family 19, subfamily A, polypeptide 1) gene (15q21.1), which consists of 9 coding exons (II-X) and is expressed in a tissue-specific manner by using different promoters (Sebastian 2001). In endometriotic tissue aromatase expression is regulated mostly by the gonad-specific promoter II (Bulun et al. 2005). Although the transcripts of aromatase have different 5'-UTRs they are spliced onto a common junction 38 bp upstream of the translation start site, meaning that the sequence encoding the open reading frame and consequently the expressed protein are identical in each case (Bulun et al. 2005).

The *CYP19A1* gene contains more than 1000 SNPs (snpper.chip.org). Three commonly studied polymorphisms in the *CYP19A1* gene include a tetra-nucleotide $(TTTA)_n$ repeat and a 3-bp (TCT) insertion/deletion (Ins/Del) polymorphism in intron 4, and a C/T SNP (rs10046) in the 3'-UTR of exon 10. These polymorphisms have been shown to correlate with aromatase activity and to influence susceptibility to oestrogen-dependent diseases (Kristensen et al. 2000; Gennari et al. 2004). Studies on their involvement in endometriosis, however, have yielded inconsistent results (Kado et al. 2002; Arvanitis et al. 2003; Huber et al. 2005; Hur et al. 2007; Vietri et al. 2009).

2.5.4. 17β-hydroxysteroid dehydrogenase type 1

A second key enzyme in oestrogen biosynthesis is 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) which converts oestrone to the biologically more active oestradiol (Figure). It is a cytosolic protein and functions as a homodimer (Labrie et al. 2000). 17β-HSD1 is abundantly expressed in different tissues, like ovaries, breasts and placenta (Peltoketo et al. 1999). Normal endometrium, however, contains only low levels of 17β-HSD1, but in eutopic and ectopic endometrium of women with endometriosis its expression is significantly up-regulated (Smuc et al. 2007), suggesting a potential role in disease development.

17β-HSD1 is encoded by the *HSD17B1* gene (17q11-q21) which contains about 130 SNPs (snpper.chip.org), including a +1954A/G SNP (Ser312Gly) in exon 6 (rs605059). The amino acid change related to this SNP does not appear to influence the catalytic or immunological properties of the 17β-HSD1 enzyme (Puranen et al. 1994), yet it has been shown to be associated with circulating oestradiol levels (Setiawan et al. 2004). Thus far only one study has investigated its role in endometriosis and reported a significant association with disease susceptibility (Tsuchiya et al. 2005).

2.6. Tissue remodelling and angiogenesis in endometriosis

According to Sampson's implantation theory, endometrial cells reflux to the abdominal cavity and implant on the peritoneum thus giving rise to the development of endometriotic lesions. This requires the involvement of different physiologic processes like cell adhesion and proliferation, tissue remodelling and neoangiogenesis to allow the ectopic growth of endometrial implants. Remodelling of the connective tissue requires both breakdown and resynthesis of extracellular matrix (ECM) components. Different enzymes can be involved in the degradation of ECM proteins, but the primary contributors are believed to be matrix metalloproteinases (MMPs) (Hulboy et al. 1997).

MMPs are a group of structurally-related extracellular and membrane bound proteinases that degrade ECM and basement membrane components throughout the body (Hulboy et al. 1997). Their activity influences several different biological processes such as embryonic development, organ morphogenesis, angiogenesis, wound healing etc (Birkedal-Hansen et al. 1993). MMPs also participate in the control of reproductive function by regulating the structural changes that occur in the ovaries and uterus throughout the menstrual cycle (Hulboy et al. 1997; Curry and Osteen 2001). The activity of MMPs is controlled by steroid hormones, cytokines, growth factors, and tissue and plasma inhibitors of metalloproteinases (Hulboy et al. 1997; Nelson et al. 2000). In endometrium, the expression of MMPs depends on the phase of the menstrual cycle: during menstruation, when tissue breakdown occurs they are highly expressed, but throughout the rest of the cycle their concentrations are low or undetectable in general (Hulboy et al. 1997). Thus, precisely controlled regulation of MMP expression is essential for normal tissue remodelling during menstrual cycle. An increase in baseline MMP levels could enhance the proteolytic activity and invasive properties of endometrial cells and might therefore favour their peritoneal implantation and the development of endometriotic lesions.

Once endometrial cells have implanted on the peritoneum the establishment of a new blood supply is a crucial event for their survival and the subsequent development of ectopic lesions. It has been demonstrated that tumour implants are not able to grow beyond a volume of 3 mm^3 unless they develop a new blood supply (Folkman 1995). It can be assumed that the same mechanism applies for the growth of endometriotic lesions. Therefore, active neo-angiogenesis and vascular remodelling are thought to be important mechanisms in the pathogenesis of endometriosis. In fact, peritoneal fluid from women with endometriosis, as well as their eutopic and ectopic endometrial tissue, have been shown to have more angiogenic activity compared to normal controls (Taylor et al. 1997; Di Carlo et al. 2009).

2.6.1. Matrix metalloproteinases 2 and 9

Endometrial stromal cells express several MMPs, including MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which seem to have an important role in endometrial ECM breakdown (Curry and Osteen 2001). These proteinases may play an active part in the establishment and progression of endometriosis, since altered MMP-2 and MMP-9 expression profiles have been reported in eutopic and ectopic endometrial tissues obtained from women with endometriosis (Chung et al. 2001; Shaco-Levy et al. 2008; Di Carlo et al. 2009) (Figure 2). In particular MMP-2 seems to correlate with endometriosis aggressiveness, as its expression in invasive colorectal endometriosis was found to be significantly higher than in superficial peritoneal lesions (Weigel et al. 2012). Also circulating MMP-2 levels are reported to be higher in women with stage III-IV disease than in those with stage I–II endometriosis (Malvezzi et al. 2012). In addition, down-regulation of both MMP-2 and MMP-9 activities by curcumin has been shown to inhibit endometriosis development in mouse models (Swarnakar and Paul 2009; Jana et al. 2012), suggesting their importance in the establishment and growth of endometriotic implants.

MMP-2 and MMP-9 are encoded by the *MMP2* (16q13-q21) and *MMP9* (20q11.2-q13.1) genes, which contain about 450 and 250 SNPs, respectively (snpper.chip.org). Some of the SNPs in the promoter regions of *MMP2* and *MMP9* have been shown to lead to changes in gene expression levels and thus could be associated with a predisposition to a variety of diseases (Zhang et al. 1999; Yu et al. 2004; Zhou et al. 2005; Kang et al. 2008a). In *MMP2*, four highly linked promoter SNPs: -1575G/A (rs243866), -1306C/T (rs243865), -790T/G (rs243864) and -735C/T (rs2285053) have been described. The minor alleles of the -735C/T and -1306C/T SNPs are associated with diminished promoter activity due to the disruption of a transcription factor *Sp1* binding element (Price et al. 2001; Yu et al. 2004). Also in the *MMP9* gene, the -1562T/C SNP (rs3918242) has been reported to influence its transcriptional activity (Zhang et al. 1999). A few genetic association studies have been carried out to find relations between these polymorphisms and endometriosis, but so far mostly on women of Asian origin, with majority reporting negative results (Shan et al. 2006; Borghese et al. 2008; Kang et al. 2008b; Han et al. 2009b).

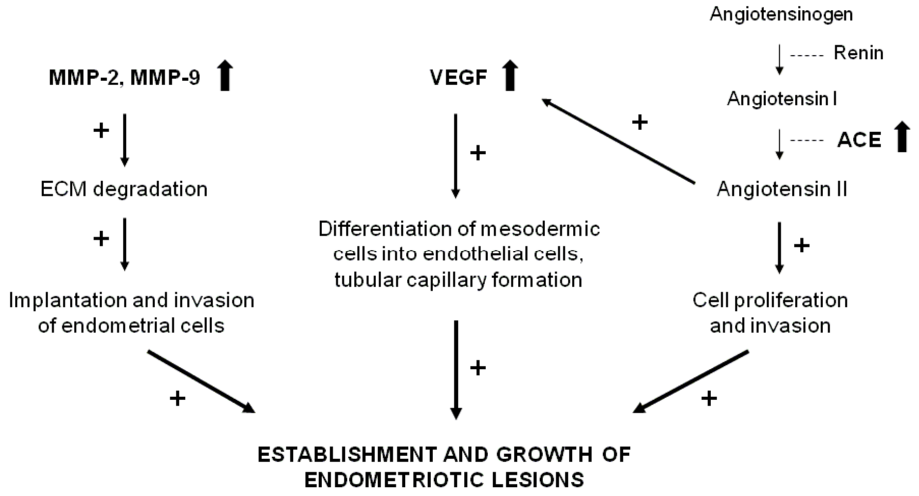

Figure 2. The possible role of matrix metalloproteinases (MMP) 2 and 9, vascular endothelial growth factor (VEGF) and angiotensin I-converting enzyme (ACE) in the development of endometriosis; ECM – extracellular matrix.

2.6.2. Vascular endothelial growth factor

Endometrial angiogenesis is controlled by sex steroids which exert their effect indirectly via numerous growth factors. One of the most important factors is vascular endothelial growth factor (VEGF), a heparin-binding homodimeric glycoprotein, which increases vascular permeability and induces endothelial cell proliferation, migration, differentiation, and capillary formation (Ferrara 2004). Different studies have shown altered VEGF mRNA expression and elevated protein levels in eutopic and ectopic endometrium as well as in the peritoneal fluid of women with endometriosis (Taylor et al. 1997; Donnez et al. 1998; Bourlev et al. 2006; Di Carlo et al. 2009). It has been suggested that after the attachment of endometrial cells, high VEGF levels could stimulate the formation of the subperitoneal vascular network and thus facilitate implantation and viability of endometrial cells (Donnez et al. 1998) (Figure 2).

VEGF is encoded by the *VEGFA* gene (6p12), which consists of eight exons and through alternative splicing produces different transcript variants, encoding either freely secreted or cell surface-bound VEGF isoforms (Ferrara 2004). The *VEGFA* gene contains over 200 SNPs (snpper.chip.org). Some of these variants, like -2578A/C (rs699947), -1154G/A (rs1570360), -634G/C (also known as +405G/C; rs2010963), and +936C/T (rs3025039) SNPs have been associated with altered expression of VEGF (Koukourakis et al. 2004; Szeto et al. 2004; Steffensen et al. 2010). Several studies have been carried out to investigate the role of these *VEGFA* polymorphisms in susceptibility to endometriosis, but the results have been inconsistent (Bhanoori et al. 2005; Kim et al. 2005a; Ikuhashi

et al. 2007; Gentilini et al. 2008a; Kim et al. 2008; Zhao et al. 2008; Cosin et al. 2009; Liu et al. 2009; Altinkaya et al. 2011).

2.6.3. Angiotensin I-converting enzyme

Angiotensin I-converting enzyme (ACE) is a major component of the renin–angiotensin system which catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II. Angiotensin II regulates blood pressure and fluid-electrolyte homeostasis, but besides its systemic effects it also modulates the angiogenic reaction and could be involved in both normal and pathological angiogenesis (Chua et al. 1998; Pupilli et al. 1999; Tamarat et al. 2002). It has been shown to stimulate cell growth and invasion, and to increase VEGF expression in endothelial cells via the angiotensin II type 1 receptor (Fujiyama et al. 2001; Tamarat et al. 2002). In human endometrium, the presence of a local renin-angiotensin system has been demonstrated (Li and Ahmed 1996). Since angiotensin II has been found to be strongly expressed around endometrial blood vessels in the late secretory phase and in the stroma and glandular epithelium during the proliferative phase, it is thought to be important in the initiation of menstruation and in angiogenesis during endometrial regeneration (Li and Ahmed 1996). As angiotensin II production depends on ACE activity, an imbalance in ACE expression could affect menstruation and might thus predispose certain women to endometriosis (Figure 2).

ACE is encoded by the *ACE* gene (17q23.3), which through alternative splicing may give rise to different isoforms. Around 300 SNPs have been identified in the *ACE* locus (snpper.chip.org), but the most studied polymorphism is a 287-bp Alu insertion/deletion (Ins/Del) polymorphism in intron 16 (rs4340). It correlates with circulating ACE level, however, its effect on blood pressure and cardiovascular diseases seems to be limited (Jeunemaitre 2008). A few studies have reported also other *ACE* polymorphisms in association with ACE expression. Two SNPs, -240A/T in the promoter region (rs4291) and +2350A/G in exon 17 (Thr776Thr; rs4343) were found to explain more than 20% of the total variance in serum ACE level and to be significantly associated with blood pressure (Zhu et al. 2001). The impact of *ACE* variants has been widely studied for example in Alzheimer's disease and in cardiovascular diseases (Sayed-Tabatabaei et al. 2006). So far, however, only a few studies have investigated their role in endometriosis, reporting conflicting results (Hsieh et al. 2005a; Hsieh et al. 2007a; Kowalczynska et al. 2011).

2.7. Apoptosis in endometriosis

Apoptosis is a process of programmed cell death that occurs in multicellular organisms and keeps balance between tissue proliferation and degradation. It can be initiated by different extra- and intracellular signals and defects in its activity are involved in the development of a variety of diseases, for example in

tumours. Similarly to neoplastic processes, diminished apoptosis appears to have a role in the pathogenesis of endometriosis. According to Sampson's theory, ectopic lesions develop from uterine endometrial cells that disseminate into peritoneal cavity during menstruation (Sampson 1927b). As retrograde menstrual flow appears to be a physiological phenomenon observed in majority of women (Halme et al. 1984), some other mechanisms favouring the survival of misplaced endometrial cells must be involved in endometriosis development. It has been shown that apoptotic activity in the eutopic endometrium of women with endometriosis lacks the normal cyclic variability and is reduced in comparison to the endometrium of disease-free women (Dmowski et al. 2001; Szymanowski 2007). Furthermore, in endometriotic lesions apoptosis is even lower than in the corresponding eutopic endometrium (Dmowski et al. 1998). This indicates that increased viability of endometrial cells may be involved in their ectopic survival, thus facilitating their implantation and the development of endometriotic lesions.

2.7.1. Survivin

Survivin is a protein that belongs to the family of inhibitors of apoptosis and its expression is regulated in a cell cycle-dependent manner. Initially, survivin expression was described in fetal tissues and placenta, and only in a few terminally differentiated tissues (Ambrosini et al. 1997), while elevated survivin levels were observed in different types of tumours, in association with reduced apoptotic activity (Altieri 2003). Later, however, several normal adult tissues and cell types were found to express survivin, although at remarkably lower levels than in cancer cells (Fukuda and Pelus 2006). The presence of survivin mRNA and protein has also been demonstrated in normal cycling endometrium (Konno et al. 2000), with its highest levels mostly reported in the proliferative phase of the cycle (Lehner et al. 2002; Nabilsi et al. 2010). Survivin expression in human endometrium appears to be regulated by sex hormones, as it increases after treatment with oestrogen and decreases with the use of progestins (Nabilsi et al. 2010).

Similarly to endometrial cancer, elevated survivin level is characteristic to non-malignant endometrial pathologies like endometrial hyperplasia (Erkanli et al. 2006) and endometriosis (Ueda et al. 2002). It has been shown that survivin expression is significantly higher in peritoneal and ovarian endometriotic lesions than in the eutopic endometrium of women with or without endometriosis (Ueda et al. 2002; Fujino et al. 2006). Early stage peritoneal lesions with high proliferative activity show higher levels of survivin in comparison to more advanced lesions, suggesting that in the first stages of lesion development survivin may be linked to the escape of endometrial tissue fragments from apoptosis (Ueda et al. 2002; Fujino et al. 2006).

Survivin is encoded by the *BIRC5* gene (17q25), which contains about 250 SNPs (snpper.chip.org). Three polymorphisms in the promoter region, -241C/T

(rs17878467), -235G/A (rs17887126) and -31G/C (rs9904341) could be associated with gene expression activity. The highly frequent -31G/C SNP is located at a CDE/CHR (cell cycle-dependent element/cell cycle homology region) repressor element (Xu et al. 2004) and the presence of the C allele has been shown to increase survivin transcription level (Jang et al. 2008; Wagner et al. 2008; Kawata et al. 2011). The data about low-frequency -235G/A and -241C/T SNP effects are more controversial revealing either no impact or slightly positive influence of minor alleles on promoter activity (Xu et al. 2004; Wagner et al. 2008; Boidot et al. 2010). The role of survivin promoter SNPs has been investigated in different neoplastic diseases (Han et al. 2009a; Jaiswal et al. 2011; Srivastava et al. 2012), but to our knowledge, there are no studies published on endometriosis thus far.

2.7.2. Anti-survivin autoantibodies

Over-expression of survivin in neoplastic tissues has been shown to stimulate humoral immunoreactivity as anti-survivin autoantibodies have been found in the sera of patients with various forms of cancer (Rohayem et al. 2000; Eto et al. 2007; Söling et al. 2007). The percentage of antibody-positive patients may vary in different tumour types (Megliorino et al. 2005). In a study on head and neck cancer patients anti-survivin autoantibodies were detected in more than 70% of cases and a positive correlation between antibody titers and disease stage was found (Eto et al. 2007). In addition, after cancer treatment a significant decrease in antibody levels was observed (Eto et al. 2007). In non-small cell lung cancer cases, instead, less than 8% of patients had anti-survivin antibodies and no correlation with tumour stage or treatment was found (Karanikas et al. 2009).

Women with endometriosis show activation of the humoral immune system, as has been evidenced by several studies reporting the presence of anti-endometrial, anti-ovarian and of other non-specific autoantibodies (lupus anticoagulant, anticardiolipin, antinuclear, anti-smooth muscle etc) in patients' sera (Vinatier et al. 1996; Reimand et al. 2001; Randall et al. 2007; Sarapik et al. 2010). In one early paper, 40-60% of women with endometriosis were reported to have elevated autoantibody titers when tested against a panel of non-specific autoantigens (Gleicher et al. 1987), while in a more recent study, using indirect immunofluorescence detection of anti-endometrial antibodies, about 80% of endometriosis patients had positive results (Randall et al. 2007). It is not clear whether these antibodies reflect a natural response to chronic tissue destruction or show a pathologic response leading to a more generalized autoimmune dysfunction (Eisenberg et al. 2012), but their presence seems to have a negative impact on the reproductive function of endometriosis patients (Eisenberg et al. 2012; Haller-Kikkatalo et al. 2012).

Taking into account the elevated humoral immunoreactivity in women with endometrios and the increased expression of survivin in endometriotic lesions, it

can be hypothesized that anti-survivin autoantibodies could be present in sera of endometriosis patients, and if it were so, their detection might be useful for minimally invasive disease diagnosis. To our knowledge, however, no data is available about anti-survivin antibodies in endometriosis patients so far.

3. AIMS OF THE STUDY

The general aim of the present study was to evaluate possible associations between different candidate genes and susceptibility to endometriosis. In addition, we wanted to evaluate if there is any immune reaction to survivin in women with endometriosis and whether anti-survivin autoantibody level in patients' sera could be used as a minimally invasive diagnostic disease marker.

Accordingly, the specific aims were the following:

1. To investigate associations between endometriosis and genetic variations in different genes involved in sex steroid biosynthesis and signalling: *ESR1*, *ESR2*, *PGR*, *HSD17B1*, *CYP19A1*.

2. To study the role of polymorphisms in matrix metalloproteinase genes *MMP2* and *MMP9* in susceptibility to endometriosis.

3. To evaluate associations between endometriosis and genetic variants in genes involved in angiogenesis: *VEGF* and *ACE*.

4. To study the effect of polymorphisms in the apoptosis inhibitor protein survivin gene *BIRC5* on the risk of endometriosis.

5. To compare the autoimmune reaction to survivin in women with and without endometriosis by measuring anti-survivin antibody level in patients' sera.

4. MATERIALS AND METHODS

The study was approved by The Ethics Review Committee on Human Research of the University of Tartu, decision nr 138/9. An informed consent was obtained from each study participant.

4.1. Study subjects

A total of 197 women (18–45 years of age) hospitalised for suspected endometriosis or for infertility work-up/treatment were recruited into the study from Tartu University Hospital's Women's Clinic and Nova Vita Clinic between February 2005 and February 2008. Of these patients, 150 had surgically and histologically confirmed endometriosis and were used in the study as 'endometriosis group' (mean age ± SD: 32.1 ± 6.1 years). Disease stages according to the American Society for Reproductive Medicine revised classification system (ASRM 1997) were as follows: stage I (minimal) – 53 patients, stage II (mild) – 39 patients, stage III (moderate) – 36 patients, and stage IV (severe) – 22 patients.

Two different control groups were used. For the genetic association studies, 199 women (30–50 years of age; mean age ± SD: 39.8 ± 5.3 years) with proven fertility (at least two children) and no medical history of endometriosis were enrolled as 'population controls' (control group A). Their DNA samples and medical data were obtained from the Estonian Genome Centre, University of Tartu.

For the study on anti-survivin antibodies, 47 women (mean age ± SD: 30.0 ± 6.1 years) recruited from Tartu University Hospital's Women's Clinic who had undergone laparoscopy and were found not to have endometriosis were used as 'gynaecological controls' (control group B).

Peripheral blood samples, taken before the operation, and a clinical data questionnaire were collected for each study participant recruited from Tartu University Hospital's Women's Clinic and Nova Vita Clinic.

4.2. Genotyping of polymorphisms

A total of 22 polymorphisms in 10 different genes, selected based on previous literature data, were analysed using different genotyping methods (Figure 3).

Genomic DNA was extracted from peripheral EDTA-blood by the salt precipitation method (Aljanabi and Martinez 1997). Prior to genotyping, polymerase chain reaction was performed. Most of the loci of interest were amplified individually, except for *CYP19A1* $(TTTA)_n$ and TCT Ins/Del, and the three *BIRC5* promoter SNPs, which were amplified together as one amplicon.

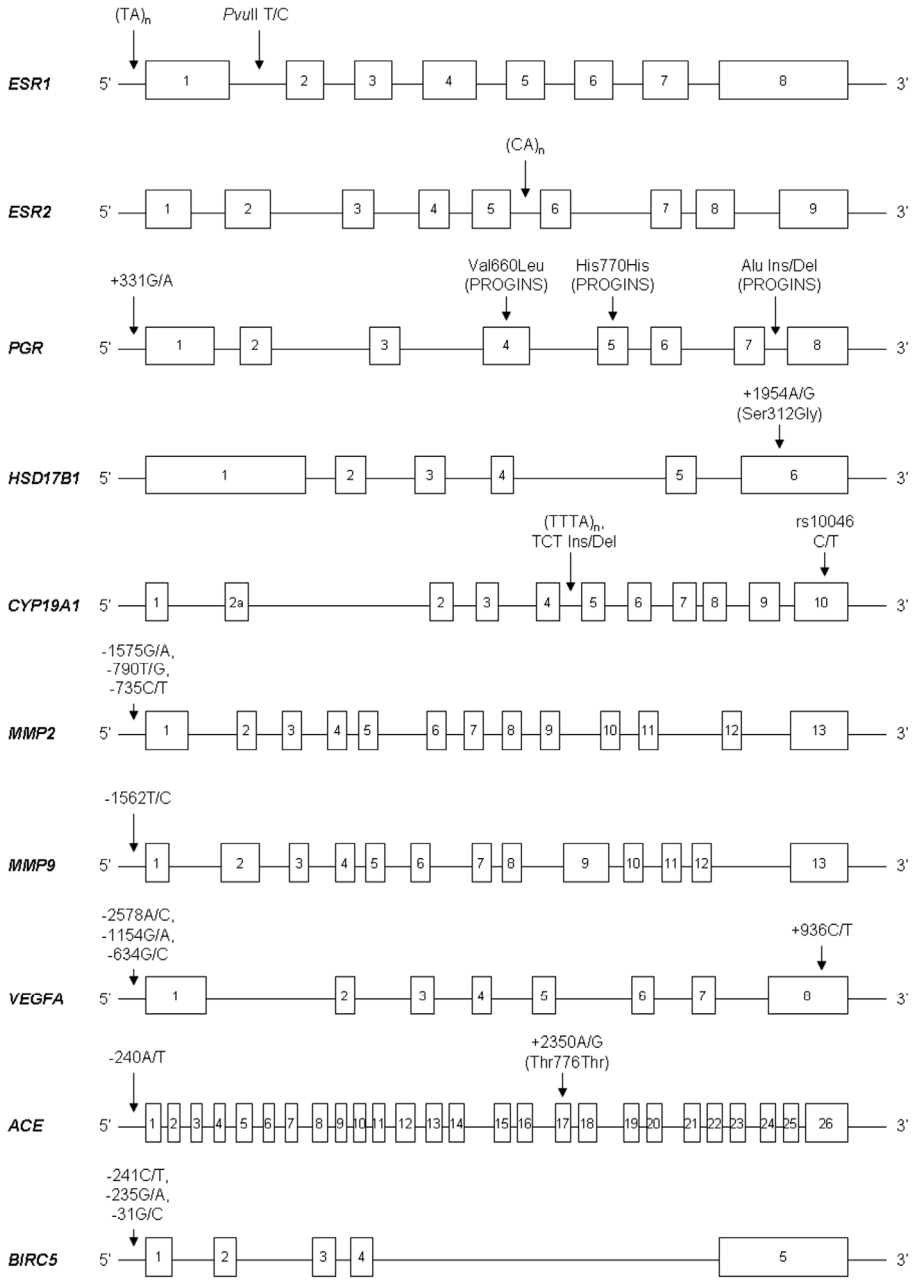

Figure 3. Genes and polymorphisms analysed in the present study.

4.2.1. *PGR* Alu-insertion genotyping

Given that the three *PGR* polymorphisms which form the PROGINS haplotype: Val660Leu, His770His and the Alu-insertion, are in very strong linkage disequilibrium (De Vivo et al. 2002; Treloar et al. 2005a), we genotyped only the Alu-insertion. The presence of the 306-bp Alu-insertion was determined by PCR followed by gel electrophoresis. Primer sequences are given in Table 2.

4.2.2. SNP genotyping

SNPs in the *ESR1*, *PGR*, *HSD17B1*, *MMP2*, *MMP9*, *VEGFA* and *ACE* genes were detected using restriction fragment length polymorphism (RFLP) method. PCR products were digested with appropriate restriction enzymes (MBI Fermentas, Vilnius, Lithuania) at 37°C overnight (Table 2). All restriction fragments were separated according to their lengths by gel electrophoresis and visualized under UV transillumination.

The *CYP19A1* C/T SNP was detected by allele-specific PCR using two different reverse primers (Table 2).

All three *BIRC5* promoter SNPs were amplified together in one amplicon and were detected by direct Sanger sequencing on an ABI 3130xl Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA).

4.2.3. Microsatellite analysis

Three microsatellite repeats: *ESR1* $(TA)_n$, *ESR2* $(CA)_n$, *CYP19A1* $(TTTA)_n$, and the *CYP19A1* TCT Ins/Del polymorphism lying 50 bp upstream from the $(TTTA)_n$ tract were genotyped using automated fragment analysis. Both *CYP19A1* polymorphisms were amplified together in one PCR amplicon. For each microsatellite, one of the PCR primers was fluorescently labelled, enabling the estimation of PCR product sizes using ABI Prism 377 automated DNA sequencer and GeneScan 2.1 software (PE Applied Biosystems, Foster City, CA, USA). GeneScan 500 ROX (PE Applied Biosystems) was used as an internal size standard. To confirm the number of repeats obtained by automated analysis, direct sequencing was performed on at least 10 cases homozygous for different lengths of each of the three microsatellite repeats.

Table 2. Primer sequences, enzymes used in RFLP analyses and DNA fragment sizes after restriction

Polymorphism	Primer sequences (5'→3')[a]	Restriction enzyme	Allele	Fragment sizes (bp)
ESR1 *Pvu*II T/C (rs2234693)	F: CTGCCACCCTATCTGTATC R: ACCCTGGCGTCGATTATCG	*Pvu*II	C T	1361 935+426
HSD17B1 +1954A/G (rs605059)	F: GACCCTGCGCTACTTCACCACCG R: TCTATCTTAATTAGCCACCCACAGCT	*Bsh*1236I	A G	263+81 186+81+77
PGR +331G/A (rs10895068)	F: GAATGGGCTGTACCGAGAGG R: CACAAGTCCGGCACTTGAGT	*Nla*IV	A G	186 131+55
MMP2 -1575G/A (rs243866)	F: ACAGCCAAGGTTTGTCACTGGG R: GTCAGTAAGGACCAGCCTAGGA	*BspH*I	G A	323 198+125
MMP2 -790T/G (rs243864)	F: GGGGTCTTTGTGACCTCGATC R: ATGTGTAAGCCTTAACTTGGCC	*Pvu*I	T G	244 224+20
MMP2 -735C/T (rs2285053)	F: ATAGGGTAAACCTCCCCACATT R: ATGTGTAAGCCTTAACTTGGCC	*Hinf*I	C T	426 253+174
MMP9 -1562C/T (rs3918242)	F: GCAGATGTTCATTGGTTAGTGAACT R: TTCTTCCTAGCCAGCCGGCATC	*Sph*I	C T	541 295+244
VEGFA -2578A/C (rs699947)	F: GGCCTTAGGACACCATACC R: CACAGCTTCTCCCCTATCC	*Bgl*II	C A	456 249+207
VEGFA -1154G/A (rs1570360)	F: TCCTGCTCCCTCCTCGCCAATG R: GGCGGGGACAGGCGAGTCTC[b]	*Mnl*I	A G	252+38 187+65+38
VEGFA -634G/C (rs2010963)	F: TTGCTTGCCATTCCCCACTTGA R: CCGAAGCGAGAACAGCCCAGAA	*Faq*I	C G	469 274+195
VEGFA +936C/T (rs3025039)	F: AGGGTTTCGGGAACCAGATC R: CTCGGTGATTTAGCAGCAAG	*Hin*1II	C T	266 211+55
ACE -240A/T (rs4291)	F: TCGGGCTGGGAAGATCGAGC R: GAGAAAGGGCCTCCTCTCTCT[b]	*Xba*I	A T	137 114+23
ACE +2350A/G (rs4343)	F: CTGACGAATGTGATGGCCGC[b] R: TTGATGAGTTCCACGTATTTCG	*Bsh*1236I	A G	122 103+19
CYP19A1 C/T (rs10046)	F: TTCTGGCTAACTGTCTGATCA R_1: GATGAGAAATGCTCCAGAGTA R_2: GATGAGAAATGCTCCAGAGTG	Allele-specific PCR		
BIRC5 -241C/T, -235G/A, -31G/C (rs17878467, rs17887126, rs9904341)	F: ATCACGGTAGTGGCCCAGTC R: CTTGAATGTAGAGATGCGGTG S: GAACTCCAGGACTCAAGTGA	Direct sequencing		
PGR Alu Ins/Del	F: GCCTCTAAAATGAAAGGCAGAAAGC R: GCGCGTATTTTCTTGCTAAATGTCTG	PCR		
ESR1 $(TA)_n$	F: AGACGCATGATATACTTCACC R: TAMRA-CCTACAACTCGATCTTCTCG	Automated fragment analysis		
ESR2 $(CA)_n$	F: FAM-GAGGTAAACCATGGTCTGTACC R: GTTGAATGAGTGGGCCTCCCT	Automated fragment analysis		
CYP19A1 $(TTTA)_n$, TCT Ins/Del	F: JOE-GGTAAGCAGGTACTTAGTTAGCTA R: CAAGGTCGTGAGCCAAGGTC	Automated fragment analysis		

[a] F, R and S indicate forward, reverse and sequencing primers, respectively; [b] Primer is modified

4.3. Anti-survivin antibody detection

Sera were collected from 98 endometriosis patients and 47 women from the control group B. Serum anti-survivin antibodies were detected by enzyme linked immunosorbent assay (ELISA). A specific ELISA Kit for Human Anti-survivin Antibody (Uscn Life Science Inc, Wuhan, China) was used according to the manufacturer's protocol. Optical density (OD) of samples was measured spectrophotometrically at wavelengths of 450 nm and 540 nm. Each sample was tested in duplicate and the mean OD value was used for further analysis. Intertest variability was corrected using the values of standard dilutions provided with the kit and of the sera used as internal controls.

4.4. Statistical analysis

Data were analysed using SPSS Statistics 17 and PASW Statistics 18 (SPSS Inc, Chicago, IL, USA). All SNPs and Ins/Del polymorphisms were tested for deviation from Hardy-Weinberg equilibrium. Frequencies of nominal variables were compared with Pearson χ^2 test. Continuous variables were analysed using student's *t* test, in case of normal distribution, and Mann-Whitney U test, in case of deviation from normal distribution. The effect of various genotypes on endometriosis risk was evaluated by logistic regression analysis with possible confounding factors included in the model.

In the analyses of microsatellites, all alleles were designated short (S) or long (L) either according to the naturally occurring bimodal distribution in case of the *ESR1* $(TA)_n$ ($S \leq 17$ TA and $L > 17$ TA repeats) and *CYP19A1* $(TTTA)_n$ repeats ($S \leq 9$ TTTA and $L > 9$ TTTA repeats), or using the median number of repeats as the cut-off in case of the *ESR2* $(CA)_n$ locus ($S \leq 22$ CA and $L > 22$ CA repeats).

Haplotype analyses were performed with Phase software, version 2.1 (Stephens et al. 2001; Stephens and Scheet 2005) and Haploview program, version 4.0 (http://www.broad.mit.edu/mpg/haploview) (Barrett et al. 2005). One thousand permutations were performed for each haplotype analysis.

A p value < 0.05 was considered statistically significant. Bonferroni-Holm correction for multiple comparisons was applied in two sub-studies (Papers I, III).

5. RESULTS

5.1. General characteristics of study groups (Papers I, II)

General characteristics of different study groups are given in Table 3. When compared to the population controls (group A), endometriosis patients were significantly taller (*t* test, $p < 0.0005$; OR = 1.06 for 1 cm increase in height, 95% CI 1.02 – 1.11, $p = 0.003$), had a lower body mass index (BMI) (Mann-Whitney U test, $p < 0.0005$; OR = 0.92 for 0.1 kg/m^2 increase in BMI, 95% CI 0.85 – 1.01, $p = 0.074$) and mean age at menarche (Mann-Whitney U test, $p = 0.003$; OR = 0.80 for 1 year increase in age at menarche, 95% CI 0.67 – 0.95, $p = 0.009$). Active smoking was less frequent among endometriosis patients and was associated with a lower disease risk (χ^2-test, $p < 0.0005$; active smoking *vs*. non-smoking: OR = 0.37, 95% CI 0.21 – 0.63, $p < 0.0005$) (Table 3).

All these differences remained statistically significant also when patients were compared to the combined control group A + B (Table 3). Between the groups used in the study of anti-survivin antibodies (98 endometriosis patients and control group B), similar tendencies were observed, but none of them reached statistical significance.

Of the 150 endometriosis patients, 104 (69.3%) were infertile: 53 women (51.0%) had primary and 51 women (49.0%) had secondary infertility (a patient was considered infertile if she had not conceived after one year of contraceptive-free intercourse). When patients were classified based on disease severity, infertility was significantly more frequent among women with stage I-II endometriosis than in those with stage III-IV disease (84.8% *vs.* 44.8%, $p < 0.0005$).

In further analysis, to assess the effect of various genotypes on endometriosis risk, all logistic regression models were adjusted for height and smoking status, as possible confounding factors. Since height and BMI are correlated with each other, only height was included as it is more constant during a woman's lifetime. Age at menarche was not used as this data was not available for all subjects.

Table 3. General characteristics of endometriosis patients and control groups A and B

	Patients (n = 150)	**Control group A** (n = 199)	**Control group B** (n = 47)	**Control group A+B** (n = 243)	***p*-value**[a]
Height (cm)					
(mean ± SD)	167.2 ± 5.7	165.4 ± 5.4	167.7 ± 6.6	165.8 ± 5.7	0.027[b]
BMI (kg/m^2)					
(mean ± SD)	22.0 ± 3.3	22.5 ± 1.8	22.7 ± 4.1	22.5 ± 2.4	<0.002[c]
Menarche[d] (years)					
(mean ± SD)	13.0 ± 1.4	13.5 ± 1.4	13.5 ± 1.5	13.5 ± 1.4	0.003[c]
Smoking (%, n)					
Active smokers	14.0 (21)	30.8 (61)	27.7 (13)	30.2 (73)	<0.0005[e]
Non/Past smokers	86.0 (129)	69.2 (137)	72.3 (34)	69.8 (169)	

[a] Patients *vs.* control group A+B; [b] *t* test; [c] Mann-Whitney U test; [d] patients n = 113; [e] χ^2-test

5.2. Genetic variation and susceptibility to endometriosis (Papers I - IV)

5.2.1. Variation in genes involved in biosynthesis and signalling of sex steroids

To evaluate the role of genes involved in biosynthesis and signalling of sex steroids in susceptibility to endometriosis nine different polymorphisms in the *ESR1*, *ESR2*, *PGR*, *CYP19A1* and *HSD17B1* genes were genotyped in women with endometriosis and in population controls (group A). All SNPs and Ins/Del polymorphisms were in HWE.

No significant differences were observed in genotype frequencies between patients and controls for the following polymorphisms: *ESR1 Pvu*II T/C, *PGR* +331G/A, *PGR* Alu Ins/Del, *CYP19A1* C/T, *CYP19A1* TCT Ins/Del, and *CYP19A1* $(TTTA)_n$ (Table 4).

The *HSD17B1* +1954A/G SNP AA and AG genotypes were more common among women with endometriosis than in the control group (84.7% *vs.* 72.9%, $p = 0.008$), and were associated with a higher disease risk (adjusted OR = 2.39, 95% CI 1.35 – 4.21, $p = 0.003$). However, when patients were divided according to disease severity, the AA and AG genotypes were only more frequent among patients with stage I–II endometriosis (90.2%; adjusted OR = 3.89, 95% CI 1.79 – 8.45, $p = 0.001$), while in women with stage III–IV disease, their frequency (75.9%) was similar to the control group. Given that the majority of patients with stage I–II endometriosis suffered from infertility, genotype distribution was analysed also based on the fertility status of study subjects, revealing an association with infertility. The AA and AG genotypes were more common among infertile women with endometriosis (88.5%; adjusted OR = 3.32, 95% CI 1.65 – 6.68, $p = 0.001$), while in patients who did

not complain of infertility their frequency (76.1%) was comparable to control subjects.

Analysis of the *ESR1* $(TA)_n$ microsatellite revealed that women carrying alleles with a higher number of TA repeats were apparently more at risk for endometriosis, since the LL and SL genotypes were more frequent among patients than in the control group (70.0% *vs*. 56.8%, $p = 0.012$), increasing the overall disease risk (adjusted OR = 1.79, 95% CI 1.12 – 2.83, $p = 0.014$). However, similarly to the *HSD17B1* +1954A/G SNP, further analysis revealed that these genotypes were only associated with stage I–II endometriosis (frequency 73.9%; adjusted OR = 2.38, 95% CI 1.35 – 4.21, $p = 0.003$), while in patients with stage III–IV disease, their frequency (63.8%) was only slightly higher than in the control group. Likewise, dividing patients according to their fertility status showed that the LL and SL genotypes were more common in women with infertility (74%; adjusted OR = 2.19, 95% CI 1.28 – 3.73, $p = 0.004$), whereas in patients without infertility their frequency (60.9%) was similar to the control group.

Within the *ESR2* gene, lower number of CA repeats correlated with a higher endometriosis risk. The SS homozygous genotype was more common in the endometriosis group than in controls (48.7% *vs*. 36.7%, $p = 0.025$; adjusted OR = 1.65, 95% CI 1.06 – 2.56, $p = 0.028$), and contrary to the abovementioned polymorphisms, it was more frequent among women with stage III–IV disease (55.2%; adjusted OR = 2.10, 95% CI 1.15 – 3.81, $p = 0.015$) and in patients without infertility (67.4%; adjusted OR = 3.56, 95% CI 1.78 – 7.10, $p < 0.0005$). Among patients with stage I-II endometriosis or in those who suffered from infertility, the SS genotype frequency (44.6% and 40.4%, respectively) was similar to controls.

Given that in this sub-study, besides comparing general characteristics of subjects, also several independent genes and polymorphisms were analysed, Bonferroni-Holm correction for multiple comparisons was used. After applying this correction, all the aforementioned associations of the *HSD17B1* +1954A/G SNP with endometriosis and infertility remained statistically significant. In case of the *ESR1* $(TA)_n$ locus, associations with stage I–II disease and with infertility in endometriosis patients reached statistical significance, while for the *ESR2* $(CA)_n$ microsatellite only the association with endometriosis without infertility remained significant.

Table 4. Genotype frequencies of *ESR1*, *ESR2*, *PGR*, *HSD17B1* and *CYP19A1* polymorphisms in endometriosis patients and controls

Genotype	Patients (n = 150) % (n)	Controls (n = 199) % (n)	*p*-value[a]
ESR1 *Pvu*II T/C			
TT	23.3 (35)	29.6 (59)	0.210
CT	50.7 (76)	51.3 (102)	
CC	26.0 (39)	19.1 (38)	
HSD17B1 +1954A/G			
GG	15.3 (23)	27.1 (54)	0.029
AG	56.0 (84)	46.7 (93)	
AA	28.7 (43)	26.1 (52)	
PGR +331G/A			
GG	86.7 (130)	82.4 (164)	0.352
AG	13.3 (20)	17.6 (35)	
AA	0.0 (0)	0.0 (0)	
PGR Alu Ins/Del			
DelDel	70.0 (105)	77.4 (154)	0.096
InsDel	28.7 (43)	19.6 (39)	
InsIns	1.3 (2)	3.0 (6)	
CYP19A1 C/T			
TT	28.0 (42)	37.7 (75)	0.130
CT	54.0 (81)	44.2 (88)	
CC	18.0 (27)	18.1 (36)	
CYP19A1 Ins/Del			
InsIns	54.7 (82)	51.3 (102)	0.344
InsDel	38.0 (57)	36.7 (73)	
DelDel	7.3 (11)	12.1 (24)	
ESR1 $(TA)_n$			
SS	30.0 (45)	43.2 (86)	0.041
SL	44.0 (66)	35.2 (70)	
LL	26.0 (39)	21.6 (43)	
ESR2 $(CA)_n$			
SS	48.7 (73)	36.7 (73)	0.026
SL	29.3 (44)	29.1 (58)	
LL	22.0 (33)	34.2 (68)	
CYP19A1 $(TTTA)_n$			
SS	34.7 (52)	35.2 (70)	0.748
SL	50.0 (75)	46.7 (93)	
LL	15.3 (23)	18.1 (36)	

[a] χ^2-test

5.2.2. Variation in matrix metalloproteinase genes

To investigate the role of matrix metalloproteinase genes in predisposition to endometriosis four SNPs in the *MMP2* and *MMP9* promoter regions were genotyped in endometriosis patients and in population controls (group A). All genotype frequencies were in HWE.

No significant differences were found in genotype frequencies of the *MMP2* -790T/G and the -1575G/A SNPs between patients and controls (Table 5). However, *MMP2* -735CC genotype was significantly more frequent among women with endometriosis than in control individuals (82.7% *vs*. 71.4%, $p = 0.014$) and was associated with an increased disease risk compared to TT and CT genotypes (adjusted OR = 1.95, 95% CI 1.13 – 3.35, $p = 0.016$). When patients were divided based on disease stage, a stronger relation between the CC genotype and stage I-II endometriosis was observed (frequency 84.8%; adjusted OR = 2.26, 95% CI 1.17 – 4.38, $p = 0.015$), while the effect on stage III–IV disease risk did not reach statistical significance (frequency 79.3%; adjusted OR = 1.57, 95% CI 0.76 – 3.24, $p = 0.219$). No differences were observed in genotype frequencies between fertile and infertile patients.

Table 5. Genotype frequencies of *MMP2* and *MMP9* polymorphisms in endometriosis patients and controls

Polymorphism	**Patients** (n = 150) % (n)	**Controls** (n = 199) % (n)	***p*-value**[a]
MMP2 -1575G/A			
GG	57.3 (86)	57.3 (114)	0.993
GA	35.4 (53)	35.7 (71)	
AA	7.3 (11)	7.0 (14)	
MMP2 -790T/G			
TT	56.7 (85)	54.3 (108)	0.797
TG	35.3 (53)	38.7 (77)	
GG	8.0 (12)	7.0 (14)	
MMP2 -735C/T			
CC	82.7 (124)	71.4 (142)	0.047
CT	16.7 (25)	27.1 (54)	
TT	0.7 (1)	1.5 (3)	
MMP9 -1562C/T			
CC	66.7 (100)	73.4 (146)	0.205
CT	29.3 (44)	25.1 (50)	
TT	4.0 (6)	1.5 (3)	

[a] χ^2-test

Haplotype analyses revealed that all three *MMP2* promoter region SNPs were in strong linkage disequilibrium (LD) and formed a single haploblock (Figure 4). Six different haplotypes with a frequency of > 1% were defined (Table 6). The most common haplotype -1575G/-790T/-735C was significantly more frequent among patients than in controls (65.8% *vs*. 56.9%, p = 0.016), but after the permutation test (n = 1000) it no longer reached statistical significance. Three haplotypes: -1575G/-790T/-735T, -1575G/-790G/-735C and -1575A/-790T/-735C occurred more often in the control group than in endometriosis patients, but after permutation test these differences remained statistically significant only for the two more rare haplotypes (Table 6).

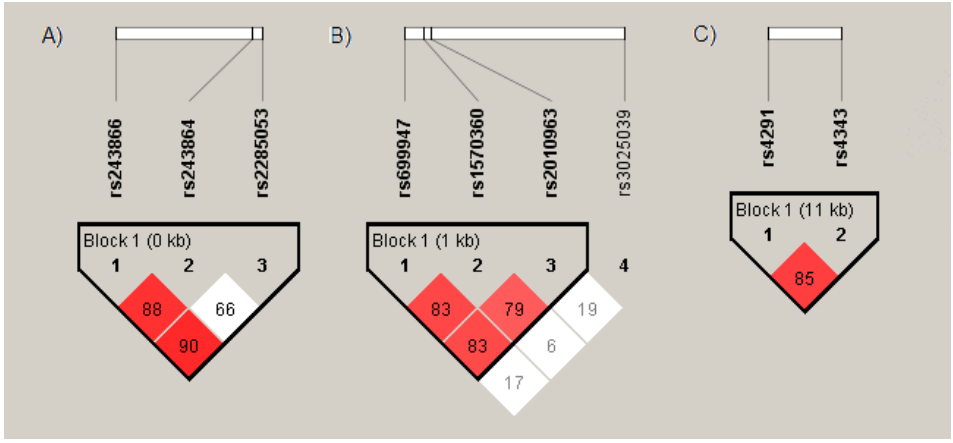

Figure 4. Haploblocks analysed in the present study: A) *MMP2* promoter haploblock -1575G/A, -790T/G, -735C/T; B) *VEGFA* haploblock -2578A/C, -1154G/A, -634G/C; C) *ACE* haploblock -240A/T, +2350A/G.

Table 6. Frequencies of *MMP2* haplotypes in endometriosis patients and controls

Haplotypes			Patients (%)	Controls (%)	*p*-value[a]
-1575 G/A	**-790 T/G**	**-735 C/T**			
G	T	C	65.8	56.9	0.016 / 0.148
A	G	C	25.1	21.3	0.256
G	T	T	8.3	13.1	0.049 / 0.282
G	G	C	0.1	3.4	0.002 / 0.019
A	T	C	0.1	3.3	0.002 / 0.011
G	G	T	0.6	1.7	0.196

[a] χ^2 test: *p*-value before / after permutation test (n = 1000)

The distribution of *MMP9* -1562C/T SNP genotypes was similar in patients and control subjects (Table 5). However, more detailed analysis revealed that the -1562TT genotype markedly increased the risk of stage III–IV endometriosis in comparison to the CC genotype (TT genotype frequency 6.9% *vs*. 1.5% in controls; adjusted OR = 7.73, 95% CI 1.53 – 38.90, $p = 0.013$). Also the combination of TT and CT genotypes was associated with an elevated risk of stage III–IV disease (TT + CT genotype frequency: 41.4% *vs*. 26.6% in controls; adjusted OR = 2.04, 95% CI 1.09 – 3.83, $p = 0.027$). Genotype frequencies were compared also in patients with and without infertility, but no significant differences were observed.

5.2.3. Variation in genes involved in angiogenesis

Four SNPs in the *VEGFA* gene and two SNPs in the *ACE* gene were genotyped and their respective haplotypes were analysed in endometriosis patients and in population controls (group A). The genotypic distribution of all *VEGFA* SNPs and of *ACE* +2350A/G SNP were in HWE, while the ACE -240A/T SNP showed a slight deviation ($p = 0.02$) from the HWE in the control group.

Both *ACE* SNPs and three *VEGFA* SNPs, -1154G/A, -634G/C and +936C/T, were distributed similarly in women with and without endometriosis (Table 7). Genotype frequencies remained comparable to controls also when patients were divided into subgroups of stage I–II and stage III–IV disease or based on their fertility status.

The *VEGFA* -2578A/C SNP CC genotype was significantly less common among patients than in controls (12.0% *vs*. 25.1%, $p = 0.002$) and was associated with a lower risk of endometriosis compared to the AA genotype (adjusted OR = 0.40, 95% CI 0.20 – 0.78, $p = 0.007$). When genotype distribution was analysed in different disease stages, similar results were obtained: compared to AA homozygous women, CC genotype carriers had a lower risk of stage I–II endometriosis (CC genotype frequency 14.1%; adjusted OR = 0.43, 95% CI 0.20 – 0.90, $p = 0.026$) and showed borderline risk reduction for stage III–IV disease (CC genotype frequency 8.6%; adjusted OR = 0.34, 95% CI 0.12 – 1.00, $p = 0.050$). However, after correcting for multiple testing only the association with the overall endometriosis risk remained statistically significant.

Table 7. Genotype frequencies of *VEGFA* and *ACE* polymorphisms in endometriosis patients and controls

Polymorphism	Patients (n = 150) % (n)	Controls (n = 199) % (n)	*p*-value[a]
VEGFA -2578A/C			
AA	37.3 (56)	30.7 (61)	0.009
AC	50.7 (76)	44.2 (88)	
CC	12.0 (18)	25.1 (50)	
VEGFA -1154G/A			
GG	34.7 (52)	39.2 (78)	0.397
GA	47.3 (71)	47.7 (95)	
AA	18.0 (27)	13.1 (26)	
VEGFA -634G/C			
GG	62.7 (94)	54.3 (108)	0.056
GC	35.3 (53)	38.7 (77)	
CC	2.0 (3)	7.0 (14)	
VEGFA +936C/T			
CC	69.3 (104)	68.8 (137)	0.838
CT	28.7 (43)	28.1 (56)	
TT	2.0 (3)	3.1 (6)	
ACE -240A/T			
AA	42.0 (63)	41.7 (83)	0.249
AT	46.0 (69)	51.3 (102)	
TT	12.0 (18)	7.0 (14)	
ACE +2350A/G			
AA	27.3 (41)	28.6 (57)	0.543
AG	46.0 (69)	49.8 (99)	
GG	26.7 (40)	21.6 (43)	

[a]χ^2-test

Haplotype analysis revealed that three *VEGFA* SNPs (-2578A/C, -1154G/A and -634G/C) were in strong LD and formed a single haploblock (Figure 4). The *VEGFA* +936C/T SNP was in low LD with other SNPs (D' < 0.2). Six different haplotypes with a frequency of > 1% were defined (Table 8). The most common haplotype -2578A/-1154A/-634G showed a higher frequency among women with endometriosis than in the control group (40.2% *vs*. 31.6%, $p = 0.018$), but after the permutation test (n = 1000) it no longer reached statistical significance. Instead, one rare haplotype -2578C/-1154A/-634G was more frequent among controls compared to endometriosis patients (3.0% *vs.* 0.4%, $p = 0.012$), but again, after the permutation test this difference became statistically in-significant. All other *VEGFA* haplotypes were distributed equally between patients and controls.

Table 8. Frequencies of *VEGFA* haplotypes in endometriosis patients and controls

Haplotypes			Patients (%)	Controls (%)	*p*-value[a]
-2578 A/C	**-1154 G/A**	**-634 G/C**			
A	A	G	40.2	31.6	0.018 / 0.071
C	G	C	18.4	22.1	0.231
C	G	G	18.1	21.1	0.322
A	G	G	21.4	17.9	0.248
C	A	G	0.4	3.0	0.012 / 0.052
A	G	C	0.4	2.0	0.067

[a] χ^2 test; *p*-value: before / after permutation test (n = 1000)

The two *ACE* SNPs (-240A/T and +2350A/G) were in strong LD (Figure 4). Four different haplotypes were defined (Table 9). The most rare haplotype -240T/+2350A was more prevalent in the control group than among patients (3.7% *vs*. 0.8%, $p = 0.014$). However, the permutation test (n = 1000) rendered this result statistically insignificant.

Table 9. Frequencies of *ACE* haplotypes in endometriosis patients and controls

Haplotypes		Patients (%)	Controls (%)	*p*-value[a]
-240 A/T	**+2350 A/G**			
A	A	49.6	49.9	0.904
T	G	34.2	29.0	0.090
A	G	15.4	17.4	0.332
T	A	0.8	3.7	0.014 / 0.055

[a] χ^2 test; *p*-value: before / after permutation test (n = 1000)

5.2.4. Variation in survivin gene

To assess the role of the *BIRC5* gene in susceptibility to endometriosis, three promoter region polymorphisms were genotyped in endometriosis patients (n = 149) and in control groups A (n = 196) and B (n = 47). All three SNPs were in HWE.

All genotype frequencies of the -241C/T, -235G/A and -31G/C SNPs were similar among patients and in population controls (group A) (Table 10). Differences in genotypic distribution remained statistically insignificant also after dividing patients based on disease severity (stage I–II and stage III–IV

disease) or their fertility status. In the control group B, genotype frequencies were slightly different than in women with endometriosis, however these differences did not reach statistical significance for any of the SNPs (Table 10).

Table 10. Genotype frequencies of *BIRC5* polymorphisms in endometriosis patients and control groups A and B

Polymorphism	Patients (n = 149) % (n)	Control group A (n = 196) % (n)	*p*-value[a]	Control group B (n = 47) % (n)	*p*-value[a]
BIRC5 -241C/T					
CC	88.6 (132)	85.7 (168)	0.288	93.6 (44)	0.321
CT	11.4 (17)	12.8 (25)		6.4 (3)	
TT	0.0 (0)	1.5 (3)		0.0 (0)	
BIRC5 -235G/A					
GG	94.0 (140)	94.4 (185)	0.866	97.9 (46)	0.288
GA	6.0 (9)	5.6 (11)		2.1 (1)	
AA	0.0 (0)	0.0 (0)		0.0 (0)	
BIRC5 -31G/C					
GG	45.0 (67)	42.3 (83)	0.845	29.8 (14)	0.149
GC	42.9 (64)	43.9 (86)		51.1 (24)	
CC	12.1 (18)	13.8 (27)		19.1 (9)	

[a] χ^2-test

5.3. Immunoreactivity to survivin (Paper IV)

5.3.1. Anti-survivin autoantibodies and endometriosis

To determine if women with endometriosis show increased autoimmunity against survivin, and whether this could facilitate the detection of endometriosis in a non-invasive manner, sera from 98 endometriosis patients (55 women with stage I–II and 43 with stage III–IV endometriosis) and 47 women from control group B were tested for anti-survivin autoantibodies. Women from group B were selected for antibody analysis because they were of the same age as endometriosis patients (30.0 ± 6.1 *vs*. 30.9 ± 6.5 years, respectively, $p > 0.05$), presented with similar gynaecological symptoms and were proved to be endometriosis-free by laparoscopic surgery.

Anti-survivin antibody levels did not differ significantly between study groups; median OD values were 0.078 and 0.119 for patients and controls, respectively ($p > 0.05$). Autoantibody levels were similar also in patients with stage I–II (median OD: 0.074) and stage III–IV (median OD: 0.104) endometriosis. Since the control group B and endometriosis patients differed slightly, though insignificantly, in the prevalence of infertility (74.5% *vs*. 59.2%, respectively, $p > 0.05$), anti-survivin antibody levels were compared

also in fertile and infertile women, but no significant differences were observed (median OD values: 0.097 *vs*. 0.080, respectively, $p > 0.05$).

Interestingly, anti-survivin autoantibody levels seemed to be influenced by the smoking status of study subjects. Analysing together all patients and controls, significantly lower median OD values were found in sera of actively smoking women (n = 32) compared to non-smokers (n = 113) (0.019 *vs*. 0.155, respectively; $p < 0.0005$). This correlation remained statistically significant also when patients and controls were analysed separately.

Since smoking seemed to influence autoantibody level, comparison of median OD values in endometriosis patients and controls was repeated including only non-smoking women (79 patients and 34 controls), which confirmed that anti-survivin antibodies were not associated with endometriosis (median ODs 0.146 *vs*. 0.187, respectively; $p > 0.05$).

5.3.2. Anti-survivin autoantibodies and variation in survivin gene

To determine whether anti-survivin antibody levels are influenced by genetic variation in the survivin gene, correlations between *BIRC5* promoter SNPs and autoantibody levels were evaluated among all non-smoking study participants.

No significant associations between the three promoter region SNPs (-241C/T, -235G/A, -31G/C) and median autoantibody levels were found. However, further analysis on elevated antibody concentrations ($OD \geq 1.0$ or $OD \geq 2.0$) revealed an association between the -235G/A SNP and high OD values. The GA genotype was significantly more frequent among women with the highest antibody level ($OD \geq 2.0$) compared to those with a lower level ($OD < 2.0$) (23.1% *vs*. 4.1%, respectively; $p = 0.008$).

6. DISCUSSION

Endometriosis is considered to be a complex trait, caused by the interaction of genetic and environmental factors. To identify the genes involved in disease development, numerous candidate gene studies and a few genome-wide investigations have been carried out, but our knowledge in this field is still incomplete. Since genetic susceptibility markers may vary in different populations, our aim was to investigate possible associations between endometriosis and some of the plausible candidate genes in the Estonian population.

For this purpose, we enrolled in the present study women with confirmed endometriosis, fertile women with no medical history of endometriosis as population controls and patients who had undergone laparoscopy and were found to be endometriosis-free as gynaecological controls. The selection of controls for studies on endometriosis is complicated since surgery is needed for the definite exclusion of endometriosis. When women are enrolled from the general population based on their self-reported medical history, some undiagnosed cases might be included in the control group, making it more difficult to detect weak associations. Then again, using women who have undergone gynaecological surgery and are proved to be endometriosis-free might form a slightly biased control sample, as they generally suffer from some other gynaecological disorder. Therefore, it might be best, if both types of controls were used. In the present study, however, we decided to concentrate mainly on the population control group.

6.1. Endometriosis and general characteristics of study subjects

Epidemiological studies on endometriosis have revealed several factors which could increase disease risk, including dysmenorrhea (as an indicator of stronger uterine contractions and increased retrograde menstruation), younger age at menarche and shorter cycle length, heavy menstrual flow, nulliparity, increased height and lower weight and BMI (Cramer and Missmer 2002; McLeod and Retzloff 2010). Also certain life style habits, like cigarette smoking or alcohol and caffeine consumption, might decrease or increase, respectively, the risk of developing endometriosis (McLeod and Retzloff 2010).

In accordance with previous literature reports, assessment of the data gathered on our study subjects revealed similar findings. A positive association was observed between endometriosis and height, while BMI was negatively correlated with disease risk. In addition, lower age at menarche was related to a higher risk of endometriosis, whereas active cigarette smoking seemed to have a protective effect.

Within the patients group, the occurrence of infertility was significantly higher among women with stage I–II endometriosis than in those with stage III–IV disease. However, it probably does not reflect a true association between the

disease stages and fertility status, as advanced endometriosis seems to correlate with higher rates of infertility (Albertsen et al. 2013). Therefore, it is more likely caused due to a diagnostic bias. Given that in the present study women who underwent laparoscopy for a suspected endometriosis or for infertility were enrolled, it is possible that among infertile patients milder asymptomatic forms of endometriosis were diagnosed, which normally would remain undiscovered.

6.2. Genetic variation influencing susceptibility to endometriosis

6.2.1. Variation in genes involved in biosynthesis and signalling of sex steroids

Considering the importance of oestrogen in the development of endometriosis (Kitawaki et al. 2002), we aimed to investigate whether genes involved in oestrogen biosynthesis and in sex steroid signalling (*ESR1, ESR2, PGR, CYP19A1, HSD17B1*) are associated with susceptibility to endometriosis in our study group. Since these genes might affect also female reproductive function, we studied their relation also to endometriosis-associated infertility.

Of the nine polymorphisms analysed, three were found to be associated with endometriosis and/or infertility: *ESR1* $(TA)_n$, *ESR2* $(CA)_n$ and *HSD17B1* +1954A/G.

Within the *ESR1* gene, we genotyped the *Pvu*II T/C SNP in intron 1 and a $(TA)_n$ microsatellite repeat in the promoter region. Both of these polymorphisms have been associated with endometriosis previously, but the results have been inconsistent (Falconer et al. 2007). In agreement with some prior studies (Wang et al. 2004; Kim et al. 2005b; Renner et al. 2006; Xie et al. 2008), we observed no association between the *Pvu*II T/C SNP and endometriosis in our cohort. This finding was confirmed also by a recent study by (Trabert et al. 2012) and by two meta-analyses which concluded that *ESR1 Pvu*II T/C SNP is not related to susceptibility to endometriosis (Hu et al. 2012; Li et al. 2012). We did, however, observe a correlation between *ESR1* $(TA)_n$ genotypes containing longer $(TA)_n$ repeats (LL and SL) and an increased risk of stage I–II endometriosis and endometriosis with infertility. This finding differs from other previous reports. Studies on Greek and Korean woman showed shorter *ESR1* $(TA)_n$ repeats to be correlated with susceptibility to endometriosis in general and with the risk of stage I–II disease, respectively (Georgiou et al. 1999; Kim et al. 2005b). Instead, in Taiwanese Chinese women, associations were found between a higher risk of stage III–IV endometriosis and three different $(TA)_n$ alleles: one short repeat (14 TA) and two longer repeats (18 and 24 TA) (Hsieh et al. 2005b). In a previous study on Estonian women exploring the effect of *ESR1* gene variants on the outcome of ovarian stimulation in infertile women, no association was observed between endometriosis and the $(TA)_n$ locus, and conversely, shorter $(TA)_n$ repeats were found to correlate with

a higher risk for unexplained infertility (Altmäe et al. 2007). This contradicts our current results, however, it is difficult to directly compare the findings of these two studies due to differences in sample size and control group selection (fertile women in the present study and women with tubal factor infertility in the previous one).

The association observed between *ESR1* $(TA)_n$ repeat length and infertility in endometriosis patients is in agreement with a previous study on fertile women and patients with unexplained infertility undergoing *in vitro* fertilisation (IVF), where longer $(TA)_n$ repeats were found to correlate with an elevated risk of infertility and with a lower oocyte fertilisation rate in IVF procedures (Ayvaz et al. 2009). In addition, carriers of long $(TA)_n$ repeats have been shown to have an elevated risk of premature ovarian failure (POF) (Bretherick et al. 2008). Likewise, other *ESR1* polymorphisms have been reported to influence female fertility (Corbo et al. 2007) and the outcome of IVF (Sundarrajan et al. 1999). Therefore, we cannot exclude that the *ESR1* $(TA)_n$ locus is actually more related to female infertility and that the observed association with stage I–II endometriosis is merely a reflection of the high frequency of infertile women in this group.

Within the *HSD17B1* gene, we studied the +1954A/G SNP in exon 6, which revealed similar associations as the *ESR1* $(TA)_n$ dinucleotide repeat. The AA and AG genotypes increased the overall risk of endometriosis, but the strongest associations were observed with stage I–II disease and infertility in endometriosis patients. The +1954AA genotype has been shown to correlate with elevated circulating oestradiol level (Setiawan et al. 2004), and thus could favour the growth of endometriotic lesions. In the only prior study on this polymorphism and endometriosis, the AA and AG genotypes correlated with an increased disease risk and also with its severity, being more frequent in women with stage III–IV endometriosis (Tsuchiya et al. 2005). Thus the results of these two studies are in part inconsistent. This could be caused by differences in study populations and sample sizes, but also by the variation in the fertility status of study subjects, as Tsuchiya and colleagues (2005) enrolled only infertile patients and controls. Considering our results and that in the previous study a significant association with endometriosis was found also using infertile controls, the *HSD17B1* +1954A/G SNP seems to be related not only with endometriosis or infertility, but more specifically with infertility in endometriosis patients. Whether this is true and which could be the molecular mechanisms behind it, needs to be elucidated by future studies.

In the *ESR2* gene, we genotyped a $(CA)_n$ microsatellite repeat in intron 5, which seemed to influence susceptibility to endometriosis and especially the risk of stage III–IV disease. However, correction for multiple testing rendered these associations statistically insignificant, which is in accordance with the previous study by Altmäe et al (2007). Still, subdividing patients according to their fertility status revealed a correlation between endometriosis without infertility and homozygosity for shorter $(CA)_n$ repeats, which remained

significant also after applying the Bonferroni-Holm correction. Among infertile patients the frequency of the SS genotype was similar to controls.

The impact of the *ESR2* $(CA)_n$ locus on gene function is not clear yet, but its length might modify gene function. Although there is no direct correlation between the number of $(CA)_n$ repeats and *ESR2* transcriptional activity (Ugai et al. 2008), it is possible that this microsatellite affects some posttranscriptional processes which influence ERβ expression (Hui et al. 2005). Significant up-regulation of ERβ and down-regulation of ERα has been reported in endometriotic lesions compared to eutopic endometrium (Fujimoto et al. 1999; Smuc et al. 2007). This leads to a decrease in ERα/ERβ ratio, suggesting that in endometriotic tissue the function of oestrogens is at least to some extent mediated via ERβ. It has been shown that ERβ suppresses the expression of ERα in endometrial and endometriotic stromal cells via binding to classic and non-classic DNA motifs in alternatively used ERα promoters (Trukhacheva et al. 2009). As ERα stimulates the production of PR, it was hypothesized that this decrease in ERα/ERβ ratio could lead to the suppression of PR expression and to progesterone resistance which has been observed in endometriosis (Bulun et al. 2006). Furthermore, ERβ might contribute to the proliferation of endometriotic lesions by regulating the cell cycle progression (Trukhacheva et al. 2009). Thus, there is several evidence of the importance of ERβ in the development of endometriosis, however, further studies are needed to clarify the exact role of ERβ and the *ESR2* $(CA)_n$ repeat in the pathogenesis of this disease.

We observed no associations between endometriosis or endometriosis-associated infertility and polymorphisms in the *PGR* and *CYP19A1* genes in the Estonian population. This is in agreement with several previous studies (Huber et al. 2005; Treloar et al. 2005a; Govindan et al. 2007; Hur et al. 2007; van Kaam et al. 2007; Gentilini et al. 2008b) as well as with one of the latest papers (Trabert et al. 2012). Also a recent meta-analysis failed to detect associations between endometriosis and polymorphisms in the *CYP19A1* gene, however, it did suggest that the *PGR* PROGINS haplotype may play a role in the risk of endometriosis (Hu et al. 2012). Still, the latter conclusion might not be completely accurate, as several studies with negative results were not included in the meta-analysis. As mentioned above, Trabert and colleagues (2012) observed no association between endometriosis and several *CYP19A1* SNPs, including the 3'-UTR C/T SNP investigated in the present study. Yet, they did find a correlation between increased risk of endometriosis and three intronic *CYP19A1* SNPs (rs1870049, rs1004982 and rs936307), that were not included in the present thesis and have not been associated to endometriosis before. Therefore, further studies are warranted to elucidate the role of *CYP19A1* variants in the development of endometriosis.

6.2.2. Variation in matrix metalloproteinase genes

In the present study, we investigated the role of two matrix metalloproteinase genes, *MMP2* and *MMP9* in susceptibility to endometriosis. Our results demonstrated that two promoter region SNPs: *MMP2* -735C/T and *MMP9* -1562C/T, as well as *MMP2* promoter haplotypes were associated with the risk of endometriosis in Estonian women.

In the only prior study investigating the *MMP2* -735C/T SNP in the development of endometriosis no significant association was found (Kang et al. 2008b). In contrast, we observed that women with the -735CC genotype had almost twice the risk of endometriosis compared to CT or TT genotype carriers and this association was particularly evident in case of stage I-II disease.

Although the relationship between *MMP2* -735C/T SNP and endometriosis is only beginning to be defined, earlier studies have reported the -735C allele to correlate with higher promoter activity and with an increased susceptibility to certain types of cancer and chronic heart failure (Vasku et al. 2003; Yu et al. 2004). Therefore, considering these previous studies along with our results, we may hypothesize that the *MMP2* -735CC genotype increases the risk of endometriosis through higher *MMP2* transcriptional and enzymatic activities facilitating the invasion and survival of endometriotic implants in ectopic locations.

The other two *MMP2* SNPs, -1575G/A and -790T/G, appear to be neutral with no effect on promoter activity (Price et al. 2001). Indeed, they were not individually associated with endometriosis in the present study. This is in accordance with a previous paper, where a minor correlation observed with the -1575G/A SNP disappeared after correcting for multiple testing (Borghese et al. 2008).

All three *MMP2* promoter SNPs were in strong LD, forming eight different haplotypes. The most common one, -1575G/-790T/-735C, was more frequent among patients, but after the permutation test it no longer reached statistical significance. Instead, two very rare haplotypes, -1575G/-790G/-735C and -1575A/-790T/-735C, were more common in the control group, suggesting a possible protective effect against endometriosis. This is in agreement with the results reported by Vašků and colleagues (2004), who found a significant association between the most common *MMP2* haplotype (-1575G/-1306C/-790T/-735C) and an elevated risk of coronary triple-vessel disease, while two rare ones, similar to our protective haplotypes, (-1575G/-1306C/-790G/-735C and -1575A/-1306C/-790T/-735C) were present only in control subjects (Vasku et al. 2004).

In vitro expression studies on the *MMP9* -1562C/T SNP have shown that the C to T transition causes the loss of a binding site for a transcription repressor protein, resulting in a significant increase in promoter activity (Zhang et al. 1999). The -1562T allele has in fact been associated with tumour aggressiveness and invasiveness (Matsumura et al. 2005; Hughes et al. 2007; Xing et al. 2007). In the present study, we observed no association between this SNP

and the overall risk of endometriosis. However, subdividing patients according to disease severity, we revealed a significant correlation between the TT and CT genotypes and stage III–IV endometriosis. Therefore, it is possible that the presence of the -1562T allele renders endometrial cells particularly aggressive and like in cancer, where it is associated with tumour invasion and metastatic spread, it could favour the development of more advanced forms of endometriosis. Our results, however, disagree with two previous studies which showed no association between the individual *MMP9* -1562C/T SNP and stage III–IV endometriosis in Asian women (Shan et al. 2006; Han et al. 2009b). Han and colleagues (2009b) reported a correlation between increased endometriosis risk and the *MMP9* -1562C/5546A haplotype, but oppositely to our findings, it included the -1562C allele. These discrepancies could be caused by population differences, therefore, further studies on Caucasian women would be needed to confirm our results.

6.2.3. Variation in genes involved in angiogenesis

We investigated the role of *VEGFA* and *ACE* gene polymorphisms and haplotypes in susceptibility to endometriosis and found evidence of a possible association with the *VEGFA* -2578A/C SNP.

In our study group, the *VEGFA* -2578CC genotype was associated with a decreased risk for endometriosis when compared to AA genotype carriers. This finding, however, is not in accordance with other two studies that have investigated the role of this SNP as a genetic determinant of susceptibility to endometriosis. In a study on Australian women no significant association was observed (Zhao et al. 2008), while in Northern Chinese women, oppositely to our results, the -2578AA genotype decreased disease risk (Liu et al. 2009). Discrepancies in the results of these three studies could be caused by ethnic differences, as the observed allele frequencies were very diverse between populations. Therefore, it might be more appropriate to compare populations with similar allele frequencies to evaluate the role of this SNP in endometriosis.

VEGFA -2578CC genotype has been shown to correlate with elevated serum VEGF levels (Steffensen et al. 2010). On the contrary, experiments on non-small cell lung cancer demonstrated that the CC genotype was associated with lower VEGF expression in cancer cells and with lower vascular density (Koukourakis et al. 2004). In accordance with both of these studies, Szeto and colleagues observed that -2578CC genotype carriers presented higher circulating VEGF levels but lower protein and mRNA expression level in peritoneal dialysis effluent, suggesting that systemic and local peritoneal VEGF production may be differently regulated (Szeto et al. 2004). Therefore, we hypothesize that the -2578A/C SNP might have a similar effect on angiogenesis in endometriotic implants and women with the CC genotype could have a decreased risk of endometriosis due to lower VEGF expression and angiogenic activity in peritoneal cavity.

No association was observed between endometriosis and other *VEGFA* SNPs, -1154G/A, -634G/C and +936C/T SNPs, in Estonian women. This is in agreement with some previous studies, while conflicts with others. For the most studied SNP, -634G/C (+405G/C), contradicting results have been reported, as positive associations have been found both with the C allele (Kim et al. 2005a; Gentilini et al. 2008a; Attar et al. 2010; Emamifar et al. 2011) and the G allele (Bhanoori et al. 2005; Altinkaya et al. 2011), whereas others reported no association (Ikuhashi et al. 2007; Zhao et al. 2008; Cosin et al. 2009). The -1154G/A SNP has been analysed only in one study, where the AA genotype was found to have a protective effect against endometriosis in Northern Chinese women (Liu et al. 2009). For the +936C/T SNP, the T allele has been reported to increase the overall disease risk (Cosin et al. 2009) or risk for stage III–IV endometriosis (Ikuhashi et al. 2007), while others have reported no association (Kim et al. 2008; Zhao et al. 2008; Liu et al. 2009). Since all these studies have been performed in different populations, ethnic variance might be one of the reasons for discrepancies in results and makes it difficult to draw any firm conclusions about the association of these individual SNPs with endometriosis. Recent meta-analyses on *VEGFA* polymorphisms concluded that only the +936C/T SNP is associated with endometriosis risk (Liang et al. 2012; Xu et al. 2012).

There is a high degree of linkage disequilibrium among the *VEGFA* up-stream SNPs, therefore we evaluated also their collective effect as a haplotype as it may have a stronger impact on endometriosis risk than single SNPs. In a previous study, some *VEGFA* -2578/-1154/-460 haplotypes were found to differ significantly in their frequency among endometriosis patients and healthy controls (Liu et al. 2009). Yet, another paper reported no association between endometriosis and -2578/-460/+405 haplotypes (Zhao et al. 2008). In agreement with the latter study, we observed no correlation between *VEGFA* haplotypes and the risk of endometriosis in Estonian women.

Circulating ACE levels have been shown to be related to *ACE* Alu I/D, -240A/T and +2350A/G polymorphisms in different populations (Zhu et al. 2001; Baghai et al. 2006; Jeunemaitre 2008). However, there are limited data about their relevance in the pathogenesis of endometriosis. The only two studies performed on Taiwan Chinese women demonstrated that the Alu-insertion, -240T allele and +2350G allele were all associated with an increased susceptibility to endometriosis (Hsieh et al. 2005a; Hsieh et al. 2007a). Since there is a strong linkage ($r^2 = 0.91$) between the Alu Ins/Del and +2350A/G polymorphisms (Kehoe et al. 2003), only the +2350A/G and -240A/T SNPs were analyzed in the present study. No significant association, however, was observed between endometriosis and *ACE* SNPs, neither individually nor in haplotype.

As for the *VEGFA* -2578A/C SNP, the discrepancy between previous and our results could be caused by very different allele frequencies in ethnically diverse study groups. The control group frequencies of the +2350G allele were 2.2% in Taiwan Chinese women (Hsieh et al. 2005a) and 46.5% in Estonian

women. Similarly, the frequencies of the -240T allele were 18.2% and 31.7%, respectively. It has been shown previously that important ethnic variation in the genetic regulation of serum ACE activity may exist (Bloem et al. 1996). In addition, there was a significant deviation from HWE (p = 0.0007) in the +2350A/G genotype distribution and a borderline deviation (p = 0.049) in the -240A/T SNP frequency among the control group in the Hsieh et al. (2005) study, which might have influenced their results to some extent.

There was a slight deviation from HWE (p = 0.02) also in our control group for the *ACE* -240A/T SNP. Since we ruled out genotyping errors, it might be that for some reason this locus is under selection and has an effect on fertility as our control group consisted solely of fertile women. However, comparing -240A/T genotype frequencies among fertile and infertile patients revealed no significant difference in our cohort. Therefore, it is difficult to say whether the selection is the principal cause of divergence from HWE in our control group. Similarly to our study, however, also Baghai et al. (2006) found different *ACE* promoter SNPs, including the -240A/T, to deviate significantly from HWE in healthy controls.

Considering the very small number of studies on *ACE* polymorphisms and endometriosis and their discordant results, future studies in genetically more homogeneous populations would be helpful to clarify the role of *ACE* gene in the pathogenesis of endometriosis.

6.2.4. Variation in survivin gene

Considering the importance of survivin in normal endometrial functioning (Nabilsi et al. 2010), its over-expression in endometriotic tissue (Ueda et al. 2002), and the reduced apoptotic properties of endometriotic cells, *BIRC5* seems an attractive candidate gene for an endometriosis susceptibility study. To evaluate possible associations between endometriosis and survivin, we analysed the distribution of three *BIRC5* promoter region polymorphisms: -241C/T, -235G/A and -31G/C in endometriosis patients and in two different control groups, population controls (group A) and gynaecological controls (group B). Although these polymorphisms appear to influence survivin expression (Xu et al. 2004; Jang et al. 2008; Wagner et al. 2008; Boidot et al. 2010; Kawata et al. 2011), in the present study, no significant difference was observed in their genotype frequencies between patients and the two control groups. Genotype distributions were similar also in stage I–II and stage III–IV disease, suggesting that an association between *BIRC5* promoter SNPs and susceptibility to endometriosis is unlikely in Estonian women. Since this is the first study about survivin polymorphisms and endometriosis, further studies in other populations would be needed to confirm these preliminary results.

6.3. Immunoreactivity to survivin

6.3.1. Anti-survivin autoantibodies and endometriosis

Survivin expression is increased in neoplastic tissues and several studies have shown that the concentration of anti-survivin antibodies is elevated in sera of cancer patients (Rohayem et al. 2000; Eto et al. 2007; Söling et al. 2007). Since endometriotic cells express more survivin than normal endometrial cells (Ueda et al. 2002; Fujino et al. 2006; Watanabe et al. 2009), we aimed to detect if this stimulates humoral immunoreactivity to survivin and whether antibodies against survivin could be used as a diagnostic marker to distinguish endometriosis patients from women with similar complaints but without the disease.

Anti-survivin autoantibody levels were measured in the sera of endometriosis patients and of gynaecological controls (group B), but no significant difference was observed. In addition, antibody levels were not influenced by disease stage nor by the fertility status. Thereby, according to our data, anti-survivin antibodies cannot be used as a biomarker for endometriosis.

Some studies on cancer patients have shown that anti-survivin antibody concentrations are not directly correlated with survivin expression levels in tumour tissues (Söling et al. 2007; Karanikas et al. 2009). This might explain, to some extent, why we observed no significant difference in anti-survivin antibody levels between women with and without endometriosis. On the other hand, as survivin is also expressed in normal endometrium, the increase in its expression in endometriotic lesions might not be sufficient to induce a stronger autoimmune response than in healthy women.

Although this was not the aim of our study, while analysing possible confounding factors, we observed a significant negative correlation between active smoking and serum anti-survivin antibody levels. This finding contradicts the results from a previous study (Rom et al. 2010), where healthy non-smoking controls had lower median anti-survivin antibody levels than heavy smokers. However, compared to our study, the enrolled individuals were considerably older, the study groups included individuals from both sexes, and most of the smokers had also had long-term asbestos exposure (Rom et al. 2010). We cannot exclude that some of these parameters may have had a modulating effect on the anti-survivin immunogenic response.

Cigarette smoking is known to cause alterations in the immune system leading to heightened constitutive inflammation, impaired responses to pathogens, suppressed anti-tumour activity and abnormal adaptive Th17 immunity. However, it is difficult to correlate reduced anti-survivin autoantibody levels directly with smoking, as in general it tends to favour autoimmune responses (Arnson et al. 2010; Lee et al. 2012). Therefore, other indirect mechanisms could be involved. Since our study group consisted only of premenopausal women, lower antibody levels in active smokers might be caused by the anti-oestrogenic effect of cigarette smoking. It has been shown that women who smoke have lower oestrogen levels than non-smoking women (Shiverick and Salafia 1999; Soldin et al. 2011) and that oestrogens stimulate survivin

expression in the endometrium and in oestrogen-sensitive cancer cells (Frasor et al. 2003; Nabilsi et al. 2010). Therefore, the anti-oestrogenic effect of smoking could lead to a reduction in survivin expression and thus to a lower autoimmune reactivity against it. However, additional research in larger study groups is required to fully reveal the impact of smoking on immunoreactivity to survivin.

6.3.2. Anti-survivin autoantibodies and variation in survivin gene

Polymorphisms in the coding region of a gene may influence the production of autoantibodies against the protein encoded by this gene, as has been shown for example for the zinc transporter 8 (ZnT8) gene. ZnT8 is a major target of autoimmunity in type 1A diabetes and the gene encoding for it, *SLC30A*, contains a nonsynonymous SNP Arg325Trp, which defines one of the protein's antigenic epitopes and thus determines the production of specific autoantibodies recognizing either 325Arg residue or the 325Trp residue (Wenzlau et al. 2008).

In the present study, only non-coding *BIRC5* SNPs were included, therefore, associations between anti-survivin autoantibodies and possible structural changes in survivin could not be assessed. However, as protein expression might be influenced by promoter region polymorphisms, we wanted to evaluate the effect of *BIRC5* -241C/T, -235G/A and -31G/C SNPs on the serum level of anti-survivin antibodies. Considering that smoking status was the major contributor to autoantibody concentrations, associations between *BIRC5* SNPs and anti-survivin antibodies were evaluated only among non-smoking women. No significant correlation was revealed between *BIRC5* genotypes and median antibody levels, but a positive association was observed between the -235A allele and higher autoantibody concentrations (OD $\geq$ 2.0). Still, this result should be considered with caution, as the -235A allele frequency was very low in our study group.

It is difficult to speculate on the biological background of this finding due to the scarce and inconsistent data about the impact of the -235G/A SNP on survivin expression. In *in vitro* gene reporter assays the -235G/A SNP has been shown to have no effect on survivin promoter activity (Xu et al. 2004; Wagner et al. 2008). However, in a recent study on breast carcinomas, a strong association between the A allele and increased survivin mRNA level was detected (Boidot et al. 2010). The -235G/A SNP seems to create a second binding-site for the transcription factor GATA-1 in the survivin promoter, which could explain increased gene expression in the presence of the -235A allele (Boidot et al. 2010), leading to elevated protein levels and a stronger antibody response.

7. CONCLUSIONS

Endometriosis is a complex gynaecological disease and is often associated also with infertility. It is caused by the interplay of different factors, including individual's genetic variations. Since the aethiopathogenesis of endometriosis is not fully understood yet, identifying genes that contribute to disease risk might help determine biological pathways and mechanisms involved in endometriosis development. Numerous association studies on plausible candidate genes have been carried out in various populations, reporting different risk alleles for endometriosis. In the present study we evaluated associations between endometriosis and ten different genes, which were selected based on their plausible role in disease development. As endometriosis is considered to be an oestrogen-dependent disease, five genes involved in sex steroid biosynthesis and signalling were included, while the other five genes participating in tissue remodelling, angiogenesis and apoptosis were chosen in view of the Sampson's implantation theory. Since the role of genetic factors in the development of endometriosis-associated infertility has received little attention thus far, all the studied variants were also evaluated in relation to the fertility status of our study subjects.

In addition, the presence of anti-survivin autoantibodies was analyzed in women with and without endometriosis, to assess whether it could be used as a possible disease marker for minimally invasive diagnosis.

Based on our result we can make the following conclusions:

1. Genetic variation in genes involved in sex steroid biosynthesis and signalling may influence both, susceptibility to endometriosis as well as infertility. More specifically, we showed that *HSD17B1* +1954A/G SNP and *ESR2* $(CA)_n$ repeat are associated with endometriosis with and without infertility, respectively, while the *ESR1* $(TA)_n$ locus appears to be more related to infertility.

2. Polymorphisms in matrix metalloproteinase genes *MMP2* and *MMP9* correlate with endometriosis risk. We showed that the *MMP2* -735C/T SNP and certain *MMP2* promoter haplotypes are associated with overall risk of endometriosis, while the presence of *MMP9* -1562C/T SNP increases susceptibility to advanced stages of endometriosis.

3. Variation in the *VEGFA* gene may influence susceptibility to endometriosis, while *ACE* polymorphisms are unlikely to affect disease risk. We showed that the *VEGFA* -2578A/C SNP is associated to overall endometriosis risk.

4. Variations in the *BIRC5* gene promoter area do not influence susceptibility to endometriosis.

5. Autoimmune reactivity against survivin is similar in women with and without endometriosis, as was revealed by the detection of autoantibodies. Therefore, anti-survivin antibodies cannot be used as a biomarker of endometriosis. However, anti-survivin antibody concentration appears to be influenced by the *BIRC5* -235G/A SNP and by cigarette smoking, showing their general importance.

In conclusion, this study identified a set of the genetic markers associated with susceptibility to endometriosis in Estonian women, and it is the first report to show a significant correlation between the *ESR2* $(CA)_n$ repeat length and endometriosis risk. Interestingly, some polymorphisms showed different distribution in patients with or without infertility. As different mechanisms have been suggested to be involved in the development of stage I–II and stage III–IV disease, the same could be hypothesized about endometriosis with and without infertility. Therefore, fertility status of endometriosis patients is an aspect that should deserve more attention in future studies, to clarify the role of genetic factors and related proteomic changes in the pathogenesis of endometriosis and infertility associated with it.

SUMMARY IN ESTONIAN

Endometrioosi geneetiline eelsoodumus

Endometrioos on krooniline günekoloogiline haigus, mille korral näärme- ja stroomarakkudest koosnev endomeetriumi taoline kude paikneb kolletena väljaspool emakaõõnt, peamiselt vaagna piirkonna peritoneumil, munasarjades ning uterosakraalligamendil. Harvem võib haiguskoldeid esineda ka kuseteedes, soolestikus, kopsudes jne. Vastavalt kollete asukohale, arvule, sügavusele ning liitelise protsessi ulatusele jagatakse endometrioos nelja staadiumisse: I – minimaalne, II – kerge, III – mõõdukas ja IV – raske. Endometrioosi peamisteks sümptomiteks on krooniline vaagnapiirkonna valu, düsmenorröa, düspareuunia ning sageli kaasneb haigusega ka viljatus. Endometrioosi on võimalik kindlalt diagnoosida ainult laparoskoopial/laparotoomial visuaalse leiu ja histoloogilise uuringu tulemuse põhjal. Mitteinvasiivse diagnostikameetodi puudumise tõttu on raske hinnata haiguse täpset esinemissagedust, kuid arvatakse, et ligikaudu 10% reproduktiivses eas olevatest naistest põeb endometrioosi. Viljatute naiste seas on esinemissagedus veelgi kõrgem, ulatudes kuni 50% juhtudest.

Endometrioosi tekkepõhjused ei ole veel selged. Välja on pakutud erinevaid hüpoteese, millest kõige levinum on implantatsiooniteooria. Selle kohaselt tekib endometrioos menstruaalvere retrograadsel liikumisel läbi munajuhade kõhuõõnde sattunud endomeetriumi rakkudest, mis kinnituvad peritoneumile ning lokaalse verevarustuse tekkimisel arenevad endometrioosi kolleteks. Selles protsessis on oluline roll ensüümidel, mis osalevad ekstratsellulaarse maatriksi remodelleerimises, nagu näiteks maatriksi metalloproteinaasid (MMPd) ning angiogeneesi stimuleerivatel faktoritel. Lisaks eelnevale, võib kollete kasvu soodustada ka endomeetriumi rakkude langenud apoptootiline aktiivsus.

Kuna menstuaalverd satub kõhuõõnde ligi 90% naistest, kuid vaid 10% esineb endometrioos, siis on tõenäoliselt haiguse tekkesse kaasatud veel teisigi mehhanisme, näiteks häired immuunsüsteemi funktsioonis. Endometrioosihaigetel on kirjeldatud NK rakkude ja T lümfotsüütide tsütotoksilise aktiivsuse langust ning põletiku tsütokiinide ja autoantikehade tootmise tõusu, mis võivad soodustada endometrioosi kollete kasvu ning lisaks mõjutada ka viljakust.

Endometrioos esineb reproduktiivses eas naistel ning enamasti taandub spontaanselt peale menopausi, seetõttu loetakse seda östrogeensõltuvaks haiguseks. Endomeetriumi kasvu reguleerivad östrogeen ja progesteroon, vastavalt kas stimuleerides või inhibeerides rakkude proliferatsiooni. Suguhormoonide retseptorid esinevad ka endometrioosikoes, mistõttu ka haiguskolded reageerivad hormonaalsele miljööle. Erinevalt emakasisesest endomeetriumist, on endometrioosi kolletes ekspresseeritud kõik östrogeenide sünteesiks vajalikud ensüümid, kaasa arvatud aromataas ja 17β-hüdroksüsteroid dehüdrogenaas tüüp 1 (17β-HSD1), mistõttu on östrogeeni lokaalne tase haiguskolletes kõrgem kui normaalses endomeetriumis.

On alust arvata, et endometrioosi tekkes on teatud roll ka geneetilistel faktoritel. Endometrioosihaige esimese astme sugulastel on haigestumisrisk ligi seitse korda kõrgem võrreldes üldpopulatsiooniga ning perekondlikel juhtudel

esineb sagedamini mõõdukat ja rasket endometrioosi kui sporaadilistel juhtudel, mis viitab võimalikule polügeensele pärandumise viisile. Endometrioosi riski mõjutavate geneetiliste faktorite kindlaks tegemine võimaldaks paremini mõista haiguse tekkepõhjuseid ja -mehhanisme ning aitaks seeläbi kaasa uute efektiivsemate diagnostika- ja ravimeetodite väljatöötamisele.

Uurimistöö eesmärgid

Käesoleva uurimistöö eesmärgiks oli analüüsida seoseid erinevate kandidaatgeenide ja endometrioosi haigestumise vahel. Lisaks soovisime uurida surviviinivastaste autoantikehade esinemist endometrioosi korral ning hinnata nende võimalikku rolli haiguse väheinvasiivses diagnostikas.

Sellest tulenevalt olid töö alaeesmärgid järgnevad:

1. Analüüsida endometrioosi haigestumise seost polümorfismidega suguhormoonide sünteesis ja toimes osalevates geenides: östrogeeni retseptorite geenid *ESR1* ja *ESR2*, progesterooni retseptori geen *PGR,* 17β-HSD1 geen *HSD17B1* ning aromataasi geen *CYP19A1*.

2. Uurida koe remodelleerimisega seotud maatriksi metalloproteinaaside 2 ja 9 geenide *MMP2* ja *MMP9* polümorfismide mõju endometrioosi tekkele.

3. Hinnata seoseid endometrioosi haigestumise ja angiogeneesis osalevate vaskulaarse endoteliaalse kasvufaktori geeni *VEGFA* ja angiotensiin-I konverteeriva ensüümi geeni *ACE* polümorfismide vahel.

4. Analüüsida endometrioosi seost apoptoosi inhibiitori surviviini geeni *BIRC5* polümorfismidega.

5. Võrrelda endometrioosiga patsientidel ja kontrollindiviididel esinevat autoimmuunreaktsiooni surviviini vastu, määrates surviviinivastaste autoantikehade esinemist uuritavate seerumites.

Materjal ja metoodika

Uuringusse kaasati 150 endometrioosi diagnoosiga naist, 199 viljakat naist üldpopulatsioonist (kontrollgrupp A) ning 47 laparoskoopia läbinud patsienti, kellel endometrioosi ei esinenud (kontrollgrupp B). Iga uuringus osaleja kohta täideti küsimustik. Hospitaliseeritud patsientidelt võeti operatsiooni eelselt 15 ml perifeerset verd, millest eraldati DNA ning seerum. Kontrollgrupp A naiste DNA-d ja kliinilised andmed saadi TÜ Eesti Geenivaramust.

Geenivariatsioonide uuringus genotüpiseeriti kõigil naistel endometrioosigrupis ning kontrollgrupis A 22 polümorfismi 10 erinevas geenis: *ESR1 Pvu*II

T/C, *ESR1* $(TA)_n$, *ESR2* $(CA)_n$, *PGR* +331G/A, *PGR* Alu Ins/Del, *HSD17B1* +1954A/G, *CYP19A1* $(TTTA)_n$, *CYP19A1* TCT Ins/Del, *CYP19A1* 3'-UTR C/T, *MMP2* -1575G/A, *MMP2* -790T/G, *MMP2* -735C/T, *MMP9* -1562C/T, *VEGFA* -2578A/C, *VEGFA* -1154G/A, *VEGFA* -634G/C, *VEGFA* +936C/T, *ACE* -240A/T, *ACE* +2350A/G, *BIRC5* -241C/T, *BIRC5* -235G/A, *BIRC5* -31G/C. *BIRC5* polümorfismid määrati ka kontrollgrupp B naistel. Genotüpiseerimisel kasutati järgnevaid meetodeid: PCR, restriktsioonanalüüs, alleelspetsiifiline PCR, sekveneerimine ja automatiseeritud fragmentanalüüs. Genotüüpide ja haplotüüpide esinemissagedust võrreldi patsientide ja kontrollindiviidide vahel. Lisaks analüüsiti geenivariatsioonide seost nii endometrioosi raskusastmega, jagades patsiendid kaheks grupiks: I–II staadium ja III–IV staadium, kui ka patsientidel esineva viljatusega.

Surviviinivastaste autoantikehade esinemine määrati ELISA meetodil 98 endometrioosiga patsiendil ning kõigil naistel kontrollgrupis B. Hinnati nii antikehade seost endometrioosiga üldiselt, kui ka haiguse erinevate staadiumide ning viljatuse esinemisega.

Uurimistöö peamised tulemused ja järeldused

Uurimistöö olulisemad tulemused on järgnevad:

1. Variatsioonid suguhormoonide sünteesi ja toimega seotud geenides võivad mõjutada nii endometrioosi haigestumise riski kui ka viljatuse teket. Antud tööst ilmnes, et *HSD17B1* geeni +1954A/G polümorfismi AA ja AG genotüübid on seotud kõrgema riskiga endometrioosi ja sellega kaasneva viljatuse tekkeks. Samas *ESR2* geeni $(CA)_n$ mikrosatelliidi lühemad alleelid on seotud endometrioosi riskiga, kuid ilma kaasneva viljatuseta. *ESR1* geeni $(TA)_n$ lookuse pikemad alleelid näivad olevat seotud pigem viljatuse kui endometrioosiga.

2. Variatsioonid MMP geenides on seotud endometrioosi riskiga. *MMP2* geeni promootorala -735C/T polümorfismi CC genotüüp suurendab haigestumise riski võrreldes TT ja CT genotüüpidega ning teatud promootorala haplotüüpidel näib olevat kaitsev efekt. *MMP9* geeni promootori -1562C/T polümorfismi TT ja CT genotüübid on seotud kõrgema riskiga III-IV staadiumi endometrioosi tekkeks.

3. Variatsioonid *VEGFA* geenis võivad mõjutada endometrioosi teket. *VEGFA* promootoralas oleva -2578A/C polümorfismi CC genotüüp on seotud madalama haigusriskiga. Antud töös uuritud *ACE* geeni variandid ei mõjuta endometrioosi haigestumist.

4. Surviviini geeni *BIRC5* promootorala polümorfismid ei ole seotud endometrioosi riskiga.

5. Humoraalne autoimmuune reaktsioon surviviini vastu on sarnane endometrioosihaigetel ja tervetel naistel, mistõttu surviviinivastaste autoantikehade määramist seerumis ei saa kasutada endometrioosi diagnostikas. Autoantikehade tase näib olevat seotud pigem *BIRC5* -235G/A polümorfismi ning suitsetamisega.

Kokkuvõttes näitavad käesoleva uuringu tulemused, et teatud variatsioonid nii suguhormoonide sünteesi ja toime kui koe remodelleerimise ja angiogeneesiga seotud geenides võivad mõjutada endometrioosi teket. Lisaks on antud töö esimene, mis kirjeldab *ESR2* geeni $(CA)_n$ mikrosatelliidi seost haigestumisriskiga. Tulemuste analüüsimine viljatutel ja viljakatel patsientidel eraldi näitas, et teatud geenivariatsioonide puhul ilmneb seos endometrioosiga sõltuvalt sellest, kas haigusega kaasneb viljatus või mitte, viidates võimalusele, et erinevad mehhanismid viivad ühe või teise haiguse vormi väljakujunemisele.

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